An Investigation into Carotid Atherosclerotic Plaque Instability

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

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Abstract

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Stroke is the leading cause of death and permanent neurological disability in the developed world and a significant burden on the NHS and wider economy. A third of all strokes are caused by thrombo-embolism from unstable carotid atherosclerotic plaques. The exact pathogenesis of plaque progression and instability is unknown. The aim of this thesis was to investigate carotid plaque instability from a clinical perspective and on a molecular level.

Patients with spontaneous embolisation detected during Transcranial Doppler (TCD) monitoring were significantly more likely to have recent symptoms and recurrent events than those patients without evidence of spontaneous embolisation. Features of unstable plaque histology including large lipid core, intra-plaque haemorrhage, plaque inflammation, neovascularisation and cap rupture all decreased with time since event from 0-28 days but then increased in prevalence thereafter. Ultrasound features found to be related to unstable plaques included Grayscale Median (GSM) <25 and plaque area >80mm². Finally a predictive model was created to identify patients with a histologically unstable plaque using clinical and ultrasound parameters.

Using whole-genome wide microarray and results validated using qRT-PCR in an independent cohort, expression of the CCL19 and CTSG genes were significantly upregulated in plaques from patients with unstable plaques graded according to 1. Clinical; 2. Ultrasound; 3. TCD microemboli and 4. Histological criteria.

Using ELISA, serum concentration of CCL19 was significantly higher in patients with clinically and histologically unstable plaques (p=0.02). Immunohistochemical staining for CCL19 demonstrated positive staining in histologically and clinically unstable plaques (P=0.03) with co-localisation to CD3 positive T-cell lymphocytes.

In conclusion there is further evidence that plaque instability is greatest in the hyper and acute period after symptom onset. CCL19 is significantly over-expressed in patients with clinically unstable carotid atherosclerotic plaques and warrants further investigation. Clinical and non-invasive ultrasound imaging criteria can be used to predict the patient with the unstable plaque.

Statement of Originality

I hereby declare that the work contained in this thesis is entirely my own, except where acknowledged, and has not been submitted for a degree to this or any other university

M. K. Salem

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List of Abbreviations

ACAS	The Asymptomatic Carotid Atherosclerosis Study
ACES	The Asymptomatic Carotid Emboli Study
ACST	Asymptomatic Carotid Surgery Trial
AFx	Amaurosis fugax
AHA	American Heart Association
Ang	Angiopoietin
ARR	absolute risk reduction
B2M	β2 microglobulin
B-Mode	Brightness Mode
BMT	Best Medical Therapy
BP	Blood Pressure
CAD	Carotid Artery Disease
CCL19	chemokine (c-c-motif) ligand 19
ССР	cathepsin cystein proteases
CCR7	CC chemokine receptor 7
CD	cluster of differentiation (cluster of designation)
cDNA	Complementary Deoxyribonucleic acid
CEA	Carotid Endarterectomy
CETC	The Carotid Endarterectomy Triallists' Collaboration
CHD	Coronary Heart Disease
CI	Confidence Interval
COX6B1	cytochrome c oxidase subunit VIb polypeptide 1
CRP	C-Reactive Protein
СТ	Computer Tomography
Ct	cycle threshold
CTSG	Cathepsin G
CVA	Cerebrovascular Accident
DAB	Diaminobenzidine
DAMP	damage-associated molecular patterns
DASL	cDNA-mediated Annealing, Selection, extension and Ligation

DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic Cells
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DWA	Discrete White Areas
EC	endothelial cell
ECST	European Carotid Surgery Trial
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immuno sorbent assay
FT	Fibrous Tissue
GAK	cyclin G associated kinase
GEO	Gene Expression Omnibus
GFP	Green Fluorescent Protein
GO	Gene Ontology
GSM	gray scale median
GUSB	β-glucuronidase
H&E	Haematoxylin and Eosin
HDL	High-density lipoprotein (HDL)
HRP	horseradish peroxidise
Hs-CRP	high-sensitivity CRP
HT	Human Tissue
ICA	Internal Carotid Artery
ICA	Internal carotid Artery
ICAM	intracellular adhesion molecule
IGFBP7	insulin-like growth factor binding protein 7
IL18	Interleukin 18
IL6	Interleukin 6
IMS	industrial methylated spirit
IMT	intimal-medial thickness
IQR	Interquartile Range
JBA	Juxta Luminal Black
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LDL	Low-density lipoprotein (HDL)

Lp(a)	lipoprotein(a)
Lp-PLA2	lipoprotein-associated phospholipase A2
MIAMI	Minimum information about a microarray experiment
MCA	Middle Cerebral Artery
MCP1	Monocyte Chemoattractant Protein-1
M-CSF	macrophage colony-stimulating factor
MHz	Megahertz
MMP	matrix metalloproteinase
MPO	Myeloperoxidase
MRC	Medical Research Council
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic acid
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NF	normalization factor
NHS	National Health Service
NICE	The National Institute for Health and Clinical Excellence
NNT	Numbers needed to Treat
NRES	National Research Ethics Service
OD	Optical Density
OPG	Osteoprotegerin
OPN	Osteopontin
OR	Odds Ratio
PAI-1	plasminogen activator inhibitor-1
PAPP-A	Pregnancy-Associated Plasma Protein A
PET	Positron emission tomography
PF4	Platelet factor 4
PFO	patent foramen ovale
PGK1	Phosphoglycerate kinase 1
PlGF	Placental Growth Factor
PRR	pattern recognition receptors
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
REC	Research Ethics Committee
RER	Rough endoplasmic reticulum

RIN	RNA Integrity Number
RNA	Ribonucleic acid
RR	Relative Risk
SAA	Serum amyloid A
SE	spontaneous embolisation
SIRPB1	Signal-regulatory protein beta-1
SLC94	Solute carrier family 9 (sodium/hydrogen exchanger), member 4
SMC	Smooth Muscle Cell
sPLA ₂ -II	Type II secretory phospholipase A
TCD	Transcranial Doppler
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF-b1	Transforming Growth Factor-b1
TIA	Transient Ischaemic Attack
TIMP4	TIMP metallopeptidase inhibitor 4
TM	thrombomodulin
TMB	Transient Monocular Blindness
TMB	3,3',5,5'-tetramethylbenzidine
t-PA	tissue plasminogen activator
TUB	Transient Unilateral Blindness
TWF2	twinfilin, actin-binding protein, homolog 2
UHL	University Hospitals Leicester
UK	United Kingdom
u-PA	urokinase plasminogen activator
US	Ultrasound
VA309	Veterans Affairs Cooperative Studies Program 309
VACSP	Veterans Affairs Cooperative Studies Program
VBA	Visual Basic Application
VCAM	vascular cell adhesion molecule
WHO	World Health Organisation
χ^2	Chi-Squared

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1.1 The History of Carotid Artery Disease

1.1.1 A Historical Perspective of Atherosclerosis

Atherosclerosis is a term derived from the Greek words *athero* (meaning gruel or paste) and *sclerosis* (hardness). Atherosclerosis is a chronic disease of arteries. It has been extensively described since the turn of the twentieth century but among the first to describe atherosclerosis was Leonardo da Vinci (1452–1519), who stated that 'vessels in the elderly restrict the transit of blood through thickening of the tunics' (Keele KD, 1973) (Davies MK, 1996).

In 1768, the symptoms of angina pectoris were described by the British doctor William Heberden (Khan IA, 2002): Some years later, in 1799, Caleb Hillier Parry, a British physician (1755–1822), made a major contribution to medicine by the recognition of the cause of angina. He conducted experiments on sheep to investigate the circulation and the effects of impairment of the vascular supply. During an autopsy he found a gritty substance in coronary arteries. His first impression was that some plaster had fallen from the ceiling, but upon closer investigation he discovered that the plaster-like substance was within the arteries themselves. Plaque rupture was reported for the first time during the autopsy of Bertel Thorvaldsen, a celebrated neoclassical Danish artist and sculptor who died of sudden cardiac death in the Royal Theatre in Copenhagen in 1844. On autopsy, his death was attributed to the rupture of an atherosclerotic plaque in the left coronary artery. It was stated that the vessel wall contained 'several atheromatous plaques, one of which quite clearly had ulcerated, pouring the atheromatous mass into the arterial lumen'.

By the end of the 18th century, two theories dominated the discussion on the pathophysiology of atherosclerosis: the thrombogenic theory by Carl von Rokitansky and the inflammatory

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theory proposed by Rudolf Virchow (Mayer C, 2006). Rokitansky proposed that the deposits observed in the inner layer of the arterial wall were derived primarily from fibrin and other blood elements rather than being the result of a purulent process. Subsequently, the atheroma resulted from the degeneration of the fibrin and other blood proteins and finally these deposits were modified toward a pulpy mass containing cholesterol crystals and fatty globules. Virchow's description of the pathogenesis of atherosclerosis was based on an indepth study of the histological characteristics of the atherosclerotic lesion in all its stages, for the first time Virchow utilised the term 'endarteritis deformans'. By this he meant that the atheroma was a product of an inflammatory process within the intima and that the fibrous thickening evolved as a consequence of a reactive fibrosis induced by proliferating connective tissue cells within the intima. He maintained that mechanical forces initiated the irritative stimulus and that the endarteritis was part of a repair mechanism. To date, Virchow's concept of local intima injury as the initiating irritative stimulus is still accepted and it has been extended to include other factors besides mechanical factors. Virchow's hypothesis formed the basis of the popular response-to injury hypothesis of Russell Ross which postulated that the lesions of atherosclerosis arise as a result of focal injury to arterial endothelium, followed by adherence, aggregation and release of platelets (Ross R, 1999).

1.1.2 A Historical Perspective of Carotid Artery Disease

Chiari in 1905 and a few years later Hunt, Moniz, and Hultquist, among others, described the possible association of carotid artery disease and stroke (Chiari H, 1905). In Prague, Chiari found thrombus superimposed on carotid artery atherosclerotic plaques of 7 patients in a series of 400 consecutive autopsies. Four of these patients had suffered cerebral embolism, and he suggested that embolic material could arise from the carotid artery and affect the brain. This could be considered the first accurate description on the pathophysiology of

cerebral embolism from the carotid artery. In 1914 Hunt reported the clinical characteristics of 20 patients with hemiplegia but did not have autopsy data (Hunt JR, 1914). He proposed that "the cerebral lesions in most stroke victims could be the effect and not the cause."

On July 7, 1927, Moniz of Portugal reported the first case of cerebral angiography at the Societe de Neurologie in Paris (Moniz E, 1927). This valuable step in stroke diagnosis allowed for the first time identification of the affected vessel before direct visualization at surgery. Surprisingly, most angiograms were performed to visualize the intracranial portion of the carotids in cases of tumours to look for abnormal displacement of arterial branches.

In 1936, Sjøqvist reported the first case of internal carotid artery occlusion documented with cerebral angiography (Sjøqvist O, 1936). In 1937, Moniz et al reported occlusion of the internal carotid artery in 4 patients diagnosed with angiography (Moniz E, 1937).

Hultquist in 1942 published the clinic-pathological correlation of patients with stroke in a series of 1400 autopsies. He performed examinations of the entire carotid circulation in 400 patients and found a 3% incidence of thrombosis.

Krayenbühl and Weber published work in 1944 on 16 patients who had carotid thrombosis and had suffered ischaemic events. Lesions were studies using angiograpphy and autopsy. 11 patients had total occlusions of the internal carotid artery. The main diagnosis considered was Buerger's disease (Krayenbühl H, 1944).

In 1951, Fisher reported the occurrence of TIAs in relation to carotid artery disease after studying over 1100 autopsy specimens (Fisher CM, 1951). Prior to this, the majority of strokes were thought to be caused by vasospasm. This was the reason why sympathectomy

was the procedure of choice in carotid artery disease and most authors kept looking for a mechanism of spasm. A postulated similarity to "migraine and vasospasm" was the argument most commonly used to explain transient visual symptoms

Fisher was also the first to associate the occurrence of loss of vision in one eye, which he termed Transient Unilateral Blindness (TUB), with ipsilateral Carotid Artery Disease (CAD). He noted that permanent blindness did not accompany the onset of hemiplegia, observing that in most instances TUB occurred before hemiplegia, only rarely presenting after the stroke was completed. Fisher found that patients did not spontaneously complain about this symptom but rather described "trouble with my eyes" or "blurred vision" on being questioned. He also emphasized the sudden onset ("like a blind pulled down") and short duration of TUB. The fundi of these patients were not studied during the episodes of TUB. Fisher reported fundi findings during TUB a few years later. One year after the publication of the 1951 article, Fisher published another study in which he changed the term TUB to Transient Monocular Blindness (TMB), probably to emphasize eye involvement and avoid confusion with unilateral loss of vision from hemianopsia (Fisher CM, 1952).

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1.1.3 A Historical Perspective of Surgery for Carotid Artery Disease

Pare in 1552 was probably the first to report the surgical treatment of a carotid artery after he performed a ligation of the common carotid artery in a patient who developed aphasia and hemiplegia after the procedure. From 1900 to 1950, embolectomy and arterectomies were performed with variable but, in general, poor results.

The first successful carotid reconstruction was completed on the basis of work by Fisher in which he stated, "Anastomosis of the external carotid artery, or one of its branches, with the internal carotid artery above the area of narrowing should be feasible." The operation was performed in Buenos Aires, Argentina on a 51-year-old businessman who suffered a right hemiplegia and left eye blindness and diagnosed a severe stenosis of the internal carotid artery by means of a left percutaneous carotid angiography (Carrea R, 1955). During surgery, "the internal carotid was cut 5 mm above the abnormal area, the external carotid was also cut at the same level and the proximal portion of the external carotid was anastomosed end to end to the distal portion of the internal carotid." Patency of the artery was confirmed postoperatively, and the patient, who remained blind but improved his strength on the right side, was followed for 27 years.

In 1954, Eastcott, Pickering, and Robb published a report of a similar operation in the Lancet (Eastcott HG, 1954). They resected a 3-cm thrombosed segment of the internal carotid artery origin and performed a direct end-to-end anastomosis between the common and the internal carotid arteries. Their patient, a 66-year-old woman who had suffered a total of 33 attacks of hemiparesis, aphasia, and amaurosis fugax, did not have any further episodes after surgery.

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On the basis of the technique of endarterectomy introduced by Cid dos Santos in 1947 for atherosclerotic disease of the aortoiliac system (Dos Santos JC 1947), the first carotid endarterectomy was performed by Strully, Hurwitt, and Blankenberg on January 28, 1953, at the Montefiore Hospital in New York on a patient who had suffered a severe stroke 2 weeks earlier (Strully KJ, 1953). Since no retrograde flow could be obtained from the cranial end, the vessel was ligated to prevent the risk of cerebral embolism. On August 7, 1953, DeBakey performed the first successful endarterectomy (DeBakey ME, 1975). The patient was a 53-year-old man with TIAs who did not have angiography before surgery "because clinical suspicion of the stenosis was strong." The patient did not have recurrence of cerebrovascular disease until his death from a myocardial infarction 19 years later.

1.2 Epidemiology of Carotid Artery Disease

1.2.1 Incidence of Symptomatic Carotid Disease/Stroke

Symptomatic carotid artery disease/stenosis causes 1/3 of all strokes. Stroke is a major health problem in the UK. It is the third leading cause of death accounting for over 56,000 deaths in England and Wales in 1999, which represents 11% of all deaths. Each year in England, the incidence (number of new cases in a specific population over a given time period) of new or recurrent strokes is 110000 and TIAs 20000. More than 900,000 people in England are living with the effects of stroke, with half of these being dependent on other people for help with everyday activities (NICE, 2008).

A study in the Scottish Borders (Syme PD, 2005) reported a higher crude incidence rate of 2.8/1000/year, which was attributed to the higher proportion of elderly subjects in the population. Although deaths from stroke have fallen in the UK over the past 40 years

(Lawler DA, 2002);(National Audit Office, 2005);(Ashton C, 2010), stroke accounted for around 46500 deaths in England and Wales in 2008 (9% of all deaths)

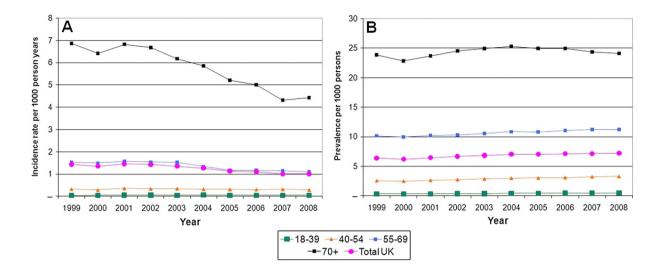


Fig 1.1 Incidence (A) and prevalence (B) of stroke in the UK adult population by age group (Kind permission of Lee S, 2011, BMJ Open)

1.2.2 Prevalence of Asymptomatic Carotid Disease/Stroke

The prevalence (total number of cases existing at a given time) of asymptomatic severe carotid artery disease based upon individual participant data meta-analysis (23706 participants) of 4 population-based studies (Malmö Diet and Cancer Study, Tromsø, Carotid Atherosclerosis Progression Study, and Cardiovascular Health Study) in severe carotid stenosis (\geq 70% NASCET) showed prevalence of severe asymptomatic carotid stenosis ranged from 0.1% (0.0% to 0.3%) in men aged <50 years to 3.1% (1.7% to 5.3%) in men aged \geq 80. For women, this prevalence increased from 0% (0.0% to 0.2%) to 0.9% (0.3% to 2.4%)(de Weerd M, 2010).

1.3 <u>Risk Factors</u>

Risk factors associated with carotid atherosclerosis and ischaemic strokes include advanced age, male sex, family history, hypertension, smoking, hypercholesterolaemia and diabetes.

Age, gender, race, ethnicity, and heredity have been identified as markers of risk for stroke. Although these factors cannot be modified, their presence helps identify those at greatest risk, enabling vigorous treatment of those risk factors that can be modified.

Non-Modifiable Risk Factors

1.3.1 Advancing Age

Age is the single most important risk factor for stroke. For each successive 10 years after age 55, the stroke rate more than doubles in both men and women (Brown RD, 1996);(Wolf PA, 1992).

1.3.2 Male Gender

Stroke incidence rates are 1.25 times greater in men, but because women tend to live longer than men, more women than men die of stroke each year.

1.3.3 Family History

An increased incidence of stroke in families has long been noted. Potential reasons are a genetic tendency for stroke, a genetic determination of other stroke risk factors, and a common familial exposure to environmental or lifestyle risks. Earlier studies suggested an increased risk for men whose mothers died of stroke and women who had a family history of stroke (Welin L, 1987). In the Framingham Study an offspring analysis revealed that both

paternal and maternal histories were associated with an increased risk of stroke (Kiely DK, 1993).

1.3.4 Ethnicity

Stroke incidence and mortality rates vary widely between racial groups. Afro-Caribbean population are more than twice as likely to die of stroke as the Caucasian population (Howard G, 1994). Between the ages of 45 and 55, mortality rates are four to five times greater for African-Americans than for Caucasians; the difference decreases with increasing age (Gillum RF, 1988). However, some race-related risk for stroke may be related to environmental factors or inherited risk factors other than race.

Potentially Modifiable Risk Factors for Ischemic Stroke

1.3.5 Hypertension

Hypertension is the single most important modifiable risk factor for ischemic stroke. Most estimates for hypertension indicate a relative risk of stroke of approximately 4 when hypertension is defined as systolic blood pressure \geq 160 mm Hg and/or diastolic blood pressure \geq 95 mm Hg.

The efficacy of antihypertensive treatment has been well established in clinical trials. In a summary of 17 treatment trials of hypertension throughout the world involving nearly 50 000 patients, there was a 38% reduction in all stroke and a 40% reduction in fatal stroke favouring systematic treatment of hypertension (MacMahon S, 1994).

1.3.6 Diabetes and Glucose Metabolism

Persons with diabetes have an increased susceptibility to atherosclerosis and an increased prevalence of atherogenic risk factors, notably hypertension, obesity, and abnormal blood

lipids. Case-control studies of stroke patients and prospective epidemiological studies have confirmed an independent effect of diabetes with a relative risk of ischemic stroke in persons with diabetes from 1.8 to 3.0. In Framingham, glucose intolerance doubled the risk of brain infarction. In addition to the role of glucose status (normal, impaired glucose tolerance, or diabetic), there are other aspects of glucose metabolism that may play a role as a risk factor for ischemic stroke—specifically hyperinsulinaemia and increased insulin resistance (the relative inability of insulin to enhance glucose disposal). Both were shown to be risk factors for ischemic stroke among subjects with normal glucose status (Shinozaki K, 1996).

1.3.7 Lipids

While hypercholesterolemia is an important modifiable risk factor for coronary heart disease, the link to ischemic stroke remains uncertain (NCEP, 1993);(Iso H, 1989). However, data clearly support the positive relation between total and LDL cholesterol and a protective influence of HDL cholesterol on extracranial carotid atherosclerosis (Heiss G, 1991). In secondary analyses, the Scandinavian Simvastatin Survival Study (4S) found a reduction of fatal or nonfatal stroke with simvastatin versus placebo (RR=.70, 95% confidence interval .52, .96), and the Asymptomatic Carotid Artery Plaque Study (ACAPS) reported fewer strokes in the lovastatin versus placebo group (5 versus 0) (4S Study, 1994). A pooled analysis of four pravastatin trials disclosed a 46% reduction in risk of stroke (P=.054) (Byington RP 1995).

1.3.8 Cigarette Smoking

Cigarette smoking increases risk (RR) of ischemic stroke nearly two times (Shinton R, 1989), with a clear dose-response relation. In both the Framingham Study and the Nurses' Health Study (Wolf PA, 1988);(Kawachi I, 1993) cessation of smoking led to a prompt

reduction in stroke risk—major risk was reduced within 2 to 4 years. This reduction in risk occurred throughout the age spans of these studies and in heavy as well as moderate smokers.

1.3.9 Alcohol

Moderate consumption of alcohol may reduce cardiovascular disease, including stroke. Recent epidemiological studies have shown a U-shaped curve for alcohol consumption and coronary heart disease mortality, with low to moderate alcohol consumption associated with lower overall mortality. In an overview analysis of stroke studies, a J-shaped association curve was suggested for the relation of moderate customary alcohol consumption and ischemic stroke (Camargo CA, 1989).

1.3.10 Lifestyle Factors (Obesity, Physical Activity, Diet, and Acute Triggers)

Various lifestyle factors have been associated with increased stroke risk. These include obesity, physical inactivity, diet, and acute triggers such as emotional stress. Obesity has been associated with higher levels of blood pressure, blood glucose, and atherogenic serum lipids, which are independent risk factors for stroke. In Framingham, obesity defined as a Metropolitan Life chart relative weight greater than 30% above average was a significant independent contributor to incidence of brain infarction in men aged 35 to 64 and women aged 65 to 94.

Moderate and heavy levels of physical activity have been associated with reduced CHD incidence. In recent years evidence supports a protective effect of moderate physical activity on stroke incidence in men and women (Kiely DK, 1994). In Framingham, physical activity was protective in men; adjusted relative risk was 0.41. However, there was no evidence of a protective effect of physical activity on risk of stroke in women. In addition, as has been found in coronary heart disease, there was no evidence that heavy physical activity conferred greater benefit than moderate levels. Physical activity exerts a beneficial influence on risk

factors for atherosclerotic disease by reducing blood pressure, weight, and pulse rate; raising HDL cholesterol and lowering LDL cholesterol; decreasing platelet aggregability; increasing insulin sensitivity and improving glucose tolerance.

Increased dietary consumption of fish, green tea, and milk were protective of stroke, whilst diets high in fat and cholesterol have been shown to be increase risk of stroke (Abbott RD, 1996).

1.3.11 Haemostatic and Inflammatory Factors

Haemostatic factors have been related to incidence of cardiovascular disease generally, and in two prospective studies fibrinogen has been linked to increased stroke risk. In Göteborg there was an independent graded relation between fibrinogen levels and incidence of stroke in 54year-old men (Wilhelmsen L, 1984). Fibrinogen has also been prospectively linked to both progression of carotid artery stenosis and risk of recurrent stroke. The mechanisms by which fibrinogen may be related to stroke risk include effects on viscosity, platelets, and atherogenesis, as well as its direct role in clot formation as the substrate for thrombin (Ernst E, 1993).

1.3.12 Homocysteine

Blood levels of homocysteine, produced from the essential amino acid methionine, can be determined by genetic factors and by intake of vitamins B6, B12, and folic acid. Numerous case-control studies have shown a strong relation between stroke and both basal and postmethionine load moderate hyperhomocysteinemia. Recently the British Regional Heart Study showed a strong, independent, and graded relation of homocysteine level to stroke risk among middle-aged men (Perry IJ, 1995). Compared with the first quartile of homocysteine,

the fourth quartile had an adjusted relative risk for stroke of 4.7 (1.1 to 20.0). Because high levels of homocysteine are both atherogenic and prothrombotic, the relation with stroke is biologically plausible and has been demonstrated in an animal model. Furthermore, although supplemental vitamins B6, B12, and folic acid may reduce blood levels of homocysteine, it has not been shown that this intervention reduces incidence of stroke (or myocardial infarction)

1.3.13 Multiple Risk Factors

Risk factors independently increase the probability of stroke and may also interact to increase the probability of stroke. Moreover, many persons have multiple borderline elevations of risk factor levels. To identify individuals at greatest risk of TIA and stroke, a risk profile was developed using 36 years of follow-up data from Framingham (Wolf PA, 1991). Genderspecific tables allow stroke probability to be determined by a point system based on age, systolic blood pressure, use of antihypertensive therapy, presence of diabetes, cigarette smoking, history of cardiovascular disease (coronary heart disease or congestive heart failure), and electrocardiographic abnormalities (left ventricular hypertrophy or atrial fibrillation). This risk profile provides a quantitative determination of probability of stroke, relative to what is average for a person of that age.

1.4 Clinical Presentation for Symptomatic Carotid Artery Disease

1.4.1 Stroke

The World Health Organisation (WHO) definition of stroke has been widely used. Stroke is defined as 'rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer, or leading to death, with no apparent cause other than of vascular origin'. This definition includes stroke due to either cerebral infarction or intracerebral and subarachnoid haemorrhage.

1.4.2 Transient Ischaemic Attack

Transient ischemic attack (TIA) is a brief episode of neurologic dysfunction resulting from focal temporary cerebral ischemia not associated with cerebral infarction. TIA was originally defined clinically by the temporary nature (<24 hours) of the associated neurological symptoms. However, the arbitrary nature of the 24-hour time limit and lack of specific pathophysiological meaning have hampered the clinical and research utility of the term "TIA." Recognition of these problems led to a change to a tissue-based definition of TIA. The change was driven by advances in neuroimaging that enabled very early identification of ischemic brain injury (Easton JD, 2009).

1.4.3 Amaurosis Fugax

Amaurosis fugax (Latin fugax meaning fleeting, Greek amaurosis meaning darkening, dark, or obscure) is a transient monocular visual loss (i.e., loss of vision in one eye that is not permanent). In 1990, the causes of amaurosis fugax were refined by the Amaurosis Fugax

Study Group, which defined five distinct classes of transient monocular blindness: embolic, hemodynamic, ocular, neurologic, and idiopathic.

With respect to embolic and hemodynamic causes, this transient monocular visual loss ultimately occurs due to a temporary reduction in retinal artery, ophthalmic artery, or ciliary artery blood flow, leading to a decrease in retinal circulation which, in turn, causes retinal hypoxia. While, most commonly, emboli causing amaurosis fugax are described as coming from an atherosclerotic carotid artery, any emboli arising from vasculature proximal to the retinal artery, ophthalmic artery, or ciliary arteries may cause this transient monocular blindness.

Amaurosis fugax may present as a type of transient ischemic attack (TIA), during which an embolus unilaterally obstructs the lumen of the retinal artery or ophthalmic artery, causing a decrease in blood flow to the ipsilateral retina. The most common source of these atheroemboli is an atherosclerotic carotid artery.

In a prospective study of patients presenting with amaurosis fugax, 70% were subsequently shown to have greater than 50% stenosis of the common or internal carotid artery (Lawrence PF, 2002).

1.5 Carotid Endarterectomy

1.5.1 The origins of carotid surgery

See 1.1.3 (page 9)

1.5.2 The Trials of Intervention in Carotid Disease

Intervention for carotid artery stenosis (carotid endarterectomy and stenting) was initiated to prevent a major stroke occurring but the paradox of the procedure is that a major risk of intervention includes causing a stroke!

Carotid endarterectomy was introduced as a logical procedure for the prevention of ischemic stroke distal to carotid artery stenosis. Although the first randomized trials of its effectiveness had negative results (Fields WS, 1970); (Shaw DA, 1984); (Kurtzke J, 1974), surgeons continued to perform carotid endarterectomy and began to report lower rates of perioperative complications (Baker WH, 1987); (Sundt TM Jr, 1990). However as the number of patients receiving intervention worldwide increased, uncertainty about the efficacy of the procedure was reflected in marked geographic variation in the rates of endarterectomy (Warlow CP, 1984). Adding to this uncertainty was the decline in the number of first and fatal strokes (Bonita R, 1990);(Kotila M, 1988);(Arraiz GA, 1989), the influence of risk-factor management in reducing strokes (Shinton R, 1989);(Klag MJ, 1989);(Garraway MJ, 1987), and emerging recognition of the efficacy of best medical therapy in preventing stroke (Antiplatelet Triallists' Collaboration, 1988). There have been 5 randomized trials of endarterectomy for recently symptomatic carotid stenosis, but the first 2 trials were small, performed >30 years ago, included a high proportion of patients with non-carotid symptoms, and did not stratify results by severity of stenosis. Only 3 trials were therefore included in forming evidence based guidelines for the treatment of carotid stenosis: North American Symptomatic Carotid Endarterectomy Trial (NASCET Trial Collaborators, 1991), European

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Carotid Surgery Trial (ECST Triallists, 1998); and Veterans Affairs trial (VACSP)(Mayberg MR, 1991). Summarised below are the major multi-centre randomised control trials providing evidence and guidelines for carotid endarterectomy in both symptomatic and asymptomatic patients with carotid artery disease.

1.5.2.1 The North American Symptomatic Carotid Endarterectomy Trial (NASCET)

The NASCET trial recruited patients between 1987 and 1996 across 50 centres in the US and Canada. Each centre had a rate of less than 6 percent for stroke and death occurring within 30 days of operation for at least 50 consecutive carotid endarterectomies performed within the previous 24 months.

Eligible patients had to be less than 80 years old, and have had a hemispheric transient ischemic attack (distinct focal neurologic dysfunction) or monocular blindness persisting less than 24 hours or a non-disabling stroke with persistence of symptoms or signs for more than 24 hours within the previous 120 days, in association with stenosis of 30 to 99 percent in the ipsilateral internal carotid artery.

Treatment consisted of anti-platelet treatment (usually 1300 mg of aspirin per day or a lower dose if necessitated by side effects) and, as indicated, antihypertensive, anti-lipid, and antidiabetic therapy was prescribed for all patients. Those assigned to surgery underwent carotid endarterectomy. Follow-up was with neurologists at 1, 3, 6, 9 and 12 months, and then at 4 monthly intervals thereafter. The average follow-up for all patients was 5 years, and all 1818 surviving patients (911 in the medical group and 907 in the surgical group) underwent final assessment in 1997. Strokes were considered disabling if patients had a Rankin score of 3 or more at 90 days. The primary intention-to-treat analysis compared medical and surgical patients in terms of the time to treatment failure (defined as a fatal or non-fatal ipsilateral stroke).

Early Termination of the Study in Patients with High-Grade Stenosis

On February 1, 1991, the trial's predetermined rule for stopping randomization was invoked because of evidence of treatment efficacy among patients with high-grade stenosis (70 to 99 percent) who underwent carotid endarterectomy. On February 21, the monitoring and executive committees agreed that (1) randomization of patients with high-grade stenosis should be stopped, (2) a summary of the results in the patients with high-grade stenosis should be communicated immediately to the participating clinicians, along with a list of all patients given medical treatment alone to whom the results might apply, (3) reports of all strokes and deaths and all patient assessments occurring before February 21 should be collected as quickly as possible for inclusion in this report, and (4) the parallel study dealing with symptomatic patients with medium-grade stenosis (30 to 69 percent) should be continued. The sponsoring agency, the National Institute of Neurological Disorders and Stroke, independently issued a peer-reviewed Clinical Alert to convey immediately a summary of these interim results to physicians across North America.

In summary the NASCET trial initiated in the mid-1980s to investigate the efficacy of CEA compared with medical treatment in patients with symptomatic carotid atherosclerotic disease. This randomized, prospective, multi-centre trial enrolled 659 patients who had had a hemispheric or retinal TIA or a non-disabling stroke within the 120 days before entry and had stenosis of 30 to 99 percent in the symptomatic (ipsilateral) carotid artery.

The study was prematurely terminated because of evidence that surgery was beneficial in this selected group of patients. At the time of study termination, patients had been followed for a mean of 18 months. Although the risk of stroke and death was higher at 30 days in the

patients treated with CEA (5.8 versus 3.3 percent with medical therapy), the following statistically significant benefits for CEA were observed at two years of follow-up:-

- A lower risk of any stroke or death (15.8 versus 32.3 percent)
- A lower risk of any ipsilateral stroke (9 versus 26 percent) (figure 2)
- A lower risk of major or fatal ipsilateral stroke (2.5 versus 13.1 percent)
- A lower risk of any major stroke or death (8.0 versus 19.1 percent) (figure 3)

The trialists concluded that CEA was highly beneficial for patients with recent TIAs or nondisabling strokes with ipsilateral stenosis of 70 to 99 percent.

In a subsequent report, the NASCET trial demonstrated that CEA also had a moderate degree of benefit for patients with 50 to 69 percent symptomatic ipsilateral stenosis compared with those receiving medical therapy (Barnett HJM, 1998). However, the overall statistical significance favouring surgery was marginal; the five-year rate of any ipsilateral stroke for endarterectomy versus medical treatment was 15.7 versus 22.2 percent (p = 0.045). Patients with stenosis of less than 50 percent did not benefit from surgery.

1.5.2.2 The MRC European Carotid Surgery Trial (ECST)

ECST was a multi-centre, prospective trial that randomly assigned 2518 patients with a nondisabling ischemic stroke, TIA, or retinal infarct due to a stenotic lesion in the ipsilateral carotid artery to best medical therapy or to surgery. The first report included 374 patients with a mild stenosis (0 to 29 percent) and 778 patients with severe stenosis (70 to 99 percent). After a three-year follow-up, the following findings were noted in the interim report:

- Patients with mild stenosis had little risk of ipsilateral ischemic stroke; possible benefits of CEA were small and were outweighed by the early risks
- At 30 days, the incidence of stroke or death was 7.5 percent in the patients with a severe stenosis who underwent CEA; the risk of these complications was not related to the severity of the stenosis
- At three years, patients treated with CEA had significant reductions in the incidence of ipsilateral ischemic stroke (2.8 versus 16.8 percent with best medical therapy alone) and in the total risk of surgical death, surgical stroke, ipsilateral ischemic stroke, or any other stroke (12.3 versus 21.9 percent). The risk varied with age and sex, with benefit being less likely in women and over a narrower range of carotid stenosis in younger patients.

A subsequent final report from ECST, based upon an ultimate total of 3024 patients followed for a mean of six years, noted two major findings:-

- CEA was beneficial for symptomatic carotid stenosis of 80 to 99 percent
- The risk of a major ischemic stroke ipsilateral to the un-operated carotid artery increased with the severity of the stenosis, particularly above 80 percent, but only for two to three years after randomization

Overall, the ECST confirmed the results of the NASCET trial, demonstrating a benefit with CEA in symptomatic patients with severe ipsilateral carotid stenosis, although age and sex were important considerations in a decision about surgery. The reduced risk of recurrent stroke associated with CEA was durable during at least 10 years of follow-up

Even in the areas where NASCET and ECST appeared to disagree (e.g. in patients with less than 80 percent stenosis), a reanalysis of the data suggests that if the same measurement criteria were used, these differences would disappear. See section 1.5.2.4 for CETC data.

1.5.2.3 The Veterans Affairs Cooperative Studies Program 309 (VA309)

The aim of the VA trial was to determine whether carotid endarterectomy provides protection against subsequent cerebral ischemia in men with ischemic symptoms in the distribution of significant (greater than 50%) ipsilateral internal carotid artery stenosis against best medical therapy alone. A prospective, randomized, multicenter trial was performed in sixteen university-affiliated Veterans Affairs medical centres. Men who presented within 120 days of onset of symptoms which were consistent with transient ischemic attacks, transient monocular blindness, or recent small completed strokes between July 1988 and February 1991 were deemed eligible and enrolled. Among 5000 patients screened, 189 individuals were randomized (Carotid endarterectomy plus the best medical care (n = 91) vs. the best medical care alone (n = 98)) with angiographic internal carotid artery stenosis greater than 50% ipsilateral to the presenting symptoms. Forty-eight eligible patients who refused entry were followed up outside of the trial. Outcome measures included a cerebral infarction or crescendo transient ischemic attacks in the vascular distribution of the original symptoms or death within 30 days of randomization. At a mean follow-up of 11.9 months, there was a significant reduction in stroke or crescendo transient ischemic attacks in patients who received carotid endarterectomy (7.7%) compared with nonsurgical patients (19.4%), or an absolute risk reduction of 11.7% (P = .011). The benefit of surgery was more profound in patients with internal carotid artery stenosis greater than 70% (absolute risk reduction, 17.7%; P = .004). The benefit of surgery was apparent within 2 months after randomization, and only one stroke was noted in the surgical group beyond the 30-day peri-operative period.

1.5.2.4 The Carotid Endarterectomy Triallists' Collaboration (CETC) analysis of the symptomatic trials (Rothwell PM, 2003)

The efficacy of CEA in patients with symptomatic carotid disease has been calculated from a pooled analysis of the major trials that included data from the ECST, NASCET, and VA trials. Pre-randomisation carotid angiograms from ECST were reassessed by the NASCET method, and outcomes were standardized to achieve comparability among the trials. Data for 6092 patients, with 35000 patient-years of follow-up, were therefore pooled. The following observations were reported:

- CEA was beneficial for patients with greater than 70 percent symptomatic stenosis (but not near occlusion). The number needed to treat (NNT) to prevent one stroke over five years for this group was 6.3, with an absolute risk reduction (ARR) of 16 percent.
- No significant benefit of CEA with near occlusion of the internal carotid artery (ICA) was observed. (Near occlusion is defined by the angiographic appearance of a collapsed ICA distal to the stenosis, accompanied by faster filling in the external carotid artery, and preferential filling of the intracranial circulation from collateral vessels). The ARR over two years with CEA for near occlusion was 5.6 percent, but the lack of benefit became apparent over five years, when the ARR was negative (-1.7 percent).

- CEA was beneficial for patients with 50 to 69 percent symptomatic stenosis. The NNT to prevent one stroke over five years in this group was 22, with an ARR of 4.6 percent.
- CEA was not beneficial for symptomatic carotid stenosis of 30 to 49 percent, and CEA was harmful for symptomatic patients with less than 30 percent stenosis.

1.5.2.5 The Asymptomatic Carotid Atherosclerosis Study (ACAS Executive Committee, 1995)

ACAS was initiated to determine whether the addition of carotid endarterectomy to aggressive medical management reduced the incidence of cerebral infarction in patients with asymptomatic carotid artery stenosis. ACAS was a prospective, randomized, multicenter trial across thirty-nine clinical sites across the United States and Canada between December 1987 and December 1993. A total of 1662 patients with asymptomatic carotid artery stenosis of 60% or greater reduction in diameter were randomized to receive daily aspirin administration and medical risk factor management or daily aspirin administration and medical risk factor management plus carotid endarterectomy; follow-up data was available on 1659. At baseline, recognised risk factors for stroke were similar between the two treatment groups. Outcome measures included initially, transient ischemic attack or cerebral infarction occurring in the distribution of the study artery and any transient ischemic attack, stroke, or death occurring in the perioperative period. In March 1993, the primary outcome measures were changed to cerebral infarction occurring in the distribution of the study artery or any stroke or death occurring in the perioperative period. After a median follow-up of 2.7 years, with 4657 patient-years of observation, the aggregate risk over 5 years for ipsilateral stroke and any perioperative stroke or death was estimated to be 5.1% for surgical patients and 11.0% for patients treated medically (aggregate risk reduction of 53% [95% confidence interval, 22% to

72%]). It was concluded that patients with asymptomatic carotid artery stenosis of 60% or greater reduction in diameter and whose general health makes them good candidates for elective surgery will have a reduced 5-year risk of ipsilateral stroke if carotid endarterectomy performed with less than 3% perioperative morbidity and mortality is added to aggressive management of modifiable risk factors.

1.5.2.6 The MRC Asymptomatic Carotid Surgery Trial (ACST)(Halliday A, 2004)

The MRC Asymptomatic Carotid Surgery Trial (ACST) assessed the long-term effects of successful CEA in patients with asymptomatic carotid stenosis. Between 1993 and 2003, 3120 asymptomatic patients from 126 centres in 30 countries were allocated equally, by blinded minimised randomisation, to immediate CEA (median delay 1 month, IQR 0.3-2.5) or to indefinite deferral of any carotid procedure, and were followed up until death or for a median among survivors of 9 years (IQR 6-11). The primary outcomes were perioperative mortality and morbidity (death or stroke within 30 days) and non-perioperative stroke. 1560 patients were allocated immediate CEA versus 1560 allocated deferral of any carotid procedure. The proportions operated on while still asymptomatic were 89.7% versus 4.8% at 1 year (and 92.1%vs 16.5% at 5 years). Perioperative risk of stroke or death within 30 days was 3.0% (95% CI 2.4-3.9; 26 non-disabling strokes plus 34 disabling or fatal perioperative events in 1979 CEAs). Excluding perioperative events and non-stroke mortality, stroke risks (immediate vs. deferred CEA) were 4.1% versus 10.0% at 5 years (gain 5.9%, 95% CI 4.0-7.8) and 10.8% versus 16.9% at 10 years (gain 6.1%, 2.7-9.4); ratio of stroke incidence rates 0.54, 95% CI 0.43-0.68, p<0.0001. 62 versus 104 had a disabling or fatal stroke, and 37 versus 84 others had a non-disabling stroke. Combining perioperative events and strokes, net risks were 6.9% versus 10.9% at 5 years (gain 4.1%, 2.0-6.2) and 13.4% versus 17.9% at 10 years (gain 4.6%, 1.2-7.9). Medication was similar in both groups; throughout the study, most were on antithrombotic and antihypertensive therapy. Net benefits were significant both for those on lipid-lowering therapy and for those not, and both for men and for women up to 75 years of age at entry (although not for older patients). In conclusion successful CEA for asymptomatic patients younger than 75 years of age reduces 10-year stroke risks. Half this reduction was in disabling or fatal strokes.

1.5.2.7 The Veterans Affairs Cooperative Study Group – efficacy of CEA for asymptomatic carotid stenosis (Hobson RW, 1993)

In order to determine the efficacy of carotid endarterectomy in patients with asymptomatic carotid stenosis a randomised control, multicenter clinical trial was performed at 11 Veterans Affairs medical centres to determine the effect of carotid endarterectomy on the combined incidence of transient ischemic attack, transient monocular blindness, and stroke. 444 men with asymptomatic carotid stenosis shown arteriographically to reduce the diameter of the arterial lumen by 50 percent or more. The patients were randomly assigned to optimal medical treatment including antiplatelet medication (aspirin) plus carotid endarterectomy (the surgical group; 211 patients) or optimal medical treatment alone (the medical group; 233 patients). All the patients at each centre were followed independently by a vascular surgeon and a neurologist for a mean of 47.9 months. The combined incidence of ipsilateral neurologic events was 8.0 percent in the surgical group and 20.6 percent in the medical group (P < 0.001), giving a relative risk (for the surgical group vs. the medical group) of 0.38 (95 percent confidence interval, 0.22 to 0.67). The incidence of ipsilateral stroke alone was 4.7 percent in the surgical group and 9.4 percent in the medical group. An analysis of stroke and death combined within the first 30 postoperative days showed no significant differences. Nor

were there significant differences between groups in an analysis of all strokes and deaths (surgical, 41.2 percent; medical, 44.2 percent; relative risk, 0.92; 95 percent confidence interval, 0.69 to 1.22). Overall mortality, including postoperative deaths, was primarily due to coronary atherosclerosis

Stenosis		CEA	BMT	ARR	NNT	Strokes prevented/1000 CEAs
<30%	n=1746	18.36%	15.71%	-2.6%	n/b	n/b
30-49%	n= 10054	22.80%	25.50%	2.6%	38	26
50-69%	n= 2312	20.00%	27.70%	7.8%	13	78
70-99%	n= 1344	17.13%	32.70%	15.6%	6	156
Near occlusion	n= 262	16.82%	15.15%	-1.7%	n/b	n/b

1.5.2.8 Summary of Evidence to Support Current Guidelines

Table 1.1 CETC (ECST, NASCET & VA studies combined and reanalysed after standardisation to NASCET angiographic measurement method (n>6000. any stroke at 5 years including operative risk. (Naylor AR, 2007)

	<u>5 Year Stroke Risk</u>				
	<u>Surgery</u>	<u>BMT</u>	ARR	RRR	<u>NNT</u>
<u>ACAS</u> (n=1662)	<u>5.1%</u>	<u>11.0%</u>	<u>5.9%</u>	<u>54%</u>	<u>17</u>
<u>ACST</u> (n=3120)	<u>6.4%</u>	<u>11.8%</u>	<u>5.4%</u>	<u>46%</u>	<u>19</u>

Table 1.2 ACAS and ACST Findings – Patients with Asymptomatic 60-99% Stenosis. (ACAS, 1995);(Halliday A, 2004).

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Chapter 2. Histological Analysis and Pathogenesis of Atherosclerotic Plaques

2.1 Arterial Intima

The intima is defined as the region of the arterial wall from and including the endothelial surface at the lumen to the luminal margin of the media. The internal elastic lamina, generally considered part of the media, denotes the border between intima and media. The thickness of arterial intima is not uniform. The arterial intima is composed of two layers. The layers may be absent or barely visible by light microscopy in segments of arteries with a very thin intima. In segments with adaptive thickening of the eccentric or diffuse type, the layers are clearly visible. The inner layer, subjacent to the lumen, has been called the proteoglycan layer because it contains an abundance of finely reticulated non-fibrous connective tissue identified as proteoglycan ground substance by electron microscopy (Richardson M, 1988). Elastic fibres are scarce here. Smooth muscle cells are of both the rough endoplasmic reticulum-rich (synthetic) and myofilament-rich (contractile) phenotypes. They occur as widely spaced single cells rather than in layers. The part of the proteoglycan layer near the endothelium contains isolated macrophages. The thicker layer underlying the proteoglycan layer (and adjacent to the media) has been called the musculo-elastic layer because of the abundance of smooth muscle cells and elastic fibres. This lower intima layer also contains more collagen than the upper layer. Smooth muscle cells are of the myofilament- rich phenotype and arranged in close layers (Stary HC, 1992)

2.2 American Heart Association Classification on Atherosclerosis

At the beginning of the century two types of intimal lesions were recognized and associated with atherosclerosis. They were called fatty streak (a thin lipid deposit in thin intima in

children) and fibrous plaque (a thick fibro-lipid lesion in adults). However, the two types of lesion were not universally accepted as an early and advanced expression of a single disease.

In the 1930s Pathologist Ludwig Aschoff was a leading proponent among those who regarded the morphologically different intimal lipid deposits of children and adults as early and late stages of one disease. Aschoff recognized two components of the disease. 1. Lipid; deposited in the intima from infancy and thereafter, Aschoff designated this stage as atherosis or atheromatosis, 2. Fibrosis (sclerosis, formation of collagen); added to the lipid in adults. Only the fibro-lipid stage was designated atherosclerosis (Aschoff L, 1930)

The Committee on Vascular Lesions of the Council on Atherosclerosis, American Heart Association (AHA), (Stary HC, 1992, 1994, 1995) described the characteristic components and pathogenic mechanisms of the normal arterial intima and of various atherosclerotic lesions. The lesions were classified by Roman numerals that indicate the usual sequence of lesion progression, grading from type I (initial lesions) to type VIII (fibrotic plaque). Varying proportions of different components (connective tissue extracellular matrix, crystalline cholesterol, cholesteryl esters, phospholipids and cells such as monocyte-derived macrophages, T lymphocytes, and smooth muscle cells) occur in different plaques, thus giving rise to a spectrum of lesions. Surface defects, hematoma, and thrombotic deposits further damage, deform, thicken, and accelerate conversion from clinically silent to overt disease.

Nomenclature and main histology	Sequences in progression	Main growth mechanism	Earliest onset	Clinical corre- lation
Type I (initial) lesion Isolated macrophage foam cells	I		From	
Type II (fatty streak) lesion Mainly intracellular lipid accumulation	(II)	Growth mainly	first decade	Clinically silent
Type III (intermediate) lesion Type II changes & small extracellular lipid pools	Ť	by lipid accumu- lation	From	
Type IV (atheroma) lesion Type II changes & core of extracellular lipid	IV		third decade	
Type V (fibroatheroma) lesion Lipid core & fibrotic layer, or multiple lipid cores & fibrotic layers, or mainly calcific, or mainly fibrotic	V	Accelerated smooth muscle and collagen increase	From fourth decade	Clinically silent or overt
Type VI (complicated) lesion Surface defect, hematoma-hemorrhage, thrombus	└→VI	Thrombosis, hematoma		

Figure 2.1. The American Heart Association's (AHA) definition and histological classification of atherosclerotic plaques. (Stary HC, 1995)

2.2.1 Simple Atherosclerotic Lesions (Stary HC, 1994)

2.2.1.1 Type 1 lesions

These early lesions are characterised by the first microscopically and chemically detectable

lipid deposits in the intima, along with the associated cell reactions. Small, isolated groups of

macrophages containing lipid droplets (macrophage foam cells) form. (Stary HC, 1987)

2.2.1.2 Type II lesions

Fatty streaks, which are visible as yellow deposits on the intimal surface of the vessel, are included here, although not all type II lesions are fatty streaks. The classification is made on the lesion's microscopic composition rather than its macroscopic appearance. Type II lesions consist of macrophage foam cells stratified in layers rather than being present only in isolated groups. Intimal smooth muscle cells now also contain lipid droplets and there is thinly dispersed lipid in the extracellular matrix. There are isolated mast cells which can influence plaque progression through the secretion of various vasoactive substances.

2.2.1.3 Type III lesions

This lesion is characterized histologically by extracellular lipid droplets. The lipid lies deep to the layers of macrophages and macrophage foam cells, replacing intercellular proteoglycans and fibres and driving smooth muscle cells apart. Similar to type II lesions, smooth muscle cells may contain lipid droplets. There tends to be more free cholesterol, fatty acid and triglyceride than in type II lesions. Type III is the intermediate stage between type II and type IV (atheroma, a lesion that is potentially symptom-producing)(Katz SS, 1976)(Small DM, 1988). In addition to the lipid-laden cells of type II, type III lesions contain scattered collections of extracellular lipid droplets and particles that disrupt the coherence of some intimal smooth muscle cells. This extracellular lipid is the immediate precursor of the larger, confluent, and more disruptive core of extracellular lipid that characterizes type IV lesions.

2.2.2 Advanced Atherosclerotic Lesions (Stary HC 1995)

Atherosclerotic lesions are considered advanced by histological criteria when accumulations of lipids, cells, and matrix components, including minerals, are associated with structural disorganization, repair, and thickening of the intima, as well as deformity of the arterial wall. Lesions considered advanced by their histology may or may not narrow the arterial lumen, may or may not be visible by angiography, and may or may not produce clinical manifestations. Such lesions may be clinically significant even though the arterial lumen is not narrowed, because complications may develop suddenly.

Advanced atherosclerotic lesions are subdivided into three main histologically characteristic types: IV, V, and VI. Type V and VI lesions have features that permit further subdivision. The features of the different advanced types and subtypes and the likely pathogenetic mechanisms related to each type are described below.

2.2.2.1 Type IV Lesions

In type IV lesions a dense accumulation of extracellular lipid occupies an extensive but welldefined region of the intima. This type of extracellular lipid accumulation is known as the lipid core. A fibrous tissue increase is not a feature, and complications such as defects of the lesion surface and thrombosis are not present. The type IV lesion is also known as atheroma. Type IV is the first lesion considered advanced in this classification because of the severe intimal disorganization caused by the lipid core. The characteristic core appears to develop from an increase and the consequent confluence of the small isolated pools of extracellular lipid that characterize type III lesions (Stary HC, 1989). Type IV lesions, when they first appear in younger people, are found in the same locations as adaptive intimal thickenings of the eccentric type. Thus, atheroma is, at least initially, an eccentric lesion

Lipid cores thicken the artery wall and are generally large enough to be visible to the unaided eye when the cut surface of the lesion is examined. Nevertheless, atheroma often fails to narrow the vascular lumen.

The usual intimal smooth muscle cells and the intercellular matrix of the deep intima are dispersed and replaced by accumulated particles of extracellular lipid. Between the lipid core and the endothelial surface, the intima contains macrophages and smooth muscle cells with and without lipid droplet inclusions. Lymphocytes and mast cells have also been identified in this region. Capillaries border the lipid core, particularly at the lateral margins and facing the lumen. Frequently macrophages, macrophage foam cells, and lymphocytes are more densely concentrated in the lesion periphery. Much of the tissue between the core and the surface endothelium corresponds to the proteoglycan-rich layer of the intima, although infiltrated with the cells just described. Formation of the lipid core precedes an increase in fibrous tissue that will subsequently change the nature of the intima above the lipid core.

In type IV lesions, the tissue layer between the lipid core and the endothelial surface is still largely the intima that preceded lesion development. When the cover of a lipid core later undergoes an increase in fibrous tissue (mainly collagen), the lesion is then labelled type V.

2.2.2.2 Type V Lesions

Type V lesions are defined as lesions in which prominent new fibrous connective tissue has formed. When the new tissue is part of a lesion with a lipid core (type IV), this type of morphology may be referred to as fibroatheroma. With these lesions, arteries are variously

narrowed, generally more than with type IV. Importantly, as with type IV lesions, type V lesions may develop fissures, hematoma, and/or thrombus (type VI lesion), and for this reason too they are clinically relevant. Sequential histological studies of the lesions of large populations indicate that reparative connective tissue forms in and around regions of the intima in which large accumulations of extracellular lipid (lipid cores) disarrange or obliterate the normal cell and intercellular matrix structure. Sometimes the new fibrous tissue accounts for more of the thickness of the lesion than does the underlying lipid accumulation. The new tissue consists of substantial increases in collagen and smooth muscle cells rich in roughsurfaced endoplasmic reticulum. In cases in which this tissue is particularly thick, some or much of it may be the remnant of thrombi that were incorporated and organized. Capillaries at the margins of the lipid core may be larger and more numerous than in type IV lesions, and they may also be present in the newly formed tissue. Lymphocytes, monocyte-macrophages, and plasma cells are frequently associated with the capillaries, and microhaemorrhages may be present around them. The architecture of some multilayered fibroatheromas could also be explained by repeated disruptions of the lesion surface, hematomas, and thrombotic deposits. Organization (fibrosis) of hematomas and thrombi could be followed by renewed accumulation of macrophage foam cells and extracellular lipid between the newly formed fibrotic layer and the endothelial surface. Lesions containing a large amount of calcium generally also have increased fibrous connective tissue, and often there is the underlying morphology of fibroatheroma. Lesions in which mineralisation is the dominant feature may be called calcific lesions. Mineral deposits may replace the accumulated remnants of dead cells and extracellular lipid, including entire lipid cores (Stary HC, 1992). Some apparently fibrotic lesions contain a small amount of lipid when processed and stained for lipid or when step sections are made through the entire lesion. The smooth muscle cells of the media adjacent to intima changed into a type V lesion may be disarranged and decreased. The media

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and adjacent adventitia may contain accumulations of lymphocytes, macrophages, and macrophage foam cells.

2.2.2.3 Type VI Lesions

Morbidity and mortality from atherosclerosis is largely due to type IV and type V lesions in which disruptions of the lesion surface, haematoma or haemorrhage, and thrombotic deposits have developed. Type IV or V lesions with one or more of these additional features are classified as type VI and may also be referred to as complicated lesions. While type VI lesions generally have the underlying morphology of type IV or V lesions, surface disruptions, hematoma, and thrombosis may be (although less often) superimposed on any other type of lesion and even on intima without an apparent lesion. Surface defects and Haematoma disruptions of the lesion surface include fissures and ulcerations (Davies MJ, 1985), but their extent and severity may differ greatly. The smallest ulcerations consist of focal loss of a part of the endothelial cell layer and are visible only under the microscope. Deep ulcerations may expose and release lipid from a lipid core. Fissures or tears of the lesion surface are of variable depth and length. Atheromatous lesions (types IV and V) are especially prone to disruptions of the lesion surface (Richardson PD, 1989)(Falk E, 1992).

It has been reported that advanced atherosclerotic lesions containing thrombi or the remnants of thrombi are frequent from the fourth decade of life on. The fissures and haematomas that underlie thrombotic deposits in many cases may recur, and small thrombi may reform many times. Repeated incorporation of small recurrent hematomas and thrombi into a lesion over months or years contributes to gradual narrowing of the arterial lumen. Some thrombi continue to enlarge and occlude the lumen of a medium-sized artery within hours or days. Functional impairment of endothelial cells or loss of small groups of endothelial cells could also facilitate thrombus formation when other predisposing conditions are present. Thrombotic deposits on lesions may also form without an apparent surface defect, haematoma, or haemorrhage.

2.2.3 The Cells and Extracellular Matrix of Histological Lesion Types IV, V, and VI

2.2.3.1 Smooth Muscle Cells

Smooth muscle cells are in part resident intimal cells that preceded the lesions and in part their progeny that arose as a response to various stimuli. The stimuli include lipid accumulation, disruption of intimal structure, damage to intimal cells and matrix, and deposits of platelets and fibrinogen, all of which may activate resident cells to produce mitogenic factors.

Intimal smooth muscle cells in close proximity to the thrombotic deposits of type VI lesions and those in type V lesions may contain very rough endoplasmic reticulum rich smooth muscle cells (Stary HC, 1990). The basement membrane rich smooth muscle cell is a smooth muscle variant found in lesion types IV, V, and VI and occasionally in type III but not in types I and II or in normal intima. These cells frequently occur in and adjacent to the lipid cores of lesions and particularly in the region between the core and the arterial lumen.

2.2.3.2 Macrophages

Driven by macrophage colony-stimulating factor (M-CSF) and probably other differentiation factors, the majority of monocytes in early atherosclerotic lesions become cells with macrophage- and/or dendritic cell-like features (Nelken NA, 1991). There is a difference in

the levels of the lesion at which macrophages without lipid droplet inclusions are found and at which macrophage foam cells are found. Macrophages without lipid droplet inclusions are more frequently located near the lumen, whereas macrophage foam cells are deeper in the intima (Stary HC, 1995). When a lipid core is present, macrophage foam cells are usually most evident along the luminal aspect and at the lateral margins of the core. Laterally, macrophage foam cells are not only more numerous, but because intima thickness is less at the lesion periphery, the foam cells are also somewhat closer to the surface. Many macrophage foam cells show ultrastructural evidence of cell injury, and some are dead and partly or wholly disintegrated.

2.2.3.3 Lymphocytes

Both T and B lymphocytes have been identified in advanced lesions by using monoclonal antibodies against CD antigens (Jonasson L, 1986). T cells are of both the T helper (CD4+) and T killer (CD8+) phenotypes and may be capable of clonal proliferation in response to appropriate antigens. There is some evidence that B lymphocytes can be stimulated to produce antibodies while resident within the lesions, although the many possible antigens must be identified and related to progression or regression of advanced lesions.

2.2.3.4 Dendritic Cells

The distinction between macrophages and dendritic cells (DCs) is a controversial topic, and it is further compounded by the likelihood of plasticity between these two types of cells (León B, 2005). DCs are defined as immune cells that internalise, process, and present antigen, leading to activation or suppression of T cells. Both pre-atherosclerotic susceptible regions of arteries and established atheromata are populated with cells that have DC-like properties. Early lesional DCs show two fundamental characteristics that were previously ascribed to early lesional macrophages: proliferation and foam cell formation (Paulson KE, 2010).

2.2.3.5 Neutrophils

In the arterial wall, defence against pathogenic damage requires a rigorous system of endothelium patrolling monocytes, resident macrophages and DCs and involves pattern recognition receptors (PRRs) sensing damage-associated molecular patterns (DAMPs) to mount an innate immune response. Early neutrophil inflammatory signals trigger intimal recruitment of monocytes, which differentiate into macrophages and internalize native and modified LDL, leading to foam cell formation. Macrophages can amplify LDL modification, promote vulnerability of advanced plaques through secretion of cytokines, proteases or procoagulant factors, undergo apoptosis and, when not effectively cleared, contribute to necrotic core formation.

During extravasation, neutrophils sequentially release preformed granule proteins. In the vascular wall, oxLDL may induce neutrophil transmigration and degranulation, which may then trigger rapid recruitment of classical monocytes usually present in hyperlipidemia-induced atherosclerosis. As short-lived cells, neutrophils rapidly become apoptotic releasing an array of signals to attract monocytes/macrophages for scavenging (Soehnlein O, 2012).

2.2.3.6 Lipid and Lipoprotein

The transfer of lipoproteins and fibrinogen from the plasma into the intima is a physiological process, but these proteins are found in advanced lesions in much larger amounts than in normal intima or in lesion types I, II, and III.

There is more extracellular lipid in type III, and in lesion types IV, V, and VI it also forms large circumscribed accumulations (lipid cores). The morphological range of the particles is similar in the diffusely scattered and circumscribed dense accumulations.

The fine structure of the extracellular particles appears identical to that of the inclusions within the cytoplasm of macrophage foam cells and smooth muscle cells. Analysis of the lipid of entire advanced lesions (those in which most lipid is extracellular) showed that cholesteryl esters were predominantly cholesteryl linoleate and thus similar to plasma low density lipoprotein (Smith EB, 1976).

2.2.3.7 Fibrinogen

The degree and extent to which fibrinogen accumulates in advanced lesions and parts of advanced lesions varies. When immunohistochemical techniques are used, the cores of advanced lesions stain for fibrinogen more extensively and intensively than any other aspect of advanced lesions except superimposed thrombi (Loukas M, 2002)

2.2.3.8 Proteoglycans

Large extracellular proteoglycans, mainly chondroitin sulfate–containing molecules have a function in arterial permeability, ion exchange, transport, and deposition of plasma materials

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such as LDL. Small extracellular proteoglycans such as dermatan sulfate–containing molecules function to regulate collagen fibrillogenesis but also bind ionically to LDL (Nigro J, 2005)

2.2.3.9 Collagen

Apart from lipid, collagen is the major extracellular component of type V lesions. The increased collagen of atherosclerotic lesions is produced by intimal smooth muscle cells. In some lipid-poor advanced lesions or parts of advanced lesions, collagen may be the major component. The major collagen type of advanced lesions is the fibrillar collagen type I. Type I collagen is especially prevalent in the fibrous cap and in vascularised regions of advanced lesions (Rekhter MD, 1993).

A significant and consistent change in the minor collagen types of advanced atherosclerotic lesions is the increase in type V collagen. This collagen is prominent in advanced lesions and increases with advancing fibrosis. Type V collagen has been reported to play a role in cell migration and may also appear to reduce the thrombogenic properties of the subendothelium. Type IV collagen is also increased in advanced lesions (Murata K, 1986).

The exact stimulus for increased collagen accumulation in atherosclerosis is unknown, although macrophage and platelet products such as transforming growth factor (TGF) upregulate collagen genes in a variety of cell types. Mechanical stresses are likely to be redistributed and modulated as lesions develop, inducing changes in matrix production in response to mechanical stimuli.

2.2.3.10 Elastin

Depending on the location and type of lesion, microscopically visible elastin fibers may be increased, decreased, or relocated. Although the smooth muscle cells of advanced lesions produce elastin, integration of the protein into a functional elastic fiber may be impaired. However, neo-formation of subendothelial, medial, and adventitial elastin does occur and may be prominent, particularly in type V lesions, where it accompanies collagen.

Elastic fibers often appear to be closely associated with lipid and calcium deposits. Lipid bound to elastic fibers may change elasticity of tissue by modifying the conformation of the elastin through hydrophobic interactions (Guantieri V, 1983).

2.2.3.11 Calcium

Atherosclerotic calcification begins as early as the second decade of life, just after fatty streak formation (Stary HC, 1990). Calcified deposits are found more frequently and in greater amounts in elderly individuals and more advanced lesions (Doherty TM, 1994). Calcium phosphate (hydroxyapatite, Ca₃[–PO₄]_{2–}×Ca[OH]₂), the predominant crystalline form in calcium deposits (Schmid K, 1980) is formed primarily in vesicles that pinch off from arterial wall cells. Attention has focused on a unique class of proteins known as Gla-containing proteins, which have a very high affinity for hydroxyapatite. Gla (gamma carboxyglutamate) is an unusual amino acid residue whose only known function is to bind calcium (Vermeer C, 1990);(Price PA, 1989). Gla proteins may be actively related to atherosclerotic calcification. They do not interfere with normal calcium homeostasis because they are not calcium chelators, but if precipitation of calcium occurs, available Gla-containing proteins would be expected to bind to the precipitate.

2.3 <u>Response to Injury Hypothesis</u>

The injury is usually a chronic low level insult to the endothelial or smooth muscle cells of the arterial wall manifesting itself in endothelial dysfunction. This leads to, among other things, lipid accumulation and leukocyte adhesion and infiltration.

2.3.1 Endothelial Dysfunction

The endothelium has three important functions that are particularly relevant to atherogenesis: (1) maintenance of a selectively permeable barrier between the intravascular space and the tissue space, (2) ability to modify and transport lipoproteins into the vessel wall, and (3) provision of a non-adherent surface for leukocytes. In accordance with the response to injury hypothesis, loss of these functions can be the most preliminary event in atherosclerosis. Injury leads to inflammatory responses that ultimately cause endothelial cell (EC) death through apoptosis. Russell Ross (1977) postulated that if the injury is chronic, the remaining viable ECs in the vessel wall will proliferate (to heal the wound) until they reach senescence, at which time the wound will not heal properly resulting in increased convection of macromolecules (e.g., LDL) from the circulation to the vessel wall.

2.3.2 Lipid Accumulation

Lipid accumulation is a major manifestation of the vascular response to injury, and there are three means by which this occurs. First, dysfunctional ECs lose their selective barrier function. Second, EC dysfunction leads to altered expression of lipoprotein receptors used to internalise and modify various lipoproteins. Certain lipoproteins, specifically oxidized LDL, perpetuate the insult by activating ECs and trigging the inflammatory cascade. A third means by which lipid accumulation may occur in response to vessel injury is that once lipids have been transported into the subintimal space, they are retained there by SMCs and macrophages that ingest lipoproteins (e.g., ox-LDL) via scavenger receptors.

2.3.3 Inflammatory Cell Infiltrate

Atherosclerosis has been described as an inflammatory process in that a major manifestation of the "response to injury" is leucocyte adhesion and infiltration. Mononuclear leukocytes have been identified in lesions in various stages of atherosclerosis. Monocytes and T lymphocytes adhere to the luminal surface of an artery where the endothelial layer is altered. Once there, they spread, migrate along the surface, and then extravasate through the endothelium into the subendothelial intimal space. This process is mediated through a variety of chemokines that allow these inflammatory cells to "home" to regions of insult or injury and adhesion molecules that provide the necessary contacts for the cells to attach and migrate from the vascular lumen into the vessel wall.

2.3.3.1 Chemokines

Atherogenic recruitment of leucocytes involves a sequence of rolling, firm adhesion, lateral migration and transendothelial diapedesis and is controlled by chemokines, which are chemotactic cytokines classified according to their conserved cysteines, and their correspondingly categorized G protein–coupled receptors (Braunersreuther V, 2007). The various leucocytes recruited during the inflammatory cascade encompass neutrophils, monocytes and T cells, but also B cells, DCs and mast cells. Considering the diversity of these cell subsets and their functions, the abundance in the chemokine system has been perceived to confer robustness and specificity. At a site of inflammation, particular leucocyte subsets may be recruited by a signature combination of chemokines engaging in heterophilic interactions, facilitated by a local repertoire of proteoglycans with differential binding

affinities for chemokine. The role of chemokines in inflammatory reactions is to become immobilized to the endoluminal surface of arteries, where they are presumed to enhance integrin adhesiveness and mediate leukocyte arrest and firm adhesion. In addition, they have been shown to promote transendothelial migration of leukocytes (Zernecke A, 2008).

2.3.3.2 Adhesion Molecules

Adhesion molecules related to the inflammatory process of atherosclerosis can be grouped into two categories, namely the selectins and the immunoglobulin adhesion molecules. Selectins are adhesion molecules that provide a loose attachment for leukocytes that allow them to roll along the luminal surface and include P-selectin, E-selectin, and L-selectin. Lselectin is constitutively expressed in leukocytes. E-selectin is expressed in activated ECs but its role in atherosclerosis has not been well established. P-selectin is not constitutively expressed by ECs but is expressed in ECs overlying active atherosclerotic plaques (Dong ZM 1998)

Immunoglobulin adhesion molecules allow leukocytes to firmly adhere to the endothelium and extravasate into the vessel wall and include intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). ICAM-1 is detected on ECs of atherosclerotic lesions and is increased by injury (Kitawaga K, 2002), whereas normal ECs show little expression. VCAM-1 is expressed in ECs overlying lipid containing human atherosclerotic lesions.

2.3.4 Role of Biomechanics in Atherosclerosis

Endothelial and smooth muscle cells of the vasculature live in a dynamic mechanical environment due to the hemodynamics of blood flow as well as the movement of the surrounding tissue beds. Cells of the blood vessel are mechanically stimulated by shear force due to contact with blood flow, strain due to pressure distension of the diameter of the vessel, and strain due to mural deformation of a vessel by its tethering to a surrounding tissue bed. In addition the direction of the shear forces alters due to blood flow reversal leading to oscillatory shear stress. The time average mean shear stress and amplitude of the oscillation are dependent upon anatomical location. Circumferential strain due to pressure distension ranges from 5 to 20% depending on arterial size and location. While every major artery in the body experiences these two mechanical stimuli, several other arteries also experience extraneous mechanical deformations due to their tethering to surrounding tissue beds.

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Chapter 3. Biological Markers of Plaque Instability

3.1 Microarray studies in carotid atherosclerosis

Microarray has been used to identify altered gene expression and subsequently biological markers of carotid atherosclerosis. Following the completion of the sequence of human genome, the major task became understanding the function of genes and their interactions. The first step in the process was the analysis of the transcriptional pattern of genes (transcriptome) in tissues and cells of the human organism, in both physiological and pathological conditions. Novel genomic approaches such as transcriptional profiling by DNA microarrays has allowed for the simultaneous analysis of over 55,000 transcripts in a single assay. DNA microarray analysis provides not only qualitative results (switched on/off genes), but also quantitative data (transcriptional level of single genes), so that subtle differences on gene activation can be detected. The transcriptome analysis using DNA microarray platforms consists of reciprocal identification and hybridization between two single chain molecules of nucleic acid based on their complementary sequences. The hybridization on the microarray can be a competitive process, for example, between RNA from a normal, reference tissue (control RNA) and RNA from pathological tissue, where expression levels of control transcripts are used as reference values for the expression levels of transcripts in the pathological sample. Transcriptional analysis can be performed on minute tissue samples obtained from biopsies and tissue fractions, cellular subpopulation or even single cells, following linear amplification of messenger RNA. Profiling cardiovascular tissue with their cellular heterogeneity has provided novel hypothesis generating findings.

Previous gene expression studies in carotid atherosclerosis using microarray technology have focussed on plaques from symptomatic versus asymptomatic patients (Randi AM, 2003);(Vemiganti R, 2005);(Dahl TB, 2007). There have also been microarray studies looking at post autopsy harvested plaques versus live tissue (Sluimer JC, 2007). Microarray studies have also been used at identifying gene expression in specific cellular subpopulations such as cap versus intima (Adams LD, 2006) and plaque versus normal arterial wall (Woodside KJ, 2003)[•] Finally most recently microarray studies have identified intra-plaque altered gene expression between stable and unstable regions (Papaspyridonos M, 2006)

3.2 Lipid accumulation

Accumulation of lipids by macrophages and smooth muscle cells plays a key role in the early stages of atherosclerosis. In atherosclerotic plaques, unstable lesions have shown to have a much greater area occupied by lipid. In addition, treatment with lipid lowering statins in patients with cardiovascular risk factors showed significant reduction in the first event rate for stroke.

3.2.1 Oxidised Low density Lipoprotein

In carotid artery disease, oxLDL has also shown to be related to carotid plaque instability (Nishi K, 2002). The oxidative modification hypothesis of atherogenesis suggests that the most significant event in early lesion formation is lipid oxidation, placing oxidized LDL (oxLDL) in a central role for the development of this disease. OxLDL has a large number of biological actions and consequences, including injuring endothelial cells (ECs), expressing adhesion molecules, recruiting leucocytes and retaining them, as well as the formation of foam cells (Jessup W, 2004). Elevated oxLDL has a role in the transition from

stable to unstable plaque with studies showing that oxLDL stimulates matrix metalloproteinase (MMP)-1 and -9 expression in human vascular EC and in monocytederived macrophages (Huang Y, 1999)(Xu XP, 1999)

3.2.2 Lipoprotein (a) (Lp(a))

A lipoprotein that has been studied in atherosclerotic disease is lipoprotein(a) (Lp(a)). High Lp(a) levels have been associated with risk for cardiovascular disease and a higher incidence of ischemic stroke (Ohira T, 2006). In relation to carotid plaque instability and stroke risk, serum Lp(a) levels are associated with ultrasound findings of carotid plaque instability (Iwamoto T, 2004). In a small observational study, serum Lp(a) was a significant independent predictor of carotid stenosis and carotid artery occlusion (Klein JH, 2008)

3.2.3 Lipoprotein-Associated Phospholipase A2

Lipoprotein-associated phospholipase (Lp-PLA₂) represents another emerging biomarker for atherosclerotic disease and is presently under intensive investigation. Lp-PLA₂, a 45.4-kDa protein, is a calcium-independent member of the phospholipase A₂ family. It is produced mainly by monocytes, macrophages, T-lymphocytes, and mast cells and has been found to be up-regulated in atherosclerotic lesions, especially in complex plaque, as well as in thin cap coronary lesions prone to rupture (Zalewski A, 2005). In the bloodstream, two-thirds of the Lp-PLA₂ plasma isoform circulates primarily bound to low-density lipoproteins (LDL), the other third is distributed between HDL and very low-density lipoproteins (VLDL) (Zalewski A, 2005);(Caslake MJ, 2000). Lp-PLA₂ may promote oxidation of LDL, and recent investigations have stressed the pro-atherogenic properties of this enzyme (Macphee CH, 1999). LDL provides a circulating reservoir, in which Lp-PLA₂ remains inactive until LDL undergoes oxidative modification. After LDL oxidation within the arterial wall, a short acyl group at thesn-2 position of phospholipids becomes susceptible to the hydrolytic action of Lp-PLA₂ that cleaves an oxidized phosphatidylcholine component of the lipoprotein particle generating two potent proinflammatory and proatherogenic mediators, namely lysophosphatidyl-choline (LysoPC) and oxidized fatty acid (oxFA) (Macphee CH, 1999). LysoPC is a potent chemoattractant for T-cells and monocytes, promotes endothelial cell dysfunction, stimulates macrophage proliferation, and induces apoptosis in SMCs and macrophages. Thus, Lp-PLA₂ may represent an important "missing link" between the oxidative modification of LDL in the intimal layer of the arterial wall and local inflammatory processes within the atherosclerotic plaque.

3.2.4 Type II Secretory Phospholipase A2

Type II secretory phospholipase A_2 (sPLA₂-II) is another well studied member of the phospholipase 2 family and is widely expressed in hepatocytes, macrophages, ECs, platelets and vascular SMCs. SPLA₂-II production is upregulated in response to proinflammatory compounds such as IL-1 β , IL-6, tumour necrosis factor (TNF)- α , INF- γ , and oxLDL (Hurt-Camejo E, 2001);(Anthonsen MW, 2000).

Possible atherogenic mechanisms of sPLA₂-II include its effects on lipoproteins which results in the release of various lipid mediators at the site of lipoprotein retention in the arterial wall, that in turn may trigger local inflammatory cellular responses. Furthermore, in arterial tissue, sPLA₂-II may also directly modify LDL particles to become more atherogenic.

3.3 Inflammation

Inflammatory cells in atherosclerotic plaques are stimulated to produce a number of biologically active molecules such as growth agonists, growth antagonists, pro- and anti-inflammatory cytokines and chemokines. The balance between pro- and anti-inflammatory activities is important in progression of atherosclerotic plaques, and inflammation can also elicit acute plaque rupture resulting in acute clinical scenarios of coronary syndromes or stroke.

An interesting phenomenon showing the relation between inflammation and atherosclerosis is the increased risk of cardiovascular disease in patients with rheumatoid arthritis (RA) that is not explained by traditional risk factors (Del Rincon ID, 2001). This also advocates an important role of inflammation in atherosclerosis and therefore, inflammatory markers in the prediction of plaque instability.

3.3.1 C-reactive protein (CRP) & High-sensitivity CRP (hs-CRP)

C-reactive protein (CRP) is a member of the pentraxin family and represents one of the most extensively studied pro-inflammatory molecules. In healthy individuals, only trace levels of CRP can be detected in the circulation. Under acute conditions, concentrations of CRP increase during the first 6 to 8 hours and can reach peak levels approaching 300 mg/L after approximately 48 hours. One of the first inflammatory biomarkers studied for prediction of atherosclerotic complications is C-reactive protein (CRP) and high-sensitivity CRP (hs-CRP). Evidence suggests that CRP may have direct pro-inflammatory effects, and contributes to the initiation, and progression of atherosclerotic lesions (Verma S, 2006). CRP has several effects that may influence progression of vascular disease, including activation and chemo-attraction of circulating monocytes, mediation of endothelial dysfunction, induction of a pro-

thrombotic state, increase of cytokine release, activation of the complement system, facilitation of extracellular matrix remodelling as well as lipid-related effects.

In carotid artery stenosis, hs-CRP correlates with morphological features of rapidly progressive carotid atherosclerosis defined by ultrasound categorization (Schillinger M, 2005). Rost et al. (2001) performed a community based prospective study with a one-time measurement of CRP in 1462 stroke- and TIA-free men and women. Elevated plasma levels of CRP could significantly predict a greater risk for a stroke.

3.3.2 Serum amyloid A (SAA)

Serum amyloid A (SAA) is an acute phase protein that can increase monocyte and macrophage cytokine production and is elevated in atherosclerotic lesions. A recent study showed that elevated SAA levels can identify patients with ischemic stroke caused by atherothrombosis (Brea D, 2009)

3.3.3 Pregnancy-Associated Plasma Protein A (PAPP-A)

PAPP-A is a metalloproteinase acting as a biomarker of inflammation by regulation of insulin-like growth factor (IGF). PAPP-A is a specific activator of insulin-like growth factor-1 (IGF-1) and acts by degrading IGF binding proteins-4 and -5, thus allowing active IGF-1 to bind to cell-surface type 1 IGF receptors (Bunn RC, 2003). IGF-1 induces cell proliferation, differentiation, migration, inflammatory cell activation, LDL-cholesterol uptake, and release of inflammatory cytokines, thus contributing to plaque progression and destabilization. In carotid plaque studies, expression of PAPP-A is elevated in specific macrophage rich plaque accumulations at the shoulder region and surrounding the lipid core (Sangiorgi G, 2006). This suggests a relationship between PAPP-A and plaque vulnerability and serum PAPP-A levels may also be used as a valuable biomarker for the detection of vulnerable plaques. In carotid artery disease, serum PAPP-A levels showed a positive predictive value for the presence of unstable plaques.

3.3.4 Cytokines

Cytokines also play an important role in atherosclerosis and a balance between pro- and antiinflammatory stimuli determines plaque progression.

3.3.4.1 Interleukin 18 (IL-18)

II-18 is a pro-inflammatory cytokine which is expressed by different cell types within the atherosclerotic plaque. IL-18 promotes the Th-1 immune response and also enhances the production of matrix metallo-proteinases (MMP's) (Ishida Y, 2004). In carotid artery plaques, IL-18 expression is especially increased in unstable lesions (Mallat Z, 2001). Serum values of IL-18 have shown to be positively correlated with carotid intima media thickness (Korshunov VA 2006).

3.3.4.2 Interleukin 6 (IL-6)

IL-6 is a 26-kDa single chain glycoprotein, produced by many cell types including activated monocytes/macrophages and endothelial cells, as well as by adipose tissue. IL-6 is a pro-inflammatory cytokine that also has pro-atherogenic properties. As with IL-18, IL-6 is produced by different cell types in the atherosclerotic plaque where it amplifies the inflammatory cascade and is also a pro-coagulant cytokine (Kerr R, 2001).

3.3.4.3 Myeloperoxidase (MPO)

Another cytokine that has been linked to atherosclerosis is Myeloperoxidase (MPO). MPO is an enzyme linked to both inflammation and oxidative stress. Myeloperoxidase (MPO), a member of the heme peroxidase superfamily, is a leukocyte-derived enzyme, and is secreted on leukocyte activation and degranulation (Nicholls SJ, 2005). In atherogenesis, MPO is involved in the oxidation process of LDL and thereby promoting foam cell formation in the vascular wall.

3.3.4.4 Transforming Growth Factor-b1 (TGF-b1)

Stable atherosclerotic carotid artery plaques show increased expression of the antiinflammatory cytokine transforming growth factor-b1 (TGF-b1) compared with unstable carotid artery plaques (Cipollone F, 2004).

3.3.4.5 Monocyte Chemoattractant Protein-1 (MCP-1)

Monocyte chemoattractant protein-1 (MCP-1) (CCL2) is the most important chemokine that regulates migration and infiltration of monocytes/macrophages. Its effects are mainly mediated through CC chemokine receptors 2 (CCR2). Endothelial Cells (ECs), monocytes, and/or SMCs express MCP-1 in response to various cytokines, growth factors, oxLDL, and CD40L (Mach F, 2001) and thus MCP-1 expression is increased in atherosclerotic lesions (Yla-Herttula S, 1991) in particular in macrophage-rich areas. MCP-1 causes chronic vascular inflammation, induces proliferation and migration of SMCs, migration of ECs, neovascularisation in plaque, oxidative stress, and thrombosis (Egashira K, 2003). Activation of the MCP-1/CCR2 pathway has also been shown to induce expression of MMPs (Yamamoto T, 2000) thus suggesting its involvement in plaque destabilisation.

3.4 Proteolysis

Proteolysis is involved in the early stages of carotid plaque development as well as in the later stages of plaque destabilization. Release of proteolytic enzymes such as matrix metalloproteinases (MMP's) and cathepsin cystein proteases (CCP's) is an important cause of cap erosion, resulting in cap rupture and thus acute neurologic events. The presence or activity of proteolytic enzymes within a plaque is not necessarily associated with instability, but an imbalance between these enzymes and their inhibitors (tissue inhibitors of metalloproteinases (TIMP's)) can lead to matrix degradation and plaque destabilization.

3.4.1 Matrix Metallo-Proteinases (MMP's)

Matrix metalloproteinases (MMPs) belong to a family of multi-domain zinc-dependent endopeptidases that promote degradation of all protein and proteoglycan-core-protein components of the extracellular matrix (ECM). Based on domain organization and substrate specificities, MMPs are grouped into collagenases (MMP1, 8, 13), gelatinases (MMP2 and MMP9), stromelysins (MMP3, 10, and 11), matrilysins (MMP7), metalloelastases (MMP12), and membrane-type (MT)-MMPs (Galis ZS, 2002). In unstable plaques there is a local increase in active MMP-9 concentration (Loftus IM, 2000). In one study, transcript levels of MMP-1 and MMP-12 were found to be higher in patients with amaurosis fugax compared to asymptomatic patients (Morgan AR, 2004). In carotid artery disease, MMP-2 and MMP-9 levels were determined in a group of 27 symptomatic and 13 asymptomatic patients undergoing carotid endarterectomy. The symptomatic group exhibited higher serum levels of MMP-2 and MMP-9. In addition MMP-9 was strongly associated with the presence of macrophages in the plaque (Alvarez B, 2004).

3.4.2 Cathepsins

Cathepsins are proteases which are distinguished by their structure, catalytic mechanism, and which proteins they cleave. Cathepsins appear to play a significant role in immune responses. Most of the members become activated at the low pH found in lysosomes. Due to altered expression and proteolytic activity (e.g. unbalanced amount between proteases and their endogenous inhibitors) and localisation (e.g. increased secretion outside the cells), deregulated cathepsins activity is thought to be a cause or contributing factor in atherosclerosis.

Cathepsin activity has been shown to either directly activate or inhibit certain cytokines. playing an important role in inflammatory responses and consequently in innate immunity. Neutrophil proteases, such as cathepsin G or proteinase-3, enhance the activity of interleukin (IL)-8, a strong neutrophil chemoattractant, activator and proinflammatory cytokine. Neutrophil serine proteases, like cathepsin G, are able to activate the proinflammatory cytokines IL-1beta and TNF-alpha and various receptors, such as epidermal growth factor receptor and protease-activated receptors (Meyer-Hoffert U. 2009). Neutrophil serine proteases, as well as cathepsins B and L, may therefore be important regulators of the inflammatory innate immune responses. The effector functions of immune cells (e.g. neutrophils) depend on the activation of granule-localised serine proteases such as cathepsin G and proteinase-3 (Colbert JD et al. 2009) These enzymes are synthesised as inactive zymogens and are activated by the aminodipeptidase cathepsin C (also known as DPPI), which removes two N-terminal residues Collagen turnover is mediated by both matrix metallo-proteinases (MMPs) and cathepsins. MMPs have been considered to be the proteases of paramount importance in the atherosclerotic plaques however other proteases such as Cathepsin K have been noted to be equally important. Cathepsin K is a lysosomal protease predominantly secreted by activated macrophages and osteoclasts. Cathepsin K has been identified in atherosclerotic plaques, and in differentiated macrophages such as epithelioid cells and multinucleated giant cells in soft tissues. Moreover, disruption of the cathepsin K gene reduces atherosclerosis progression suggesting the proteolytic activity of cathepsin K to be important for the pathogenesis of atherosclerosis (Lutgens E, 2006)(Liu J, 2004).

Cathepsin L which is a cysteine protease activated by legumain has been shown to be involved involved in death of macrophages, necrotic core formation and development of atherosclerotic plaque instability. Levels of legumain were found to be twice as high in regions of unstable carotid atherosclerotic plaques (Mattock KL, 2010)(Li J, 2009).

3.5 Angiogenesis

Microvessels are present in the normal arterial adventitia, where they supply the vessel wall with oxygen and nutrients. In atherosclerotic plaques, the formation of microvessels has been recognized as a contributing factor to plaque destabilization and rupture. In carotid artery plaques, microvessel content is related to intra-plaque haemorrhage, plaque vulnerability and symptomatology (Mofidi R, 2001).

The mechanisms underlying plaque angiogenesis are thought to be driven by hypoxia, reactive oxygen species and inflammation (Sluimer JC, 2008). Extravasation of inflammatory cells and red blood cells through microvessel leakage are important contributors to plaque progression resulting in a high risk of intraplaque haemorrhage. Damage to fragile blood vessels causes adhesion of various cell types resulting in inflammatory and proteolytic activity.

The balance between pro- and anti-angiogenic factors determines initiation of neoangiogenesis and evaluation of these pro- or anti-angiogenic stimuli may be helpful in identifying vulnerable atherosclerotic plaques.

3.5.1 Placental Growth Factor (PIGF)

Placental growth factor (PIGF) represents another important candidate biomarker of plaque instability. PIGF, a member of the cysteine-knot family of growth factors, is a \approx 50-kDa angiogenic protein, demonstrating an \approx 40% amino acid sequence similarity to vascular endothelial growth factor (VEGF). PIGF was initially discovered in the placenta, which represents a primary source of its synthesis; further it is expressed in the heart, lungs and thyroid (Iyer S, 2002) and was found to be upregulated within early and advanced atherosclerotic lesions (Luttun A, 2002). Besides its physiological functions during pregnancy, PIGF also possesses potent pro-atherogenic properties such as proliferation and migration of ECs and smooth muscle cells (SMCs), chemotactic recruitment of circulating monocytes and macrophages into atherosclerotic lesions, and upregulation of several cytokines such as, e.g., TNF- α (Autiero M, 2003).

3.5.2 Platelet factor 4 (PF4)

Platelet factor 4 (PF4), a chemokine released by activated platelets, is an example of a proangiogenic factor. PF4 levels in carotid plaques correlated between lesion severity and symptomatology (Pitsilos S, 2003).

3.5.3 Angiopoietin 1 (Ang-1) and Angiopoietin 2 (Ang-2)

Whereas Ang-1 is known to stabilize vessels by maximizing interactions between endothelial cells and their surrounding, Ang-2 leads to loosening of these interactions. Vulnerable

plaques with high vessel density have higher Ang-2 expression, making them more susceptible to intra-plaque haemorrhage (Post S, 2008)

3.6 <u>Hypoxia</u>

Hypoxia is one of the mechanisms involved in the regulation of neo-angiogenesis in physiological and pathological conditions including atherosclerosis. In atherosclerotic plaques, hypoxia can occur due to diminished oxygen diffusion capacity through calcified and thickened vessel walls or higher oxygen consumption due to activated immune cells. The exact role of hypoxia in the progression of atherosclerosis is still unknown. Besides angiogenetic influences, hypoxia may also be pro-inflammatory and anti-fibrotic. In the clinical setting, there are correlations found between hypoxia in carotid artery plaques and the presence of macrophages, angiogenesis and thrombus formation (Sluimer JC, 2004). In vitro studies in macrophages and other immune cells have shown that hypoxia increases the production of cytokines and MMP's.

3.7 Apoptosis

Apoptosis plays an important role in the initiation and progression of atherosclerosis and has been recognized as a feature of advanced human atherosclerotic plaques. The advanced atherosclerotic plaque contains a necrotic core of dead cells and debris. Both smooth muscle cells and inflammatory cells die because of the process of programmed cell death or apoptosis.

The significance of apoptosis can have different patho-physiological outcomes. On one side, apoptosis of inflammatory cells may slow down the inflammatory reaction because the

Introduction

number of cytokine producing immune cells is decreasing. This way, the unstable cellularrich plaque can change into a more stable hypo-cellular plaque with possibly less collagen breakdown. Alternatively, accumulation of dead cells results in enlargement of the necrotic core of the plaque. In addition, the programmed death of vascular smooth muscle cells results in weakening of the fibrous cap creating an unstable plaque that is prone to plaque rupture. The combination of an increased necrotic core and a thin fibrous cap is an important determinant in plaque instability.

Annexin 5 is a biological marker of apoptosis that has been studied in atherosclerotic lesions. Exogeneous radiolabelled annexin 5 was detected in symptomatic carotid artery plaques (Kietselaer BL, 2004). Whether apoptosis is an ongoing process in development of an atherosclerotic plaque or whether it is associated with the acute complications of atherosclerosis through plaque rupture remains to be determined.

3.8 Thrombosis

Thrombomodulatory factors have been implicated in carotid plaque instability. Unstable carotid artery plaques express a wide array of thrombomodulatory factors such as tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), plasminogen activator inhibitor-1 (PAI-1), tissue factor (TF), tissue factor pathway inhibitor (TFPI), and thrombomodulin (TM). Expression of thrombomodulatory factors is higher in unstable plaques compared to stable plaques in the acute stage (Sayed S, 2009).

In a large series of carotid endarterectomy specimens, thrombotic activity was seen in 74% and 35% of patients with ischemic stroke and TIA's respectively, and in only 14% of asymptomatic patients. In stroke patients, thrombotic activity was seen until several months after the first cerebrovascular event (Spagnoli LG, 2004). These findings suggest that

thrombotic activity plays a crucial role in plaque rupture and the pathogenesis of stroke. In addition, patients with thrombotically active plaques stay at risk for future neurological events caused by micro-emboli from the thrombotically active plaque.

3.9 Calcification

Vascular calcification is an important manifestation of atherosclerosis, which is tightly regulated by promoters and inhibitors. Many key regulators of bone formation and bone structural proteins are expressed in atherosclerotic plaques (Boström K, 1993);(Shanahan CM, 1994).

Approximately 15% of carotid artery plaques contain calcifications, but the influence of calcification in plaque stability is controversial. In carotid artery stenosis, the presence of calcification in the plaque has shown to be associated with fewer symptoms of stroke and transient ischemic attacks (Hunt JL, 2002). It has therefore been suggested that calcification in carotid artery plaques may be a plaque-stabilizing factor and protective of acute neurologic events. Serum osteopontin (OPN) levels have been shown to correlate with carotid artery intima thickness (Kurata M, 2006). In one study, osteoprotegerin (OPG) and OPN levels in patients with stable and unstable carotid artery stenosis and healthy subjects were compared (Kadoglou NP, 2008). Symptomatic patients had higher OPG and OPN levels compared to asymptomatic patients and healthy subjects.

Chapter 4. Ultrasound Imaging the 'Unstable' Carotid

Atherosclerotic Plaque Contents

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<u>Chapter 4. Ultrasound Imaging the 'Unstable' Carotid</u> Atherosclerotic Plaque

4.1 Introduction

Historically, the burden of atherosclerosis was determined by angiographic measurement of stenosis and luminal diameter (ESCT Triallists, 1991);(NASCET Collaborators, 1991). However, it has become clear that plaque rupture and subsequent embolisation of plaque particles as well as thrombosis, rather than stenosis, precipitates most acute ischemic events. Plaques at risk of rupture and thrombosis have been defined as vulnerable plaques or 'unstable' plaques. Unstable plaques are histopathologically characterised by a combination of active inflammation, large lipid core, thin fibrous cap, intraplaque haemorrhage, and neovascularisation (Alsheikh-Ali AA, 2010);(Naghavi M, 2003);(Fleiner M, 2004);(Staub D, 2010).

In the last decades, major advances have been made in the field of non-invasive imaging of vessel anatomy, arterial wall morphology, plaque composition, and metabolic processes, allowing non-invasive evaluation of atherosclerotic plaque vulnerability. High-resolution accurately depict a flow limiting stenosis in the carotid arteries (Wardlaw JM, 2006). Ultrasound in the clinical setting has a superior temporal and spatial resolution over other imaging modalities. However, transcutaneous ultrasound is limited to the evaluation of the superficial vascular system. Multiple ultrasound parameters that give an indication of plaque characteristics and composition have been investigated and summarised in Table 4.1. The parameters, echolucency, heterogeneity (combined echolucent and echogenic plaque), and

border irregularity, have been studied to identify their respective histological substrate. A summary of studies on ultrasound and their outcomes is displayed in Table 4.2 and Table 4.3.

Vasa vasorum derived intraplaque neovascularisation is an important feature in plaque development. The vasa vasorum are functional end arteries, present in the vessel wall of large arteries, supplying the vessel wall with nutrients and removing waste products, thus sustaining plaque inflammation and growth. The neovascularisation are known to be of poor structural integrity, which results in an increased risk for intraplaque haemorrhage and rupture (Moreno PR, 2004)(Doyle B, 2007).

Histological Finding	<u>Ultrasound Imaging</u>
Ulceration	Irregular Border
Lipid Core	Echolucent, Homogenous
Intraplaque Haemorrhage	Echolucent, Heterogenous
Fibrous Plaque	Echogenic, Homogenous
Calcification	Echogenic, Heterogenic
Thrombus	Echolucent
Inflammation	Echolucent
Neovascularisation	Contrast Enhancement

Table 4.1. Histological findings and the related findings with ultrasound imaging

Author	Number of	Number in	Ultrasound	Histological	Correlation
	Patients	Analysis	Assessment	Assessment	Correlation
El- Barghouty 1996	52	52 arteries	Grayscale Median	% Fibrous Tissue	$ \rho = 0.412 (P = 0.0003) $
				% lipid and hemorrhagic composition	$\rho = -0.352 (P = 0.0381)$
Grønholdt 1998	58	58 arteries	Grayscale median	% fibrous tissue	r = 0.29 (P < 0.03)
				% lipid content	r = -0.30 (P < 0.02)
Tegos 2000	67	71 plaques	Grayscale median	Necrotic core size	r = 0.1 (P = 0.37)
Aly 2000	17	17 arteries	Grayscale mean	% fibro-calcified	$\rho = 0.80 (P = 0.002)$
Grønholdt 2001	38	38 plaques	Grayscale median	% plaque lipid	r = -0.31 (P = 0.06)
				% plaque haemorrhage	r = -0.31 (P = 0.07)
				% plaque calcification	r = 0.30 (P = 0.07)
				% plaque fibrous tissue	r = 0.28 (P = 0.09)
Grønholdt 2002	106	106 arteries	Grayscale median	Macrophage density	r = -0.31 (P = 0.002)
			Grayscale mean	Macrophage density	r = -0.29 (P = 0.004)
Ciulla 2002	19	19 arteries	Grayscale mean	% fibrous tissue	r = 0.80 (P < 0.0001)
			Broad band	% fibrous tissue	r = 0.83 (P < 0.001)
			Skewness	% fibrous tissue	r = 0.79 (P < 0.0001)
			Kurtosis	% fibrous tissue	r = 0.72 (P < 0.0006)
Puato 2003	88	26 arteries	Grayscale mean	Smooth muscle cells	r = 0.449 (P = 0.021)
				Macrophages	r = 0.415 (P = 0.035)
				SMC/(SMC+Mac rophages)	r = 0.611 (P < 0.001)
Lal 2006	42	45 arteries	PDA % blood	% blood	$\rho = 0.60 (P = 0.0001)$
			PDA % lipid	% lipid	$\rho = 0.82 (P = 0.0001)$
			PDA % calcium	% calcium	ρ = 0.82 (P < 0.0001)

			PDA % fibromuscular	% fibromuscular	$\rho = 0.49 (P = 0.007)$
			Lipid core size	Lipid core size	$\rho = 0.58 (P = 0.031)$
			Least distance to	Least distance to	$\rho = 0.60 (P =$
			lumen	lumen	0.021)
Shah 2007	15	17 arteries	Contrast	CD 31	$\rho = 0.68 (P =$
	10	17 urterres	enhancement	00 51	0.002)
				CD 43, von	
				Willebrand factor	$\rho = 0.50 (NS)$
				or haemosiderin	

Table 4.2. Summary of the studies reporting the correlation between ultrasound and

histology

	Patients					
Author	in analysis	Number in analysis	Ultrasound assessment	Histological Finding	Sensitivity	Specificity
Widder 1990	161	165 arteries	Irregular plaque border	Ulceration	75% (12/16)	64% (58/91)
			Echolucent plaque	Intraplaque haemorrhage	34% (33/96)	36% (25/69)
			Echolucent plaque	Intraplaque haemorrhage or atheromatous debris	51% (65/12)	68% (25/37)
Comerota 1990	109	126 arteries	Ulceration	Ulceration	47% (36/76)	86% (43/50)
Feeley 1991	51	51 arteries	Gray-Weale classification	>80% fibrous content	94%	67%
Hatsukami 1994	24	24	Heterogenic and echolucent	Haemorrhage	53% (18/34)	76% (47/62)
				Foam cells	50% (23/46)	80% (40/50)
				Necrotic core	43% (26/60)	63% (29/46)
				Cholesterol clefts	40% (17/42)	70% (38/54)
				Speckled calcium	51% (22/43)	79% (42/53)
				Dense calcium	39% (25/64)	75% (24/32)
ECPSG 1995	270	119 arteries	Irregular plaque border	Ulceration	47% (27/57)	63% (39/62)
Sitzer 1996	43	39 arteries	Ulceration	Ulceration	33% (6/18)	76% (16/21)
			Ulceration or irregular border	Ulceration	94% (17/18)	33% (7/21)
			Heterogenic plaque	Ulceration	61% (11/18)	52% (11/21)
			Calcification	Ulceration	39% (7/18)	62% (13/21)
Kardoulas 1996	36	36 arteries	Irregular plague border	Ulceration	64% (9/14)	68% (15/22)
Kagawa 1996	64	48 arteries	Irregular plaque border	Irregular plaque border	97% (36/37)	81% (9/11)
Noritomi 1997	15	15 plaques	Thrombus	Thrombus	90% (9/10)	80% (4/5)
AbuRahma 1998	135	111 arteries	Irregular plaque border	Intraplaque haemorrhage	81% (57/70)	85% (35/41)
			Heterogenic plaque	Intraplaque haemorrhage	76% (53/70)	85% (35/41)
Schulte- Altedornebur g 2000	44	46	Echolucency, heterogeneity, and border regularity in 7 grades	Cholesterol <1%	40%	92%
				Fatty necrosis <1%	63%	87%

I		1 1				1
				Fibrosis >33%	50%	97%
				Thrombosis <10%	35%	89%
				Calcium > 19% < 33%	38%	92%
				Cholesterol >10%	77%	66%
				Haemorrhage >10%	76%	67%
				Fibrosis <3%	71%	68%
				Calcification >33%	68%	94%
Kawasaki 2001	12	12	Backscatter index	Thrombus	80%	91%
				Lipid pool or intimal hyperplasia	84%	78%
				Fibrotic tissue	80%	85%
				Mixed lesions	85%	87%
				Calcification s	89%	91%
Denzel 2003	15	15 arteries	GSM <35	Soft plaque	40% (2/5)	70% (7/10)
			GSM 35-65	Combined plaque	60% (3/5)	80% (8/10)
			GSM >65	Hard plaque	40% (2/5)	70% (7/10)
Sztajzel 2005	28	31 arteries	Heterogenic plaque	Thin fibrous cap	77% (17/22)	22% (2/9)
			Low GSM values dominant in whole plaque	Lipid core at luminal surface	74% (14/19)	17% (2/12)
				Thin fibrous cap	45% (10/22)	67% (6/9)
			Low GSM values dominant at plaque surface	Lipid core at luminal surface	53% (10/19)	75% (9/12)
				Thin fibrous cap	73% (16/22)	67% (6/9)
				Lipid core at luminal surface	84% (16/19)	75% (9/12)
Saba 2007	237	103 arteries	Ulceration	Ulceration	38%	92%
Watanabe 2008	57	54 arteries	Soft plaque	At-risk plaque	75% (18/24)	63% (19/30)

Table 4.3 Summary of the studies reporting the sensitivity and specificity of ultrasound for plaque characterization

4.2 Border Regularity

Of those studies listed in the table, eight investigated the detection of irregularities in the luminal border of the plaque and detection of ulceration. Ulceration of a plaque exposes the thrombogenic interior of the plaque to the lumen and thus may precipitate clinical events. The European carotid plaque study group (1995) used B-mode ultrasound to detect plaque ulcerations with a sensitivity of 47% and a specificity of 63%. Five studies (Widder B, 1990)(Comerota AJ, 1990)(Sitzer M, 1996)(Kardoulas DG, 1996)(Saba L, 2007) investigated the addition of Doppler techniques to B-mode ultrasound for the detection of ulcerations. The results showed a large variance with a slight improvement in mean sensitivity (60%, range 38%-94%) and specificity (74%, range 33%-92%). Kagawa et al.(1996) demonstrated that border irregularities on B-mode ultrasound, rather than just ulceration, could be detected with a high sensitivity (97%) and specificity (81%)

Two studies (Sitzer M, 1996)(Aburahma AF, 1998) compared border regularity with the plaque composition. The presence of an irregular border on ultrasound was shown to predict an intraplaque haemorrhage with a sensitivity of 81% and a specificity of 85%, while conversely, heterogeneity and calcification on ultrasound had a poor predictive value for the presence of ulceration.

4.3 <u>Echolucency</u>

Several studies have reported on the relationship between echolucency and plaque composition. It has been shown that echolucent plaques contain significantly higher amounts of "soft" constituents (lipids, intraplaque haemorrhage), while echogenic plaques contain more fibrous tissue and calcifications (Kardoulas DG, 1996)(Grønholdt ML, 1997)(Droste DW, 1997)(Grønholdt ML, 2002).

To improve the detection of plaque components, quantification of echolucency has been developed by gray scale median (GSM), pixel distribution analysis, and integrated backscatter analysis. GSM measurements are made from digitized ultrasound images normalized to the GSM of blood (GSM 0-5) and adventitia (GSM 180-200). Subsequently, the echolucency in the region of interest is expressed in a 256 gray-tone range where 0 is black and 256 is white. A strong correlation was found between the GSM and the amount of fibro-calcified tissue in the plaque. However, no correlation was found between the GSM and other tissue components (El-Barghouty NM, 1996)(Grønholdt ML, 1998)(Aly S, 2000)(Tegos TJ, 2000)(Ciulla M, 2002). The use of GSM resulted in low sensitivities (range 40%-60%) for the detection of soft (GSM <35), combined (GSM 35-65), and calcified (GSM >65) plaques (Denzel C, 2003).

4.4 <u>Heterogeneity</u>

The division of plaques in homogeneous and heterogeneous showed that heterogeneous plaques contain significantly more calcifications, while no difference in the amount of "soft" tissues was found (Grønholdt ML, 1997);(Sztajzel R, 2005). The evaluation of plaque heterogeneity improved the sensitivity (76%) and specificity (85%) for the detection of

intraplaque haemorrhage over Echolucency (Aburahma AF, 1998). The evaluation of plaque heterogeneity predicted fibrous cap thickness ($<80 \mu m$) and the location of the lipid core with sensitivities of 77% and 74% and specificities of 22% and 17%, respectively (Sztajzel R, 2005).

4.5 Combined Echolucency and Heterogeneity Scales

A number of scales combining echolucency and heterogeneity have been developed with the Gray-Weale scale as the most consistently used (Gray-Weale AC, 1988). The use of these combined scores resulted in varying sensitivities ranging from 39% to 94% and specificities ranging from 57% to 80% for the detection of various components (Kardoulad DG, 1996);(Feeley TM, 1991);(Hatsukami TS, 1994). The use of a more elaborate scale with 7 grades combining echolucency, heterogeneity, and the surface regularity showed only a slight improvement (Schulte-Altedorneburg G, 2000). It has been proposed that the lack of accuracy is due to the large number of components investigated and the number of "soft" components identified by echolucency. The use of a score combining all "soft" components for the detection of "at-risk" plaques was suggested as a strategy to increase the diagnostic accuracy (Schulte-Altedorneburg G, 2000). This strategy resulted in a sensitivity of 75% and a specificity of 63% for the detection of at-risk plaques (histologically defined as <70% fibrous tissue or calcification, with a lipid core or intraplaque haemorrhage)(Watanabe Y, 2008).

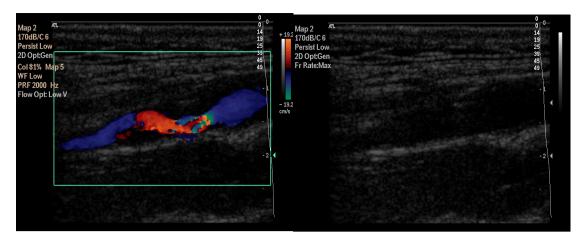


Figure 4.1 B-mode imaging (BMI) including the use of colour doppler

4.6 Vasa Vasorum

Ultrasound contrast agents have paved the way for plaque characterization with ultrasound. Ultrasound contrast agents consist of acoustically active microbubbles with a diameter of 3 to 4 μ m. When exposed to an ultrasound field, these microbubbles expand and contract rhythmically, producing strong backscattered signals that can be detected by conventional ultrasound systems. Furthermore, microbubbles also produce a specific nonlinear signal that helps differentiate them from surrounding tissues (DeMaria AN, 2006);(Kaufmann BA, 2007). Because ultrasound contrast agents are pure intravascular tracers, contrast-enhanced ultrasonography allows for the assessment of the amount of blood contained in the microvasculature within the region of interest. Contrast-enhanced ultrasound uniquely visualizes the intraplaque microvascularisation. Ultrasound contrast agent, small enough (1-5 μ m) to pass through the capillary system. This has led to microbubbles being investigated for the visualization of the microvasculature in atherosclerotic plaques (Figure 4.2) (Schinkel AF, 2010);(Feinstein SB, 2010). Coli et al (2008) have shown that plaques with high contrast

enhancement have a significantly higher intraplaque vasa vasorum density, while in comparison, vasa vasorum density did not significantly differ between high- and low-grade stenotic plaques (stenosis >70%) or between echolucent and echogenic plaques. However, echolucent plaques had a significantly higher degree of contrast enhancement compared with echogenic plaques. Shah et al (2007) found a strong correlation between contrast enhancement and CD-31-stained vasa vasorum in the plaque, indicating angiogenesis-derived vasa vasorum, rather than pre-existing vasa vasorum. Further studies are needed to determine the diagnostic accuracy of contrast-enhanced ultrasound for the detection of intraplaque neovascularisation.

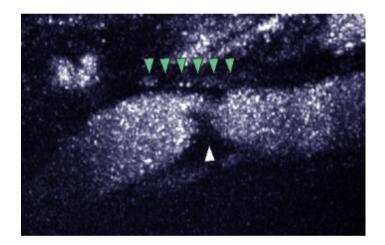


Figure 4.2 Contrast enhanced ultrasound of carotid intra-plaque neovascularisation in longitudinal section; the microbubbles are seen both within the internal carotid artery lumen and within the carotid atheromatous plaque (green arrows). Furthermore, there is a region of plaque which is not perfused by microbubble contrast (white arrow). (Shalhoub J, 2010)

4.7 Conclusion

Ultrasound can provide information about plaque components such as lipid core, intraplaque haemorrhage, inflammation, and neovascularisation that are related to plaque vulnerability. The use of ultrasound for the qualitative detection of border regularity, echolucency, and heterogeneity is promising; however, the accuracy of ultrasound in detecting individual plaque components and characteristics still shows large variability.

The quantification of echolucency resulted in a clear improvement for the detection of various plaque components. The use of microbubble contrast may improve the detection of ulcerations by enhancing the contrast between the lumen and the vessel wall. The use of microbubble contrast shows a significant correlation with neovascularisation and might be an interesting new marker for plaque vulnerability.

Chapter 5. Transcranial Doppler Monitoring of the Middle

Cerebral Artery in Carotid Artery Disease

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Chapter 5. Transcranial Doppler Monitoring of the Middle

Cerebral Artery in Carotid Artery Disease

5.1 Introduction

Transcranial Doppler (TCD) is a sensitive technique for real-time detection of spontaneous embolisation (SE). SE have been detected in a number of clinical conditions: carotid artery stenosis, aortic arch plaques, atrial fibrillation, myocardial infarction, prosthetic heart valves, patent foramen ovale, valvular stenosis, during carotid surgery, surgery on open heart, stent implantation, percutaneous transluminal angioplasty and angiography, and in patients with migraine and patent foramen ovale (PFO). Patients who have detectable SE are considered as high-risk patients for stroke.

Consensus on SE detection by TCD has been established (Consensus Committee 1995). SE can be identified as short lasting (<0.01-0.03 s), unidirectional intensity increase, and intensity increase (>3 dB) within the Doppler frequency spectrum; intensity increase is focused around 1 frequency. SE appear randomly within the cardiac cycle and produce a "whistle," "chirping," or "clicking" sound when passing through the sample volume.

TCD is a very convenient tool to monitor intracranial circulation. The optimal time of monitoring depends on the clinical entity. The embolic activity is highest in the first couple of hours after stroke; however, SE may be detectable days and weeks after cerebrovascular incidents which means that those patients are under higher risk for stroke (Sliwka U 1994)(Forteza AM 1996)(Demarin V 1997)(Markus HS 1994)(Van Zuilen EV 1994).

Although technological improvement in the area of SE detection has recently developed, it is still impossible to reliably distinguish the composition of emboli (particles of fat, platelet

Introduction

aggregates, or particles of atheroma). Differentiation between solid and gaseous microemboli is based on the principle that solid emboli reflect more ultrasound at higher frequency, whereas the opposite is the case for gaseous emboli. This principle is used in multi-frequency TCD instrumentation where the vessels are insonated simultaneously with 2.5 and 2.0 MHz and can be used for the differentiation between gaseous and solid emboli (Russell D 2006). A recent study has shown that there is a significant relationship between low- and high-intensity SE, indicating that many SE routinely rejected because of their low intensity are real and may predict future occurrence of high-intensity SE (Telman G 2011).



Figure 5.1 Intra-Operative TCD Monitoring during a Carotid Endarterectomy

5.2 Symptomatic Carotid Disease

Carotid artery stenosis is a well-known source of cerebral SE (Sliwka U 1994);(Forteza AM 1996);(Ries S 1995);(Hutchinson S 2002);(Molloy J 1999);(Vassileva E 1999). Systematic review of the literature showed that SE can be detected in 43% of patients with symptomatic carotid stenosis; presence of one SE indicated an increased risk of future events (OR 7.5, 95% confidence interval (CI): 3.6–15.4, P<0.0001) for symptomatic patients (Ritter MA 2008). A meta-analysis of the literature revealed that SE are most frequent in large artery disease, less frequent in cardioembolic stroke, and infrequent in lacunar stroke. For symptomatic carotid stenosis, SE predicted stroke alone (OR, 9.57; P=0.02) and stroke/TIA (OR, 6.36; P<0.0001). In acute stroke, SE predicted stroke alone (OR, 2.44;) and stroke/TIA (OR, 3.71; P=0.02). A high frequency of SE immediately after carotid endarterectomy predicted stroke alone (OR, 24.54; P<0.00001) and stroke/TIA (OR, 32.04; P<0.00001). The meta-analysis suggests that SE predict stroke risk in acute stroke, symptomatic carotid stenosis, and postoperatively after carotid endarterectomy (King A, 2009).

After carotid endarterectomy, SE disappear or the frequency is significantly lower (Siebler M 1993);(Van Zuilen EV, 1995). However, patients with clinically significant postoperative microembolism have an approximately 15 times higher risk of ipsilateral stroke or TIA (Abbott AL, 2007). Administration of dual antiplatelet therapy (clopidogrel 75 mg plus aspirin 75 mg) prior to CEA reduces postoperative embolisation and thromboembolic events (Sharpe RY, 2010).

5.3 <u>The Value of Transcranial Doppler Monitoring in Asymptomatic</u> <u>Patients – A Systematic Review and Meta-Analysis</u>

5.3.1 Background

Asymptomatic carotid artery disease is known to be more benign and the benefit in intervening in patients with asymptomatic carotid stenosis in preventing future strokes is much less pronounced. Two large randomised controlled trials, the Asymptomatic Carotid Atherosclerosis Study (Executive Committee ACAS, 1995) and the Asymptomatic Carotid Surgery Trial (Halliday A, 2004) evaluated the benefit of carotid endarterectomy in preventing death or stroke and showed that the 5 year stroke risk was 5.1% and 6.4% with CEA compared to 11% and 11.8% with only best medical therapy respectively. More recent studies (Marquardt L, 2010);(Naylor AR, 2011) have shown that with more advanced medical therapy and aggressive treatment of cardiovascular risk factors the annual risk of stroke in patients with asymptomatic carotid artery stenosis treated conservatively is less than 1%.

Asymptomatic extra-cranial carotid artery disease does however remain a major cause of strokes, with only 15% of all strokes preceded by a TIA. Therefore a high risk subgroup of asymptomatic patients with carotid artery stenosis exists and identifying these patients for carotid endarterectomy or stenting remains an important research goal.

Several studies have been performed looking at the value of monitoring the middle cerebral artery using Transcranial Doppler to detect spontaneous embolisation. Detection of spontaneous embolisation could be used to predict the stroke risk in patients with

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asymptomatic carotid disease and thus identifying a high risk group that would benefit from carotid endarterectomy over best medical therapy alone.

The aim of this meta-analysis and systematic review is to determine if patients with asymptomatic carotid artery stenosis had a higher risk of ipsilateral ischaemic event if they were found to have spontaneous embolisation during pre-operative TCD monitoring.

5.3.2 Methods

An electronic search was performed by two independent researchers using PubMed, Medline and Embase databases between Jan 1 1990 and May 1 2011. All articles in English that reported results in humans were included. Search terms including the use of truncated terms were (TCD OR Transcranial Doppler OR ultrasound OR ultrasonography) AND (Spontaneous Embolisation OR embolic signals OR emboli) AND Asymptomatic. Abstracts were reviewed and full versions were obtained for articles fulfilling the criteria. References were also searched from papers meeting the inclusion criteria. Data from original prospective cohort studies have been included here. See Figure 5.2 for PRISMA flow chart. A metaanalysis was performed using a generic inverse variance fixed-effects model. Identification 212 citations identified 8 Additional records through database identified through searches with limits reference sourcing applied 93 duplications deleted Screening 109 records excluded 127 records screened 18 full text articles assessed for eligibility Eligibility 12 studies included in qualitative analysis 8 studies included in Included systematic review

Figure 5.2 PRISMA chart for systematic review

5.3.3 Results

A total of 212 citations were retrieved from the database and further 8 from source referencing. 93 citations were found to be duplicates and excluded.127 records were then screened by the single investigator (MS). 109 records were deleted after not meeting the initial inclusion criteria. This left 18 records for full inspection. After reviewing the selected titles, abstracts and references for only publications relating to TCD monitoring of the middle cerebral artery in patients with asymptomatic carotid artery stenosis a total of 8 papers were selected which have been described here in chronological order.

In 1995 Siebler et al reported on 64 patients with high grade (70-90%) clinically asymptomatic stenosis. Transcranial Doppler monitoring was performed for 1 hour at each patient visit, and independently analysed by a blinded observer using strict criteria. In 48 patients that remained in the study 8 were found to have evidence of spontaneous embolisation and five of these patients went on to develop ipsilateral ischaemic symptoms. A significant association was found between evidence of spontaneous embolisation and occurrence of ischaemic events. (p=0.005, OR 31; 95% CI 3-302).

In 1999 Molloy et al. examined 42 asymptomatic patients prospectively using TCD monitoring for 1 hour. All subjects were followed up until the occurrence of a stroke, TIA, death, or end of the study period. Mean follow up was 258 days (range 2 to 774) days. 12 (28.6%) of the 42 patients were found to have spontaneous embolisation. Spontaneous embolisation was found to occur more frequently in patients with a 71-90% stenosis. No patients with a stenosis >90% had spontaneous embolisation. Two patients of 42 suffered

ipsilateral cerebral ischaemic events (1 stroke, 1 TIA). Both patients who suffered an ischaemic event were found to be spontaneously embolising during the initial TCD recording. A significant association was found between spontaneous embolisation and subsequent stroke and TIA risk in the asymptomatic group using a Kaplan-Meier nonparametric survival analysis (P=0.007).

In 2002 Orlandi et al. reported on 21 asymptomatic patients with >70% stenosis. Each study participant underwent 60 minutes of TCD monitoring of the middle cerebral artery to detect spontaneous embolisation according to international consensus guidelines. Monitoring was repeated for each participant at 6, 12, 18 and 24 months together with clinical examination to detect symptoms of a cerebral ischaemic event including Amaurosis Fugax, TIA or a stroke. Six patients (28.6%) had evidence of spontaneous embolisation during at least one period of monitoring. Five of these six patients became symptomatic after the positive embolic signals were found (2 TIAs, 3 strokes). In three cases spontaneous embolisation continued to be detected after symptom onset.

The results of the Asymptomatic Stenosis Embolus Detection (ASED) Study were published in 2005. ASED was an Australian multicentre prospective observational cohort study that evaluated the risk of spontaneous embolisation in asymptomatic patients and subsequent risk of stroke or TIA. Between May 1996 and December 2000 202 patients with 240 asymptomatic arteries were found to fit the inclusion criteria and recruited into the study. Six of 60 arteries scanned were positive for spontaneous embolisation and had an ipsilateral carotid stroke/TIA compared with 12 of 171 arteries negative for spontaneous embolisation (OR 1.47; 95% CI 0.43-4.48; p=0.624). As the results from this study were not significant, Introduction

this study found that the detection of spontaneous embolisation in asymptomatic patients did not aid in identifying a high risk group of patients who would benefit from intervention.

In 2005 Spence et al. published results from a study including 319 asymptomatic patients of whom 210 were available for analysis at 2 years (59 patients had suffered a TIA >18 months earlier and were classed as asymptomatic) for the presence of spontaneous embolisation. Transcranial Doppler monitoring was performed for 1 hour, on two occasions a week apart and on an annual basis. Spontaneous emboli were present in 32 (10%) of patients at baseline. At 1 year of follow up only 1.4% of patients who had no evidence of spontaneous embolisation at baseline had spontaneous embolisation detected compared to 34.4% of patients who had spontaneous embolisation detected in only 1% of patients who had no evidence of spontaneous embolisation at baseline to 39.4% who were positive at baseline. (P=0.004). Among patients who were found to have spontaneous embolisation, 15.6% went on to have a stroke compared to only 1% in those patients that had no spontaneous embolisation (95% CI 4.1 - 79; P<0.00001).

In 2009 Zhang et al. looked at 62 patients with asymptomatic carotid stenosis between 50-99% evaluating the presence of spontaneous embolisation and subsequent incidence of TIA and stroke during a 1 year follow up period. Spontaneous embolisation was detected in 10/62 patients at baseline. TIA/stroke occurred more frequently in patients with spontaneous embolisation than those without spontaneous embolisation (p = 0.009).

Spence et al published results in 2010 of a study looking at 468 patients with >60% asymptomatic carotid artery stenosis who has baseline TCD measurements. Comparisons were made between patients recruited pre 2003 (199 patients) and post-2003 (269 patients) after the introduction of an aggressive medical therapy programme. The prevalence of spontaneous embolisation was 12.6% in patients before 2003 compared to 3.7% in patients after 2003 (P < .001). After adjusting for age, sex, smoking, systolic blood pressure, serum cholesterol, and cholesterol to high-density lipoprotein cholesterol ratio, the difference remained significant (P = .02). In all patients the 2 year risk of stroke was 1.8% in patients without baseline evidence of spontaneous embolisation compared to 18.4% in patients with spontaneous embolisation detected at baseline (p < 0.001)

The ACES (The Asymptomatic Carotid Emboli Study) study by Markus et al. in 2010 was a prospective observational study in patients with asymptomatic carotid stenosis of at least 70% from 26 centres worldwide. In order to detect spontaneous embolisation patients underwent two 1 hour TCD recordings from the ipsilateral middle cerebral artery at baseline and then 1 hour recording at 6, 12 and 18 months. Patients were followed up for a total of 2 years. The primary end point was ipsilateral stroke and TIA. Secondary end points included any stroke and stroke and cardiovascular death. TCD recordings were analysed by investigators blinded to clinical details. 477 patients were found to be eligible and recruited into the study after appropriate power calculations. 467 patients had a baseline TCD recoding of sufficient quality for analysis. Spontaneous embolisation was present in 77 of 467 patients at baseline. The hazard ratio for the risk of ipsilateral stroke and transient ischaemic attack from baseline to 2 years in patients with embolic signals after controlling for the presence versus absence of anti-platelet therapy was 2.39 (95% CI 1.12 -5.1; p=0.025). The hazard ratio for ipsilateral

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stroke alone at two years after adjusting for anti-platelet therapy was 5.9 (1.68-20.72; p=0.006). The absolute annual risk of stroke or TIA was 7.13% in patients with embolic signals compared to 3.04% in patients without embolic signals. The absolute annual risk of ipsilateral stroke alone was 3.62% in patients with spontaneous embolisation compared to 0.7% in those without spontaneous embolisation.

A meta-analysis of the 8 published series involving 1674 patients showed a hazards ratio for the risk of ipsilateral stroke for those with spontaneous embolisation on TCD monitoring compared to those without was 8.58 (95% CI 4.77-15.44); P<0.0001), with no heterogeneity between studies (p=0.41).

Study	Spontaneous Embolisation Detected	Spontaneous Embolisation Not Detected	
Abbott ¹²	2/60 (3.34%)	4/171 (2.3%)	
Molloy ¹⁰	1/12 (8.3%)	0/30 (0%)	
Orlandi ¹¹	3/6 (50%)	0/15 (0%)	
Siebler ⁸	1/8 (12.5%)	1/56 (1.8%)	
Spence ¹³	5/32 (15.6%)	3/287 (1%)	
Zhang ¹⁴	3/10 (30%)	1/52 (1.9%)	
Markus ¹⁶	5/77 (6.5%)	5/390 (1.3%)	
Spence ¹⁵	8/37 (21.6%)	10/431 (2.3%)	
TOTAL	28/242 (11.6%)	24/1432 (1.7%)	

Table 5.1 Risk of Ipsilateral Stroke in Patients with and Without Spontaneous Embolisation on TCD Monitoring

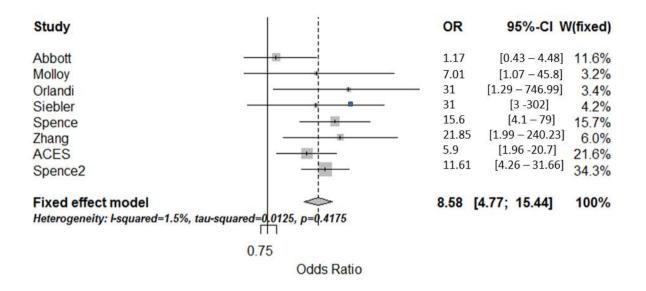


Fig 5.3 Forest Plot of Meta-Analysis Data. Results show the weight of each study as represented by the size of mark. Shift to right of centre represents size of risk (odds ratio) of ipsilateral stroke if spontaneous emboli are detected. (Salem MK, 2011)

5.3.4 Discussion

Management of asymptomatic carotid artery disease remains a controversial issue, with wide variation in practice between clinicians. There are no clear guidelines and although the landmark evidence from two large randomised control trials (ACAS, 1995); ACST 2004) suggested carotid endarterectomy significantly prevented more strokes at 5 years than best medical therapy alone. The current consensus amongst clinicians is that carotid endarterectomy for patients with asymptomatic carotid artery stenosis (>60%) should be reserved for men, with a life expectancy greater than 5 years and should only be performed in centres where the procedural risks associated with carotid endarterectomy are less than 3%.

In the eight studies described above, the definition of having spontaneous embolisation varied. In five of the studies (Molloy J, 1999);(Orlandi G, 2002);(Abbott AL, 2005);(Zhang C, 2009);(Markus HS, 2010) an artery was deemed to be spontaneously embolising if one or more embolic signals were detected during the monitoring period. In the other three studies (Siebler M, 1995);(Spence JD, 2005);(Spence JD, 2010) there had to be at least two embolic signals to be deemed positive.

Molloy et al studied both symptomatic and asymptomatic patients with extra cranial carotid artery disease. The investigators analysing the TCD recording were blinded to clinical status. A significant association was found in both symptomatic (p=0.02) and asymptomatic (p=0.007) patients who had spontaneous embolisation and who subsequently went on to have cerebral ischaemic events. In the small cohort of asymptomatic patients, a key finding was that in patients with >90% stenosis there was no detection of spontaneous embolisation suggesting that a tight stenosis conferred some protection against future ischaemic events in asymptomatic patients.

In the study by Orlandi et al. 6 (28.6%) patients were found to be spontaneously embolising during at least one TCD monitoring. 5 of these 6 patients went on to develop cerebral ischaemic symptoms with all developing symptoms after the detection of spontaneous embolisation. This group of patients were followed up bi-annually for 2 years after initial baseline recordings were taken. Symptoms always occurred within 6 months of the detection of spontaneous embolisation. 3 of the 5 patients with spontaneous embolisation and ischaemic symptoms were found to be still spontaneously embolising in the following six month monitoring visit but not 12 months after. This suggested that a subgroup of asymptomatic patients with highly unstable plaques exist and that periods of spontaneous embolisation from carotid atherosclerotic plaques could be due to periods of acute plaque destabilisation in otherwise stable plaques, periods of which cause symptoms. The temporal trend seen in this study mirrors that seen in other studies (Siebler M, 1995) looking at spontaneous embolisation in asymptomatic patients with risks higher in the immediate period (<6 months since detection of spontaneous embolisation) and studies looking at silent spontaneous embolisation in symptomatic patients (Siebler M, 1993)(Markus HS, 1995). This lends support to the argument that asymptomatic patients with spontaneous embolisation should be treated like symptomatic patients with urgent treatment conferring greatest benefit. In the ACES study the hazard ratios between risks over a 2 year follow up and within 6 months were similar suggesting that risk was not short term.

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In the ASED study although there were more ipsilateral cerebral ischaemic events amongst asymptomatic patients who were detected to have spontaneous embolisation during TCD monitoring, there was no statistical significance seen in the results. It was felt that the study was underpowered, and more conclusive analysis could not be performed due to the low levels of detection of spontaneous embolisation, 60 out of 231 (26%) arteries monitored and the low level of cerebral ischaemic events seen (18 in 202 patients), which amounted to an annual ipsilateral stroke/TIA and stroke alone rate of 3.1% and 1.0% respectively. The overall reduction in stroke rate was attributed to a worldwide fall in stroke rate due to aggressive treatment of vascular risk factors. The study also highlighted again that very severe stenosis (>90%) served to offer some protection against spontaneous embolisation. This was a very labour intensive study which also highlighted the early difficulties in bringing TCD monitoring and analysis to the patient bedside. The study which involved biannual TCD monitoring showed a 75% sensitivity level in detecting an artery that was not spontaneously embolising when TCD monitoring was performed on >6 occasions. This suggested that arteries that were defined as negative for spontaneous embolisation could be managed conservatively as the likelihood of becoming unstable and symptomatic was small.

The effect of aggressive medical therapy including anti-platelet therapy on the detection of spontaneous embolisation was highlighted by Spence et al. study (2010), which demonstrated that asymptomatic patients with extra-cranial artery disease treated with aggressive medical therapy had a significantly lower incidence of spontaneous embolisation during TCD monitoring. Dual anti-platelet therapy using aspirin and clopidogrel has now been shown (Wong KS, 2010);(Sharpe RY, 2010) to reduce the rate of spontaneous embolisation in symptomatic pre and post operative patients. Its effect in asymptomatic patients has yet to be shown in a large trial. The ACES study was the largest multi centre study to address the issue

of the benefit of TCD monitoring in asymptomatic patients with carotid artery disease. Results were adjusted for the use of statins and anti-platelet therapy, and showed a significant association between the detection of spontaneous embolisation and subsequent risk of ipsilateral stroke or TIA. In the study by Siebler et al. (1995) 64% of all patients were on anti-platelet therapy and 80% of those with positive spontaneous embolisation who went on to have ischaemic events were on anti-platelet therapy.

The meta-analysis performed on the above studies showed a significant association between detection of spontaneous embolisation during TCD monitoring and subsequent risk of ipsilateral stroke with no heterogeneity in the results (p=0.41).

The detection of spontaneous embolisation in asymptomatic patients suggests the presence of plaque instability and identifies those patients that need urgent intervention. If a patient is not found to have spontaneous embolisation on repeated TCD monitoring (>6 occasions), this would identify a low risk group of patients which is as important at identifying as the high risk group. Patients in the low risk group are unlikely to ever spontaneously embolise from their plaque, and can be treated safely with best medical therapy and aggressive management of vascular risk factors.

The findings from this review suggest that TCD monitoring can be used as a simple and quick diagnostic tool for patients with asymptomatic carotid artery stenosis. TCD monitoring is a non-invasive, simple test that can be performed at the bed side or in the clinic. All of these prospective cohort studies have been performed using professionals trained at detecting

spontaneous embolisation during TCD monitoring according to international consensus guidelines. Ensuring vascular scientists and ultrasonographers are trained at interpreting TCD data is essential before TCD monitoring becomes a widespread clinical tool used in the decision making of patients with asymptomatic and symptomatic carotid artery disease.

The next generation of automated TCD monitoring machines have come a long way from the original designs (Van Zuilen EV, 1995), the newer systems are being trialled and once results are published on the sensitivity and specificity of these automated systems (Lipperts MG, 2009);(Cowe J, 2006) in asymptomatic patients, the use of TCD monitoring at baseline and regular review follow ups will become essential clinical practice.

Chapter 6. The Rapid Assessment and Triage of Patients with

Symptomatic Carotid Artery Disease

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<u>Chapter 6. The Assessment and Triage of Patients with</u> <u>Symptomatic Carotid Artery Disease</u>

6.1 Introduction

Evidence from a large meta-analysis suggested that the highest risk of stroke after suffering a transient ischaemic attack/minor stroke was in the first 7-14 days (Giles MF, 2007). In addition, pooled data from the ECST, NASCET and VA trials suggested that patients undergoing expedited carotid endarterectomy (CEA) gained greater benefit than patients with significant delays to surgery (Rothwell PM, 2003). Performing CEA within 2 weeks in patients with a NASCET 50-99% stenosis (ECST 70-99%) would prevent 185 ipsilateral strokes at 5 years per 1000 CEAs, while only 8 strokes would be prevented at 5 years if surgery was delayed by >12 weeks (Rothwell PM, 2004);(Naylor AR, 2007). Accordingly guidelines from the European Vascular Surgery Society (Liapis CD, 2009) and the UK National Institute for Health and Clinical Excellence (NICE, 2008) recommended that CEA should be performed within 14 days of onset of symptoms unless contra-indicated. The UK National Stroke Strategy (Department of Health, 2008) however recommended that CEA should be regarded as an emergency procedure and should be performed within 48 hours of TIA or minor stroke. However despite evidence suggesting that there is considerable benefit in intervening early, there remained concerns about the logistics of how a 48hr or 14 day target could be achieved and whether expedited CEA was associated with a significant increase in the procedural risk. Urgent CEA is still considered by many surgeons to significantly increase the procedural risk (Rockman CB, 2006);(Rerkasem K, 2009) possibly to the extent that any benefit (in terms of late stroke prevention) may be negated.

This chapter reviews the logistics of introducing a new rapid access carotid surgery service with specific regard to delays to surgery and any effect on the procedural risk.

6.2 The Pre-2008 Leicester System

In an earlier study (Brown C, 2009) our unit in Leicester had showed that a surgeon with an interest in carotid surgery could not make simple modifications to practice (use of ad-hoc cancelled theatre sessions, ad-hoc cancellation of non-urgent cases, altering the way referrals were processed) and then expect to radically reduce delays to surgery. The previous study concluded that this could only be achieved by a radical re-organisation of practice amongst stroke physicians, hospital managers and surgeons. In the past patients with symptomatic carotid disease were referred to the Vascular Surgery Unit by their family doctor, local/regional Stroke Physicians, Accident & Emergency Department or Eye Casualty. In a review of practice, it was noted that there were a multitude of reasons why delays were introduced into the system. These included; lack of patient awareness, no rapid access TIA clinic, limited single visit imaging (i.e. CT and Duplex Ultrasound), administrative referral delays, no prioritisation on waiting lists and a reluctance for surgeons to ask colleagues to take on CEA patients in order to expedite their operation.

6.3 Service Reconfiguration

In October 2008, a complete service reconfiguration took place which is summarised below.

6.3.1 Rapid Access TIA Clinic

All patients with a suspected TIA/minor stroke attend their family doctor or accident & emergency and have a referral faxed to the TIA clinic where this was rapidly triaged. Any patient with an ABCD2 score of 0-3 is scheduled to be seen in the neurovascular clinic within 7 days. However, any patient with an ABCD 2 score of 4-7 is seen either the same day or the following morning. Risk factor modification is commenced in the TIA clinic, where Duplex ultrasound and either CT or MR brain imaging was also performed. Optimum medical therapy (antiplatelet, antihypertensive, anti-arrhythmic, statin therapy etc.) is commenced in the TIA clinic. Any patient with a NASCET 50-99% stenosis is then discussed with the on-call vascular surgeon and immediately transferred to the Leicester Vascular Surgery Unit unless there are contra-indications such as severe co-morbidity, dementia or advanced malignancy. See figure 6.1.

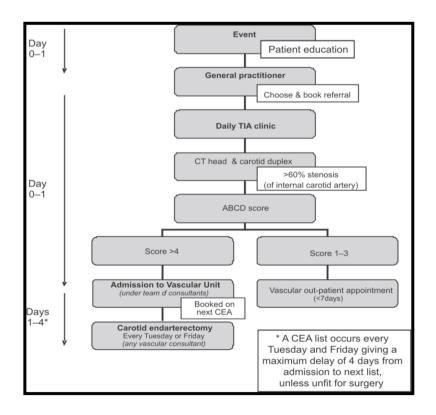


Figure 6.1 New Fast Track Symptomatic Carotid Endarterectomy Service. (Brown C, 2009)

6.3.2 Vascular Surgery Unit

Following transfer, all patients undergo a second Duplex assessment (in line with HTA guidelines (Wardlaw JM, 2006)) and also undergo 30 minutes transcranial Doppler (TCD) recording to identify those with spontaneous embolisation (i.e. those with an unstable carotid plaque). Patients with uncontrolled hypertension start treatment as soon as possible with the aim of getting the systolic BP below 160mmHg wherever possible. It is the aim of the service to only delay CEA if the hypertension does not respond to treatment and remains excessively high (>190mmHg systolic BP).

The unit also aims to offer expedited CEA to all patients who present with a minor stroke provided they fulfilled the following criteria; Rankin score 0-2, no internal carotid artery (ICA) occlusion, no evidence of haemorrhage on CT/MRI, infarction <1/3 of the MCA territory, patient not obtunded. Patients with a Rankin score \geq 3 only undergo urgent CEA if they had recurrent symptoms while in hospital and then only after discussion with the stroke physicians and anaesthetists.

The Vascular Surgery Unit maintains two half day operating lists (Tuesday/Friday) for performing expedited CEA. CEA procedures are performed by the consultant allocated to that particular list (i.e. this could be different to the consultant admitting the patient). The emergency theatre is only used if these lists are already filled. All patients receive 300mg aspirin and 40mg simvastatin in the TIA clinic, in addition to other appropriate secondary prevention therapy, and these are then continued throughout the pre-operative period, although the aspirin dose is reduced to 75mg. All patients received 75mg clopidogrel the night before surgery in addition to their regular aspirin.

6.3.3 Carotid Endarterectomy

The CEA procedure has remained essentially unchanged since 1991 (general anaesthesia, routine patching, routine shunting, systemic heparinisation (unfractionated) and distal intimal tacking sutures) (Gaunt ME, 1996) A preliminary clamp of the internal carotid artery is performed to minimise embolic events during manipulation of the carotid bifurcation if there is evidence of SE during the dissection phase. TCD monitoring is undertaken following induction of general anaesthesia using a fixed 2 MHz head probe and continued throughout the procedure. Completion angioscopy was performed with a flexible hysteroscope (Olympus 1070-48). Unit policy was to repair all intimal flaps >3 mm and aspirate any residual thrombi from the lumen.

Prior to August 2006, all patients underwent 3 hours of TCD monitoring after CEA and any patient with a high rate of embolisation was given incremental dose intravenous Dextran (Lennard M, 1999) After August 2006, all patients received 75mg clopidogrel the night before surgery (in addition to routine aspirin). Only two patients have required post operative Dextran (out of 420 patients) and routine post operative TCD monitoring has now ceased as no benefit has been shown (Sharpe RY, 2010).

Following surgery the patient is nursed in theatre recovery for three hours and then transferred back to the vascular ward, pending discharge on day 2. There are written guidelines for managing hypertension in the post-operative period (Naylor AR, 2009)

6.3.4 Procedural Complications

Thirty day outcomes include any stroke (ipsilateral and contralateral hemisphere), Myocardial Infarction (MI) or death within 30 days of the procedure. Stroke is defined as any new (or worsened) focal neurological deficit lasting for more than 24 hours. All patients received a routine neurological examination at 30 days by a consultant stroke physician. Any patient suspected of having suffered a procedural stroke underwent a CT scan, extra-cranial Duplex ultrasound examination and intracranial transcranial Doppler ultrasound assessment as soon as possible after onset of symptoms.

A myocardial infarction is defined by the presence of two of the following three criteria; specific cardiac enzymes (Troponin I) more than twice the upper limit of normal; history of chest discomfort for at least 30 minutes; or the development of specific abnormalities (e.g. Q waves, ST elevation) on a 12 lead electrocardiograph.

6.3.5 Data Collection

Data is collected prospectively by a single investigator (MS) through 'direct face to face' contact with patients, and through analysis of documentation in clinical case notes and in discussion with the admitting consultant vascular surgeon. Statistical analysis was performed using SPSS (v16).

6.4 Results of Service Change

One hundred and nine symptomatic patients from Leicestershire that underwent a CEA and were referred via the TIA clinic were included in the analysis. Twenty seven presented with a minor stroke (five with residual neurological deficit), 74 with a TIA, and eight with Amaurosis Fugax (Figure 6.2). Figure 6.2 also details the median times from onset of the index event to surgery and from the date of referral to surgery. Overall the median delay from index symptom to CEA was 9 days (95% CI 7.5 days to 10.5 days), while the median delay from referral to surgery was 4 days (95% CI 3 days to 5 days). There was no significant difference in delay to CEA depending on mode of presentation (Stroke, TIA, etc). Figure 6.3 summarises the cumulative delay from referral to undergoing surgery from three consecutive time periods. The curves for 2006 and 2007 represent the effect of a surgeon with an interest in carotid disease trying to make modifications in practice to speed up treatment (Brown C, 2009). As can be seen, this had little impact in 2007. However following the total reconfiguration of services in 2008, there was a dramatic reduction in the delay from referral to surgery (90% operated on within 14 days of referral). The mean length of hospital stay preoperatively was two days (range 0-12) following introduction of the reconfigured service, compared to only one day before the service was introduced.

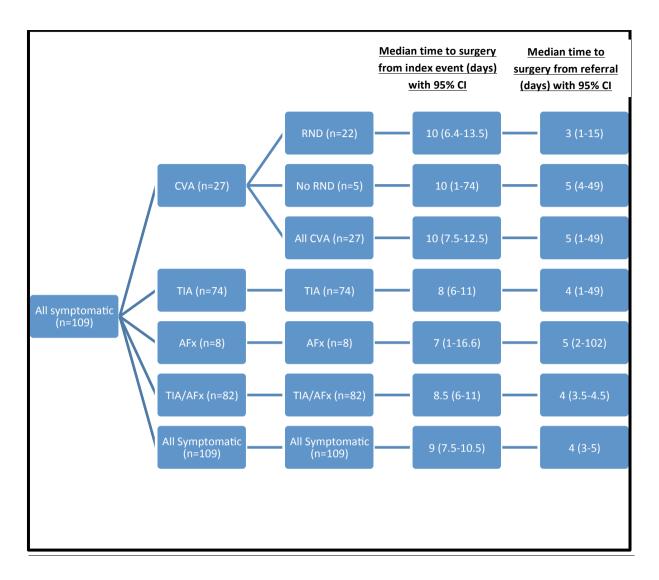


Figure 6.2 Median Time to Carotid Endarterectomy (CEA) in Relation to Timing of Index Event and Timing of Referral. (Salem MK, 2011)

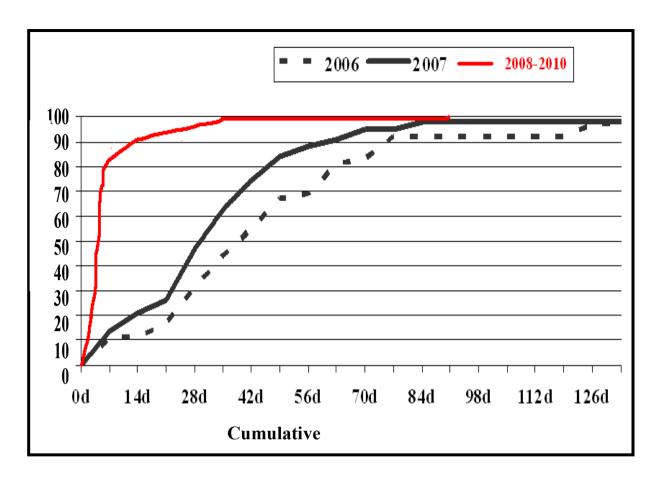


Figure 6.3 Cumulative delay (days) from date of referral to undergoing Carotid Endarterectomy (CEA). (Salem MK, 2011).

Tables 6.1 and 6.2 present the delays to surgery in greater detail. Overall 78% of patients underwent CEA within 14 days of the index event (table 6.1), while 90% were treated within 14 days of referral to the vascular team (table 2). This compares to only 11% and 21% of patients being treated within 14 days of referral in 2006 and 2007 respectively (Brown C, 2009). Only two patients underwent surgery within 48 hours of the index event, but 44% underwent CEA within seven days of the index event.

Table 6.3 presents the reasons for delay for the 25 patients who underwent CEA greater than14 days after the index event.

Time from Index Event to surgery	Number of patients Treated (Total n=109)
Under 48 Hours	2 (1.8%)
3-7 Days	45 (41.3%)
8-14 Days	37 (33.9%)
>14 Days	25 (22.9%)

Table 6.1 Delay to surgery in relation to timing from index event

Time from Referral to surgery	Number of patients Treated (Total n=109)
Under 48 Hours	21 (19.2%)
3-7 Days	64 (58.7%)
8-14 Days	13 (11.9%)
>14 Days	11 (10%)

Table 6.2 Time from date of referral to surgery

Reason	n=
Delay in presentation	9
Patient under OP review – clinical status changed from asymptomatic to symptomatic	7
Unfit for anaesthesia due to URTI	2
No surgeon available	1
Delay in transfer from Stroke Unit	3
Delay in referral	2
Patient cancelled/refused operation	1

Table 6.3 Reasons for delay for patients undergoing CEA >14 days after index event

Tables 6.4, 6.5 and 6.6 summarise the procedural risks, stratified for mode of presentation, timing to surgery and whether the patient had recurrent events after admission to the vascular unit. Overall there were no deaths within 30 days, but two patients suffered a stroke (1.83%) and one patient suffered an MI (0.92%). One patient who suffered a peri-operative stroke was transferred to the Vascular Surgery Unit having suffered a Rankin 3 stroke. He then had recurrent TIAs following admission and had spontaneous embolisation during TCD monitoring. After a review of his case, we proceeded to CEA. He suffered an intra-operative stroke, it having not been possible to insert a shunt during the procedure. It is assumed he extended the ischaemic penumbra around his infarct. The second patient suffered a non-disabling embolic stroke in the contralateral hemisphere on day 24 after surgery. This patient had also undergone CEA within 7 days of his index event (TIA).

Presentation	Death	Ipsilateral CVA	Contralateral CVA	Any CVA	Any Death/CVA	MI
Stroke with residual deficit (n=22)	0	1 (4.55%)	0	0	1 (4.55%)	0
Stroke without residual deficit (n=5)	0	0	0	0	0	0
All Strokes (n=27)	0	1 (3.7%)		0	1 (3.7%)	
TIA (n=74)	0	0	1 (1.35%)	1 (1.35%)	1 (1.35%)	1 (1.35%)
TIA/AFx (n=82)	0	0	1 (1.22%)	1 (1.22%)	1 (1.22%)	1 (1.22%)
All Symptomatic (n=109)	0	1 (0.92%)	1 (0.92%)	2 (1.83%)	2 (1.83%)	1 (0.92%)

Table 6.4 30-Day Death and Stroke Rate after CEA for Symptomatic Subgroups

Delay	Death	Ipsilateral CVA	Contralateral CVA	Any CVA	Any Death/CVA	MI
Index symptom to CEA (1-7 days) (n=47)	0	1 (2.13%)	1(2.13%)	2 (4.26%)	2 (4.26%)	0
Index symptom to CEA (8-14 days) (n=37)	0	0	0	0	0	1 (2.7%)
Index symptom to CEA (>14 days) (n=25)	0	0	0	0	0	0

Table 6.5 30-Day Death and Stroke Rate after CEA in Relation to Timing of Index Event

	Death	Ipsilateral CVA	Contralateral CVA	Any CVA	Any Death/CVA	MI
>1 event after admission to vascular unit(n=13)	0	1 (7.69%)	0	1 (7.69%)	1 (7.69%)	0
No further events after index event(n=96)	0	0	1 (1.04%)	1 (1.04%)	1 (1.04%)	1 (1.04%)

Table 6.6 30-Day Death and Stroke Rate after CEA in Patients Presenting with Recurrent Events Pre-operatively

Patients presenting with a stroke had a non-significantly increased risk of post-operative death/stroke (3.7%) as compared with 1.2% in patients presenting with a TIA or Amaurosis Fugax. Similarly, while both post-operative strokes occurred in the 47 patients undergoing CEA within 7 days of the index event, this was not statistically significantly higher than the 0% in 37 patients undergoing CEA between 8 and 14 days and within the 25 patients undergoing CEA after >14 days had elapsed. In addition, patients who suffered recurrent events following transfer from the TIA clinic incurred a 7.7% risk of death/stroke (1/13), but

this was not significantly higher than the 1% observed in 96 patients with no recurrent neurological events prior to surgery.

6.4 Discussion

The timing of carotid endarterectomy for symptomatic carotid artery disease remains an important benchmark for secondary stroke prevention. The landmark evidence for intervening in patients with extra cranial symptomatic carotid artery disease came from The European Carotid Surgery trial (ECST, 1991) and The North American Symptomatic Carotid Endarterectomy Trial (NASCET, 1991), which demonstrated benefit for treating patients who reported carotid territory symptoms in the preceding six months and who had a NASCET 50-99% stenosis of the ipsilateral carotid artery (ECST 70-99%).

The impetus for undertaking more rapid intervention came from two recently published metaanalyses by Giles (2007) and Wu (2007). These meta-analyses showed that the risk of stroke after a TIA was highest in the first seven days. This and other natural history studies, has driven the move towards expedited intervention as recommended by the latest European Vascular Society, NICE and The UK National Stroke Strategy guidelines.

Conventional teaching has however taught that the risk of stroke after a TIA is only about 1-2% at seven days and 2-4% at 28 days. The meta-analyses by Giles and Wu (which were based on natural history studies that utilised 'face to face' follow up of the entire population of TIA patients) demonstrated that at 48 hours the risk of stroke increased to 6.7%, and was 10% at seven days. This represents a fourfold increase in the early risk of stroke after a patient presented with their index TIA.

Since the service reconfiguration, the workload of the Vascular Surgery Unit has increased. In order to balance the need to see patients urgently and have the service capabilities to meet the demand, the urgency with which patients are referred and then seen is now based upon the ABCD2 scoring system. Ensuring that patients who have an ABCD2 score of greater than 4 are assessed on the same (or next) day in the rapid access TIA clinic remains an important step in triaging high risk patients. This ABCD2 is a validated scoring system for assessing the risk of early recurrent stroke after presenting with a TIA or minor stroke (Johnston SC, 2007). The scoring system based on 5 independent clinical variables; Age (>60 years scores 1), Blood pressure (Systolic BP >140mmHg or Diastolic BP >90mmHg scores 1), Clinical Symptoms (Unilateral weakness scores 2, speech impairment without weakness scores 1), Duration of symptoms (Duration >60mins scores 2, duration 10-59 minutes scores 1) and presence of **D**iabetes (scores 1). This gives a total score between 0-7. Studies have shown that patients who present with a score of 0-1 have a 1% and 1.2% risk of stroke at 48 hours and seven days. Those with a score of between 4-5 have a stroke risk of 4.1% and 5.9% at 48 hours and 7 days respectively, while a score of between 6-7 predicts a stroke risk of 8.1% at 48 hours and 12% at seven days. A parallel study by Giles et al (2007) also demonstrated similarly high risks of recurrent stroke in patients presenting with a minor stroke. Our cohort of patients included both high and low risk ABCD2 patients. No significant difference in outcome was seen between high and low risk ABCD2 patients, though this may be due to the small number of patients with a recorded complication.

The above natural history studies all focussed on TIA patients irrespective of the extent of carotid disease. A recent single centre study (Ois A, 2009) evaluated the hyperacute risk of stroke after TIA in patients with a carotid artery stenosis of 50-99%. They observed that in patients with 50-99% stenoses, the risk of stroke was 17% at 72 hours, 22% at 7 days and

25% at 14 days. It was also observed that patients with a 70-99% stenosis had a significantly higher risk of stroke than those with 50-69% stenosis.

However while there may be good epidemiological reasons for intervening early, surgeons remain concerned that this will lead to significant increases in procedural risks (Rockman CB, 2006); (Rerkasem K, 2007) despite the publication of studies suggesting that this is not always likely to be the case. Results from the Carotid Endarterectomy Triallists Collaboration study (Rothwell PM, 2004) demonstrated no significant difference in the procedural risk in the early period, although patients presenting with symptoms within 48 hours or with crescendo TIAs were excluded from the analysis. The 30 day death and stroke rate following CEA in published series where patients presented with crescendo TIAs or a non-disabling stroke and then underwent CEA within 14 days found that the procedural risk was less than 8% with a low risk of haemorrhagic transformation from an ischaemic infarct (Ricco JB, 2000)(Paty PSK, 2004)(Dorigo W, 2007)(Aleksic M, 2006)(Karkos CD, 2007)(Sbarigia E, 2006)(Rantner B, 2006). This level of procedural risk may be seen by some to be too high, however further analysis of the CETC data has allowed researchers to model the number of strokes prevented per 1000 CEAs at 5 years in relation to the procedural risk and delay to surgery. The evidence from this analysis suggest that surgical intervention within 14 days with a procedural risk of less than 10% would still prevent more strokes at 5 years than if surgery was delayed by four or more weeks and then performed with a procedural risk of 0% (Rothwell PM, 2004)

There are, however, a number of logistical issues that still need to be addressed in order to deliver the optimal service. In the past, patients were admitted either on the day before

surgery, or on the morning of the procedure. Following our service reconfiguration, the mean delay from admission to surgery increased to two days (0-12). There were several reasons for this (currently the subject of an on-going audit) including; poor control of hypertension, admission over the weekend or other untreated medical co-morbidities. In addition it has been our experience that the prevalence of severe post operative hypertension has increased in patients undergoing expedited surgery, probably reflecting the greater prevalence of patients with poorly controlled hypertension pre-operatively. However, this has not caused an increase in post-operative complications, probably because the unit has written guidelines in all clinical areas for dealing with hypertension as soon as it develops.

In conclusion, and contrary to the intuitive beliefs of many surgeons, this study has shown that there has been no significant increase in the procedural risk in symptomatic patients undergoing expedited CEA in the hyperacute period. This may of course, be down to chance (due to relatively small numbers) but it is more likely to be due to the unit's policy of rapid implementation of best medical treatment in the TIA clinic, dual anti-platelet therapy immediately prior to surgery, intra-operative TCD monitoring, completion angioscopy and rigorously enforced guidelines for managing post-operative hypertension. Future studies will ascertain whether these low risks can be maintained.

Chapter 7. Aims and scope of study

Although extensive research has been undertaken into the pathogenesis of carotid plaque instability many of these processes remain unclear. More recently the response to injury hypothesis and localised plaque inflammation and rupture has led to investigations into changes in gene expression levels locally.

This study will allow me to investigate biochemical, genetic and ultrasound imaging changes for patients with histologically and clinically unstable plaques. Firstly using histological analysis and grading of plaques, ultrasound, TCD data and clinical data will be correlated to histological features of plaque instability to identify key histological features and temporal trends in plaque morphology. I will investigate the biological markers related to unstable carotid plaques, and relate these to plaque histology and clinical symptoms. We will identify where these are acting within the atherosclerotic plaque. I will utilise novel duplex ultrasonography analysis software to determine whether these measures relate to the biochemical markers of instability identified in the laboratory studies. The secondary aim of this project is to identify circulating markers of plaque instability.

The hypothesis is:-

That there is altered gene expression, biological markers or ultrasound features in patients with high risk clinically and histologically unstable carotid atherosclerotic plaques when compared to patients with low risk carotid atherosclerotic plaques.

Chapter 8. Study Design, Materials & Methods

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Chapter 8. Aims, Study Design, Materials & Methods

8.1 Study Design

I assessed histological and ultrasound features of plaque instability and linked these to clinical and TCD evidence of plaque instability. Data was collected to create a model to predict the unstable plaque using non-invasive measures that could be simply performed in the clinic or at the bed-side.

To search for pathways associated with carotid plaque instability I conducted a two-stage study of gene expression in carotid atherosclerotic plaques, followed up with serum protein quantification and plaque immunohistochemical analysis of positive findings from the gene expression studies. The gene expression studies consisted of an initial discovery study using whole genome expression arrays and a validation study in an independent cohort.

8.2 <u>Ethics</u>

The study was authorised by the Leicestershire, Northamptonshire and Rutland Ethics Committee and patients were entered after giving informed consent. The National Research Ethics Service (NRES) Research Ethics Committee (REC) reference was:07/H0406/151. The local University Hospitals Leicester (UHL) Research and Development reference was UHL 10369.

Ethical consideration was given to consenting of patients into the study and secure storage of personal information. Patients were recruited after receiving the information sheet and being given time to consider enrolment. The initial data sheet record (see Appendix I, page 288) was the only source of information that allowed the patient to be identifiable through name and date of birth. This was stored in a secure folder in a locked office on the university site.

Entry into the building was only allowed with approved access. All patients were given a unique identifying number that was written on this record. Patient data was also transferred and stored on a stand-alone computer with no network or internet access. This was kept in the above mentioned office and was not removed. Access to the computer was through several password checks. Biological tissue samples were only identified by the unique patient number. Once processed, they were stored in a locked freezer and kept in the above mentioned building.

8.3 Patient Selection

All symptomatic patients with a >50% NASCET and asymptomatic patients with a >70% NASCET extra-cranial carotid artery stenosis who were admitted to the Leicester Vascular Surgery unit for CEA were considered for inclusion. All had been referred via a consultant Stroke Physician (mostly via the rapid access TIA Clinic which was established in October 2008)(Salem MK, 2011).

8.3.1 Rapid Access TIA Clinic

See section 6.3.1 for full details. Briefly, patients with a suspected TIA were triaged using the ABCD2 scoring system. Any patient with an ABCD2 score of 0-3 were scheduled to be seen in the neurovascular clinic within 7 days. However, any patient with an ABCD 2 score of 4-7 was seen either the same day or the following morning. Any patient with a NASCET 50-99% stenosis was then discussed with the on-call vascular surgeon and immediately transferred to the Leicester Vascular Surgery Unit unless there were contra-indications such as severe comorbidity, dementia or advanced malignancy.

8.3.2 Vascular Surgery Unit

See section 6.3.2(page 103). Following transfer, all patients underwent a repeat Duplex assessment and 30 minutes transcranial Doppler (TCD) recording to identify those with spontaneous embolisation.

The Vascular Surgery Unit maintains two half day operating lists (Tuesday/Friday) for performing expedited CEA. CEA procedures were performed by the consultant allocated to that particular list (i.e. this could be different to the consultant admitting the patient). The emergency theatre was only used if these lists were already filled. All patients received 300mg aspirin and 40mg simvastatin in the TIA clinic, in addition to other appropriate secondary prevention therapy, and these were then continued throughout the pre-operative period, although the aspirin dose was reduced to 75mg. All patients received 75mg clopidogrel the night before surgery in addition to their regular aspirin.

8.3.3 Transcranial Doppler Monitoring of the Middle Cerebral Artery to Detect Spontaneous Embolisation (SE)

Transcranial Doppler ultrasound (TCD) is capable of detecting microembolic material, known as spontaneous emboli (SE), both gaseous and solid, within the intracranial cerebral arteries. Although these emboli may be clinically silent, they may be clinically important by indicating an increased risk of stroke.

As soon as possible after admission (same day if admitted before 5pm or next day if admitted after 5pm) in symptomatic patients or at the time of pre-assessment for asymptomatic patients, a 30 minute period of TCD monitoring was performed using a commercially available TCD machine (Sonara TCD System from Viasys Healthcare) with the 2-MHz head probe held in place by an external fixation device. Insonation of the ipsilateral MCA of the affected artery was achieved via the temporal acoustic window at a depth of 50 to 60mm using a 2-MHz pulsed Doppler transducer. The sample volume (generally 8-13 mm), power and gain were adjusted to ensure an optimal embolic signal to background signal relationship. Each embolic signal was recorded and categorised using the Consensus Committee Criteria (Consensus Committee, 1995) to differentiate between true emboli and other high intensity transient signals (HITS), including artefacts. Patients were classed as having evidence of SE if one or more true emboli were detected in the 30 minute monitoring period. Each TCD recording was made by a trained vascular technologist who was blinded to data regarding timing of the clinical event in symptomatic patients.

8.3.4 Carotid Endarterectomy (Figure 8.1)

CEA was performed using a standard technique, unchanged since 1991 (general anaesthesia, routine patching, routine shunting, systemic heparinisation (unfractionated) and distal intimal tacking sutures).(Gaunt ME, 2006). See section 6.3.3 (page 104)

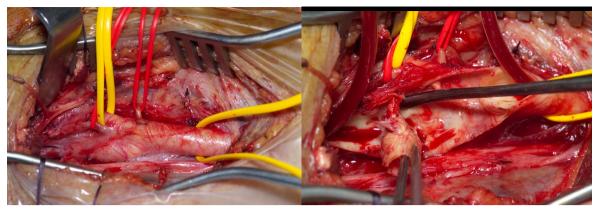


Figure 8.1a

Figure 8.1b

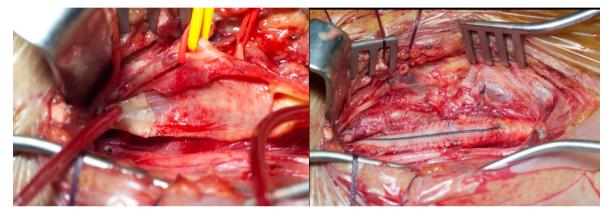


Figure 8.1c



Carotid Endarterectomy performed under general anaesthetic. Figure 8.1a – slinging of common and external carotid artery (yellow slings) and superficial thyroid artery (red sling). Figure 8.1b – Harvesting plaque after arteriotomy (shunt in situ). Figure 8.1c – Artery after plaque excised. Figure 8.1d – patch closure of arteriotomy.

8.4 Data Collection

Pre-operative clinical data were recorded by a single investigator (MS) through face to face questioning of patients and after review of medical notes and in-patient investigations. Demographic data included; age, sex, vascular risk factors (hypertension, hyperlipidaemia, diabetes mellitus and smoking), pre-operative medication and in symptomatic patients time

from index event and most recent event. See data proforma (appendix 1 page 288).

8.5 **Duplex Imaging**

All Duplex examinations were undertaken by experienced ultrasonographers and stenosis severity was measured using consensus criteria consistent with the NASCET method (See Figure 8.2) of measurement. The second duplex image taken after admission to the Vascular Surgery Unit was used for analysis. Enhanced, normalised and non-normalised images were used for blinded off-line analysis.

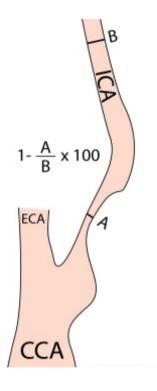


Figure 8.2 The NASCET uses the formula 1 - (A/B), where A is the residual luminal diameter at the stenosis and B is the luminal diameter at a visible, disease-free point above the stenosis. Kind permission Ota H et al. Radiographics 2005;25:1141-1158

8.5.1 Image normalization and measurements of Gray-Scale Median (GSM) and Juxta-Luminal Black Areas (JBA)

The "Plaque Texture Analysis software" (Iconsoft International Ltd, Greenford, London, UK), is a dedicated research software package and has five modules: (1) image normalization; (2) measurements (Intima Media Thickness (IMT), plaque thickness and areas); (3) pixel density standardization (this is essential for texture features that are pixel-density-dependent); (4) image crop (outlining the plaque and saving it as a separate file); and (5) feature extraction (including Gray-Scale Median). Normalization was performed using the "image normalization" module. First, a sample of "blood" was selected from the vessel lumen avoiding areas of "noise". Next, using the zoom facility the brightest part of adventitia adjacent to the plaque was magnified at least twice and the middle two fourths were selected. Pressing the "features extraction" button in this window or using the "feature extraction" module produced a variety of texture features which were automatically calculated including GSM and saved in a database that could be opened by Microsoft Office Excel (Microsoft Inc, Redmond, Wash).

Parameters measured using the Iconsoft Plaque Texture Analysis Software included 1. Juxta-Luminal Black Area; 2. Discrete White Areas; 3. Type of Plaque; 4. Plaque area and percentage area and 5. GSM.

The definitions and reproducibility of texture features used in this study are given below.

8.5.2 Gray Scale Median (GSM).

This is the median of the gray values of all the pixels in the plaque image (El-Atrozy T, 1998);(Tegos TJ, 2000);(Sabetai MM, 2000). Computer-assisted GSM analysis is less operator-dependent and has been claimed to have better reproducibility than visual

characterization (Iwamoto T, 2003). In one reproducibility study of 35 plaques measured by two observers, the inter-observer mean difference of GSM was 3.6, the within-subject standard deviation was 13.6 and the intra-class correlation coefficient was 0.93 (Griffin M, 2007). In another reproducibility study looking at 610 plaques inter- and intraobserver agreement on GSM classification was substantial, with k values (95% CI) of 0.77 (0.73 to 0.80) and 0.79 (0.75 to 0.84), respectively (Fosse E, 2006).

8.5.3 Plaque Type

Plaque types were scored automatically by the software according to a modified Geroulakos classification (Geroulakos G, 1993);(Nicolaides AN, 2005)

- **Type 1.** Uniformly echolucent (black): < 15% of the pixels in the plaque area were occupied by pixels with gray scale values > 25.
- **Type 2.** Mainly echolucent: pixels with gray scale values > 25 occupy 15-50% of the plaque area.
- **Type 3.** Mainly echogenic: pixels with gray scale values > 25 occupy 50-85% of the plaque area.
- **Type 4 or 5.** Uniformly echogenic: pixels with gray scale values > 25 occupy > 85% of the plaque area.

Because of the low event rate in plaque types 4 and 5 as previously demonstrated (Sabetai MM, 2000) and because the software cannot distinguish between them, these plaque types have been grouped together in this study.

8.5.4 Plaque area

This was calculated by the software using the distance scale on the side of the image frame for calibration and the plaque area outlined by the operator. It was expressed in mm². In a reproducibility study involving 50 plaques the inter-observer intra-class correlation coefficient was 0.73. A threshold of 80mm² was used to identify a large plaque area, previously associated with development of symptoms (Spence JD, 2006).

8.5.5 Discrete White Areas (DWA)

The presence of DWA is defined as areas with pixels having gray scale values >124 (colored red by the software for easy visual identification) not producing acoustic shadowing in plaque types 1-3. DWAs which are non calcified white areas (absence of acoustic shadow) within black areas have been previously associated with the development of symptoms (Carra G, 2003).

8.5.6 Juxtaluminal Black Areas

Juxtaluminal black area without visible echogenic cap (JBA). The largest juxtaluminal black area of the contoured image (i.e. area with pixels having a gray-scale less than 25) without a visible echogenic cap (i.e. pixels with gray-scale value equal or higher than 25) was outlined by the mouse and expressed as mm^2 . This was automatically calculated by the software using the on-screen mm scale from each ultrasonic image. The larger value was used in cases where there were two plaque components with black areas. A JBA >6mm² was defined as being large.

8.6 Sample Processing

8.6.1 Blood

Peripheral venous blood was drawn pre-operatively the evening before surgery into pyrogenfree tubes containing no anticoagulant. Tubes were incubated in an upright position at room temperature for 30 to 45 minutes to allow clotting. Tubes were then centrifuged for 15 minutes at 2000g. The supernatant (serum) was aspirated at room temperature and placed into a micro-centrifuge tube. Samples were re-centrifuged and aspirated again to remove any remaining insoluble matter. Serum was aliquoted into cryovials and all samples were stored at -80°C for batch analysis. Samples were stored for between 6-30 months before being processed. Serum was chosen for use in ELISA studies as per manufacturer's recommendations.



Figure 8.3 Processing of blood to retrieve serum

8.6.2 Carotid Atherosclerotic Plaques

The aim of processing immediately in theatre was to provide tissue optimally processed and stored for downstream use. Carotid plaques harvested during surgery were rinsed in normal saline and divided longitudinally in theatre. One half was immediately placed in formalin for 24 hours at room temperature and subsequently embedded in paraffin for histological grading and immunohistochemical analysis. The other half was immediately suspended in RNA*later*® (Ambion, Europe) for 24 hours at room temperature, then drained, snap frozen and stored in liquid nitrogen for later RNA extraction. Figure 8.4 demonstrates fresh harvested samples rinsed and divided.



Figure 8.4 Fresh plaque rinsed and divided in theatre

8.7 RNA extraction from carotid plaques

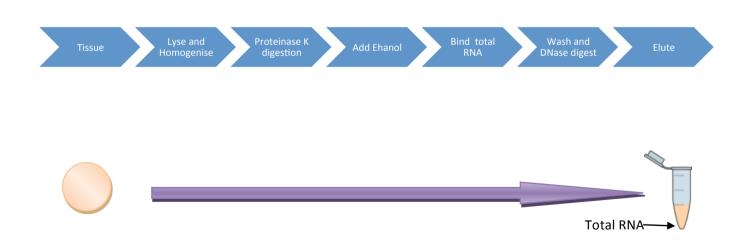
RNA extraction was optimised to give the highest quality RNA free from degradation and genomic DNA. Genomic DNA can affect the interpretation of microarray and qRT-PCR results by competing for reagents and introducing background signals.

RNA extraction from carotid atherosclerotic plaques needs attention to detail in minimising carry-over of genomic DNA and degradation by free RNAses. The heterogenous cellular nature of the plaque and presence of micro-necrotic regions around/within intra-plaque haemorrhage contain abundant RNAses. RNAses are released after the start of the homogenisation process and can quickly affect RNA quality. This led us to try two established techniques for RNA extraction.

Methods

8.7.1 Qiagen RNeasy Fibrous Tissue Mini Kit (See Figure 8.1)

Initially RNA was extracted using a standard manufacturer's (Qiagen) kit. As total RNA purification from fibrous tissues, such as skeletal muscle, heart, and atherosclerotic tissue, can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. RNeasy Fibrous Tissue Kits are supplied with proteinase K, which digests these proteins. Tissue samples are first lysed in buffer and then diluted before being treated with proteinase K. Debris is pelleted by centrifugation, and the supernatant is removed. The supernatant is mixed with ethanol and then centrifuged through an RNeasy spin column, where RNA binds to the silica membrane. Traces of DNA that may co-purify with the RNA are removed by DNase treatment on the silica membrane. DNase and any contaminants are efficiently washed away, and high-quality total RNA is eluted in RNase-free water.



RNeasy Fibrous Tissue Mini Procedure

Figure 8.5 Qiagen RNeasy Fibrous Tissue Mini Kit Work Flow.

8.7.2 Trizol (phenol) Reagent RNA Extraction

100mg of tissue was cut into 1mm^2 pieces with a sterile scalpel. Using a mortar and pestle, tissue was ground to a fine powder in liquid nitrogen and then transferred to a Dupont tube on ice. 1ml of homogenising buffer (Trizol®) was used per 100mg of wet tissue. Tissue was homogenised in Trizol using a power homogeniser. The homogenised sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Between samples all tubes and the homogeniser blade were thoroughly cleaned and washed using DEPC water and RNase Zap ® to prevent carry over and contamination of RNAses. 0.2 ml of chloroform per 1 mL of TRIzol® reagent was added and the tube was shaken vigorously by hand for 15 second and then incubated for 2–3 minutes at room temperature. Samples were centrifuged at 12,000 × g for 15 minutes at 4°C. This resulted in a triphase appearance (a lower red phenolchloroform phase, an interphase, and a colourless upper aqueous phase). RNA remains exclusively in the aqueous phase.

The upper aqueous phase is \sim 50% of the total volume. The aqueous phase of the sample was removed by angling the tube at 45° and pipetting the solution out, avoiding drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.

The aqueous phase was placed into a new tube in order to proceed to the RNA Isolation. 0.5 mL of 100% isopropanol was added to the aqueous phase, per 1 mL of TRIzol® and the sample incubated at room temperature for 10 minutes. The sample was then centrifuged at $12,000 \times g$ for 10 minutes at 4°C. The RNA was often invisible prior to centrifugation, and then forms a gel-like pellet on the side and bottom of the tube. The supernatant was removed from the tube, leaving only the RNA pellet. The pellet was washed with 1 mL of 75% ethanol per 1 mL of TRIzol® Reagent used in the initial homogenization. The sample was vortexed briefly, then centrifuged at 7500 × g for 5 minutes at 4°C. Following this the wash was discarded and the RNA pellet was allowed to air dry for 5–10 minutes. The RNA pellet is

then re-suspended in RNAse free water. Finally clean up was performed using the Qiagen RNeasy Mini Kit which included a double DNAse step to prevent genomic DNA carry forward.



Figure 8.6 Trizol extraction of RNA

8.7.3 RNA Quality and Quantity

The integrity and concentration of the extracted total RNA was tested using an RNA 6000 Nanodrop LabChip Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA was stored at -80°C for later batch analysis. The full protocol is available in appendix III

8.8 Gene Expression Analysis

8.8.1 Whole Genome Microarray (see Figure 8.7)

For the initial gene expression study, microarray analysis was performed using Illumina cDNA-mediated Annealing, Selection, extension and Ligation (DASL) assay. This microarray platform was chosen as it provided accurate profiling of RNA that had partial degradation during the RNA extraction protocol as demonstrated during the qualitative and quantitative assessment of each sample. Each RNA sample was hybridized to its own microarray in isolation, sample pooling was not used. Total RNA was converted to cDNA using biotinylated oligo-dT₁₈ and random nonamer primers. Two assay-specific oligonucleotides were designed to interrogate a single contiguous 50 nucleotide (nt) sequence on each cDNA. Each of these oligonucleotides consisted of two parts: an upstream-specific

oligonucleotide (USO) containing a 3' gene-specific sequence and a 5' universal PCR primer sequence (P1), while the downstream-specific oligonucleotide (DSO) contained a 5' genespecific sequence and a different 3' universal PCR primer sequence (P2'). The USOs and DSOs were designed with an average T_m of 65°C and 58°C, respectively and an average length of 22 and 20 nt, respectively. Using this approach, a total of 24526 oligonucleotide pairs (probes) were designed and pooled, which together constituted the whole genome DASL assay pool (DAP), corresponding to 18626 unique genes, based on well-annotated content derived from the National Center for Biotechnology Information Reference Sequence Database (Build 36.2, Release 22)). The DAP was then annealed to the targeted cDNAs during a 16 h temperature gradient (70 to 30°C) incubation followed by enzymatic extension and ligation steps, as previously described (Fan JB, 2004). Ligated products were PCRamplified and labeled with a universal Cy3-coupled primer after which single-stranded labeled products were precipitated and then hybridized to whole genome gene expression BeadChips as previously described (Kuhn K, 2004). BeadChips were then scanned on a BeadArray[™] Reader using BeadScan software (v3.2), during which fluorescence intensities were read and images extracted. Scanned data were then uploaded into GenomeStudio® software (v1.1), via the gene expression module (WG-DASL Assay) for further analysis. All of the microarray data are MIAME compliant and have been submitted to GEO (Accession Number: GSE17599). Detailed descriptions of all data and protocols were submitted to the ArrayExpress public repository (http://www.ebi.ac.uk/miamexpress/login.htm, accession number: E-MEXP-3683) as per MIAMI guidelines (Brazma A, 2001) and are available in appendices III and IV. Cubic spline normalisation was performed. Genes differentially expressed were identified by filtering, using the following criteria: (1) an Illumina diff score greater than 13 or less than -13 (equivalent to a P value <0.05) and (2) a 1.3-fold difference in the mean signal between groups in each separate analysis.

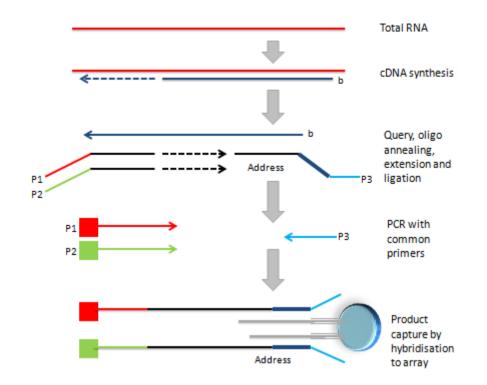


Figure 8.7 Whole Genome HT DASL: Summary of Assay. The DASL Assay monitors gene expression with probe groups to query total RNA-generated cDNA target sequences.

8.8.2 Expression Validation Using Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Polymerase Chain Reaction (PCR) was devised during the 1980s and has revolutionised molecular biology. PCR is a standard procedure that allows for the amplication of a small amount of template DNA (or cDNA) into large quantities in a few hours. This is performed by mixing the DNA with primers on either side of the DNA (forward and reverse), *Taq* polymerase (of the species *Thermus aquaticus*, a thermophile whose polymerase is able to withstand extremely high temperatures), free nucleotides (dNTPs for DNA, NTPs for RNA), and buffer. The temperature is then alternated between hot and cold

to denature and re-anneal the DNA, with the polymerase adding new complementary strands each time.

The advent of real-time PCR allows investigators to actually view the increase in the amount of DNA as it is amplified. TaqMan® real-time PCR was chosen for this study.

The TaqMan® probe consists of two types of fluorophores, which are the fluorescent parts of reporter proteins (Green Fluorescent Protein (GFP) has an often-used fluorophore). While the probe is attached or unattached to the template DNA and before the polymerase acts, the quencher (Q) fluorophore (usually a long-wavelength coloured dye, such as red) reduces the fluorescence from the reporter (R) fluorophore (usually a short-wavelength coloured dye, such as green). It does this by the use of Fluorescence (or Förster) Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. The reporter dye is found on the 5' end of the probe and the quencher at the 3' end.

Once the TaqMan® probe has bound to its specific piece of the template DNA after denaturation (high temperature) and the reaction cools, the primers anneal to the DNA. *Taq* polymerase then adds nucleotides and removes the Taqman® probe from the template DNA. This separates the quencher from the reporter, and allows the reporter to emit its energy. See Figure 8.8 below.

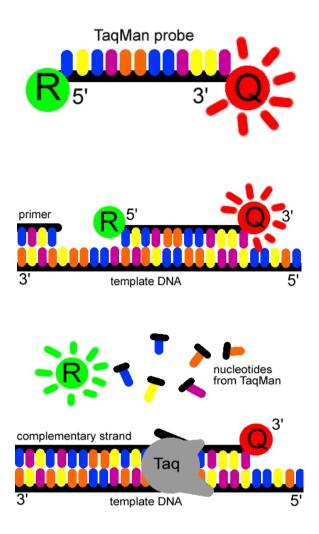


Figure 8.8 The red circle represents the quenching dye that disrupts the observable signal from the reporter dye (green circle) when it is within a short distance. The TaqMan® probe binds to the target DNA, and the primer binds as well. Because the primer is bound, *Taq* polymerase can now create a complementary strand. The reporter dye is released from the extending double-stranded DNA created by the Taq polymerase. Away from the quenching dye, the light emitted from the reporter dye in an excited state can now be observed.

The light emitted from the dye in the excited state is received by a computer and quantified. The more times the denaturing and annealing takes place, the more opportunities there are for the Taqman® probe to bind and, in turn, the more emitted light is detected. The results from the computer are shown on a graphical display, showing PCR cycles on the X-axis and a logarithmic indication of intensity on the Y-axis. Methods

Detailed description of the solutions, reagents and protocol for the qRT-PCR is noted in appendices III and IV. Total RNA was reverse transcribed into cDNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems). After addition of master mix to the RNA the thermal cycler was set to 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and then to 4° for ∞. Once cDNA was obtained, the cDNA was added to a 384-well custom plate ordered from Applied Biosystems containing probes of genes of interest. Once TaqMan master mix had been added the plate was run on a Roche Light Cycler 480 at the following programme; 1. Hold at 50°C for two minutes; 2. Hold at 95°C for 10 minutes; 3. PCR - 40 cycles (melt 95°C 15 seconds; anneal/extend 60°C for one minute). All samples were run in triplicate.

The initial hold at 50°C for two minutes is to allow the activity of uracil-N glycosylase (UNG) in the master mix. UNG is a pure, nuclease free, 26-kDa recombinant enzyme encoded by the Escherichia coli uracil-N glycosylase gene which has been inserted into an E. Coli. UNG acts on single- and double-stranded dU-containing DNA by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, and creates an alkali-sensitive apyrimidic site in the DNA. Apyrimidic sites block replication by DNA polymerases. The enzyme has no activity on RNA or dT-containing DNA. UNG incubation at 50 °C is necessary to cleave any dU-containing PCR carryover products. Tenminute incubation at 95 °C is necessary to substantially reduce UNG activity and for initial denaturation.

Gene expression data were analysed by normalisation against the geometric mean of the expression of the 3 housekeeper genes (B2M, GUSB and PGK1) showing the most stable expression from a panel of six housekeeping genes as described by Vandesompele et al

(2002). In order to determine the best housekeeping (internal control) gene, an experiment was performed on RNA extracted from carotid plaques under variable experimental conditions with variable RNA quality. Standard qRT-PCR was performed as per section 8.8.2 with 5 housekeeping genes chosen. For every housekeeping gene we determined the pairwise variation with all other control genes as the standard deviation of the logarithmically transformed expression ratios, and defined the internal control gene-stability measure M as the average pairwise variation of a particular gene with all other control genes. Genes with the lowest M values have the most stable expression. Assuming that the control genes are not co-regulated, stepwise exclusion of the gene with the highest M value results in a combination of two constitutively expressed housekeeping genes that have the most stable expression in the tested samples. For simple calculations, the relative quantification data of each housekeeping gene is used. To calculate this relative quantification data the highest Ct value is subtracted from all other Ct values for each gene measured. Hence each Ct value has been transformed in to a "delta CT" value, with the highest deltaCT value as 0. All other values are less than 0. Applied to each data point is the equation $2^{(-\text{delta Ct})}$. Hence all data is expressed relative to the expression of the least expressed gene. Values are entered into a Visual Basic Application (VBA) for Microsoft Excel - termed geNorm. This software automatically calculates the gene-stability measure M for all control genes in a given set of samples and enables elimination of the worst-scoring housekeeping gene (that is, the one with the highest M value) and recalculation of new M values for the remaining genes.

In order to measure expression levels accurately, normalization by multiple housekeeping genes instead of one is required. Consequently, a normalization factor based on the expression levels of the best-performing housekeeping genes must be calculated. For accurate averaging of the control genes, it is proposed to use the geometric mean instead of the

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arithmetic mean, as the former controls better for possible outlying values and abundance differences between the different genes. It is recommend the minimal use of the three most stable internal control genes for calculation of an RT-PCR normalization factor (NF*n*,*n* = 3). The geNorm program calculates Pairwise variation (Vn/n + 1). This is an analysis between the normalization factors NF*n* to determine the number of control genes required for accurate normalization.

8.9 Enzyme Immunoassay (ELISA)

ELISAs enable absolute quantification of specific proteins through comparison of the optical density (OD) of the unknown specimen to the OD of a standard curve of known concentrations. The assay employs an antibody specific for the protein of interest coated on a 96-well plate. Standards and samples are pipetted into the wells and the protein of interest present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and colour develops in proportion to the amount of protein bound. The Stop Solution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm.

Serum levels of the proteins of interest were measured by ELISA using a commercially available kit (Abcam). Each ELISA plate measured the amount/concentration of one specific protein within a number of samples. A known concentration range was made from serial dilutions of the standard protein at a fixed concentration. A positive control was provided by the manufacturer. Negative controls of the buffer without serum were used to determine to the background light absorbance of the solutions.

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The intra-assay coefficient of variation (CV) was 7%. This was calculated first by calculating the CV for each standard which was the standard deviation/mean x100, and then taking mean value for each. The inter-assay CV was 9%, calculated as above but the mean CV of each standard taken over several plates and days was calculated.

Pre-coated 96-microtitre plates, coated with antibodies specific to the protein under investigation, were incubated with the samples or standard for a fixed period of time (2.5 hours at room temperature) to allow binding of the antibodies and specific proteins within the samples/standards. Each sample/standard was measured in duplicate. The samples/standards were aspirated off and the wells were washed four times. A secondary biotinylated detection antibody, which recognised a second epitope on the protein, was incubated in the wells for a fixed time period (1 hour at room temperature) to allow binding between antibody and protein. The unbound antibody was removed and the wells were washed thoroughly. Finally each well was incubated with a HRP-Streptavidin solution for 45 minutes at room temperature. This allowed the streptavadin to bind to the biotin, and then the wells were washed to remove any unbound HRP-Streptavidin (See Figure 8.9).

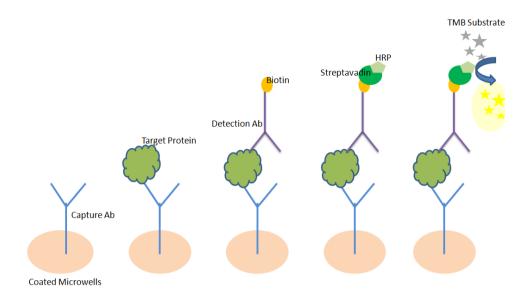


Figure 8.9 Summary of ELISA assay

The amount of immobilised peroxidise in each well was directly proportional to the amount of protein captured within each well and was measured by a colorimetric reaction between the HRP and its chromagen substrate TMB (3,3',5,5'-tetramethylbenzidine). In the presence of peroxidise this colourless substrate turns blue. This reaction is stopped by the addition of 0.2M sulphuric acid resulting in a yellow colour change (See Figure 8.10) which was read at 450nm using a microtitre plate spectrophotometer. All wells were background corrected and a standard curve was constructed from the OD of the standards of a known concentration. The concentrations of the unknown samples were interpolated from the standard curve. Full protocol and solutions are in appendices III and IV.

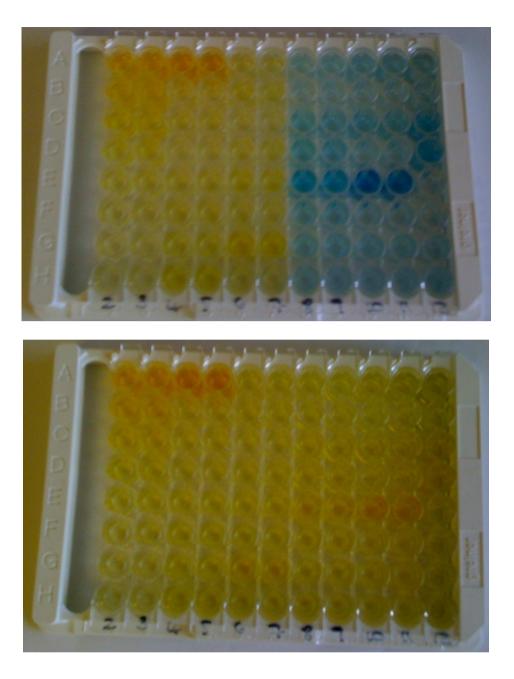


Figure 8.10 Example of ELISA plate. After the addition of the stop solution (0.2M sulphuric acid) the solution turns from blue to yellow. The concentration of the unknown samples (columns 3-12) are determined from a standard curve constructed from the OD values of the standards (columns 1-2) versus their concentration.

8.10 Histopathology

8.10.1 Tissue Sectioning for Histology

As described in section in 8.4.2, the carotid atherosclerotic plaque had been fixed in 10% formalin for 24 hours prior to paraffin embedding. 5µm transverse sections were taken from the paraffin block at the site of maximal stenosis and placed onto silicone coated slides and dried at 37°C overnight. Paraffin embedded tissue was used in preference to frozen sections as structural integrity is thought to be better maintained with no impact on results of staining (Shi SR, 2008).

8.10.2 Haematoxylin and Eosin Staining (H&E)

Most cells are colourless and transparent, and therefore histological sections have to be stained to make the cells visible. General staining of the tissue specimen was achieved through Haematoxylin and Eosin Staining (H&E). Haematoxylin in complex with aluminium salts is cationic and acts as a basic dye. It is positively charged and can react with negatively charged, basophilic cell components, such as nucleic acids in the nucleus. These stain blue as a result. Eosin is anionic and acts as an acidic dye. It is negatively charged and can react with positively charged, acidophilic components in the tissue, such as amino groups in cytoplasmic proteins. Collagen and elastin being positively charged proteins attract the negatively charged eosin and stain pink. The mounted tissue sections were de-waxed by passing sequentially through three washes of xylene, then rehydrated by two washes in 99% industrial methylated spirit (IMS), one wash in 95% IMS and finally washed in water. All washes were done for 1 minute. This process is known as 'taking to water'. The slides were immersed in Mayers's haematoxylin for 5 minutes and the unbound stain was washed off in running water. The sections were dehydrated by 1 minute immersions

in one bath of 95% IMS and two baths of 99% IMS. The slides were finally immersed in three successive baths of xylene and cover-slipped using DPX mounting media.

Using Haematoxylin and Eosin staining, histological specimens were analysed and a semiquantitative 3 or 4 point score (Table 8.1) was assigned to show the presence and/or amount of features of plaque instability, based on the AHA histological classification of advanced atherosclerotic lesions and a well validated previously published scoring system (Lovett JK, 2004). Features analysed included; intra-plaque haemorrhage, lipid core size, chronic plaque inflammatory infiltrate, chronic cap inflammatory infiltrate, neovascularity, amount of foam cells, and evidence of cap rupture. Intra-plaque haemorrhage was defined as evidence of erythrocytes within the plaque causing disruption of plaque architecture as defined by Bassiouny (1989). Lipid core was defined as an area of amorphous material containing cholesterol clefts. Rupture was defined as a clear communication between lipid core and lumen with a break in the fibrous cap. A grade of 2 or 3 for each of these features demonstrated histological instability.

Specific cell markers and proteins of interest were studies through specific immunohistochemical staining.

	Grade 0	Grade 1	Grade 2	Grade 3
Haemorrhage	No Haemorrhage	Small	Large	
macmonnage	No Haemonnage	Haemorrhage Haemorrhage		-
Thrombus	No Thrombus	Small Thrombus	Large Thrombus	-
Lipid Core	No Lipid Core	Small Lipid Core	Large Lipid Core	-
Fibrous Tissue	Very Little	Approx 50%	Predominantly	
FIDIOUS FISSUE	Fibrous Tissue	Fibrous Tissue	Fibrous Tissue	-
Chronic Plaque	None	Occasional cells	2-5 groups >50	>5 groups >50
Inflammation	None	or one group >50	2-3 groups ~30	or 1 group >500
Chronic Cap	None	<10 cells in cap	10-50 cells in	>50 cells in cap
Inflammation	Ivone		cap	> 50 cens in cap
Acute Plaque	None	Occasional cells	2-5 groups >50	>5 groups >50
Inflammation	INOILE	or one group >50	2-5 groups > 50	or 1 group >500
Acute Cap	None	<10 cells in cap	10-50 cells in	>50 cells in cap
Inflammation	Ivone		cap	> 50 cens in cap
Foam Cells	None	<50 cells	>50 cells	-
Neovascularity	None	<10/section	>10/section	-
Cap Rupture	Intact	Probably Intact	Probably	Definitely
	intact	i iooaoiy intact	Ruptured	Ruptured
Overall Stability	Stal	ble	Unstable	

Table 8.1 Semi-Quantitative Scoring System for Histological Grading

Figures 8.11-8.15 show a number of examples of histological features identified from H&E staining. Examples include a stable plaque with predominantly fibrous tissue (Figure 8.11) and also examples of unstable plaques with large lipid core (Figures 8.12-8.13), intra-plaque haemorrhage, cap rupture and surface thrombus. (Figures 8.14-8.16).

		0	1	2	3
	Haemorrhage	۷			
	Thrombus	۷			
	Lipid Core	۷			
A CONTRACTOR OF THE OWNER	Fibrous Tissue			۷	
4 FT	Chronic Plaque Inflammation	۷			
FI C	Acute Plaque Inflammation	۷			
and the second se	Acute Cap Inflammation	۷			
	Foam Cells		۷		
	Neovascularity		۷		
	Cap Rupture	٧			
	Overall Stability	۷			

Figure 8.11 Stable plaque on H&E staining showing intact cap (C) and predominantly fibrous tissue (FT) and underlying intima (I) (x10)

2"		0	1	2	3
	Haemorrhage		٧		
	Thrombus		٧		
	Lipid Core			v	
Contraction of the second	Fibrous Tissue	٧			
ALLON LA LA LA	Chronic Plaque Inflammation			v	
	Chronic Cap Inflammation			v	
The Mark Mark	Acute Plaque Inflammation		٧		
	Acute Cap Inflammation			v	
E A REALINE	Foam Cells			v	
	Neovascularity		٧		
	Cap Rupture			v	
	Overall Stability			v	

Figure 8.12 Unstable plaque on H&E staining showing large lipid core (x10)

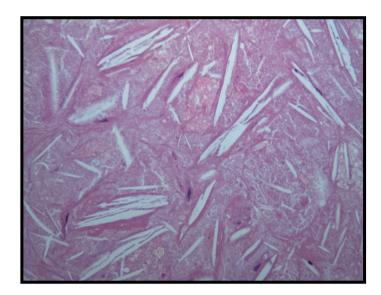


Figure 8.13 Unstable plaque on H&E staining showing large lipid core (x40)

The second se		0	1	2	3
	Haemorrhage			۷	
	Thrombus			۷	
14.45	Lipid Core		۷		
	Fibrous Tissue		۷		
	Chronic Plaque Inflammation			۷	
Star Star Star Star Star	Chronic Cap Inflammation			۷	
	Acute Plaque Inflammation		۷		
	Acute Cap Inflammation		۷		
	Foam Cells			۷	
	Neovascularity			۷	
	Cap Rupture			۷	
	Overall Stability			۷	

Figure 8.14 Unstable plaque on H&E staining showing thrombus formation (x10)

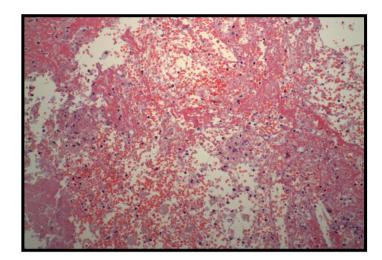


Figure 8.15 Unstable plaque on H&E staining showing thrombus formation (x20)

The second s		0	1	2	3
mu	Haemorrhage			٧	
	Thrombus			٧	
	Lipid Core		٧		
L. L	Fibrous Tissue	v			
A CARLON AND AND AND AND AND AND AND AND AND AN	Chronic Plaque Inflammation			٧	
	Chronic Cap Inflammation				٧
	Acute Plaque Inflammation		٧		
21	Acute Cap Inflammation		٧		
	Foam Cells			٧	
	Neovascularity			٧	
ALL AND AND AND AND	Cap Rupture				٧
	Overall Stability			٧	

Figure 8.16 Unstable plaque on H&E staining showing cap rupture (arrow), intraplaque haemorrhage (IPH) and surface thrombus (ST) (x10)

Methods

8.10.3 Immunohistochemistry

Immunohistochemistry was used to identify specific cell types and the localised expression of certain proteins. The inflammatory infiltrate was characterised through the use of specific primary antibodies against CD markers uniquely expressed on specific cell types. The regional expression of proteins of interest was visualised using specific antibodies against these proteins.

Formalin fixation can lead to masking of antigens and prevent their exposure to antibodies. In order to counter this, various antigen retrieval methods are used to improve detection by increasing accessibility to the antigen. In order to ascertain the optimum antigen retrieval method for our chosen proteins/cell markers, an experiment was performed to compare Heat-induced epitope retrieval using a water bath and microwave, and either a basic (Tris-EDTA, pH 9.0) or acidic (sodium citrate, pH 6.0) buffer solution. Results were then compared to enzymatic antigen retrieval using Trypsin. In each case we found that using an acidic buffer (pH 6.0) and heating using a waterbath provided optimum results in our tissue and in control tissues. Table 8.2 below demonstrates the antigen retrieval method used and primary antibody concentration for each protein marker of interest.

Immune Cell Type/Protein Marker	Cell-Specific CD Marker	Primary Antibody	Antigen Retrieval Method
T-Cell	CD3	Monoclonal Mouse anti- human CD3	Sodium Citrate Buffer (pH 6.0) Microwave
Macrophage	CD68	Monoclonal Mouse anti- human CD68	Sodium Citrate Buffer (pH 6.0) Microwave
Cathepsin G	Cathepsin G	Sheep polyclonal anti- human Cathepsin G	Sodium Citrate Buffer (pH 6.0) Microwave
CCL19	CCL19	Monoclonal Mouse anti- human CCL19	Sodium Citrate Buffer (pH 6.0) Microwave

Table 8.2 Primary Antibodies Used During Immunohistochemistry

Sections underwent antigen retrieval using 100mM sodium citrate buffer (pH 6). Tissues were blocked using serum-free blocking agent and stained for identified proteins (R&D/DAKO labs). Primary antibodies were detected using biotinylated anti-rat secondary antibodies (R&D). Secondary antibodies were detected using streptavidin-alkaline phosphatase ABC system. Diaminobenzidine (DAB) was used as the chromagen. The sections were counterstained with hematoxylin. Omission of the primary antibody served as a negative control. Tonsillar tissue was used as a positive control. Protein staining for antibodies was scored as absent or present.

8.11 Statistical Analysis

Microarray study analysis was performed according to current guidelines using Illumina GenomeStudio (v1) software. QRT-PCR-validated mRNA expression of selected genes between stable and unstable groups was assessed using paired two-tailed Student's t-test. ELISA results were analysed using a Mann Whitney U Test. T-Test and χ^2 was used to compare baseline characteristics between patient groups. In order to create a predictive model using clinical, ultrasound and histological criteria univariate and then a stepwise multivariate analysis was performed to identify independent associations between patient groups and histological and ultrasound features identified. A P value of <0.05 were deemed significant. Except where stated all statistical analyses were performed using SPSS, version 18.

8.12 <u>Summary of Study</u>

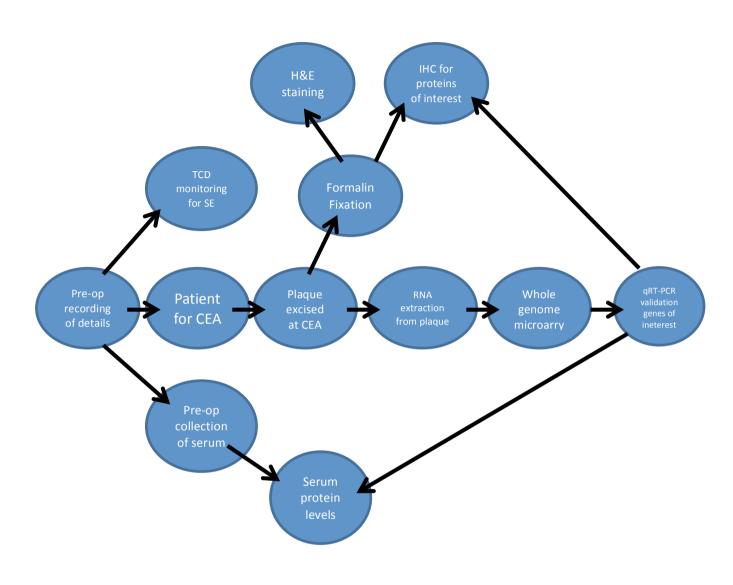


Figure 8.17 Flow diagram of study

Chapter 9 Spontaneous Cerebral Embolisation in Asymptomatic

and Recently Symptomatic Patients with TIA/Minor stroke

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<u>Chapter 9. Spontaneous Cerebral Embolisation in Asymptomatic</u> <u>and Recently Symptomatic Patients with TIA/Minor stroke</u>

9.1 Introduction

Embolisation of atherothrombotic material from an extracranial carotid stenosis is responsible for up to one third of all strokes (Henry JM, 2000). Recent evidence suggests that the highest risk of recurrent stroke (after the index transient ischaemic attack (TIA) or minor stroke) is during the first seven days (Giles MF, 2007). This has been the impetus behind the move towards expedited/emergency carotid endarterectomy (CEA).

The detection of emboli in the ipsilateral middle cerebral artery (MCA) using Transcranial Doppler (TCD) has been associated with a nine-fold increase in stroke risk (Valton L, 1998) However, to date few large scale studies have evaluated the prevalence of spontaneous embolisation in the acute and hyperacute period after onset of a TIA or minor stroke and then correlated this with the risk of early recurrent cerebrovascular symptoms. In our unit in Leicester, following a reconfiguration of vascular surgery and stroke medicine services in October 2008, a rapid access TIA service was established operating everyday of the year as described in Chapter 6 (Salem MK, 2010). This service has ensured that over 78% of patients referred through the TIA clinic underwent CEA within 14 days of the index event, and 43% within 7 days.

The hypothesis underlying the current study was that TCD-detected spontaneous embolisation would be more prevalent in patients who were acutely symptomatic and that these patients would have a higher rate of early recurrent cerebral ischaemic events. This would further support the drive towards expedited CEA in acutely symptomatic patients with carotid artery disease.

The aims of this study and chapter was to correlate patterns of spontaneous embolisation (SE) with the risk of suffering recurrent cerebral ischaemic events (new onset Stroke, TIA, or episode of Amaurosis Fugax) following admission to hospital and prior to undergoing surgery

The hypothesis is that there are differences in the rates of spontaneous embolisation as detected by Transcranial Doppler (TCD) monitoring of the middle cerebral artery in patients in the hyperacute period after onset of TIA/Minor stroke and that there is subsequent difference in the rates of early recurrent ischaemic events in this cohort

9.2 Materials and Methods

9.2.1 Patient Selection

Patients undergoing CEA between August 2008 and March 2010 (including asymptomatic patients) were included in the study after giving informed consent. Patients were included in the study if they were undergoing a carotid endarterectomy for a critical stenosis (>50% NASCET) of the carotid artery during the study period. Patients were excluded if they were found to have any other cause of embolic disease e.g. atrial fibrillation, or if there was not an accessible cranial window allowing transcranial Doppler monitoring of the ipsilateral middle cerebral artery. One hundred and seventy two patients underwent CEA during the study

period. Sixteen patients were excluded (no temporal window for TCD) leaving 156 for inclusion in the study (symptomatic n=123, asymptomatic n=33).

9.2.2 Assessing Symptomatic Status and Performing TCD Monitoring

Patients were defined as being clinically symptomatic if they reported a stroke, TIA or amaurosis fugax in the territory of the ipsilateral stenosed internal carotid artery within the preceding 6 months. As soon as possible after admission (same day if admitted before 5pm or next day if admitted after 5pm) in symptomatic patients or at the time of pre-assessment for asymptomatic patients, a 30 minute period of TCD monitoring was performed. See section 8.3.3. Briefly using a commercially available TCD machine (Sonara TCD System from Viasys Healthcare) with the 2-MHz head probe held in place by an external fixation device. Insonation of the ipsilateral MCA was achieved via the temporal acoustic window at a depth of 50 to 60mm using a 2-MHz pulsed Doppler transducer to detect true emboli. Patients were classed as having evidence of SE if one or more true emboli were detected in the 30 minute monitoring period. Each TCD recording was made by a trained vascular technologist who was blinded to data regarding timing of the clinical event in symptomatic patients. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) (v16).

9.2.3 Best Medical Therapy

In the 33 asymptomatic patients, aspirin and statin therapy was started in the outpatient clinic. In the 123 recently symptomatic patients, aspirin and statin therapy was started as soon as possible after onset of symptoms. All patients seen in the TIA clinic received 300mg aspirin and 40mg simvastatin, in addition to other appropriate secondary prevention therapy, and these were then continued throughout the pre-operative period, although the aspirin dose was reduced to 75mg. All patients received 75mg clopidogrel the night before surgery in addition to their regular aspirin. Patients who were not referred through the Leicester TIA clinic (i.e.) from outside of Leicestershire, and who had not been started on aspirin and statin therapy from the referral centre (n=7) were commenced on aspirin (75mg) and simvastatin (40mg) on admission to the vascular surgery unit.

9.2.4 Recurrent Cerebral Ischaemic Event

A recurrent cerebral ischaemic event was defined as any patient suffering a stroke, TIA or episode of amaurosis fugax between admission to the vascular surgery unit and time of surgery. Patients referred from the TIA clinic received a routine neurological examination at 30 days post-operatively by a consultant stroke physician. Any patient suspected of having suffered a pre/intra/post-procedural stroke underwent a CT scan, extra-cranial Duplex ultrasound examination and intracranial transcranial Doppler ultrasound assessment as soon as possible after onset of symptoms and was reviewed by a stroke physician. All patients were seen by a consultant vascular surgeon prior to surgery on a daily ward round and a repeat neurological examination was performed on patients found to have SE or suspected of having a recurrent cerebral ischaemic event.

9.3 <u>Results</u>

Between August 2008 and March 2010, 156 patients undergoing CEA with an accessible TCD window were entered prospectively into the study. One hundred and twenty three were recently symptomatic {stroke n=29 (24%), TIA n=73 (59%), amaurosis fugax n=21 (17%)}. Thirty three were neurologically asymptomatic. Patient demographics are detailed in Table 13.1. There was no significant difference in co-morbidities or gender between symptomatic and asymptomatic patients. In symptomatic patients the median time to surgery (from the index event) was 12 days (95% CI 10 days to 14 days) and was 8 days (95% CI 6 days to 9 days) from the most recent clinical event to CEA.

Overall, in all symptomatic patients (n=123) the median time to surgery from admission to undergoing CEA was 2 days (95% CI 1-4 days). In symptomatic patients with recurrent events (n=18) the median time to surgery from admission was 1 day (95% CI 1-2 days). In symptomatic patients without a recurrent cerebral ischaemic event (n=105) the median time to surgery from admission was 2 days (95% CI 1-7).

Patient Demographics	Symptomatic (n=123)	Asymptomatic (n=33)	P Value
Male	87 (71%)	24 (73%)	0.50
Age (median)	72 (37- 94)	66 (52-87)	0.02
Hypertension	83 (67%)	23 (70%)	0.50
Hypercholesterolaemia	97 (79%)	23 (70%)	0.90
Diabetes Mellitus	25 (20%)	9 (27%)	0.90
Ischaemic Heart Disease	26 (21%)	7 (21%)	0.80
Ex/Current Smoker	91 (74%)	28 (85%)	0.10
On Anti-platelet therapy following admission	123 (100%)	33 (100%)	>0.99
Anti-platelet therapy pre-admission	116 (94.3%)	31 (94%)	>0.99
On Statin therapy following admission	123 (100%)	33 (100%)	>0.99
On Statin therapy pre- admission	116 (94.3%)	31 (94%)	>0.99

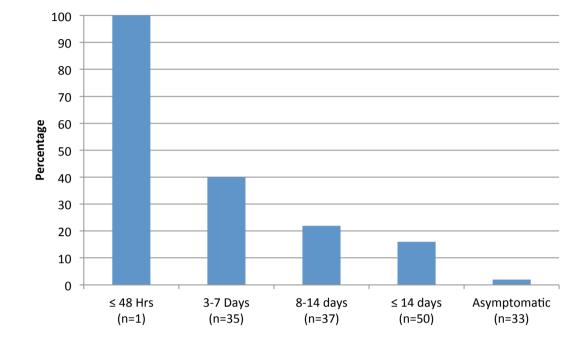
Table 9.1 Patient Demographics

This study found that symptomatic patients had a significantly higher prevalence of SE than asymptomatic patients (31/123 (25%) vs. 2/33 (6%)) (OR 5.2 95% CI 1.2 – 23.1) (P=0.02).

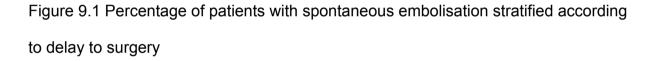
When analysed according to whether surgery was performed less than 7 days vs. More than 14 days of the index event, the prevalence of SE was significantly higher in those treated within the earlier time frame {15/36(42%) vs. 8/50(16%) (OR 3.8 95% CI 1.4 – 10.2) (P=0.01)}.

Spontaneous embolisation was detected in 1/1 (100%), 14/35 (40%), 8/37 (22%) and in 8/50 (16%) of patients presenting within 48 hours, 3-7 days, 8-14 days and greater than 14 days

respectively from the index clinical event. Spontaneous embolisation occurred in only 2 (6%)



of asymptomatic patients. See Figure 9.1



Out of 31 patients found to be spontaneously embolising, seven (22.6%) reported recurrent cerebrovascular events following admission as opposed to 11/92 patients (11.9%) who had no evidence of spontaneous embolisation (OR 2.2 (95% CI 0.8-6.1))(p=0.2). In 30 minutes of TCD monitoring, the mean rate of SE was 4.5 in the cohort that went on to have a recurrent cerebral ischaemic event compared to 3.3 in the cohort that did not (P=0.376).

Figure 9.2 details the degree of stenosis and rates of spontaneous embolisation in patients who were symptomatic and asymptomatic. In patients who were recently symptomatic, figure 9.2 also details the median delay from index symptom to CEA and the median delay from the most recent symptom to undergoing CEA. These data were then correlated with the risk of suffering a recurrent cerebral ischaemic event following admission but before undergoing

surgery.

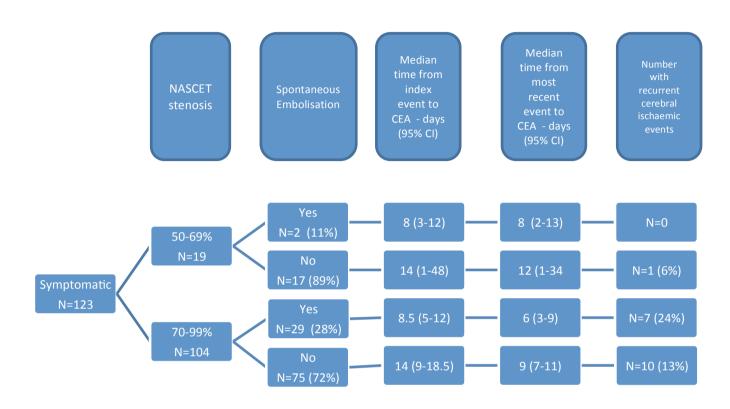


Figure 9.2 Relationship between degree of carotid stenosis determined by duplex ultrasound, presence of spontaneous emboli and timing of emboli to clinical event in recently symptomatic patients

Overall, 18/123 symptomatic patients (14.6%) suffered recurrent cerebral ischaemic events between the time of admission and undergoing surgery, though none of these were strokes and the secondary (recurrent event) was noted to be the same as the primary event (Transient Ischaemic Attack n=14, Amaurosis Fugax n=4). Patients reported events occurring prior to, during and after TCD monitoring. Patient demographics are detailed in Table 9.2 for those recently symptomatic patients that had recurrent cerebral ischaemic events after transfer to the vascular surgery unit. There was no significant difference in co-morbidities, age or gender between symptomatic patients that suffered a recurrent cerebral ischaemic event and those that did not suffer a recurrent cerebral ischaemic event after admission. Spontaneous embolisation was detected in 3/7 (43%) symptomatic patients who were commenced on aspirin and statin on admission but prior to TCD monitoring compared to 28/116symptomatic patients (24%) who were on aspirin and statin therapy prior to admission (OR 2.3 (95% CI 0.5 - 11.1)(P=0.4). In the cohort of patients who were commenced on aspirin and statin on admission and found to have TCD evidence of SE, no recurrent cerebral ischaemic events occurred.

Patient Demographics	Recurrent Cerebral Ischaemic Event (n=18)	No recurrent Cerebral Ischaemic Event (n=105)	P-Value
Male	14 (78%)	73 (70%)	0.6
Age (median years)	70 (52-87)	73 (37-94)	0.9
Hypertensive	13 (72%)	71 (68%)	0.8
Hypercholesterolaemia	16 (89%)	83 (79%)	0.5
Diabetes Mellitus	2 (11%)	23 (22%)	0.4
Ischaemic Heart Disease	7 (39%)	21 (20%)	0.1
Ex/Current Smoker	13 (72%)	77 (73%)	>0.99
On Anti-platelet therapy following admission	18 (100%)	105 (100%)	>0.99
Anti-platelet therapy pre-admission	18 (100%)	98 (93%)	0.6
On Statin therapy following admission	18 (100%)	105 (100%)	>0.99
On Statin therapy pre- admission	18 (100%)	98 (93%)	0.6

Table 9.2 Demographics for Symptomatic Patients Presenting with/without RecurrentCerebral Ischaemic Events after Admission to the Vascular Surgery Unit

When all 123 recently symptomatic patients with a 50-99% stenosis are combined, the presence of SE was associated with a non-significant two fold increase in the risk of recurrent cerebrovascular events {OR 2.2 (95% CI 0.8 - 6.1)} (P=0.2 Fisher's Exact test).

Figure 9.3 correlates the prevalence of SE and its relationship with recurrent cerebral ischaemic events relative to delay to surgery from the index event in recently symptomatic patients with a 50-99% (n=123) stenosis.

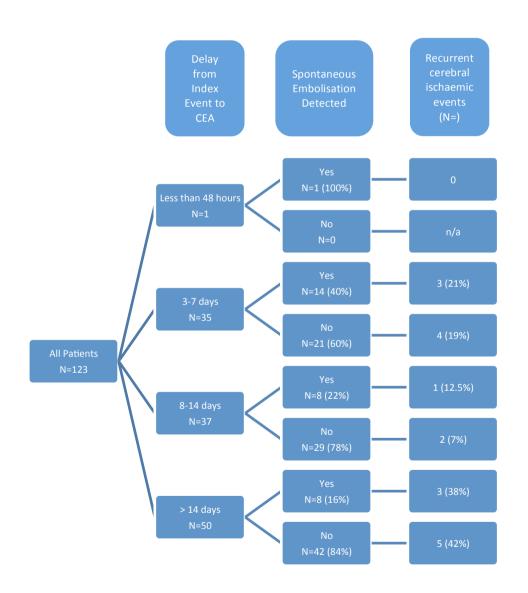


Figure 9.3 Spontaneous embolisation in relation to time from index presenting symptom to CEA in recently symptomatic patients with a 50-99% Stenosis

9.4 Discussion

This is one of the first studies to correlate the prevalence of SE in the hyperacute period after suffering a TIA or minor stroke and to then correlate patterns of SE with recurrent cerebral ischaemic events.

This study observed that symptomatic patients had a significantly higher prevalence of SE than asymptomatic patients (P=0.02). In addition, this study observed that the prevalence of SE was closely related to the recency of both the index clinical symptom and the most recent clinical event. There was a significantly higher incidence of SE in patients treated within 7 days compared to those treated after 14 days from the index event (P=0.01). It should be noted that patients found to have TCD evidence of SE were prioritised for urgent surgery either on the next available CEA list (twice weekly) or on the emergency list if space was not available on the next available CEA list.

The third finding of this study was the relationship between SE, delays to surgery and the risk of recurrent cerebral ischaemic events prior to CEA. Overall, 18/123 symptomatic patients (14.6%) suffered recurrent cerebral ischaemic events between the time of admission and undergoing surgery, though none of these were strokes (Transient Ischaemic Attack = 14, Amaurosis Fugax = 4). The prevalence of recurrent events was higher in patients with 70-99% stenoses {17/104 (16%)} than in those with 50-69% stenoses {1/19 (5.3%)} (p=0.3).

Overall, the prevalence of recurrent cerebral ischaemic events was twice as high in patients with TCD evidence of SE $\{7/31 (23\%)\}$ than in those with no evidence of SE $\{11/92 (11.9\%)\}$ (OR 2.15 (95% CI 0.75 – 6.1)(P=0.2) but this did not reach statistical significance.

So how should these results be interpreted? This study has shown a non-significant trend towards SE being predictive of recurrent cerebral ischaemic events, especially in patients with a 70-99% stenosis. It may be that a significant result might have become evident with greater numbers which is one of the study limitations, but the fact that only 30 minutes of pre-operative TCD monitoring was performed may have missed patients who were truly embolising (i.e. a type II error).

Reducing delays in admission and therefore surgery after the index clinical event remains an important benchmark for secondary stroke prevention. We have previously described in Chapter 6 (Salem MK, 2010) that after the introduction of a rapid access TIA clinic in October 2008, more than 43% of patients in our unit underwent surgery within 7 days of their index clinical event. The findings and clinical impact of this study is limited by the delay in admission from index clinical event. Enhancing patient education, and improving links between primary care and secondary care, and between stroke physicians and vascular surgeons is ensuring this delay is reduced.

Although no strokes occurred prior to surgery in this particular series, these findings are otherwise entirely consistent with natural history studies demonstrating the higher risk of recurrent stroke and TIA in the hyperacute period after suffering a TIA (Giles MF, 2007). The landmark evidence for intervening in patients with extra cranial symptomatic carotid artery disease came from The European Carotid Surgery trial (ECST, 1991) and The North American Symptomatic Carotid Endarterectomy Trial (NASCET, 1991), which demonstrated benefit for treating patients who reported carotid territory symptoms in the preceding six months and who had a NASCET 50-99% stenosis of the ipsilateral carotid artery (ECST 70-99%).

Conventional teaching has taught that the risk of stroke after a TIA is only about 1-2% at seven days and 2-4% at 28 days (Hankey GJ, 1996);(Wolf PA, 1999). The impetus for undertaking more rapid intervention came from two recently published meta-analyses by Giles and Wu. These meta-analyses showed that the risk of stroke after a TIA was highest in the first seven days. The meta-analyses by Giles and Wu (which were based on natural history studies that utilised 'face to face' follow up of the entire population of TIA patients) demonstrated that at 48 hours the risk of stroke increased to 6.7%, and was 10% at seven days. This represents a fourfold increase in the early risk of stroke after a patient presented with their index TIA when compared to conventional teaching. Analysis of the Carotid Endarterectomy Triallists Collaboration (CETC) (Rothwell PM, 2004) data has allowed researchers to model the number of strokes prevented per 1000 CEAs at 5 years in relation to the procedural risk and delay to surgery. The evidence from this analysis suggest that surgical intervention within 14 days with a procedural risk of less than 10% would still prevent more strokes at 5 years than if surgery was delayed by four or more weeks and then performed with a procedural risk of 0%.

For these reasons, there is a drive towards urgent/emergency treatment of TIA patients. While guidelines from the European Vascular Surgery Society (ESVS Guidelines, 2009) and the UK National Institute for Clinical Excellence (NICE, 2008) now recommend that CEA should be performed within 14 days of onset of symptoms unless contra-indicated, the ultimate goal of the UK National Stroke Strategy (2008) is that CEA should be regarded as an emergency procedure and should be performed within 48 hours of a TIA or minor stroke

The use of pre-operative TCD monitoring and detection of SE allows for individualised patient management, in order to prioritise treatment for those patients at highest risk of a recurrent cerebral ischaemic event. The study serves as a pilot study which can now lead to a powered study with the possibility of two-time point TCD measurements to determine intrapatient variation in rate of SE in time.

In summary, therefore, this study lends further support to natural history studies which suggest that the risk of recurrent cerebral ischaemic events is highest in the first few days after onset of symptoms and the higher rates of SE detected during these time periods support on-going thrombo-embolisation (despite anti-platelet therapy) being the principle cause of these recurrent events. The hypothesis for this study can thus be accepted. These data support the move towards expedited CEA in patients who present with TIA or minor stroke

Chapter 10. Patients with Recurrent Ischaemic Events from

Carotid Artery Disease Have a Large Lipid Core and Low GSM

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<u>Chapter 10. Patients with Recurrent Ischaemic Events from</u> <u>Carotid Artery Disease Have a Large Lipid Core and Low GSM</u>

10.1 Introduction

Over the last five years, there has been a drive towards performing expedited carotid endarterectomy (CEA) within the hyperacute period after onset of symptoms. This is because the risk of recurrent stroke is highest within the first two weeks after onset of symptoms (Giles MF, 2007) and evidence suggests that delays beyond this time period reduce the cumulative benefit conferred by surgery. We have described in Chapter 6 that it is possible to reconfigure the entire treatment pathway for TIA/minor stroke patients so that they can undergo expedited surgery without incurring a significant increase in the procedural risk (Salem MK, 2010) The main changes in practice (compared to previous) were that patients were rapidly triaged for clinic attendance through their ABCD2 score, Aspirin and Statin therapy was physically started in the TIA Clinic (as recommended by the EXPRESS Study (Rothwell PM, 2007)) and patients with an ipsilateral 50-99% stenosis were transferred directly from the TIA Clinic to the Vascular Surgery Unit for urgent surgery.

One consequence of this move towards expedited surgery was that the Unit encountered a greater proportion of patients suffering recurrent cerebral ischaemic events following admission and before undergoing surgery, suggesting that they had highly unstable plaques. Previous studies have identified that ultrasound analysis can detect differences in plaque morphology and can be correlated with symptomatic status (Reilly LM, 1983);(Biasi GM, 1998).

The aim of this chapter was to determine whether the histology or ultrasonographic features in these patients with highly 'unstable' plaques somehow differed from other 'recently symptomatic patients' awaiting surgery.

The hypothesis is that symptomatic patients who have recurrent ischaemic events have differences in their plaque histology and/or ultrasound appearance.

10.2 Methods

10.2.1 Patient Selection

All recently symptomatic patients with a >50% NASCET extra-cranial carotid artery stenosis who were admitted to the Leicester Vascular Surgery unit for CEA were considered for inclusion. All had been referred via a Stroke Physician (most via the rapid access TIA Clinic which was established in October 2008), although a relative minority were referred some time after the index event. This was usually because the patient presented late to the referring Stroke Physician.

10.2.2 Best Medical Therapy and Surgery

In the TIA Clinic, the consultant Stroke Physician took responsibility for optimising risk factors. Each patient was commenced on statin therapy (Simvastatin 40mg daily), antiplatelet therapy (300mg loading dose of Aspirin plus 200mg Dipyridamole followed by Aspirin 75mg daily, in addition 75mg clopidogrel was given the night before surgery) and antihypertensive therapy as appropriate. Carotid surgery was performed as soon as possible following

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admission, provided there were no contra-indications. If a patient suffered a recurrent ischaemic event, every effort was made to perform CEA as soon as possible thereafter.

10.2.3 Duplex Imaging.

Carotid Duplex Ultrasound imaging was performed in the Rapid Access TIA clinic and then again following admission to the Vascular Unit in accordance with HTA guidelines (Wardlaw JM, 2006). All Duplex examinations were undertaken by experienced ultrasonographers and stenosis severity was measured using consensus criteria consistent with the NASCET mode of measurement. The second duplex image taken after admission to the Vascular Surgery Unit was used for analysis. Enhanced, normalised and non-normalised images were used for blinded off-line analysis by a single investigator (AN). For full details see section 8.5

10.2.3.1 Plaque Analysis and measurements of GSM and JBA

Using the "Plaque Texture Analysis software" (Iconsoft International Ltd, Greenford, London). Normalisation was performed using the "image normalization" module. See Figure 14.1 below. Parameters measured using the Iconsoft Plaque Texture Analysis Software included 1. Juxta-Luminal Black Area; 2. Discrete White Areas; 3. Type of Plaque; 4. Plaque area and percentage area and 5. GSM. The definitions and reproducibility of texture features used in this study are given below.

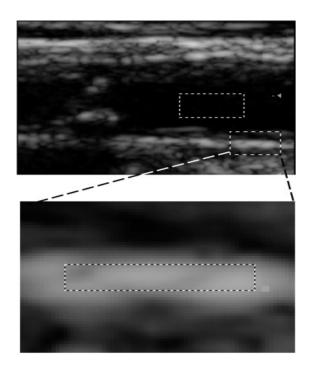


Figure 10.1 Image normalisation using a sample of 'blood' free from noise and a sample from the inner two-fourths of the brightest part of the adventitia adjacent to the plaque. Courtesy of A. Nicolaides.

1. Gray Scale Median (GSM) (see Figures 10.2 and 10.3)

This was the median of the gray values of all the pixels in the plaque image.

2. Plaque Type (see Figures 10.2 and 10.3)

Plaque types were scored automatically by the software according to a modified Geroulakos

classification.

- **Type 1.** Uniformly echolucent (black): < 15% of the pixels in the plaque area were occupied by pixels with gray scale values > 25.
- **Type 2.** Mainly echolucent: pixels with gray scale values > 25 occupy 15-50% of the plaque area.
- **Type 3.** Mainly echogenic: pixels with gray scale values > 25 occupy 50-85% of the plaque area.

• **Type 4 or 5.** Uniformly echogenic: pixels with gray scale values > 25 occupy > 85% of the plaque area.

3. Plaque area (see Figures 10.2 and 10.3)

This was calculated by the software using the distance scale on the side of the image frame for calibration and the plaque area outlined by the operator. It was expressed in mm²

Discrete White Areas (DWA) (see Figures 10.2 and 10.3)

The presence of DWA defined as areas with pixels having gray scale values >124 (colored red by the software for easy visual identification) not producing acoustic shadowing in plaque types 1-3. DWAs which are non calcified white areas (absence of acoustic shadow) within black areas have been previously associated with development of symptoms (Carra G, 2003).

Juxtaluminal Black Areas (see Figures 10.2 and 10.3)

Juxtaluminal black area without visible echogenic cap (JBA). The largest juxtaluminal black area of the contoured image (i.e. area with pixels having a gray-scale less than 25) without a visible echogenic cap (i.e. pixels with gray-scale value equal or higher than 25) was outlined by the mouse and expressed as mm^2 . This was automatically calculated by the software using the on-screen mm scale from each ultrasonic image. A JBA >6mm² was defined as being large.

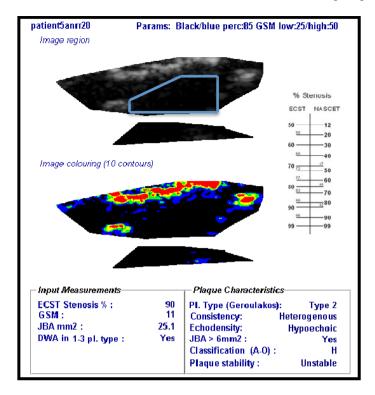


Figure 10.2. 'Unstable' plaque detected on ultrasound analysis. Juxta luminal black area outlined in blue. DWAs are bright red areas within the black lumen.

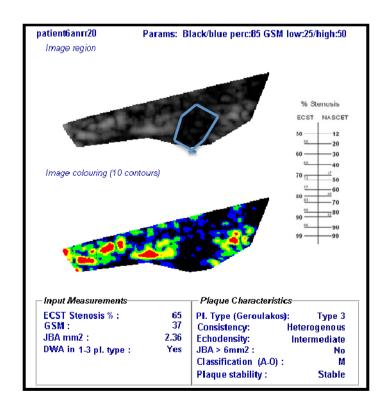


Figure 10.3. 'Stable' Plaque features detected on ultrasound analysis. Juxta luminal black area outlined in blue. DWAs are bright red areas within the black lumen.

A summary of the ultrasound findings that the Iconsoft software provides is given in table 10.1. This illustrates how to differentiate stable and unstable for each feature.

Ultrasound Feature	'Stable'	'Unstable'
GSM	>25	<25
Juxta-luminal Black Area	<6mm ²	>6mm ²
Discrete White Areas	No	Yes
Plaque Type	3&4	1&2
Plaque Area	<80mm ²	>80mm ²

Table 10.1 Ultrasound scoring based on Iconsoft Plaque Texture Analysis Software.

10.2.4 Carotid Endarterectomy

Carotid surgery was performed under general anaesthesia with routine patching, routine shunting, systemic heparinisation (unfractionated) and distal intimal tacking sutures as described in section 8.3.4

10.2.5 Recurrent Clinical Event

A recurrent cerebral ischaemic event was defined as any further episode of stroke, TIA or amaurosis fugax between the time of admission to the Vascular Surgery Unit and undergoing surgery. Any patient suffering a recurrent event underwent a repeat CT scan, extra-cranial Duplex ultrasound examination and transcranial Doppler (TCD) monitoring to exclude ongoing embolisation.

10.2.6 Histopathology

As described in section 8.10, plaques harvested during CEA were divided longitudinally and randomly one half fixed immediately in formalin for 24 hours and then paraffin embedded. A 5µm transverse section was taken from the paraffin block and stained using haematoxylin and eosin.

Histological specimens were analysed by two independent Histopathologists (KW, DM) who were blinded to the clinical and ultrasound findings.

10.2.6.1 Histological Features

For each plaque, a semi-quantitative 3 or 4 point score (Table 8.1) was assigned to show the presence and/or amount of features of plaque instability based on the AHA histological classification of advanced atherosclerotic lesions (Stary HC, 1995) and a well validated previously published scoring system (Lovett JK, 2004). Features analysed included; intraplaque haemorrhage, haemosiderin deposition, surface thrombus, lipid core size, nodular calcification, proportion of fibrous tissue, acute and chronic plaque inflammatory infiltrate, acute and chronic cap inflammatory infiltrate, neovascularity, amount of foam cells, and evidence of cap rupture.

10.2.7 Statistical Analysis

T-Test and χ^2 was used to compare baseline characteristics between patients with recurrent events and those without. A stepwise multivariate analysis was performed to identify independent associations between patients with recurrent events and histological and ultrasound features identified above. Statistical analysis was performed using SPSS (v16).

10.3 <u>Results</u>

One hundred and fifty eight patients were included in the study. All had complete histological data, however, 13 ultrasound images from the early part of the study were found to be unsuitable for computerised analysis and 12 sets of images were lost during transfer from the mobile scanner to the vascular studies unit. This left 133 duplex ultrasound images for direct comparison against histological features.

Seventy five patients (47%) underwent surgery within seven days of suffering their most recent clinical event, 43 (27%) within 8-14 days, 17 (11%) within 15-28 days, while >29 days had elapsed in 23 patients (14%). This, therefore, provided a broad spectrum of patients with regard to delays to surgery ranging from the hyperacute period (<14 days, n=118), intermediate (15-28 days, n=17) and late (>29 days, n=23). The median delay from admission to imaging and undergoing surgery in patients with recurrent events was 1 day (0-4) in those with no recurrent events prior to surgery.

Twenty patients suffered recurrent cerebral ischaemic events after admission to the vascular unit; stroke n=1 (5%); TIA n=16 (80%) and Amaurosis Fugax n=3 (15%). All recurrent events were the same as the presenting event. Baseline patient characteristics are listed in Table 10.2.

Chapter 10. Patients with Recurrent Ischaemic Events From Carotid Artery Disease Have a Large Lipid Core and Low GSM

Patient Demographics	No Recurrent Event (n=138)	Recurrent Event (n=20)	P Value
Male	97 (70%)	15 (75%)	0.60
Age (median years)	73 (37-94)	70 (52-87)	0.90
Hypertensive	94 (68%)	14 (70%)	0.80
Hypercholesterolaemia	109 (79%)	18 (90%)	0.50
Diabetes Mellitus	23 (30%)	2 (10%)	0.40
Ischaemic Heart Disease	21 (27.6%)	8 (40%)	0.10
Ex/Current Smoker	101 (73%)	14 (70%)	>0.99
On Anti-platelet therapy following admission	105 (100%)	20 (100%)	>0.99
Anti-platelet therapy pre-admission	132 (96%)	20 (100%)	0.60
On Statin therapy following admission	138 (100%)	20 (100%)	>0.99
On Statin therapy pre- admission	132 (96%)	20 (100%)	0.60
Median Time from Index Event to Undergoing Surgery	14 days (2-180)	11 days (3-35)	0.08

Table 10.2 Demographics for symptomatic patients presenting with/without recurrent cerebral ischaemic events after admission to the vascular surgery unit

Table 10.3 shows the comparison between duplex imaging features in patients with recurrent symptoms and those without symptoms. A plaque area $>80 \text{mm}^2$ was more common in patients with recurrent symptoms, but this did not reach statistical significance. Discrete white areas were found equally in both groups. Univariate analysis of ultrasound based criteria showed type 1/2 plaque (OR 5.79 95% CI 1.8-18.5; P=0.005), an echolucent plaque (GSM <25) (OR 4.68, 95% CI 1.58 to 13.8, P=0.03) and positive juxta-luminal black area (OR 3.56, 95% CI 1.1 to 11.4, P=0.05) were significantly more common in patients with recurrent events as opposed to those patients without recurrent events.

Ultrasound Feature	No Recurrent Event n=115 (86.4%)		Recurrent Ev	P Value	
GSM (<25)	25	21.7%	9	50%	0.03
Juxta-luminal Black Area (>6mm ²)	33	37%	11	61.1%	0.049
Discrete White Areas (Yes)	66	57.3%	10	55.5%	>0.99
Plaque Type (1-2)	23	25.8%	10	55.5%	0.005
Plaque Area (>80mm ²)	43	37.3%	8	44.4%	0.6

Table 10.3 Ultrasound plaque features present in plaques from patients with recurrent events Vs non- recurrent events

Table 10.4 shows the comparison between histological features in patients with recurrent symptoms and those with no recurrent symptoms. Features including any haemorrhage, any thrombus, chronic plaque inflammation, chronic cap inflammation, acute plaque inflammation and cap rupture were more prevalent in the recurrent symptomatic group, but did not reach statistical significance. Interestingly, predominantly fibrous tissue, acute cap

inflammation and marked vascularity were more common in the *non-recurrent* symptomatic group. Univariate analysis of histological features showed that a large lipid core was significantly more common in patients with recurrent events than the cohort without recurrent events (OR 10.8, 95% CI 1.4 to 83, P=0.004).

Histological Feature	No Recurrent Event n=138 (87.4%)			rent Event (12.6%)	P Value
Any Haemorrhage	92	66.6%	16	80%	0.3
Any Thrombus	47	34%	9	45%	0.45
Large Lipid Core	89	64.4%	19	95%	0.002
Predominant Fibrous Tissue	20	14.4%	0	0%	0.07
Chronic Plaque Inflammation	118	85.5%	19	95%	0.47
Chronic Cap Inflammation	58	42%	11	55%	0.33
Acute Plaque Inflammation	9	6.5%	2	10%	0.63
Acute Cap Inflammation	9	6.5%	1	5%	>0.99
Many Foam Cells	63	45.6%	8	40%	0.81
Marked Vascularity	115	83.3%	16	80%	0.75
Cap Rupture	66	47.8%	11	55%	0.63

Table 10.4 Histological features present in plaques from patients with recurrent events vs. non- recurrent events

In a multivariate stepwise analysis of histological features and ultrasound features only a large lipid core (OR 4.00, 95% CI 1.07 to 14.83, P=0.042) and low GSM (OR 6.21, 95% CI 1.86 to 20.4, P=0.003) were independently predictive of an increased risk of recurrent events.

10.4 Discussion

Previous studies have correlated plaque histology with delays from onset of symptoms to surgery (Redgrave JN, 2006) but few have included sufficient numbers of patients in the hyperacute period after onset of symptoms (<7 days), while none have evaluated histological and ultrasonographic features in those suffering recurrent symptoms between admission and undergoing surgery.

Recent meta-analyses have suggested that the risk of recurrent stroke is highest in the first few days after onset of symptoms (Giles MF, 2007);(Wu CM, 2007) and much higher than was previously thought (Hankey GJ, 1996);(Wolf PA, 1999). These data refer to 'all TIA patients', but Ois et al (2009) have suggested that the early risk of recurrent stroke may be even higher in patients with significant ipsilateral 50-99% carotid stenoses. In the current series, 75% of patients underwent surgery within 14 days of their most recent event and 13% of patients suffered recurrent ischaemic events before they could undergo surgery, suggesting that the early period after onset of symptoms remains a high risk time. Interestingly, all had been started on statin and antiplatelet therapy in the TIA Clinic and the median delay from admission to CEA in patients with recurrent symptoms was 1 day (0-12), while the delay for those without recurrent symptoms was also 1 day (0-4). All but one of the recurrent events were TIAs or Amaurosis Fugax and 70% had evidence of spontaneous embolisation compared to 38% of those with no recurrent symptoms.

If we assume that the presence of recurrent symptoms following admission (despite antiplatelet and statin therapy) is a marker of a highly unstable plaque, it is interesting to observe the histological and ultrasonographic correlates, both of which were scored independently without any knowledge of clinical demographics. On univariate analysis (Table 14.4), the plaques of patients with recurrent symptoms after admission showed that the only statistically significant histological feature was the presence of a large lipid core (OR 10.8, 95% CI 1.4 to 83, P=0.004). The following features were not associated with an increased risk of recurrent symptoms; any haemorrhage, any thrombus, predominant fibrous tissue, chronic plaque inflammation, chronic cap inflammation, acute plaque inflammation, acute cap inflammation, many foam cells, marked vascularity and cap rupture.

Similarly, the plaques of patients with recurrent symptoms had a significantly higher prevalence of echolucent plaque (GSM <25) (OR 4.68, 95% CI 1.58 to 13.8, P=0.03), large juxta-luminal black areas (>6mm²)(OR 3.56, 95% CI 1.1 to 11.4, P=0.05) and type 1 or 2 Plaques (OR 5.79 95% CI 1.8-18.5; P=0.005). Interestingly, the presence of discrete white areas and large plaque area (>80mm²) were not associated with an increased risk of recurrent symptoms (Table 14.4).

Following multivariate analysis, the following key histological and ultrasonographic features were associated with an increased risk of suffering recurrent cerebral ischaemic events; a large lipid core (OR 4.00, 95% CI 1.07 to 14.83, P=0.042) and low GSM (OR 6.21, 95% CI 1.86 to 20.4, P=0.003).

So how might these data influence future clinical practice? It is all very well knowing that a large lipid core correlates with an increased risk of recurrent events, but this histological

feature is of limited predictive value to the surgeon who is trying to decide upon the best management strategy. However, because Duplex ultrasound is so versatile and readily accessible, the positive correlation with TCD embolisation and computerised plaque features might facilitate changes in practice.

In those centres who already undertake expedited CEA in the majority of their patients and who manage to do so without encountering recurrent symptoms before surgery, clearly there is little that needs to be changed. However, this situation probably does not apply to the majority of vascular centres who currently struggle to see large numbers of patients quickly and then get those with significant ipsilateral stenoses processed and submitted to surgery within days of onset of symptoms. In the latter type of unit, the data from this study would suggest that if there is any likelihood of delays entering the patient pathway (i.e. no spaces on existing elective theatre lists or weekend approaching), consideration might be given to performing an emergency CEA in the small cohort of recently symptomatic patients who have evidence of a low GSM (<25), in association with either a juxtaluminal black area exceeding 6mm² on computerised plaque analysis or TCD evidence of spontaneous embolisation.

Few studies have evaluated histological and ultrasonographic features in such a large cohort of TIA/minor stroke patients who underwent surgery in the hyperacute period after onset of symptoms and none have looked specifically at characteristics associated with recurrent symptoms, making direct comparison with other studies difficult. These data do suggest, however, that in larger cohorts of patients, it may be possible to develop a scoring system (similar in principle to ABCD2 (Johnston SC, 2007)) for triaging patients based on TCD and

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computerised ultrasonographic features. If this were possible, it would enable surgeons to identify a cohort of 'ultra' high risk patients who merited emergency CEA (i.e. <24 hours), while others might be allowed to wait 48-72 hours for expedited surgery.

The hypothesis in this study that symptomatic patients who have recurrent ischaemic events have differences in their plaque histology or ultrasound appearance to those symptomatic patients that have a single event can be accepted.

Chapter 11. Features of unstable carotid plaque during and after

the hyperacute period following TIA/stroke

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<u>Chapter 11. Features of unstable carotid plaque during and after</u> <u>the hyperacute period following TIA/stroke</u>

11.1 Introduction

Natural history studies have shown that the early risk of stroke after TIA/minor stroke is significantly higher than was previously thought (Giles MF, 20070;(Wu CM, 2007) This has led to the current drive towards performing CEA in the hyperacute period after onset of symptoms. It is assumed that the higher risk of early stroke is associated with a highly unstable carotid plaque with overlying surface disruption and thrombus formation.

Previous studies (Imparato AM, 1983);(Lusby RJ, 1982);(Avril G, 1991)(Svindland A, 1988) have correlated histological features from resected carotid atherosclerotic plaques following CEA, however few have analysed plaque histology in the very early time period after onset of symptoms (e.g. first 7 days).

The aim of this study and chapter was to test the hypothesis that patients undergoing CEA within the hyperacute period after onset of symptoms would exhibit a greater prevalence of unstable histological plaque features (Stary HC, 1995) that would then diminish with time.

The hypothesis is that patients undergoing CEA within the hyperacute period after onset of symptoms would exhibit a greater prevalence of unstable histological plaque features than patients having delayed surgery

11.2 Methods

11.2.1 Patient Selection (see section 8.3)

All recently symptomatic patients with a >50% NASCET extra-cranial carotid artery stenosis who were admitted to the Leicester Vascular Surgery unit for CEA were considered for inclusion. All had been referred via a consultant Stroke Physician (mostly via the rapid access TIA Clinic which was established in October 2008). Our aim was to offer CEA as soon as possible after onset of symptoms. However, a proportion of patients were referred after 14 days had elapsed. As a baseline for comparison all asymptomatic patients undergoing CEA in our unit were also recruited into the study for comparison.

11.2.2 Carotid Endarterectomy

Carotid surgery was performed under general anaesthesia with routine patching, routine shunting, systemic heparinisation (unfractionated) and distal intimal tacking sutures. Endarterectomy was performed via a linear arteriotomy and the plaque removed with the minimum of trauma possible. (See section 8.3.4 for full details)

11.2.3 Histopathology

Plaques harvested during CEA were divided longitudinally and one half randomly fixed immediately in formalin for 24 hours and then paraffin embedded. A 5-micrometre transverse section was taken from the paraffin block and stained using haematoxylin and eosin. Histological specimens were analysed by two independent Histopathologists (KW, DM) who were blinded to the clinical data. (See section 8.10 for full details) For each plaque, a semi-quantitative 3 or 4 point score (Table 8.1) was assigned to show the presence and/or amount of features of plaque instability. Features analysed included; intraplaque haemorrhage, lipid core size, chronic plaque inflammatory infiltrate, chronic cap inflammatory infiltrate, neovascularity, amount of foam cells, and evidence of cap rupture.

11.2.4 Statistical Analysis

T-test and χ^2 was used to compare baseline characteristics between patients in each group. Statistical analysis was performed using SPSS v16 (Chicago, USA).

11.3 Results

Two hundred patients were included in the study (121 TIA patients, 38 Stroke patients, 41 Asymptomatic)

Seventy six patients (48%) underwent surgery within seven days of suffering their most recent clinical event, 43 (27%) within 8-14 days, 17 (11%) within 15-28 days, while >29 days had elapsed in 23 patients (14%). This, therefore, provided a broad spectrum of patients with regard to delays to surgery ranging from the acute period (<14 days, n=119), intermediate (15-28 days, n=17) and late (>29 days, n=23).

Demographic data for patients in the study stratified by timing and delays to surgery are shown in Table 11.1. All groups were matched for age, sex and vascular risk factors.

Chapter 11. Features of unstable carotid plaque during and after the hyperacute period following TIA/stroke

	0-7 days (n=76)	8 - 14 days (n=43)	15-28 days (n=17)	>28 days (n=23)	Asymp (n=41)	P value
Age (Median years)	75	75	72	70	67	0.76
Male	55 (73%)	27 (63%)	12 (71%)	17 (74%)	30 (73%)	0.64
Hypertension	56 (76%)	27 (63%)	12 (71%)	15 (65%)	28 (68%)	0.55
High lipids	62 (83%)	31 (72%)	12 (71%)	13 (57%)	27 (66%)	0.07
Diabetes	20 (27%)	10 (23%)	3 (18%)	3 (13%)	10 (24%)	0.54
Heart disease	13 (18%)	5 (12%)	3 (18%)	5 (22%)	10 (24%)	0.71
Ex-Smoker	35 (47%)	21 (49%)	11 (65%)	13 (57%)	25 (61%)	0.53
Current Smoker	20 (27%)	5 (12%)	6 (35%)	3 (13%)	8 (20%)	0.08
Aspirin*	64 (87%)	33 (77%)	17 (100%)	22 (96%)	40 (98%)	0.048
Statin	63 (84%)	37 (86%)	15 (88%)	19 (83%)	38 (93%)	0.95
TIA	63 (84%)	26 (60%)	10 (59%)	20 (87%)	n/a	0.003
Stroke	11 (15%)	17 (40%)	7 (41%)	3 (13%)	n/a	0.003
Single Event	32 (42%)	28 (65%)	11 (65%)	15 (65%)	n/a	0.04
Multiple Events	44 (58%)	15 (35%)	6 (35%)	8 (35%)	n/a	0.04

Table 11.1 Demographics for all symptomatic patients grouped by time delay to surgery from most recent event

*If intolerant to aspirin, patients offered clopidogrel or dipyridamole.

The number of histologically unstable features identified in each plaque stratified according to the delay to surgery from most recent ischaemic event is shown in figure 11.1. In patients in the acute group (symptoms within 14 days) 87/119 (73%) had 5 or more unstable features

detected in their plaque. In comparison in patients who underwent delayed surgery only 22/40 (55%) had 5 or more unstable features (p=0.04). As a baseline comparison only 9/41 (22%) asymptomatic patients had 5 or more unstable features compared to the 109/159 (69%) of all symptomatic patients (OR 7.8 95% CI 3.442 - 17.4546 P<0.0001)

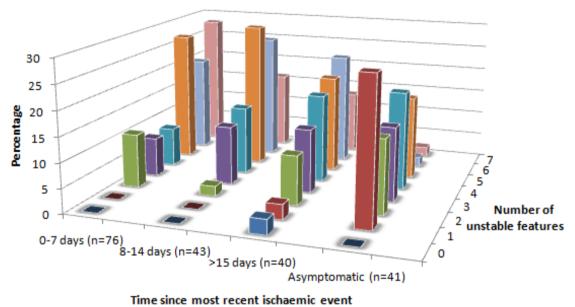


Figure 11.1 Number of unstable features in each histological plaque stratified by time from most recent event to CEA

The prevalence of each individual histologically unstable feature stratified by timing and delay to surgery are shown in Table 11.2. There is a sustained decline in the number of histologically unstable features detected in the 136 patients undergoing delayed surgery between <7 days, 8-14 days and 15-28 days. However there was then an upsurge in 6 of the7 unstable features in patients who underwent delayed surgery >28 days from the most recent event, compared to those operated on within 15-28 days from most recent event. Only chronic cap inflammation continued to show a sustained decline. Notably; a large lipid core

significantly increased from 7/17 (41%) at 9-28 days compared with 18/23 (78%) in patients undergoing surgery after >28 days had elapsed (p=0.002). As a baseline comparison, when looking at individual features in the asymptomatic group all features showed lower prevalence than in the symptomatic group.

	Haemorrhage	Lipid	Plaque Chronic	Cap Chronic	Many Foam	Marked	Cap
	macmorriage	Core	Inflammation	Inflammation	Cells	Vascular	Rupture
0-7 days (n=76)	54 (71%)	60 (79%)	68 (89%)	34 (45%)	40 (53%)	61 (80%)	42 (55%)
8 to 14 days (n=43)	30 (70%)	24 (56%)	37 (86%)	21 (49%)	17 (40%)	38 (88%)	21 (49%)
9-28 days (n=17)	10 (59%)	7 (41%)	12 (71%)	7 (41%)	4 (24%)	12 (71%)	6 (35%)
>28 days (n=23)	15 (65%)	18 (78%)	21 (91%)	7 (30%)	10 (43%)	21 (91%)	9 (39%)
Asymptomatic (n=41)	10 (24%)	22 (53%)	26 (63%)	7 (17%)	9 (22%)	31 (75%)	17 (41%)
P Value	< 0.0001	0.002	0.003	0.02	0.01	0.26	0.388

Table 11.2 Histologically Unstable Plaque Features Present in Plaques from Patients Stratified by Delay to Surgery

The prevalence of each individual histologically unstable feature stratified by timing and delay to surgery and by symptom status is shown in table 11.3. A similar trend is seen in both patients who have TIAs and strokes as their presenting symptom with a sustained decline in the number of histologically unstable features detected in patients undergoing delayed surgery between <7 days, 8-14 days and 15-28 days. However there was then an upsurge in 5 of the7 unstable features in patients who underwent delayed surgery >28 days from the most recent event, compared to those operated on within 15-28 days from most recent event in TIA and stroke patients. Only chronic cap inflammation continued to show a sustained decline in both stroke and TIA patients. In TIA patients the prevalence of cap rupture demonstrated a

non-significant trend of increasing in the delayed group. In comparison stroke patients showed a non-significant decline in the prevalence of cap rupture in the delayed surgery group

		Days since most recent ischaemic event to CEA					
Histological Feature	Event	0-7 (TIA n=65 Stroke n=11)	8 - 14 (TIA n=26 Stroke n=17)	9-28 (TIA n=10 Stroke n=7)	>28 (TIA n=18 Stroke n=5)	P-Value	
Haemorrhage	TIA	26 (40%)	7 (27%)	3 (30%)	7 (38%)	0.65	
macmorriage	Stroke	4 (36%)	9 (53%)	1 (14%)	2 (40%)	0.36	
Large Lipid	TIA	50 (77%)	16(62%)	4 (40%)	14 (78%)	0.06	
Laige Lipiu	Stroke	9 (82%)	8 (47%)	3 (43%)	4 (80%)	0.16	
Plaque	TIA	57 (88%)	22 (84%)	8 (80%)	16 (89%)	0.89	
Chronic Inflammation	Stroke	10 (91%)	15 (88%)	4 (57%)	5 (100%)	0.13	
Cap Chronic	TIA	30 (46%)	11 (42%)	4 (40%)	6 (33%)	0.8	
Inflammation	Stroke	4 (36%)	10 (59%)	3 (43%)	1 (20%)	0.4	
Many foam	TIA	33 (51%)	8 (31%)	2 (20%)	7 (39%)	0.14	
cells	Stroke	7 (64%)	9 (53%)	2 (29%)	3 (60%)	0.51	
Marked	TIA	51 (78%)	23 (88%)	6 (60%)	16 (89%)	0.19	
Vascularity	Stroke	9 (82%)	15 (88%)	6 (86%)	5 (100%)	0.78	
Can Buntura	TIA	36 (55%)	15 (58%)	4 (40%)	8 (44%)	0.66	
Cap Rupture	Stroke	5 (45%)	6 (35%)	2 (29%)	1 (20%)	0.76	

Table 11.3 Histologically Unstable Plaque Features Present in Plaques from Patients Stratified by Delay to Surgery and Symptom Type (TIA vs. Stroke)

The prevalence of each individual histologically unstable feature stratified by timing and delay to surgery and by number of events (single vs. multiple) is shown in table 11.4. In patients who had delayed presentation (>28 days from most recent event to CEA (n=23)), 15/23 (65%) had experienced just a single event, whilst 8/23 (35%) described two or more events. Notably marked neovascularity was noted to rise significantly in the delayed group in patients who had multiple events.

Histological	Number	Days sin	ce most recent	ischaemic even	t to CEA	
Feature	of events	0-7 (Single n=32 Multiple n=44)	8 to 14 (Single n=28 Multiple n=15)	9-28 (Single n=11 Multiple n=6)	>28 (Single n=15 Multiple n=8)	P-Value
Haemorrhage	Single	11 (34%)	13 (46%)	2 (18%)	7 (43%)	0.34
macmonnage	Multiple	18 (41%)	3 (20%)	2 (33%)	2 (25%)	0.08
Large Lipid	Single	22 (69%)	14 (50%)	5 (45%)	12 (80%)	0.13
Laige Lipiu	Multiple	36 (82%)	10 (67%)	2 (33%)	5 (63%)	0.07
Plaque	Single	28 (88%)	24 (86%)	8 (73%)	14 (93%)	0.5
Chronic Inflammation	Multiple	38 (86%)	13 (87%)	3 (50%)	7 (88%)	0.14
Cap Chronic	Single	15 (47%)	16 (57%)	6 (55%)	5 (33%)	0.49
Inflammation	Multiple	18 (41%)	5 (33%)	1 (17%)	2 (25%)	0.6
Many foam	Single	22 (69%)	13 (46%)	3 (27%)	5 (33%)	0.04
cells	Multiple	17 (39%)	4 (27%)	1 (17%)	5 (63%)	0.25
Marked	Single	24 (75%)	24 (86%)	10 (91%)	14 (93%)	0.34
Vascularity	Multiple	35 (80%)	14 (93%)	1 (17%)	7 (88%)	0.001
Cap Rupture	Single	18 (56%)	13 (46%)	5 (45%)	7 (47%)	0.84
	Multiple	22 (50%)	8 (53%)	1 (17%)	2 (25%)	0.25

Table 11.4 Histologically Unstable Plaque Features Present in Plaques from Patients Stratified by Delay to Surgery and Number of Events (Single vs. Multiple)

11.4 Discussion

A number of studies have correlated plaque histology with symptom status (Imparato AM, 1983);(Lusby RJ, 1982);(Avril G, 1991);(Svindland A, 1988), risk of recurrent ischaemic events (Salem MK, 2012) and more recently with delays from onset of symptoms to surgery (Peeters W, 2009);(Redgrave JN, 2006). Few, however, have included a sufficiently large number of patients undergoing CEA in the hyperacute period after onset of symptoms. Our study is, therefore, one of the first to look at standardised histologically verified unstable features in plaques taken from patients undergoing surgery at various points from the most recent ischaemic event, including a large proportion in the hyperacute period (symptoms <7 days).

In the current series, 76% of symptomatic patients underwent surgery within 14 days of their most recent event and 73% demonstrated 5 or more highly unstable histological features. This was statistically greater than the 55% of patients who underwent delayed surgery (p=0.048), adding weight to the hypothesis that the early period after onset of symptoms remains a high risk time.

To date the largest most comprehensive study of carotid plaque histology in relation to timing from clinical event was performed by Redgrave et al (2006). 526 recently symptomatic patients undergoing carotid endarterectomy had their plaques analysed for histological features using the same semi quantitative score used in this paper. Symptomatic patients were classed according to aetiology of ischaemic event; Stroke or TIA. Time since most recent event was divided according to the following periods; 0-30 days, 31-90 days, 91-180 days

and >180 days. In patients presenting with a stroke; plaque inflammation, plaque macrophages, cap rupture and unstable features showed a strong decline from the time of stroke out to 180 days. However, a similar trend was not observed in TIA patients. Overall they observed that in TIA patients' features of plaque instability persisted, suggesting a more chronic course of inflammation whilst in stroke patients the features of instability reduced with time suggesting a more acute event. In our study when we stratified for type of ischaemic event and timing from surgery we found that intraplaque haemorrhage, large lipid core, chronic plaque inflammation, marked neovascularity and many foam cells showed similar decline and then upsurge in stroke and TIA patients. Chronic cap inflammation continued to decline in both groups. Only cap rupture showed any difference between cohorts based upon type of event, whereby TIA patients in the delayed group had an upsurge in the prevalence of cap rupture whilst this was seen to continue to fall in the stroke cohort.

Peeters et al (2009) evaluated the plaques of 804 patients (Stroke=204, TIA=426, Asymptomatic=174) undergoing carotid endarterectomy and assessed plaque histology in relation to timing between most recent event and surgery. Time periods used in the study were identical to the Oxford study. Peeters showed that in patients who presented with a stroke, macrophage staining decreased nearly two-fold over time, with levels in the >180 day group similar to that observed in asymptomatic patients. They also found smooth muscle cell (SMC) levels rose over time, again reaching levels similar to asymptomatic patients in the group presenting after 180 days. Interestingly; Peeters showed that in patients presenting with a stroke and TIA, the percentage of plaques having a large lipid core increased in those patients who had the greater delay before surgery; i.e. very similar to the findings in this study.

In comparison to the two studies mentioned above our study aimed to focus on the first 30 days after an ischaemic event and to look at changes in this period. We had a relatively larger cohort of patients in the hyperacute period and smaller numbers in the following groups, however this reflects accurately the case load of patients presenting for CEA in our unit. Asymptomatic patients have been included but only for comparison as a baseline. The highly significant p trend values for each individual unstable feature shown in Table 11.2 is a reflection of including asymptomatic patients in the analysis.

As expected, the current study showed that there was a sustained decline (with time over the first 28 days) in the prevalence of intra plaque haemorrhage, large lipid core, chronic plaque inflammation, large number of foam cells, marked vascularity and cap rupture. Chronic cap inflammation showed a small increase in prevalence from the hyperacute period (45%) to the acute period (49%), before continuing to decline in those delayed between 15-28 days and >28 days. These features are what one might have expected based on previous studies.

However, all six features of plaque instability (intra plaque haemorrhage, large lipid core, chronic plaque inflammation, large number of foam cells, marked vascularity and cap rupture) which had initially shown a sustained decline in prevalence out to day 28, were then noted to increase in prevalence in patients who underwent surgery after 29 days had elapsed. This was most obvious when looking at large lipid core, where the prevalence increased from 41% to 78% prevalence (p=0.02).

I do not believe there has been a selection bias in the patients referred for delayed CEA in having more unstable features as the majority of these patients 15/23 (65%) presented with just a single event and no significant difference was seen in the type of unstable features and number of unstable features seen (Table 11.4). Analysis of patients in this delayed group who were not on a statin agent 4/23 (17%) compared to those who were on a statin agent 19/23 (83%) showed no significant difference between prevalence of unstable features seen.

So how should these findings be interpreted? Studies (Carr S, 1996);(Falk E, 1995);(Seeger JM, 1995) have shown that a high risk plaque characteristically demonstrates a large lipid core, thin or ruptured cap and marked inflammation in response to healing. This tends to reduce with time. Statins are known to have anti-inflammatory effects on plaques (Arnaud C, 2005). In patients who have a delayed CEA, it may be that either best medical therapy was not commenced (many patients in the delayed group had delayed presentation) or patients had poor compliance with medication, leading to a secondary increase in the prevalence of unstable histological features. It could also mean that the introduction of antiplatelet and statin therapy has meant that these patients with unstable histological features have remained asymptomatic. These findings may also reflect normal plaque progression whereby in the hyperacute period, there is a vulnerable large lipid core and plaque rupture, followed by discharge of the large lipid core (showing a decrease in plaques with this feature in the 8-28 days group) and then secondary re-accumulation of the lipid core thereafter.

The limitations of this study include; 1. Not having sufficient details on the duration of use of aspirin and statin therapy prior to CEA for patients in each group; 2. The relatively small numbers of patients in the delayed group, which is a result of changing practice to expedite wherever possible intervention for symptomatic patients.

Results

In conclusion this study has shown that plaques excised from patients in the hyperacute period demonstrate the greatest number of unstable features and so the original hypothesis can be accepted. Unstable plaque features diminished with time (out to 28 days), but then increased. It is, as yet, unclear as to the clinical significance of this phenomenon. Further work can be focussed on establishing the cause of these changes and using imaging techniques to identify patients with delayed presentation who may have unstable plaques.

<u>Chapter 12. Identifying the Unstable Plaque in the Clinic – A New</u> <u>Model</u>

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<u>Chapter 12. Identifying the Unstable Plaque in the Clinic – A New</u> <u>Model</u>

12.1 Introduction

Recently updated national and international guidelines (Liapis CD, 2009);(Brott TG, 2011);(NICE 2008);(National Stroke Strategy, 2008) for treating patients with carotid artery stenosis still rely primarily on degree of stenosis, clinical symptoms and in asymptomatic patients' co-morbid status of the patient.

Thrombo-embolism from an unstable carotid plaque defined as having several of the following features; thin or ruptured cap, evidence of a large lipid core, intra-plaque haemorrhage, surface thrombus, cap and plaque inflammation, marked neovascularisation and foam cells (Golledge J, 2000);(Stary HC, 1995) is still the major cause of strokes. Therefore, identifying patients pre-operatively based upon histologically unstable plaques and prioritising them for intervention remains an important research area in stroke prevention.

Advanced imaging techniques with and without the use of contrast (Millon A, 2012) such as computer tomography (CT)(Adraktas DD, 2012) magnetic resonance imaging (MRI) (Parmar JP, 2012), positron emission tomography (PET) and more recently infra-red spectroscopy (Wallis de Vries BM, 2008) have all been used in addition to plain duplex imaging to try and identify the unstable plaque. With these imaging modalities however, there remains to be good concordance with histological results and the costs involved mean that they are not widely used in routine practice.

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The aim of this study and chapter was to determine if a model could be created to identify patients with histologically highly unstable plaques using clinical data and B-Mode duplex images easily obtained in the clinic or at the bedside.

The hypothesis is that there is a difference in clinical or ultrasound criteria between patients with histologically stable and unstable plaques, and so a statistical model can be created.

12.2 Methods

12.2.1 Patient Selection (see section 8.3)

Patients were included in the study if they were undergoing a carotid endarterectomy for a critical stenosis (>50% NASCET) of the carotid artery.

Patients were defined as being clinically symptomatic if they reported a stroke, TIA or Amaurosis Fugax in the territory of the ipsilateral stenosed internal carotid artery within the preceding 6 months.

As soon as possible after admission (same day if admitted before 5pm or next day if admitted after 5pm) in symptomatic patients or at the time of pre-assessment for asymptomatic patients, a 30 minute period of TCD monitoring was performed using a commercially available TCD machine (Sonara TCD System from Viasys Healthcare) with the 2-MHz head probe held in place by an external fixation device. Insonation of the ipsilateral MCA was

achieved via the temporal acoustic window at a depth of 50 to 60mm using a 2-MHz pulsed Doppler transducer.

Patients were classed as having evidence of SE if one or more true emboli were detected in the 30 minute monitoring period or during the dissection phase of the operation. Each TCD recording was made by a trained vascular technologist who was blinded to data regarding timing of the clinical event in symptomatic patients. Following admission, all patients underwent a second Duplex assessment in line with HTA guidelines (Wardlaw JM, 2006).

12.2.2 Duplex Imaging

All Duplex examinations were undertaken by experienced ultrasonographers and stenosis severity was measured using consensus criteria consistent with the NASCET mode of measurement. The second duplex image taken after admission to the Vascular Surgery Unit was used for analysis. Enhanced, normalised and non-normalised images were used for blinded off-line analysis. Full details can be found as described in section 8.5 and 14.2.3. Table 16.1 shows the ultrasound features detected using the computer software programme and the cut-off values to determine 'unstable' from 'stable'.

Ultrasound Feature	'Stable'	'Unstable'	
GSM	>25	<25	
Juxta-luminal Black Area (JBA)	<6mm ²	$\geq 6 \text{mm}^2$	
Discrete White Areas (DWA)	No	Yes	
Plaque Type	3&4	1&2	
Plaque Area	<80mm ²	≥80mm ²	

Table 12.1 Ultrasound scoring based on 'Iconsoft' Plaque Texture Analysis Software

12.2.3 Carotid Endarterectomy

Carotid surgery was performed under general anaesthesia with routine patching, routine shunting, systemic heparinisation (unfractionated) and distal intimal tacking sutures. Endarterectomy was performed via a linear arteriotomy and the plaque removed with the minimum of trauma possible as described in 8.3.4.

12.2.4 Histopathology

Plaques harvested during CEA were divided longitudinally and randomly one half fixed immediately in formalin for 24 hours and then paraffin embedded. A 5um transverse section was taken from the paraffin block and stained using haematoxylin and eosin.

Histological specimens were analysed by two independent Histopathologists (KW, DM) who were blinded to the clinical and ultrasound findings. See section 8.10 for full details.

12.2.4.1 Histological Features

For each plaque, a semi-quantitative 3 or 4 point score (Table 8.1) was assigned to show the presence and/or amount of features of plaque instability. Features analysed included; intraplaque haemorrhage, haemosiderin deposition, surface thrombus, lipid core size, nodular calcification, proportion of fibrous tissue, acute and chronic plaque inflammatory infiltrate, acute and chronic cap inflammatory infiltrate, neovascularity, amount of foam cells, and evidence of cap rupture

12.2.5 Statistical Analysis

Statistical comparisons of baseline characteristics between patients with stable and unstable plaques were performed using the two-tailed student t-test for comparison of continuous variables, and χ^2 analysis for comparison of discrete variables Multivariate analysis was performed using logistic regression. On the basis of the univariate and multivariate analyses, a model was designed using logistic regression to assess the relationship between the number of risk factors present and the presence of an unstable plaque. A result was considered statistically significant with a P value of <0.05. Statistical analysis was performed using SPSS v16 (SPSS Chicago III.).

12.3 Results

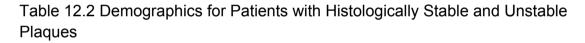
Two hundred patients were included in the study (121 TIA patients, 38 Stroke patients, 41 Asymptomatic). However, twenty ultrasound images from the early part of the study were found to be unsuitable for computerised analysis and thirteen sets of images were lost during

transfer from the mobile scanner to the vascular studies unit. This left one hundred and sixty seven duplex ultrasound images for direct comparison against histological features.

Of the 159 symptomatic 76 patients (48%) underwent surgery within seven days of suffering their most recent clinical event, 43 (27%) within 8-14 days, 17 (11%) within 15-28 days, while >29 days had elapsed in 23 patients (14%). This, therefore, provided a broad spectrum of patients with regard to delays to surgery ranging from the acute period (<14 days, n=119), intermediate (15-28 days, n=17) and late (>29 days, n=23). Symptomatic patients who had a most recent ischaemic event within 14 days of surgery were significantly more likely to have an unstable plaque (OR 1.8 95% CI 1.01 to 3.3 p=0.047)

Patients demographics (Table 12.2) were well matched between the groups who were found to have unstable plaques compared to those found to have stable plaques, the only significant difference was those with stable plaques were more likely to have hypertension and being treated with anti-hypertensive's (OR 2.4 95% CI 1.2 to 5; p=0.01).

Patient Demographics	Stable n=68 (34%)	Unstable N=132 (66%)	P-Value
Male	39 (57%)	102 (77%)	0.008
Age (median)	73 (48-90)	72 (66-96)	0.97
Hypercholesterolaemia	49 (72%)	96 (73%)	1
Diabetes Mellitus	18 (26%)	28 (21%)	0.3
Ischaemic Heart Disease	11 (16%)	35 (27%)	0.2
Hypertension	54 (79%)	85 (64%)	0.01
Ex/Current Smoker	50 (74%)	97 (74%)	0.86
On Anti-platelet therapy following admission	68(100%)	132 (100%)	>0.99
On Statin therapy following admission	68 (100%)	132 (100%)	>0.99
Symptomatic	50 (74%)	109 (83%)	0.2
Asymptomatic	18 (26%)	23 (17%)	
Recent Ischaemic Event <2 weeks	33 (49%)	85 (64%)	0.04



Comparison between duplex imaging features in patients with histologically stable and unstable plaques is described in Table 16.2. Discreet white areas were more common in patients with stable plaques, but this did not reach statistical significance. Univariate analysis of ultrasound based criteria showed a type 1/2 plaque (OR 7.8 95% CI 3.3 to 18.9; P<0.001), an echolucent plaque (GSM <25) (OR 3.01 95% CI 1.2 to 7.4; P=0.01), positive juxta-luminal black area (OR 2.8, 95% CI 1.4 to 5.7, P=0.004) and a large plaque area (>80mm²)(OR 3.3 95% 1.5 to 7.2) were significantly more common in patients with histologically unstable plaques as opposed to those patients with histologically stable plaques

Ultrasound Feature	Stable N=59 (35%)		Unstable N=108 (65%)				Odds Ratio (95% CI)	P Value
GSM (<25)	7	(12%)	31	(28%)	3.01 (1.2 to 7.4)	0.01		
Juxta-luminal Black Area (>6mm ²)	14	(24%)	50	(46%)	2.8 (1.4 to 5.7)	0.004		
Discrete White Areas (Yes)	37	(63%)	58	(55%)	1.4 (0.7-2.8)	0.3		
Plaque Type (1- 2)	7	(12%)	55	(51%)	7.8 (3.3 to 18.9)	<0.001		
Plaque Area (>80mm ²)	10	(17%)	43	(40%)	3.3 (1.5 to 7.2)	0.003		

Table 12.3 Ultrasound Plaque Features Present in Histologically Stable and Unstable Plaques

Patients with spontaneous embolisation detected during Transcranial Doppler monitoring were significantly more likely to have an unstable plaque than those patients without evidence of spontaneous embolisation (OR 2.2, 95% CI 1.1 to 4.2, P=0.02).

In a multivariate stepwise analysis (table 16.3) of clinical, ultrasound and transcranial data an echolucent plaque (GSM <25) (OR 2.94 95% CI 1.01 to 8.62; p=0.02), large plaque area (>80mm²) (OR 2.74 95% CI 1.16 to 6.46; p=0.02) and recent symptoms (most recent even within 14 days) (OR 2.3 95% CI 1.01 to 5.14; p=0.04) were independently predictive of an

increased risk of having a histologically unstable plaque. Interestingly the presence of spontaneous embolisation during 30 minutes pre-operative TCD monitoring was not found to have a significant association after multiple regression analysis.

Feature	Odds Ratio (95% CI)	P Value
Symptoms <2 weeks	2.30 (1.01- 5.14)	0.04
Low GSM (<25)	2.94 (1.01 - 8.62)	0.02
Plaque Area (>80mm ²)	2.74 (1.16 - 6.46)	0.02

Table 12.4 Significant features obtained through multiple logistic regression analysis

12.3.1 Development of a model

On the basis of the result of univariate and multivariate analyses, the following three risk factors were entered into a logistic regression analysis: recent symptoms (<2 weeks), echolucent plaque (GSM <25), and large plaque area (>80mm²). The presence of one, two, and three risk factors showed a statistically significant effect on the presence of unstable carotid plaque when compared with no risk factors present.

Using regression analysis and the logit equation:-

$$Z = B0 + B1X1 + B2X2 + B3X3 \dots + BkXk$$

When entering the values obtained from our analysis we deduce the following equation:-

Z = -0.32 + 0.817(symptoms under 2 weeks) + 0.981(GSM <25) + 0.969(Plaque area >80mm²)

Risk of having an unstable plaque with any of these features can then be calculated from the following equation:-

Risk = $1/1 + e^{-z}$

Using the values from the regression analysis, this provides the following data that can be added to a tabular spreadsheet as below:-

	All 3 risk factors	Recent Symptoms and low GSM	Recent Symptoms and Large plaque area	Large plaque Area	No risk factors
Constant	-0.322	-0.322	-0.322	-0.322	-0.322
Timing Constant (B1)	0.817	0.817	0.817	0	0
GSM Constant (B2)	0.981	0.981	0	0	0
Plq Area Constant (B4)	0.969	0	0.969	0.969	0
Z	2.445	1.476	1.464	0.647	-0.322
e^-z	0.086726	0.22855	0.231309	0.523614	1.379885
1+e^-z	1.086726	1.22855	1.231309	1.523614	2.379885
1/1+e^-z	0.920195	0.813968	0.812144	0.656334	0.420188
Risk	92%	81%	81%	66%	42%

Table 12.5.Excel spreadsheet to calculate risk based on having recent symptoms, low GSM and large plaque area. Figures given betas and constants calculated from regression analysis.

Risk Factors					
Recent Symptoms (<2 weeks)	Yes	Yes	Yes	Yes	No
Low GSM (<25)	Yes	Yes	No	No	No
Large Plaque Area (>80mm ²)	Yes	No	Yes	No	No
Number of Risk Factors	3	2	2	1	0
Risk	92%	81%	81%	66%	42%

This has been summarised simply below:-

Table 12.6 Summary of Model

If no risk factors were present, the prevalence of carotid stenosis was 42%. This increased to 66% with one risk factor, 82% with two risk factors, and 92% with three risk factors

12.4 Discussion

This is the first study to look at simple readily available clinical data and B-Mode ultrasound images to assess the risk of patients with known carotid artery stenosis of having a histologically unstable plaque. Given that the risk of having a major stroke from an unstable carotid plaque is high, there has been a trend towards identifying high risk patients in need of urgent intervention.

Current national and international guidelines for the treatment of symptomatic carotid artery disease are based upon evidence from large meta-analyses have shown that the highest risk of a stroke in patients with carotid artery stenosis is in the acute period after a TIA or minor

stroke. In asymptomatic patients data for intervention shows benefit though overall less than those seen who are symptomatic. Despite strong evidence for the use of novel imaging techniques to detect unstable plaque, there has not been consistent results in identifying histologically unstable plaques (Walker LJ, 2002);(Lovett JK, 2005) and so, evidence based guidelines utilising image analysis does not extend beyond the use of imaging to determine grade of stenosis.

I showed that patients presenting within 14 days of an ischaemic event were significantly more likely to have an unstable plaque (OR 1.8 95% CI 1.01-3.3 p<0.05) which adds to the large bank of data that shows these patients are at highest risk of a stroke following a TIA or minor stroke.

When analysing image features on univariate analysis a type 1/2 plaque (OR 7.8 95% CI 3.3-18.9; P<0.001), an echolucent plaque (GSM <25) (OR 3.01 95% CI 1.2-7.4; P=0.01), positive juxta-luminal black area (OR 2.8, 95% CI 1.4 to 5.7, P=0.004) and a large plaque area (>80mm²)(OR 3.3 95% 1.5-7.2) were significantly more common in patients with histologically unstable plaques as opposed to those patients with histologically stable plaques. Discreet white areas as a feature were not predictive of an unstable plaque and in fact had a non-significant trend towards being more prevalent in stable plaques.

I was also able to show that the detection of spontaneous embolisation during TCD monitoring was significantly associated with a histologically unstable plaque (OR 2.2, 95% CI 1.1 to 4.2, P=0.02). Evidence of spontaneous embolisation during transcranial doppler

monitoring of the middle cerebral artery has been extensively shown in the literature to be a risk factor for ischaemic events (Valton L, 1998)

Following multivariate analysis, the following key clinical and ultrasonographic features were associated with an increased risk of having a histologically unstable plaque data; 1. An echolucent plaque (GSM <25) (OR 2.94 95% CI 1.01 – 8.62; p=0.02); 2. A large plaque area (>80mm²) (OR 2.74 95% CI 1.16 – 6.46; p=0.02) and 3. Recent symptoms (most recent even within 14 days) (OR 2.3 95% CI 1.01 - 5.14; p=0.04)

Scoring methods for prediction of a stroke following TIA or minor stroke include the ABCD2 score (5 point system), The Essen Stroke Risk Score (ESRS) (10 point system) and the Stroke Prognosis Instrument II (SPI-II) score (a 15 point system). All have shown to have predictive value in patients who present with a TIA or minor stroke, though limitations have been found including poor short term predictive value (ESRS and SPI-II) and not being specific to patients with critical carotid artery stenosis (all). Furthermore, no scoring system exists which utilises any form of imaging, or is able to predict which patients have histologically unstable plaques

Ultrasonography performed by well-trained, experienced technologists provides accurate and relatively inexpensive assessment of the carotid arteries. The technique is truly non-invasive and does not involve venipuncture or exposure to ionizing radiation or potentially nephrotoxic contrast material. B-Mode ultrasound has been previously used to identify high risk plaques by identifying plaque echolucency which has been linked to future ischaemic events (Gronholdt M, 2001);(Polak JF, 1998);(Reiter M, 2008) and more recently with advanced plaque texture analysis software (Kakkos SK, 2007) though this has not yet been applied in the wider clinical setting.

The limitations of this study include having approximately 14% of images unavailable for use, although we know that numbers are missing equally from both groups and reflects the early images obtained and the training required for optimal image capture. The limited duration of TCD monitoring may have meant that some patients who were embolising were missed (Type II error), though 30 minutes pre-operative has been our unit's standard practice for many years. A further limitation may be considered as the absence of macroscopic plaque analysis in judging plaque instability and corresponding this to how a plaque was scored overall microscopically. As whole specimens were immediately processed in the operating theatre, plaque analysis would have to be done at the time of harvesting and in that environment the introduction of bias would be much higher, as all clinical details would be known.

In conclusion this study has proven that the hypothesis can be accepted and that a predictive model based upon readily available data can be used to predict which patients are likely to have the histologically 'unstable' plaque.

So how might these data influence future clinical practice? For the clinician who is trying to decide upon the best management strategy with a patient with carotid artery stenosis because duplex ultrasound is so versatile and readily accessible a simple duplex scan would be able to

determine plaque area and echolucency with GSM score. This predictive model once validated on a larger independent cohort, would give a risk score that could be used to triage patients according to risk category, which could help vascular centres plan their lists accordingly.

Chapter 13. Whole Genome Expression Profile of Carotid

Atherosclerotic Plaques

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<u>Chapter 13. Whole Genome Expression Profile of Carotid</u> Atherosclerotic Plaques

13.1 Introduction

Carotid atherosclerosis remains a major health problem in the western world, with greater than one third of all strokes thought to be caused from thrombo–embolism from an unstable carotid atherosclerotic plaque (Barnett HJ, 2000). The patho-genetic steps leading to atherosclerotic plaque formation, progression to an unstable plaque and ultimately thrombo embolism remain a complex and poorly understood phenomenon, thought to result from altered gene expression and environmental triggers.

Plaque instability is usually determined by a combination of clinical, physiological and histological parameters with the highest risk of ischaemic stroke occurring in the first 14 days after an index event such as transient ischaemic attack (TIA) (Giles MF, 2007);(Wu CM 2007). Spontaneous embolisation detected by transcranial doppler (TCD) ultrasound of the middle cerebral artery is associated with a significantly increased risk of early ischaemic recurrence in patients with carotid stroke or TIA (Valton L, 1998). In addition, histological characterisation of the carotid plaque can identify high risk subgroup but there is poor correlation between pre-operative imaging and post-operative plaque analysis (Walker LJ, 2002);(Lovett JK, 2005).

Despite progress in the clinical management of patients with symptomatic carotid artery disease, the molecular mechanisms which lead to plaque instability remain poorly understood. Elucidation of the pathways leading to plaque instability and identification of markers of instability may permit the stratification and prioritisation of patients for surgical intervention. In addition, this may also identify targets for treatment to avoid surgery.

In this chapter we aimed to determine gene expression profiles associated with plaque instability using whole genome microarray analysis of plaques harvested during CEA. We aimed to perform multiple analyses based upon multiple definitions of the 'unstable' plaque using clinical, histological, ultrasound and transcranial doppler monitoring data criteria. This approach was chosen as we have previously shown that there is no single clear definition of the 'unstable' plaque, so using multiple analyses would allow us to find genes consistently differentially expressed.

The hypothesis is that there are significant differences in the expression of genes between atherosclerotic plaques that are 'stable' and those that are 'unstable'

13.2 Methods

Consecutive patients undergoing carotid endarterectomy in our unit were recruited into the study. See section 8.3 (Patient Selection).

13.2.1 Definition of Unstable Plaques

Patients were deemed to have unstable plaques based upon clinical, ultrasound, TCD and histological evidence. Clinical criteria for instability were defined as any ischaemic event (TIA/stroke/amaurosis fugax) within the 14 day period prior to CEA. TCD criteria for instability were defined as evidence of spontaneous embolisation during pre-operative monitoring or during the dissection phase of CEA prior to plaque harvest. Ultrasound criteria for instability were any plaque that showed a Gray Scale Median (GSM) score ≤ 25 .

Histological instability was defined according to a modified well defined, well validated AHA atherosclerotic scoring system (See Table 8.1). Patients were grouped according to each of these criteria independently and separate analyses were performed for each definition of instability.

24 carotid atherosclerotic plaques were initially used. The baseline characteristics and demographics of the patients chosen for this study are shown in Table 9.1. Fresh specimens of plaque were immediately longitudinally divided in theatre, stored in RNA later for 24 hours at 4°C and then drained, snap frozen in liquid nitrogen and subsequently stored at -80°C for batch analysis.

13.2.2 Gene Expression Analysis

RNA extraction was performed as described in section 8.7. The initial gene expression study microarray analysis was performed using Illumina cDNA-mediated Annealing, Selection, extension and Ligation (DASL) assay which allows for expression profiling of partially degraded RNA. Detailed descriptions of all data and protocols were submitted to the ArrayExpress public repository (http://www.ebi.ac.uk/miamexpress/login.htm, accession number: E-MEXP-3683) as per MIAMI guidelines (Brazma A, 2001). Each RNA sample was hybridized to its own microarray in isolation, sample pooling was not used. (See Appendix IV for full protocol).

Four separate analyses were performed comparing stable and unstable plaques based upon clinical (symptoms within 14 days vs. >14 days), TCD (evidence of spontaneous

embolisation versus no detection of spontaneous embolisation), ultrasound (GSM <25 vs. >25) and histological criteria (unstable histological classification versus stable histological classification)

Patient Demographics	N=24
Male Female	15 (63%) 9 (27%)
Age (median)	69 (52-86)
Hypertension	17 (71%)
Hypercholesterolaemia	17 (71%)
Diabetes Mellitus	4 (17%)
Ischaemic Heart Disease	4 (17%)
Ex/Current Smoker	18 (75%)
On Anti-platelet therapy pre-operatively	24 (100%)
On Statin therapy pre-operatively	24 (100%)

Table 13.1. Demographics for Patients Used in Microarray Study

As described in chapter 8.7 all specimens were ground in liquid nitrogen, homogenised in Trizol reagent and had RNA extracted as per protocol. (See Appendix IV).

13.3 Results

RNA was initially extracted using a Qiagen RNeasy Fibrous Tissue Mini Kit as described in section 8.7.1 on the first 24 consecutive samples. An example of the output from the RNA 6000 Nanodrop showing concentration is shown in Figure 13.1

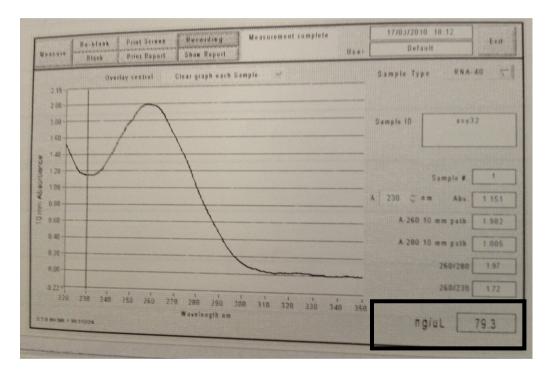


Figure 13.1 Graphical representation of quantitative assessment of RNA concentration.

An example of the RNA integrity output chart produced using the Agilent Bioanalyzer is shown (Figure 13.2). Each RNA sample is individually analysed and given a unique RNA integrity number (RIN) with values from 1-10.

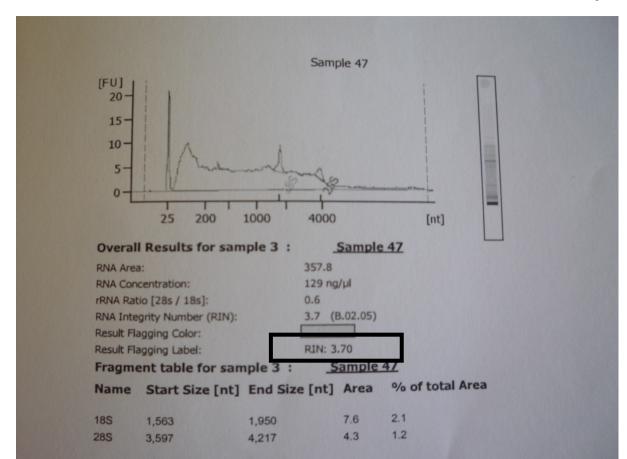


Figure 13.2 Graphical representation of qualitative assessment of RNA concentration. RIN value is highlighted

Initial RNA extraction using the commercial kit yielded smaller volumes, of lower concentration and inferior RNA quality as required for microarray and so the decision was made to use the modified Trizol extraction. Results of the initial RNA extraction process using the Qiagen RNeasy fibrous tissue mini kit compared to the modified Trizol process are shown in Table 13.2.

	RNA Extraction Protocol			
	Qiagen RNeasy Kit (n=24)	Modified Trizol Technique (n=24)		
Concentration (Median 95% CI)	88ng/µl (64-111)	246ng/µl (98-445)		
Quantity (per sample)	20µ1	60µl		
RIN (Median 95% CI)	4.2 (3.3-6.2)	7.4 (6.8 - 8.7)		

Table 13.2 RNA quality and quantity results using different extraction protocol

For quality assessment validation of the RNA extraction protocol, RNA was extracted from 2 patients using vein and plaque harvested at the same time during a carotid endarterectomy procedure using a vein patch. In both cases when using the same protocol for extraction of RNA using the modified Trizol method the RIN values of RNA extracted were higher than corresponding plaque as shown in Table 13.3

Sample	Concentration (RIN Value)		
	Carotid Plaque	Vein	
1	246ng/µl (6.9)	362ng/µl (8.4)	
2	167ng/µl (6.6)	293ng/µl (8.2)	

Table 13.3 Comparison of vein and carotid plaque RNA using the modified TRIzol process

Results

The initial microarray discovery study was performed on RNA extracted using a modified Trizol technique from 24 patients (Table 13.1). Of these 24 patients, 9/24 were unstable based upon clinical criteria, 9/24 were unstable on ultrasound criteria, 6/24 were unstable on TCD criteria and 7/24 were unstable on histological criteria. There were no patients with all four criteria for instability. RIN values for each of the plaques showed no significant difference when analysed according to stability group. Each plaque RNA used in the microarray study had a RIN value greater than 6.5.

For patients with clinical instability, 177 genes were differentially expressed, with CCL19 and COX6B1 being the top up and down differentially expressed, respectively. For ultrasound instability 1973 genes were differentially expressed, with GAK and SLC9A4 being the top up and down differentially expressed respectively. For TCD instability, 2294 genes were differentially expressed with TWF2 and SLC9A4 being the top up and down expressed respectively and for histological instability, 134 genes were differentially expressed with CTSG and TIMP4 being the top and down expressed respectively. A summary table (Table 13.4) is shown together with heat maps showing the top 10 up and down expressed genes from each analysis

Analysis	Gene Symbol	Gene Name	Fold Change	P-Value
	CCL19	chemokine (C-C motif) ligand 19	1.71	<0.001
Clinical	COX6B1	cytochrome c oxidase subunit VIb polypeptide 1	-6.02	<0.01
	GAK	cyclin G associated kinase	1.44	<0.01
Ultrasound	IGFBP7	insulin-like growth factor binding protein 7	-1.48	<0.01
Transcranial	SLC94	Solute carrier family 9 (sodium/hydrogen exchanger), member 4	1.84	<0.01
Doppler	TWF2	twinfilin, actin-binding protein, homolog 2	-1.4	<0.05
	CTSG	Cathepsin G	1.38	< 0.05
Histology	TIMP4	TIMP metallopeptidase inhibitor 4	-1.39	<0.01

Table 13.4 Microarray results showing top up and down regulated genes from each analysis

Plaque According to Clinical Criteria

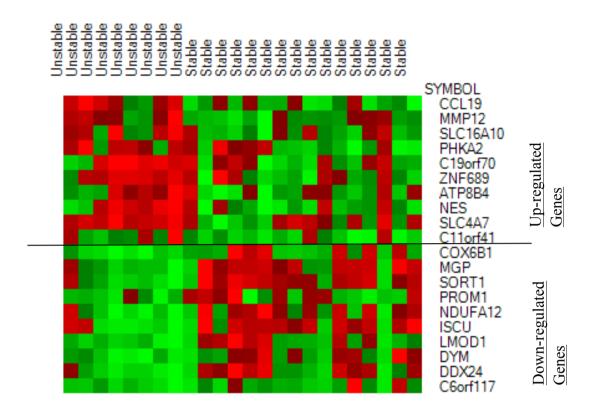


Figure 13.3 Heat map Showing 10 most up and down regulated genes according to clinical criteria. This heat map pictorially demonstrates the most up and downregulated genes found according to the clinical criteria analysis. Each row demonstrates a single gene and each column a separate sample. The unstable samples (plaques from patients with symptoms in the last 2 weeks) are located to the left of the heat map, and those from stable samples (plaques from asymptomatic patients and those who have had symptoms greater than 14 days from surgery) are to the right.



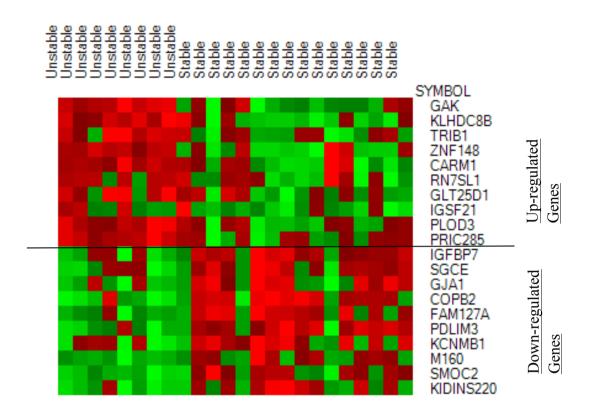


Figure 13.4 Heat map Showing 10 most up and down regulated genes according to ultrasound criteria. This heat map pictorially demonstrates the most up and down-regulated genes found according to the ultrasound criteria analysis. Each row demonstrates a single gene and each column a separate sample. The unstable samples (plaques from patients with US images showing plaque GSM <25) are located to the left of the heat map, and those from stable samples (plaques from patients with US images showing plaque GSM <25) are located to the left of the heat map, and those from stable samples (plaques from patients with US images showing plaque GSM <25) are to the right.

Plaque According to TCD Criteria

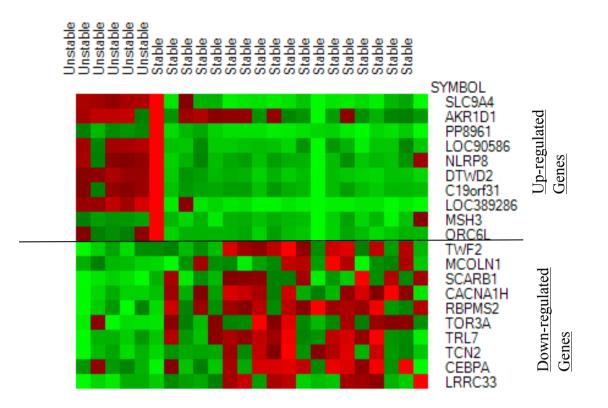


Figure 13.5 Heat map Showing 10 most up and down regulated genes according to TCD criteria. This heat map pictorially demonstrates the most up and down-regulated genes found according to the TCD criteria analysis. Each row demonstrates a single gene and each column a separate sample. The unstable samples (plaques from patients with TCD evidence of spontaneous embolisation from the plaque) are located to the left of the heat map, and those from stable samples (plaques from patients with TCD evidence of no spontaneous embolisation from the plaque) are to the right.

Plaque According to Histological Criteria

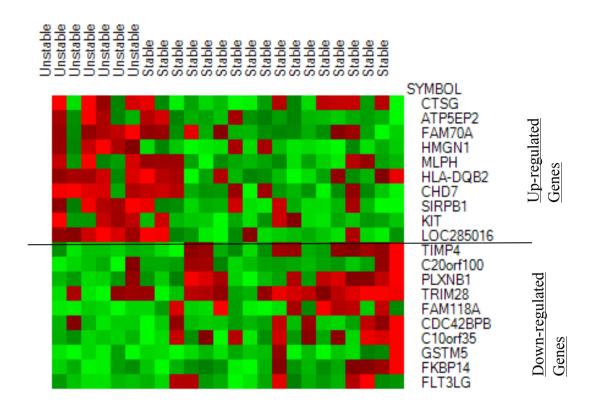


Figure 13.6 Heat map Showing 10 most up and down regulated genes according to histological criteria. This heat map pictorially demonstrates the most up and down-regulated genes found according to the histological criteria analysis. Each row demonstrates a single gene and each column a separate sample. The unstable samples (plaques from patients with histologically unstable features) are located to the left of the heat map, and those from stable samples (plaques from patients with histologically stable features) are to the right.

Initial investigation sought genes that were significantly up or down-regulated in all four analyses. No genes were found to match these criteria. 8 genes were found in 3 out of 4 analyses showing concordance with direction and fold change. The table below shows these genes identified.

Gene Name	Gene Symbol	Analysis							
		Clinical		Ultrasound		TCD		Histology	
		Fold Change	P Value	Fold Change	P Value	Fold Change	P Value	Fold Change	P Value
chemokine (C-C motif) ligand 19	CCL19	1.71	< 0.001	1.37	< 0.05	1.22	>0.05	1.45	< 0.05
cyclin G associated kinase	GAK	1.35	< 0.05	1.44	< 0.05	1.53	< 0.05	-1.2	>0.05
Cathepsin G	CTSG	1.35	< 0.05	-1.05	>0.05	1.2	< 0.05	1.38	< 0.01
Signal-regulatory protein beta-1	SIRPB1	1.36	<0.05	-1.02	>0.05	1.34	< 0.05	1.42	<0.01
insulin-like growth factor binding protein 7	IGFBP7	1.04	>0.05	-1.37	<0.05	-1.31	<0.05	-1.35	<0.05
twinfilin, actin- binding protein, homolog 2	TWF2	1.51	<0.05	1.35	<0.05	1.38	<0.01	-1.2	<0.05
matrix metalloproteinase- 11	MMP11	1.42	<0.01	1.61	< 0.05	1.13	>0.05	1.1	<0.05
matrix metalloproteinase- 11	MMP12	1.4	<0.05	1.32	<0.05	1.05	>0.05	1.44	<0.05

Table 13.5 Genes significantly dys-regulated in multiple analyses (minimum 3 of 4).

13.4 Discussion

Chronic atherosclerotic disease is often complicated by conversion into an acute stage. This is characterized by thrombo-embolism evolving from atherosclerotic plaques or by thrombotic vessel occlusion. In coronary arteries this leads to the acute coronary artery syndrome. Similarly, stable atherosclerotic plaques of extracranial vessels, mainly the ICA, can convert into a source of thrombo-embolism with subsequent transient ischemic attacks or stroke.

Whole genome wide microarray has become an established and powerful research technique in the field of biomedical sciences and is approaching a quarter of a century since being first used (Augenlicht LH, 1987). Since the completion of the human genome project 'Whole Genome Microarray' studies have given rise to the ability of surveying over 18,000 known genes simultaneously from a little over 2micrograms of RNA. The results of microarray studies have allowed for the search of differentially expressed genes in different patient groups or disease states and using sophisticated pathway analysis software the genetic pathways leading to observed phenotypes.

This is the first study to use a whole genome microarray technique to study the differential gene expression in unstable plaques classified according to timing from index event, plaque histology, ultrasound criteria and evidence of spontaneous embolisation.

We initially performed RNA extraction using a commercially available spin column kit, however we found the yield, concentration and quality of the RNA to be inferior to when we used a Trizol technique with double DNAse clean up step. Therefore we decided that all samples used in the microarray would be extracted using this protocol to minimise bias.

RNA extraction from carotid plaques is fraught with difficulties in ensuring RNA is not degraded during the extraction process. Minimising RNA degradation required strict adherence to protocols countering the effect of free RNAses. In order to minimise any degradation, samples were immediately processed in the operating theatre, rinsed with sterile normal saline, and stored in RNA Later for 24 hours before being snap frozen in liquid nitrogen. In the laboratory there was a specific area for RNA extraction. I found that RNA from carotid plaques still remained heavily degraded despite all of these quality assurance steps. In order to ensure the protocol was correct, I extracted RNA from a vein sample from the same patient as a carotid plaque specimen on the same day using the same protocol. The result showed higher quality RNA from vein than from carotid plaque which confirmed that harvested carotid plaques have an abundant number of RNAses that begin to degrade RNA within the plaque immediately. After consultation with researchers in the University of Leicester department of Genetics a decision was made to use an Illumina DASL as the microarray of choice. DASL microarray was initially designed to be used for formalin fixed tissue samples that were subsequently used in gene expression and microarray studies. DASL microarray has been well shown to be able to identify altered gene expression in tissue samples with degraded RNA.

This study has demonstrated that analysing using clinical instability criteria, 177 genes were differentially expressed, with CCL19 and COX6B1 being the top up and down differentially expressed, respectively. When analysing using ultrasound criteria 1973 genes were

differentially expressed, with GAK and SLC9A4 being the top up and down differentially expressed respectively. When analysing using TCD criteria, 2294 genes were differentially expressed with TWF2 and SLC9A4 being the top up and down expressed respectively and when using histological instability criteria, 134 genes were differentially expressed with CTSG and TIMP4 being the top and down expressed respectively.

As this was the first study to use multiple analyses on the same samples this study looked at genes that overlapped in different analyses. There were no patients that had all four criteria of instability. When looking at genes that had a significant up or down fold change no genes were found to be consistently expressed in all analyses. However, 8 genes were identified that consistently matched in 3 out of 4 analyses. These were CCL19, GAK, CTSG, S1RPB1, IGFBP7, TWF2 and MMP11 and MMP12.

Of the genes identified as being the most highly up or down regulated genes in each analysis or being found in multiple analyses, CCL19, CTSG, IGFBP7, MMP11, MMP12 and TIMP4 have all been shown to have a role in atherosclerosis. CCL19 has previously been shown to have a role in plaque destabilisation in atherosclerosis (Damås JK, 2007) through its role in cytokine production in the chemokine signalling pathway. CTSG has been shown to have a possible role in atheroma formation through angiotensin generating enzyme and the decreased production of smooth muscle cells (Legedz L, 2004). IGFBP7 has been shown to have a link to regulation of angiogenesis, through vascular endothelial cells that contain unique storage organelles, designated Weibel-Palade bodies (WPBs), that deliver inflammatory and haemostatic mediators to the vascular lumen in response to agonists like thrombin and vasopressin (van Breevoort D, 2012). MMP11 has shown to have proteolytic activity within the plaque (Dollery CM, 2006) but not through direct matrix-degradation, but

instead through degradation of serpins, relieving serine proteinases and some cathepsins from inhibition (Schönbeck U, 1999). MMP12 transcript levels have been shown to be significantly increased in ruptured plaques compared with lesions without cap disruption (P=0.001) and also significantly higher in plaques from symptomatic patients, than in those from asymptomatic patients (Morgan AR, 2004). In advanced atherosclerotic lesions, TIMP4 was detected around the necrotic lipid cores of plaques and in areas with an abundant inflammatory infiltrate (Koskivirta I, 2006). GAK (Shimizu H, 2009) has a role in regulating the cell cycle but no link has been made to atherosclerosis or the unstable plaque.

Previous gene expression studies in carotid atherosclerosis using similar microarray technology have identified differential expression between 1. Symptomatic versus asymptomatic patient groups (Randi AM, 2003);(Vemiganti R, 2005);(Dahl TB, 2007); 2. Post autopsy versus live tissue (Sluimer JC, 2007) 3. Cap versus intima (Adams LD, 2006);4. Plaque versus normal arterial wall (Woodside KJ, 2003) and 5. Intra-plaque stable and unstable regions (Papaspyridonos M, 2006) however limitations of some of these studies have included small numbers used for both array work and for validation, and in some studies, pathway specific arrays were examined, and therefore a whole transcriptomic approach was not used.

Based on the results of this initial discovery study the null hypothesis should be rejected. There is altered gene expression between plaques deemed stable and unstable. Multiple analyses have been performed in order to seek genes consistently being over or under expressed between 'stable' and 'unstable' groups. Genes have been identified that are involved in inflammation, angiogenesis, proteolysis and cell signalling as potential genes of

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interest. This initial discovery study was devised to allow us to focus in on further genes of interest that can be validated further using real time quantitative PCR (qRT-PCR). This is the focus of Chapter 14.

The limitations of this study are that whole plaque specimens were used in all experiments. The purpose of this was historical gene expression studies in carotid plaques have evaluated subgroup cell types and populations, and by avoiding this targeted selection of cells we have avoided introducing potential bias into the analysis. Another limitation was that due to the heterogenous nature of human plaque specimens it was not possible to apply multiple testing correction to microarray results, therefore as per the manufacturers recommendation, genes that had a fold change greater than or less than 1.3 were placed in hierarchical order of significance and top genes investigated in further detail. In this particular study pathway analysis was not performed as only 8 genes were found to be significantly up and down regulated in more than 3 of 4 analyses, so the decision was made to focus on these genes in follow up studies. A further limitation of this study is related to how plaques were histologically graded as unstable. As discussed in the conclusion of chapter 12, macroscopic plaque analysis was not used in determining histological plaque instability. The reason for not performing macroscopic plaque scoring was reduce risk of bias. Any macroscopic scoring would have to be performed in the operating theatre, as this is where processing immediately occurs, and so any investigator scoring would have all patient and clinical details to hand, including urgency of procedure and evidence of intra-operative microembolisation as detected by TCD monitoring.

Following this investigation, the aim is to validate the gene expression findings using qRT-PCR (See Chapter 14).

Chapter 14. qRT-PCR validation of Expression Profile of Carotid

Atherosclerotic Plaques

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<u>Chapter 14. qRT-PCR validation of Expression Profile of Carotid</u> <u>Atherosclerotic Plaques</u>

14.1 Introduction

Identifying the pathways leading to plaque instability and identification of markers of instability may permit the stratification and prioritisation of patients for surgical intervention. In addition, this may also identify targets for treatment to avoid surgery.

In Chapter 13, using a novel whole genome wide microarray platform we were able to identify the altered gene expression profile across the whole transcriptome at a single point in multiple samples of carotid atherosclerotic plaques that were deemed to be 'unstable' according to clinical, ultrasound, histological and TCD criteria when compared to 'stable' plaques. Single analysis for each criterion allowed us to identify the most up and down regulated genes and combining analyses allowed us to identify genes that were consistently altered

The genes CCL19, GAK, SLC94 and CTSG were the most upregulated genes according to clinical, ultrasound, TCD and histological criteria respectively. The genes COX6B1, IGFBP7, TWF2 and TIMP4 were the most down-regulated genes according to clinical, ultrasound, TCD and histological criteria respectively. In addition we identified MMP11, MMP12, TRIB1 and SIRPB1 as genes that were consistently, significantly up-regulated across analyses.

In this chapter therefore these 12 genes were chosen as genes of interest to be carried forward and used in the validation study. The validation would be carried out on a cohort of larger samples that included the 24 samples used in the original microarray study.

The hypothesis is that there are significant differences in the expression of any of the 12 genes between atherosclerotic plaques that are 'stable' and those that are 'unstable' based on any criteria.

14.2 Methods

Consecutive patients undergoing carotid endarterectomy in our unit were recruited into the study. See section 8.3 Patient Selection.

14.2.1 Definition of Unstable Plaques

This was as described in section 13.2.1 (page 216)

14.2.2 Replication Study in Independent Cohort

In addition to the 24 carotid atherosclerotic plaques obtained for the initial microarray study, a further 96 samples were obtained making a total of 120 samples used for validation. The baseline characteristics and demographics of the 96 additional patients chosen for this study are shown in Table 14.1 below.

Only genes shown to be differentially expressed in the microarray study and subsequently confirmed by qRT-PCR validation were taken forward for replication in a larger independent cohort.

Patient Demographics	N=96
Male	72 (75%)
Age (median)	71 (36-87)
Hypertension	71 (74%)
Hypercholesterolaemia	70 (73%)
Diabetes Mellitus	21 (22%)
Ischaemic Heart Disease	22 (23%)
Ex/Current Smoker	77 (80%)
On Anti-platelet therapy pre-operatively	96 (100%)
On Statin therapy pre-operatively	96 (100%)
Spontaneous Embolisation during pre-operative TCD Monitoring	36 (37.5%)
Histologically Unstable Plaque	58 (60%)
Recent Symptoms within 14 Days	54 (56%)

Table 14.1 Demographics for patients used in qRT-PCR validation study

14.2.3 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) using Taqman Probes

Full description of the qRT-PCR process and protocol is available in section 8.8.2 and in Appendix IV. qRT-PCR was performed using TaqMan probes with all samples run in triplicate. Gene expression data were analysed by normalisation against the geometric mean of the expression of the 3 housekeeper genes (B2M, GUSB and PGK1) showing the most stable expression from a panel of five housekeeping genes as described by Vandesompele et al (2002). In order to determine the best housekeeping (internal control) gene, an experiment was performed on RNA extracted from carotid plaques under variable experimental conditions with variable RNA quality. Standard qRT-PCR was performed as per section 8.8.2 with five housekeeping genes chosen. For simple calculations, the relative quantification data of each housekeeping gene is used. To calculate this relative quantification data the highest Ct value is subtracted from all other Ct values for each gene measured. Hence each Ct value has been transformed in to a "delta CT" value, with the highest deltaCT value as 0. All other values are less than 0. Applied to each data point is the equation $2^{(-\text{delta Ct})}$. Hence all data is expressed relative to the expression of the least expressed gene. Values are entered into a Visual Basic Application (VBA) for Microsoft Excel - termed geNorm. This software automatically calculates a gene-stability measure 'M 'for all control genes in a given set of samples and enables elimination of the worst-scoring housekeeping gene (that is, the one with the highest M value) and recalculation of new M values for the remaining genes.

In order to measure expression levels accurately, normalization by multiple housekeeping genes instead of one is required. Consequently, a normalization factor based on the expression levels of the best-performing housekeeping genes must be calculated. For accurate averaging of the control genes, it is proposed to use the geometric mean instead of the arithmetic mean, as the former controls better for possible outlying values and abundance differences between the different genes. It is recommend the minimal use of the three most stable internal control genes for calculation of an RT-PCR normalization factor (NF*n*,*n* = 3). The geNorm program calculates Pairwise variation (Vn/n + 1). This is an analysis between the normalization factors NF*n* to determine the number of control genes required for accurate normalization. The arrowhead in the graph identifies the optimal number of control genes for normalization.

14.3 Results

The initial microarray discovery study described in Chapter 9 was performed on 24 patients (Table 13.1).

For the validation in the larger independent cohort, 96 patients were recruited (Table 14.1). Of these 96 patients, 54/96 (56%) were unstable based upon clinical criteria, 25/96 (26%) were unstable on ultrasound criteria, 36/96 (38%) were unstable on TCD criteria and 58/96 (60%) were unstable on histological criteria.

Following quality control, all samples had a RNA Integrity Number (RIN) of greater than five and were deemed suitable for qRT-PCR analysis. RIN values for each of the plaques showed no significant difference when analysed according to classification of stability. Figures 14.1 and 14.2 Shows the results of the experiment performed to determine the number and optimal housekeeping genes to use for normalisation.

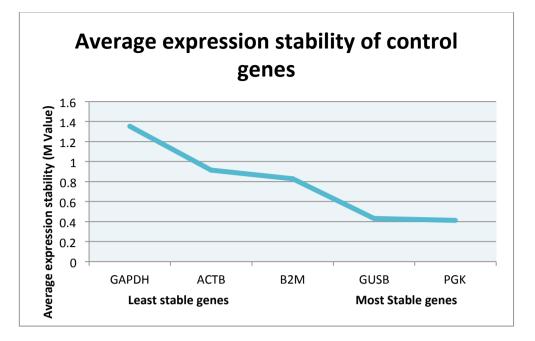


Figure 14.1 Identification of most stable genes to be used as 'housekeeping' genes

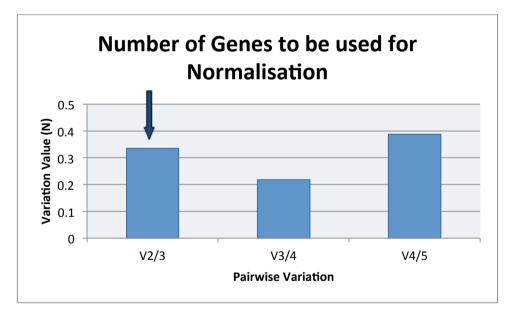


Figure 14.2 GeNorm data showing number of genes to be used as housekeepers. The arrowhead in the graph identifies the optimal number of control genes for normalization From the results shown above it was deduced that for normalisation 3 housekeeping genes would be used which would be B2M, GUSB and PGK.

Table 14.2 shows the results of the validation of the qRT-PCR when compared to the original microarray findings and when performed in the larger independent cohort. The most differentially expressed genes found on microarray were validated using qRT-PCR on the same 24 samples used in the microarray. If results were found to be validated with a significance value (p<0.05) then these genes were sought for using an independent cohort of 96 samples. Depending on the type of analysis performed (Clinical, Ultrasound, Spontaneous Embolisation, Histological) the same criteria were applied to each gene in the independent cohort. The microarray results were validated by qRT-PCR in the original 24 samples for all genes in terms of direction of fold change (up or down regulated) and significance except SLC9A4 and SIRPB1. These two genes were therefore not analysed in the independent cohort.

Gene Name	Microarray Study Fold Change (P- Value)	qRT-PCR Validation of Microarray Sample (P-Value)	qRT-PCR Validation on Independent Cohort (P-Value)		
CLINICAL INSTABILITY					
CCL19	1.71 (<0.001)	1.67 (0.03)	1.89 (0.02)		
COX6B1	-6.02 (<0.01)	-1.4 (0.04)	1.04 (0.866)		
ULTRASOUND INSTABILITY					
GAK	1.44 (<0.01)	1.53 (0.02)	1.2 (0.48)		
IGFBP7	-1.48 (<0.01)	-1.57 (0.04)	2.49 (0.08)		
	SPONTANEOUS	EMBOLISATION			
SLC9A4	1.84 (<0.01)	1.69 (0.18)	n/a		
TWF2	1.37 (<0.01)	1.06 (0.04)	1.03 (0.922)		
	HISTOLOGICA	L INSTABILITY			
CTSG	1.38 (<0.01)	2.02 (0.04)	6.05 (0.03)		
TIMP4	-1.45 (<0.01)	-1.14 (0.01)	-1.42 (0.15)		
GENI	GENES DYSREGULATED IN MULTIPLE ANALYSES				
MMP11	1.52 (<0.05)	1.63 (0.03)	4.6 (0.06)		
MMP12	1.41 (<0.05)	1.54 (0.02)	1.75 (0.08)		
SIRPB1	1.42 (<0.01)	1.69 (0.16)	n/a		

Table 14.2 qRT-PCR Validation of Chosen Genes

The validation study was replicated in an independent larger cohort of 96 patients undergoing CEA for all the genes shown to be validated in the first experiment (CCL19, COX6B1, GAK, IGFBP7, TWF2, CTSG, TIMP4, MMP11 and MMP12). In this larger replication study, the significant up-regulation of both CCL19 (fold change 1.89; P-value 0.02) and CTSG (fold change 6.05, P-value 6.05) was confirmed.

14.4 Discussion

Although microarrays are an excellent tool for initial target discovery, there is a recognized variability in microarray results depending on the user, platform and the quality of specimen (RNA) used. It is also very expensive for day to day diagnostic purposes and equipment is not readily available. Therefore it is necessary to validate the results of the microarray using qRT-PCR. Results which are validated using qRT-PCR can be trusted to be accurate. The amount of specimen and the quality of RNA needed for qRT-PCR validation is also less than what is required for microarray, making it a potentially more accurate and accessible platform when carried forward for clinical applications.

Several variables need to be controlled for in gene-expression analysis, such as the amount of starting material, enzymatic efficiencies, and differences between tissues or cells in overall transcriptional activity. Various strategies have been applied to normalize these variations. Under controlled conditions of reproducible extraction of good-quality RNA, the gene transcript number is ideally standardized to the number of cells, but accurate quantification of cells is often impossible, for example when starting with solid tissue. Drawbacks to the use of 18S or 28S rRNA molecules as standards are their absence in purified mRNA samples, and their high abundance compared to target mRNA transcripts. The latter makes it difficult to accurately subtract the baseline value in real-time RT-PCR data analysis. To date, internal

Results

control genes are most frequently used to normalize the mRNA fraction. This internal control known as the housekeeping gene - should not vary in the tissues or cells under investigation, or in response to experimental treatment. However, many studies make use of these constitutively expressed control genes without proper validation of their presumed stability of expression. Studies have shown that housekeeping gene expression - although occasionally constant in a given cell type or experimental condition - can vary considerably (Warrington JA, 2000);(Thellin, O, 1999);(Suzuki T, 2000).

I performed a preliminary experiment to determine the ideal housekeeper genes for our study based on a number of samples with variable RNA that best represented our sample cohort. I determined that B2M, GUSB and PGK1 were the ideal combination of house-keeping genes to use as internal controls for data normalisation.

Using qRT-PCR I have shown that our microarray findings are validated with 100% concordance in directionality of fold change (up/down regulated) and 82% concordance with significant results. Only SLC9A4 and SIRPB1 did not significantly validate the results of the microarray, though both showed a similar trend.

This therefore allowed me to take forward nine genes to be validated in an independent cohort. In this cohort 8 out 9 genes showed identical directionality of fold change, giving a concordance rate of 89%. Only IGFBP7 was up-regulated in the larger cohort (p=n/s) whilst being shown to be a down regulated gene in the microarray and initial validation study.

CCL19 (fold change 1.89; P-value 0.02) and CTSG (fold change 6.05, P-value 0.03) were shown to have significant fold changes matching the microarray and initial validation study both being up-regulated in the 'unstable' larger independent cohort. These were also identified in Chapter 13 as two of the eight genes on the microarray to be up-regulated in multiple analyses.

I have previously described that CCL19 has previously been shown to have a role in plaque destabilisation in atherosclerosis (see page 227) through its role in the cytokine production in the chemokine signalling pathway.

CCL19 gene is one of several CC cytokine genes clustered on the p-arm of chromosome 9. Cytokines are a family of secreted proteins involved in immunoregulatory and inflammatory processes. Chemokine (C-C motif) ligand 19 (CCL19) is a small cytokine belonging to the CC chemokine family that is also known as EBI1 ligand chemokine (ELC) and macrophage inflammatory protein-3-beta (MIP-3-beta). The CC cytokines are proteins characterized by two adjacent cysteines and which act via G-protein-coupled cell surface receptors and are expressed by lymphocytes and macrophages and are best known for their chemotactic and proinflammatory effects.

The role of CCL19 has been documented in the chemokine signalling pathway that ultimately leads to further cytokine production, cellular growth and differentiation and apoptosis. It also influences the transendothelial migration of leucocytes pathway.

Results

CTSG gene codes the protein Cathepsin G. Cathepsin G is a 225-aminoacid cationic, neutral serine protease. Cathepsin G is synthesised in neutrophils, monocytes and mast cells. Cathepsin G has a pro-peptide which is cleaved by a cysteine protease, dipeptidyl peptidase (Cathepsin C), to yield the mature enzyme. The mature enzyme is kept in its active form in azurophilic granules of neutrophils and in peroxidase-sensitive granules of macrophages (Korkmaz B, 2008). In vascular disease, SMCs transform to a synthetic phenotype and this is accompanied by expression of cathepsin G (Hu WY, 2003). Cathepsin G has been shown to have a possible role in atheroma formation through angiotensin generating enzyme and the decreased production of smooth muscle cells (Legedz L, 2004).

Cathepsin G activity can cause the degradation of various ECM molecules either directly or indirectly. Cathepsin G hydrolyzes glycoproteins such as elastin, fibronectin, thrombospondin and von Willebrand factor (Bonnefoy A, 2000). Collagen is degraded by collaboration of collagenase and cathepsin G. Cathepsin G activates collagenase which can hydrolyse and denature collagen that, in turn, can be further degraded by Cathepsin G (Capodici C,1989). Cathepsin G can indirectly degrade ECM by activating MMPs such as MMP-1, MMP-9 and MMP-10. Cathepsin G in cardiomyocytes induces apoptosis by activating protein phosphatase and SHP2, which dephosphorylates focal adhesion kinases (FAK) and results in down-regulation of the pro-survival signal, protein kinase B (AKT) (Rafiq K, 2008). Cathepsin G has a potent inflammatory function and enhances neutrophil and monocyte chemotaxis by removing the N- terminal pro-peptide from CCL15 resulting in its activation (Richter R, 2005). Another pro-inflammatory function of cathepsin G is maturation of II-33, which drives cytokine release from various inflammatory cells (Lefrancais E, 2012).

Results

In this follow up study using real time polymerase chain reaction to measure gene expression between stable and unstable plaques I have been able to validate the findings of my microarray results and when tested on a larger cohort of 96 patients I have been able to show the significant upregulation of CCL19 and CTSG gene in 'phenotypically' unstable plaques. I have proved the hypothesis that there is altered gene expression between atherosclerotic plaques deemed stable and unstable.

The limitations of this study are that as per the microarray study, whole plaque specimens were used in all experiments. The purpose of this was that historical gene expression studies in carotid plaques have evaluated subgroup cell types and populations, and by avoiding this targeted selection of cells we have avoided introducing potential bias into the analysis. The numbers included in this study reflect the collection period of the study and as a validation study was deemed to be appropriate after seeking statistical advice from the Department of Health Sciences, University of Leicester.

In conclusion, I have now identified two genes that merit further investigation as a potential marker of instability and equally a target to prevent the progression of stable to unstable atherosclerotic plaque. Following this investigation, I aim to identify the protein product of the CCL19 and CTSG gene within the plaque and in matching serum samples and this is the focus of chapter 15.

<u>Chapter 15. Protein Quantification of CCL19 and Cathepsin G in</u> <u>the 'Unstable' Carotid Atherosclerotic Plaque</u>

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<u>Chapter 15. Protein Quantification of CCL19 and Cathepsin G in</u> <u>the 'Unstable' Carotid Atherosclerotic Plaque</u>

15.1 Introduction

It is well recognised that the 'unstable' carotid atherosclerotic plaque histologically has a predominantly inflammatory infiltrate, large lipid core, neovascularisation, a thin or ruptured cap and evidence of surface ulceration and thrombus formation. This is thought to occur due to local environmental triggers and changes in gene expression.

In Chapter 13 I have previously performed a whole-genome wide microarray study looking at the entire transcriptome of unstable plaques for altered gene expression when compared to the transcriptome of stable plaques. With microarray I identified several altered genes to be consistently upregulated in atherosclerotic plaques deemed unstable according to clinical, histological, ultrasound and TCD criteria. I validated our findings using qRT-PCR on an independent cohort of patients with similar criteria and found two genes in our panel to be consistently up-regulated in these unstable plaques. The two genes identified were CTSG which codes for the protein Cathepsin G and CCL19 which codes for the protein chemokine (c-c-motif) ligand 19.

In this chapter I aimed to determine if the protein products of these two differentially expressed genes were present in the plaque and/or circulation. A primary goal for identifying markers of instability is that they may permit the stratification and prioritisation of patients for surgical intervention and may also to identifying targets for treatment to avoid surgery.

The hypothesis is that there are significant differences in the protein expression of Cathepsin G and chemokine (c-c-motif) ligand 19 between atherosclerotic plaques that are 'stable' and those that are 'unstable' based on clinical and histological grading of instability

15.2 Methods

Consecutive patients undergoing carotid endarterectomy in our unit were recruited into the study. See section 8.3 Patient Selection.

15.2.1 Definition of Unstable Plaques

Clinical criteria for instability were defined as any ischaemic event (TIA/stroke/amaurosis fugax) within the 14 day period prior to CEA. Histological instability was defined according to a modified well defined, well validated AHA atherosclerotic scoring system (See table 8.1).

15.2.2 Sample Processing

Peripheral venous blood was drawn pre-operatively the evening before surgery into pyrogenfree tubes containing no anticoagulant. Serum was aspirated from the blood after centrifugation as per section 8.6.1.

Carotid atherosclerotic plaques harvested during surgery were rinsed in normal saline and divided longitudinally. One half was immediately placed in formalin for 24 hours and subsequently embedded in paraffin for histological grading and immunohistochemical analysis. See section 8.6.2.

15.2.3 Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded carotid plaques. 24 plaques were selected from the extremes of the clinical stability/instability group (12 stable (asymptomatic patients), 12 unstable (most recent symptom within 14 days)) and from extremes of the histologically graded plaques group {12 stable (Type 0/1 Plaques), 12 unstable (Type 3 Plaques)). Staining was also performed for CD68 (macrophage marker) and CD3 (T-Cell Marker)

15.2.4 Enzyme Immunoassay

Protein serum levels were measured by ELISA using a commercially available ELISA kit (Abcam). Serum was incubated on microtitre plates with biotinylated monoclonal antibodies for relevant proteins. Streptavidin-peroxidase and substrate were added, and absorbance was measured at 450 nm. Full details of the protocol are available in section 8.9 and appendix IV.

15.2.5 Statistical Analysis

Immunohistochemical staining was scored using χ^2 . ELISA results were analysed using a Mann Whitney U Test. T-Test and χ^2 was used to compare baseline characteristics between patient groups. All statistical analyses were performed using SPSS, version 18.

15.3 <u>Results</u>

15.3.1 Immunohistochemistry

Patient Demographics	N=24
Male	14 (58%)
Age (median)	66 (60-81)
Hypertension	20 (83%)
Hypercholesterolaemia	20 (83%)
Diabetes Mellitus	3 (13%)
Ischaemic Heart Disease	7 (29%)
Ex/Current Smoker	16 (67%)
On Anti-platelet therapy pre-operatively	24 (100%)
On Statin therapy pre-operatively	24 (100%)
Spontaneous Embolisation during pre-operative TCD Monitoring	14 (31%)
Histologically Unstable Plaque	12 (50%)
Recent Symptoms within 14 Days	10 (42%)

Table 15.1 Demographics for Patients Used in Immunohistochemistry Study

15.3.1.1 CCL19

CCL19 staining localised within the plaque core and cap region in 14/24 (58%) of all plaques. CCL19 staining was significantly greater in 'unstable' plaques (n=10/12) compared to 'stable' plaques (n=4/12) (OR 10; 95% CI 1.4-69; p=0.03). Figures 15a and 15b show examples of a clinically and histologically 'unstable' plaque and that of a clinically and histologically 'stable' plaque (Figures 15a and 15b)

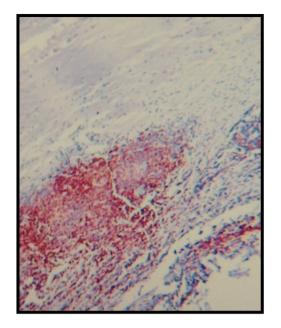




Figure 15a CCL19 staining (red) within the plaque core of an 'unstable' plaque (X10). Figure 15b Absent CCL19 staining within the plaque of a 'stable' plaque (X10)

15.3.1.2 Cathepsin G

Cathepsin G staining occurred around the core, cap and shoulder region in 17/24 (71%) including those classed as clinically and histologically 'unstable' (n=11/12; 92%) and in plaques classed clinically and histologically 'stable' (n=6/12; 50%). There was no statistical significance in staining between the groups. Figure 15.3 shows staining of Cathepsin G within the core of a plaque classed as histologically 'unstable'.

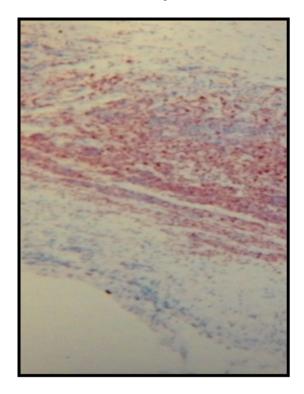


Figure 15.3 Cathepsin G staining red within the plaque of an 'unstable' plaque (X10)

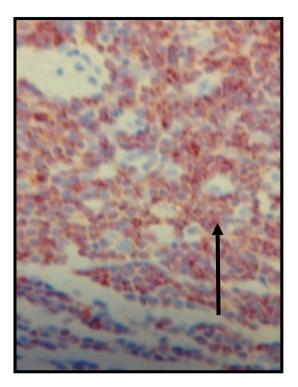
15.3.1.3 CD68 and CD3

In order to search for co-localisation of our expressed proteins we performed staining of CD3 as a T-cell marker and CD68 as a marker of macrophages. We found that there was co-localisation of CD3 positive T-cell lymphocytes in the core region, around where CCL19 was expressed (see figure 15.4). CD68 staining occurred non-discriminately and did not appear to co-localise with Cathepsin G or CCL19.

Chapter 15. Protein Quantification of CCL19 and Cathepsin G in the 'Unstable' Carotid Atherosclerotic Plaque

(B)

(A)



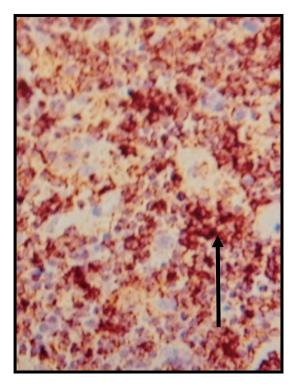


Figure 15.4. Plaque stained for CCL19 (A) and CD3 (B). CCL19 is shown to colocalise with T-cell lymphocytes.

15.3.2 ELISA Protein Quantification of CCL19 and CTSG in Serum

ELISA was performed on serum samples from 36 patients. {n=18 clinically unstable (most recent symptom within 14 days), n=18 clinically stable (asymptomatic patients)} Demographic data for patients used in this study are shown in Table 15.2. Of the 36 serum samples taken from these patients 16/36 had histologically unstable plaques.

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Patient Demographics	N=36
Male	28 (78%)
Age (median)	68 (36-83)
Hypertension	21 (58%)
Hypercholesterolaemia	21 (58%)
Diabetes Mellitus	7 (19%)
Ischaemic Heart Disease	8 (22%)
Ex/Current Smoker	28 (78%)
On Anti-platelet therapy pre-operatively	36 (100%)
On Statin therapy pre-operatively	36 (100%)
Spontaneous Embolisation during pre-operative TCD Monitoring	11 (31%)
Histologically Unstable Plaque	16 (44%)
Recent Symptoms within 14 Days	18 (50%)

Table 15.2 Demographics for Patients Used in ELISA Study

Of the 36 serum samples taken from these patients 16/36 had histologically unstable plaques. There was a significantly higher concentration of CCL19 in the acutely symptomatic 'unstable' group (median 417pg/ml, 95% CI 244-677pg/ml) compared to the clinically 'stable' group (median 288pg/ml, 95% CI 138-316pg/ml) p=0.02 (Figure 15.5). Cathepsin G was not identified in any serum samples including samples taken from those patients with histologically unstable plaques. The sensitivity values for the enzyme immunoassay were 2 pg/mL and 20 pg/mL respectively. The intra-assay and inter-assay coefficients of variation were <10% for all enzyme immunoassays.

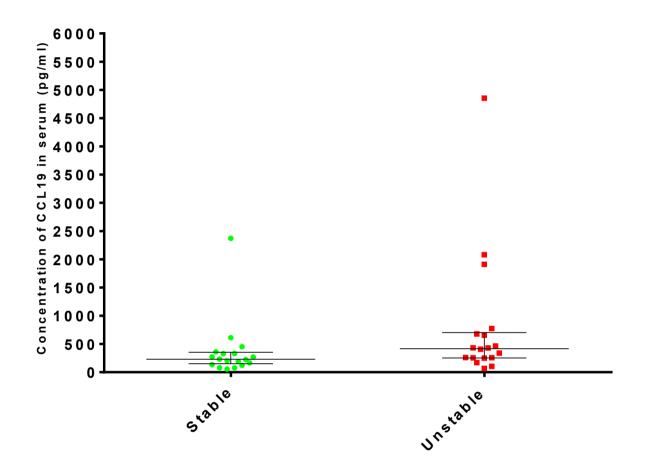


Figure 15.5 Graphical Representation of CCL19 Protein Concentration in Serum from Patients with Clinically 'Stable' (symptoms >2 weeks) Plaques versus Clinically 'Unstable' (symptoms <2 weeks) Plaques. P=0.02

15.4 Discussion

Understanding the dynamic patho-genetic changes that cause a 'stable' atherosclerotic plaque to undergo changes and become an 'unstable' plaque at high risk of causing thrombosis and distant embolism remains poorly understand. Using whole genome microarray, and validating the results using qRT-PCR on an independent cohort we have previously shown that the genes CCL19 and CTSG are significantly upregulated in plaques that are 'clinically' and 'histologically' unstable.

In this study using an independent cohort of patients I have been able to demonstrate the protein expression of CCL19 and Cathepsin G (protein product of the CTSG gene) within the plaque cap and core in clinically and histologically unstable plaques. CCL19 was found to be significantly expressed in plaques from patients that had recent clinical symptoms (symptoms within the last 14 days prior to surgery) and that were histologically 'unstable'. CTSG was also found to be expressed more predominantly in these 'unstable' plaques however results failed to reach significance. I have shown that CCL19 is co-localised with CD3 positive T-cell lymphocytes in the cap and core regions of the plaque. I have also demonstrated that CD68 expression is found in this region, and is reflective of macrophage activity within the plaque.

In order to determine the protein expression I also performed ELISA analysis on serum from an independent cohort of patients, to ascertain if CCL19 or Cathepsin G were a circulating protein. The independent cohort of patients were matched for basic demographic data, with half (n=18) having acute symptoms (symptoms within 7 days prior to surgery) and half (n=18) being asymptomatic. 16/36 (44%) plaques from this independent cohort were found to be histologically unstable. In this study I found levels of CCL19 were significantly higher in serum from patients who were recently symptomatic compared to those who were asymptomatic

CCL19 is a small cytokine belonging to the CC chemokine family that is also known as EBI1 ligand chemokine (ELC). The CC cytokines are proteins characterized by two adjacent cysteines and which act via G-protein-coupled cell surface receptors and are expressed by lymphocytes and macrophages and are best known for their chemotactic and proinflammatory effects. The chemokine CCL19 is a chemoattractant for different types of cells, including natural killer cells (Kim CH, 1993) as well as T and B lymphocytes (Kim CH, 1998). MIP-3β/CCL19 acts through CC chemokine receptor 7 (CCR7) and is a more forceful chemoattractant of lymphocytes than MIP-3α/CCL20 and other CC chemokines (Yoshida R, 1997).

The role of CCL19 has been documented in the chemokine signalling pathway that ultimately leads to further cytokine production, cellular growth and differentiation and apoptosis and also influences the transendothelial migration of leucocytes pathway. A previous study (Damås JK, 2007) has shown a possible link between CCL19 and plaque destabilisation through recruitment of T-cells and macrophages.

Previous studies into human carotid artery plaques have shown T-cell and macrophage infiltration similar to atherosclerosis-prone laboratory animals (Hansson GK, 2005);(Stemme S, 1992);(Carr SC, 1997);(Jander S, 1998);(Cipollone F, 2001);(Nadareishvili ZG, 2001).

Comparison between symptomatic and asymptomatic endarterectomy specimens have revealed that plaque areas covered by inflammatory cells were significantly larger in symptomatic ICA plaques versus asymptomatic ones (Hansson GK, 2005);(Jander S, 1998);(Cipollone F, 2001);(Nadareishvili ZG, 2001) indicating that acute inflammation could also be involved in plaque destabilization.

Acute T-cell and macrophage infiltration is most likely facilitated by an increased expression of cell adhesion molecules on the luminal surface of ICA stenoses (DeGraba TJ, 1998). Interestingly, also the number of dendritic cells (DC) that present antigens to T cells and thereby activate them to proliferate were more numerous in symptomatic than asymptomatic plaques (Stoll G, 2006). Previous studies have linked CCL19 as a DC chemokine to patients with symptoms versus asymptomatic patients (Erbel C, 2007).

Pro-inflammatory cytokines such as IL-18 that can activate T cells and macrophages are expressed at higher levels in symptomatic ICA plaques (Mallat Z, 2001) providing evidence that the local cytokine milieu influences plaque stability. Conversely, stable atherosclerotic ICA plaques exhibited increased expression of the anti-inflammatory cytokine transforming growth factor- β -1 compared with unstable ICA plaques (Cipollone F, 2004).

A limitation of this study is that relatively small numbers have been used in each experiment. This however best reflects the patients recruited during the study period. It was decided that a well conducted approach with rigorous methodology would include performing validation at each step in an independent population. In this study this did not affect the outcome as I was still able to get statistically significant results looking at CCL19 expression in both serum using ELISA and plaque using immunohistochemistry. Whilst Cathepsin G was not detected in any serum sample, suggesting this is not a circulating protein in any disease state, with larger numbers it may be proven that Cathepsin G expression is statistically higher in histologically unstable plaques, as this trend was shown in this study. Another limitation is that due to the nature of tissue, there was limited tissue available after RNA extraction, so although we were able to perform immunostaining for proteins of interest, accurate protein quantification of expressed genes could not be assessed on the tissue. A final limitation of this study is related to absence of IgG controls during the immunostaining process. Although the omission of the primary antibody was used as a negative control, in this study it was not replaced with an IgG to confirm the negative control findings.

With reference to CCL19, the hypothesis that there is a significant differences in the protein expression between atherosclerotic plaques that are 'stable' and those that are 'unstable' based on clinical and histological grading of instability can be accepted. With reference to Cathepsin G , the hypothesis that there is significant differences in the protein expression between atherosclerotic plaques that are 'stable' and those that are 'unstable' based on clinical and histological grading of instability should be rejected.

In conclusion, we have evaluated the role of CCL19 in acutely symptomatic plaques with histologically unstable features at high risk of stroke. This study has shown through immunostaining that CCL19 protein is localised around the cap and core region of the plaque and through ELISA protein expression in serum is higher in acutely symptomatic patients and that the level of the circulating chemokine could be used as potential biological marker of plaque instability. Its pro-inflammatory effects maybe orchestrated through chemotaxis, cell

signalling particularly G-Protein Coupled Receptor signalling and its involvement in the

transendothelial migration of leucocytes pathway.

<u>Chapter 16. Whole Genome Expression Profiling of Carotid</u> <u>Atherosclerotic Plaques from Asymptomatic Patients – A</u> <u>discovery study for future work</u>

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<u>Chapter 16. Whole Genome Expression Profiling of Carotid</u> <u>Atherosclerotic Plaques from Asymptomatic Patients – A</u> discovery study for future work

16.1 Introduction

Carotid atherosclerotic plaque instability is characterised by a large lipid core and plaque inflammation, leading to cap rupture, surface ulceration and thrombus formation. The pathogenesis of plaque instability is poorly understood but is thought to result from a combination of altered gene expression in conjunction with local and systemic inflammation.

Guidelines exist for the management of patients with symptomatic carotid stenosis (NICE, 2008);(National Stroke Strategy, 2008)(ECST 1991);(NASCET, 1991) however with increasing evidence that advanced and aggressive medical therapy is reducing the annual risk of stroke in patients with asymptomatic stenosis (Spence JD, 2010), guidelines for intervention in asymptomatic patients are currently under review (Rudarakanchana N, 2009). However; a small but important cohort of patients with asymptomatic carotid disease will go on to suffer a stroke. It would, therefore, be very useful to identify these patients who might then benefit from intervention.

To date, few gene-profiling studies have been performed in asymptomatic patients where the profiling is correlated with histological patterns of plaque instability; i.e. in patients who have not yet reported neurological symptoms. My hypothesis was that asymptomatic patients with histologically unstable plaques would have an altered gene expression profile compared to histologically stable plaques from asymptomatic patients. If true, this would open up a new

means of developing novel management strategies in asymptomatic patients with carotid disease.

The hypothesis is that there are significant differences in the expression of genes between atherosclerotic plaques that are histologically 'stable' and those that are histologically 'unstable' in asymptomatic patients.

16.2 Methods

Asymptomatic patients were included in the study if they were undergoing carotid endarterectomy (CEA) in the presence of a 70-99% carotid stenosis (NASCET) using colour duplex imaging. Prior to surgery, all patients were commenced on statin therapy (Simvastatin 40mg daily), antiplatelet therapy (Aspirin 75mg daily) and antihypertensive therapy as appropriate (unless contraindicated). In addition 75mg clopidogrel was given the night before surgery (Sharpe RY, 2010). 30 minutes of TCD monitoring was performed and recorded using the Consensus Committee Criteria (Consensus committee, 1995).See section 8.3 Patient Selection.

16.2.1 Data Collection

Pre-operative clinical data were recorded by a single investigator (MS) through face to face questioning of patients and following review of medical notes and in-patient investigations. Demographic data recorded included; age, sex, vascular risk factors (hypertension, hyperlipidaemia, diabetes mellitus and smoking) and pre-operative medication. See Appendix II.

16.2.2 Carotid Endarterectomy

Carotid Endarterectomy was performed under general anaesthesia with routine patching, routine shunting, systemic heparinisation (unfractionated) and distal intimal tacking sutures (see section 8.3.3)

16.2.3 RNA extraction from carotid plaques

See section 8.7 and Appendix IV for full details.

16.2.4 Histopathology

Histological specimens were analysed independently by two histopathologists (KW, DM) who had clinical and demographic data blinded.

16.2.4.1 Histological Features

For each plaque, a semi-quantitative 3 or 4 point score (Table 8.1) was assigned to show the presence and/or amount of features of plaque instability based on the AHA histological classification of advanced atherosclerotic lesions (Stary HC, 1995) and a well validated previously published scoring system (Lovett JK, 2004) Features analysed included; intraplaque haemorrhage, haemosiderin deposition, surface thrombus, lipid core size, nodular calcification, proportion of fibrous tissue, acute and chronic plaque inflammatory infiltrate, acute and chronic cap inflammatory infiltrate, neovascularity, number of foam cells, and evidence of cap rupture. See section 8.10 for full details.

Based upon the score for each of these features in each plaque an overall stability rating was given by the histopathologists as 'stable' or 'unstable'. Unstable plaques demonstrated many

or all of these features whilst stable plaques demonstrated none, allowing for direct comparison between two groups.

16.2.5 Gene Expression Analysis

Gene expression study microarray analysis was performed using Illumina cDNA-mediated Annealing, Selection, extension and Ligation (DASL) assay which allows for expression profiling of partially degraded RNA and covers approximately 24,000 genes. Detailed descriptions of all data and protocols were submitted to the ArrayExpress public repository (http://www.ebi.ac.uk/miamexpress/login.htm, accession number: E-MEXP-3683) as per MIAMI guidelines (Brazma A, 2001). Genes differentially expressed were identified by filtering, using the following criteria: (1) an Illumina diff score greater than 13 or less than -13. The Diff Score is a transformation of the p-value that provides directionality to the pvalue based on the difference between the average signal in the reference group vs. the comparison group. The formula is: Diff Score = 10*sgn(µcond-µref)*log10p. A diff score of 13 is equivalent to a P value 0.05. The second criterion used was a 1.3-fold difference in the mean signal between groups in each separate analysis.

Hierarchical clustering, gene-class testing and KEGG pathway analysis for functional pathways and gene ontology (GO) categories was performed by using DAVID software (Huang a W, 2009). Gene class testing and clustering is an exploratory tool for looking at associations within gene expression data and allows for hypothesis generation and selection of genes for further consideration. Gene ontology is a bioinformatics initiative to unify representation of gene and gene products across species. Gene ontology covers three domains; 1. Cellular component, the parts of a cell or its extracellular environment; 2.Molecular function, the elemental activities of a gene product at the molecular level and 3. Biological process, operations or sets of molecular events with a defined beginning and end, pertinent to the functioning of integrated living units: cells, tissues, organs, and organisms. KEGG pathway analysis is used to assess which pathways are over-represented in a given set of genes. This allows for identification of pathways affected by sets of genes which have altered expression.

16.2.6 Statistical Analysis

Microarray study analysis was performed according to current guidelines using Illumina GenomeStudio (v1) software. T-Test and χ^2 was used to compare baseline characteristics between patients in each group. Statistical analysis was performed using SPSS v16 (Chicago, USA).

16.3 <u>Results</u>

18 consecutive patients undergoing carotid endarterectomy for asymptomatic carotid artery stenosis were initially recruited into the study. Nine patients were excluded as RNA quality {RNA Integrity Number (RIN) <5} was not suitable for microarray study. Table 16.1 shows results for the RNA samples used in the microarray study. RNA was extracted using a modified Trizol protocol as described in section 8.7.

	Samples used in microarray (n=9)
Concentration (Median, 95% CI)	289ng/µl (113-430)
Volume (per sample)	60µ1
RNA Integrity Number (RIN) Value (Median 95% CI)	7.1 (6.3-8.2)

Table 16.1 RNA quantity and quality used in the microarray study

Of the nine patients included, three were classed as unstable and six were defined as stable based upon histological criteria. Full patient demographics for the patients in this study are shown in Table 16.2

Patient Demographics	Stable Plaque (n=6)	Unstable plaque (n=3)	P Value
Male	5 (83%)	2 (67%)	>0.99
Age (median)	70 (53-75)	64 (60-71)	0.74
Hypertension	5 (83%)	2 (67%)	0.8
Hypercholesterolaemia	4 (67%)	2 (67%)	>0/99
Diabetes Mellitus	1 (17%)	0 (0%)	>0.99
Ischaemic Heart Disease	1 (17%)	0 (0%)	>0.99
Ex/Current Smoker	6 (100%)	2 (67%)	0.33
On Anti-platelet therapy	6 (100%)	3 (100%)	>0.99
On Statin therapy	6 (100%)	3 (100%)	>0.99
Evidence of Spontaneous Embolisation During TCD Monitoring	1 (17%)	1 (33%)	>0.99

Table 16.2 Demographics for Asymptomatic Patients Presenting with Stable/Unstable Plaques

16.3.1 Gene Expression Studies

Comparing the stable and the unstable plaques, 346 differentially expressed genes (>1.3 fold, P<0.05) were identified. Of these 293 were down-regulated and 53 were up-regulated

16.3. 2 Functional Annotation Clustering

The significantly differentially expressed genes contributed to four functional annotation clusters with an enrichment score greater than 1.3. Full details are shown in table 16.2 below. These functional annotation clusters relate to The Src Homology 2 (SH2) motif and domain, which are involved in intracellular signalling; biological processes involved in response to wounding and inflammation; biological processes involved in protein maturation, processing and cleavage; and cellular components related to the endoplasmic reticulum. Benjamini refers to a Benjamini-Hochberg q-value, similar to a p-value corrected for multiple hypothesis testing using the false discovery rate.

Enrichment Score 1.7							
Term	Count	P-Value	Benjamini				
RT	9	0.00094	0.09				
SH2 motif	7	0.0017	0.01				
SH2	7	0.002	0.095				
SH2 domain	6	0.006	0.09				
	Enrichment Score 1.5						
Term	Term Count P-Value Benjamini						
Response to wounding	18	0.0016	0.01				
Immune response	20	0.0025	0.01				
Defence response	18	0.0054	0.099				

Inflammatory response	11	0.0074	0.099					
Enrichment Score 1.3								
Term	Count	P-Value	Benjamini					
Protein Maturation	8	0.0006	0.01					
Protein Processing	6	0.0048	0.09					
Protein Maturation by peptide bond cleavage	3	0.045	0.01					
	Enrichment Score 1.3							
Term	Count	P-Value	Benjamini					
Endoplasmic Reticulum lumen	6	0.0023	0.01					
Short sequence motif: Prevents secretion from ER	5	0.0027	0.01					
Protein Folding	8	0.0031	0.01					
Endoplasmic reticulum, targeting sequence	4	0.005	0.01					
Isomerase	6	0.0072	0.09					
Endoplasmic reticulum part	10	0.027	0.098					

Table 16.2 Functional Annotation Clustering – Enrichment Scores

16.3.3. Gene Ontology (GO) Processes

When genes were analysed according to the GO processes, the differentially expressed genes contributed to 35 different GO biological processes, 14 different GO molecular functions and 8 different GO cellular components which are shown below in Table 16.3. The number of

differentially expressed genes annotated under each of these GO processes varied from 2 to 55. The top 10 GO biological processes involved transcription, regulation of transcription, polymerase II promoter, response to wounding, immune response, response to wounding, vesicle mediated transport, defence response, protein kinase cascade and inflammatory response.

Category (GO tern)	Term	Count	Percentage	p-value	Benjamini
Biological Function	regulation of transcription	55	15.8	0.086	0.09
Biological Process	transcription	48	13.8	0.04	0.09
Biological Process	regulation of transcription from RNA polymerase II promoter	21	6	0.045	0.09
Biological Process	immune response	20	5.7	0.025	0.01
Biological Process	response to wounding	18	5.2	0.016	0.01
Biological Process	vesicle-mediated transport	18	5.2	0.037	0.01
Biological Process	defense response	18	5.2	0.054	0.09
Biological Process	protein kinase cascade	12	3.4	0.078	0.09
Biological Process	inflammatory response	11	3.2	0.074	0.09
Biological Process	positive regulation of immune system process	10	2.9	0.03	0.01
Biological Process	positive regulation of developmental process	10	2.9	0.072	0.09
Biological Process	muscle organ development	9	2.6	0.041	0.09
Biological Process	actin cytoskeleton organization	9	2.6	0.052	0.09
Biological Process	actin filament-based process	9	2.6	0.071	0.09
Biological Process	Protein Maturation	8	2.3	0.06	0.01
Biological Process	protein folding	8	2.3	0.031	0.01
Biological Process	regulation of cytokine production	8	2.3	0.051	0.09

Diological 1 1 0ccss	protein processing	0	1.7	0.040	0.07
Biological Process	protein processing positive regulation of cell	6	1.7	0.048	0.09
Biological Process	activation	0	1./	0.055	0.09
Biological Process	immune effector process	6	1.7	0.09	0.01
Biological Process	positive regulation of growth	5	1.4	0.063	0.09
Biological Process	sphingolipid biosynthetic process	4	1.1	0.02	0.01
Biological Process	membrane lipid biosynthetic process	4	1.1	0.025	0.01
Biological Process	ceramide biosynthetic process	3	0.9	0.031	0.01
Biological Process	membrane budding	3	0.9	0.035	0.01
Biological Process	sphingoid biosynthetic process	3	0.9	0.035	0.01
Biological Process	antigen processing and presentation of peptide antigen via MHC class I	3	0.9	0.039	0.01
Biological Process	retrograde vesicle-mediated transport, Golgi to ER	3	0.9	0.058	0.09
Biological Process	multicellular organismal catabolic process	3	0.9	0.084	0.09
Biological Process	collagen metabolic process	3	0.9	0.096	0.01
Biological Process	antigen processing and presentation of peptide antigen	3	0.9	0.096	1.0
Biological Process	positive regulation of keratinocyte migration	2	0.6	0.05	0.09
Biological Process		2	0.6	0.05	0.09

	regulation of keratinocyte migration				
Cellular Component	endoplasmic reticulum lumen	6	1.7	0.023	1
Cellular Component	cell surface	14	4	0.026	0.98
Cellular Component	perinuclear region of cytoplasm	12	3.4	0.034	0.96
Cellular Component	cell fraction	31	8.9	0.051	0.97
Cellular Component	insoluble fraction	25	7.2	0.058	0.96
Cellular Component	transcription factor complex	9	2.6	0.064	0.095
Cellular Component	membrane fraction	24	6.9	0.067	0.093
Cellular Component	intracellular organelle lumen	45	12.9	0.087	0.096
Molecular Function	enzyme binding	22	6.3	0.00049	0.091
Molecular Function	complement binding	3	0.9	0.0087	0.085
Molecular Function	protein dimerization activity	17 4.9		0.036	1
Molecular Function	specific RNA polymerase II transcription factor activity	4	1.1	0.036	0.098
Molecular Function	carbohydrate binding	12	3.4	0.05	0.098
Molecular Function	protein kinase binding	7	2	0.05	0.096
Molecular Function	ligase activity, forming carbon-nitrogen bonds	9	2.6	0.052	0.095
Molecular Function	complement component C1q binding	2	0.6	0.054	0.093
Molecular Function	acid-amino acid ligase activity	8	2.3	0.066	0.95
Molecular Function	RNA polymerase II	9	2.6	0.069	0.94

	transcription factor activity				
Molecular Function	small conjugating protein ligase activity	7	2	0.079	0.093
Molecular Function	protein homodimerization activity	11	3.2	0.08	0.93
Molecular Function	ubiquitin-ubiquitin ligase activity	2	0.6	0.08	0.091

Table 16.3 Gene Ontology Processes

16.3.4 KEGG Pathway Analysis

When looking at genes involved in KEGG pathways, differentially expressed genes were identified to contribute to the chemokine signalling and spliceosome pathway.

16.4 Discussion

This is the first study to evaluate the gene expression profile of carotid atherosclerotic plaques harvested during carotid endarterectomy from asymptomatic patients with histologically stable as opposed to histologically unstable plaques. Histologically unstable features (traditionally seen in recently symptomatic patients) include; plaque haemorrhage, marked cap and plaque inflammation, large lipid core, surface thrombus, marked vascularity, many foam cells and cap rupture. Histologically stable plaques that did not demonstrate these features were chosen as the control as this would allow for direct comparison in gene expression profiles. This study used a whole genome approach to characterise the gene-expression profile of the histologically unstable asymptomatic plaque compared to the histologically stable asymptomatic plaque. As in chapter 13, there were difficulties in obtaining high quality RNA for microarray. These problems were adjusted early on in the study by using a modified TRIzol technique. This provided better quality and quantity RNA. To ensure the most accurate results were obtained, a cut-off RIN value of >5 was required for samples to be included in the study.

346 differentially expressed genes were identified. Expression of the majority of genes (293) was decreased, whilst expression of 53 genes was increased.

Using functional gene clustering and gene ontology terms we were able to identify clusters of genes involved in intracellular signalling, response to wounding and inflammation, protein maturation, transcription and protein kinase cascade.

KEGG pathways analysis allowed us to identify eight genes involved in the chemokine signalling pathway and six genes involved in the spliceosome pathway

Previous studies using microarray in a similar number of specimens have compared plaques from symptomatic against asymptomatic (Razuvaev A, 2011);(Dahl TB, 2007);(Ijas P, 2007);(Vemuganti R, 2006);(Vemuganti R, 2005);(Türeyen K, 2006). Papaspyridonos et al (2006) looked at histologically 'stable' versus 'unstable' plaques but samples included patients who were both symptomatic and asymptomatic. Results

Previous microarray studies comparing carotid atherosclerotic plaques in symptomatic and histologically 'unstable' plaques have alluded to the role of angiogenesis (Türeyen K, 2006), including hypoxia driven angiogenesis (Sluimer JC, 2007), inflammation (Razuvaev A, 2011);(Dahl TB, 2007), cytokine and chemokine signalling (Haley KJ, 2000), apoptosis (Oksala N, 2010);(Martinet W, 2002) alterations in lipid metabolism (Vemyganti R, 2006), osteogenesis, proteolysis and collagenolysis (Murillo CA, 2009), and changes in iron metabolism in plaque progression (Ijas P, 2007).

This study further adds evidence regarding the role of altered expression of genes related to inflammation, proteolysis and chemokine signalling, but also suggests an additional role for disturbances in transcription, spliceosome pathway and cellular components related to the endoplasmic reticulum. As the majority of the genes are down-regulated 293/346 (85%) it is likely that down-regulation of many of the genes identified are contributing to plaque instability. It is also possible that varying degrees of altered gene expression can cause variable plaque instability.

The limitations of this study are similar to those in Chapter 13. These limitations include the use of whole plaque specimens in all experiments. The purpose of this was that historical gene expression studies in carotid plaques have evaluated subgroup cell types and populations, and by avoiding this targeted selection of cells we have avoided introducing potential bias into the analysis. By using whole plaque specimens and individual RNA samples on each array the heterogeneity of specimens meant that when multiple testing correction was applied no significant values were detected. On the recommendation of the array manufacturer, after omitting multiple testing correction our gene list was placed in hierarchical order and top genes used based on p-value. In view of this single analysis we

looked at gene clusters and pathways and not individual genes. This study was meant to serve as a discovery study to provide genes and pathways of interest for further large scale studies.

In conclusion, this study has identified genes with altered expression which are involved in immune, inflammatory, cell signalling and protein defining processes between histologically unstable and stable plaques within neurologically asymptomatic patients. Functional analysis of these candidate genes will be needed to determine if the differential expression is a precursor to symptom onset. Once validated, these genes may become targets for innovative medical treatments in the future or (in conjunction with novel US/MRI/PET imaging features or plasma biomarkers) could help identify asymptomatic patients with histologically, unstable plaques that would benefit from surgical intervention. This work will require evaluation in experimental models of unstable carotid plaque to separate cause from effect.

Chapter 17. Conclusions and Future Work

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Chapter 17. Conclusions and Future Work

17.1 Introduction

Worldwide, stroke is the second leading cause of death, responsible for 4.4 million (9 percent) of the total 50.5 million deaths each year. It is the third leading cause of death in the UK accounting for over 56,000 deaths in England and Wales in 1999, which represents 11% of all deaths. Each year in England, The incidence (number of new cases in a specific population over a given time period) of new or recurrent strokes is 110000 and TIAs 20000. More than 900,000 people in England are living with the effects of stroke, with half of these being dependent on other people for help with everyday activities (NICE, 2008).

Greater than 1/3 of all strokes are caused by thrombo-embolism from an unstable carotid atherosclerotic plaque. In this thesis I aimed to look at patho-genetic causes of plaque instability. I also aimed to correlate clinical, ultrasound and TCD data to histologically unstable plaques.

Using data obtained from transcranial doppler monitoring of the middle cerebral artery we showed the detection of spontaneous embolisation was significantly greater in symptomatic patients than asymptomatic patients. The prevalence of SE was closely related to the recency of both the index clinical symptom and the most recent clinical event and finally the prevalence of recurrent cerebral ischaemic events was twice as high in patients with TCD evidence of SE.

Conclusions

When I analysed the plaques from patients whom had recurrent events after admission to the vascular surgery unit, we found that patients that had a large lipid core on histology and low GSM on plaque ultrasound were more significantly more likely to have recurrent events, suggesting a role for measuring GSM in all patients in order to prioritise intervention.

When looking at how the histological composition of plaques changed from time of most event, I showed as might be expected that features of plaque instability, including large lipid core, marked inflammation, many foam cells, marked vascularity and cap rupture, there was a sustained decline in prevalence out to day 28. This added to the current body evidence that the acute period is the 'high risk' period for recurrent events. Interestingly after day 28 there was an increase in all features of plaque instability suggesting that in patients who have delayed surgery there the natural progression maybe that plaques re-develop 'unstable' features and that best medical therapy may possibly be preventing onset of symptoms, or it may possibly be that compliance of best medical therapy is poor in this delayed group and so there is re-accumulation of unstable features.

Finally as the gold standard for measuring 'plaque instability' remains to be histology, using data from 200 patients having carotid endarterectomy I was able to create a statistical model using clinical and ultrasound data to predict which patients have histologically unstable plaques. I was able to show that patients that have had recent symptoms (<14 days) and have a low GSM (<25) and large plaque area (>80mm²) have a 92% chance of having a histologically unstable plaque. This model once validated can be used in units in the clinic or at the bedside to help prioritise patients for urgent intervention.

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Conclusions

Using whole genome microarray that measures the entire transcriptome (24,000) genes at a single time point I was able to demonstrate the upregulation of several genes related to 'unstable' plaques graded according to clinical, ultrasound, TCD and histological criteria. I have found several new genes never previously associated with atherosclerosis, including GAK, SIRPB1 and TWF2. In an independent cohort we showed CCL19 and CTSG genes related to inflammation and proteolysis were significantly upregulated in histologically and clinically 'unstable' plaques.

I performed protein quantification studies to demonstrate the protein expression of these genes within the plaque that showed Cathepsin G (protein product of CTSG) was expressed in most plaques, which may reflect its role in leucocytes within the plaque. We found that the CCL19 protein was significantly expressed in plaques that were histologically unstable and from patients with recent symptoms (<14 days). CCL19 was expressed around the cap and core region. Finally using a final independent cohort we were able to show that serum samples from acutely symptomatic patients (symptoms under 7 days) had significantly higher levels of CCL19 than matched samples from asymptomatic patients. The expression of Cathepsin G was not detected in serum suggesting a localised effect within plaque only.

In a discovery study, I showed that in plaques harvested from clinically asymptomatic patients using whole genome microarray, I have been able to show that, that plaques that are histologically unstable, there is altered expression of genes related to inflammation, proteolysis and chemokine signalling, but also suggests an additional role for disturbances in transcription, spliceosome pathway and cellular components related to the endoplasmic

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reticulum. This is the first study to look at gene expression in plaques from patients who were asymptomatic but subsequently found to have histologically 'unstable' plaques.

17.2 Future Work

Future work can be directed to the validation of further genes highlighted through the whole genome microarray studies including the study using plaques from clinically asymptomatic patients.

Future laboratory work also includes an investigation into primarily cultured cells obtained from carotid plaques to develop a laboratory model for testing potential therapeutic agents to reduce plaque instability. Carotid plaques harvested from study participants with symptomatic disease will be split into two parts. One part will be used for explant culture and placed directly in DMEM (Dulbeccos modification of Eagles medium), the second part will be subjected to enzymatic digestion with Pronase followed by formal cell culture. Aliquots of supernatant will be removed from each culture at one, two, three and four weeks, snap frozen and stored for later analysis in batches. Culture supernatant will be analysed by ELISA for the proteins identified in our experiment and subsequent validation experiments. This would allow the identification of those markers that remain expressed in culture over time and the optimal type of culture that best reflects the in vivo plaque. Once this culture model has been developed a series of experiments will be performed to determine the effects of blocking the expression of CCL19 and its effects on plaque instability and the effects of other pharmacotherapeutic agents including statins, , angiotensin II receptor agonist, angiotensin converting enzyme and cyclo-oxygenase inhibitor Further experiments will be based on proteomic analysis of plasma samples collected from the study participants over the course of the study. Using MALDI-TOF mass spectroscopy a proteome screen will be performed in each patient. Groups will be compared using not only clinical indicators of plaque instability but also according to ultrasound, transcranial doppler microembolic counts and histological evidence of plaque instability.

Finally using the model described in chapter 12 we hope to validate this on a new cohort of patients in a prospective study, with data ultimately leading to the commencement of a randomised control trial whereby patients with all features of instability undergo immediate surgery and those predicted to have a low chance of plaque instability remain on best medical therapy.

Appendix I

Data Collection Proforma

ID			DOB:			Gender: Ma	le/Female
Mode of Admiss	ion: Elect	ive/Emerg	ency				
Risk factors:	DM	HT	LIP	MI/Ang	Renal,	Smoking (Yes/No/Ex)	
Symptomatic: Y	ſes	No					
Time from prese Actual days:	ntation to	surgery (weeks): N	V/A <2	2-4 5-8	9-12 >12	
Time from most Actual days:	recent eve	nt to surg	ery (weeks	6): N/A	<2 2-4	5-8 9-12 >12	
Total No of Even	its:						
Nature of Event:	Stroke	No RND RND		TIA		AFx	
Duplex Stenosis:	Left:		Right:				
BP and time/loca	ntion of me	asuremei	ıt:				
Chronic Antipla	telet thera	py:-		There	2017		Started within 1 week

Therapy	Started within 1 week
None pre-op	
Aspirin	
Aspirin & Dipyridamole	
Clopidogrel	
Other:	

Spontaneous Emboli count (30 mins pre-op): Nicolaides Criteria Duplex plaque section:

- GSM:
- Plaque Volume:
- Dark Area Close to Lumen:
- Discrete White Areas:
- Number of Discrete White Areas:
- Type of Plaque:
- Percentage Dark Area:

Complications:-

Complication	Intra-op Aetiology	Post op (<30 Days) Actiology
Death		
Disabling CVA		
Non Disabling CVA		

Fresh Weight: ABCD2 Score Referrer Index event Referred Seen Op Home

Appendix II

Patient Information and Consent Sheet

Patient information sheet number 2.5 Dated 24/06/2010

An investigation into carotid plaque instability

Principal Investigator Mr Matthew J Bown MD MRCS Lecturer in Surgery University of Leicester/University Hospitals of Leicester NHS Trust Contact telephone number: 0116 2523252

We would like to invite you to take part in a research study, which will improve our understanding of carotid artery disease and hopefully lead to improvements in its future treatment. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully before you decide whether or not you wish to take part in this study. Ask us if there is anything that is not clear or if you would like more information.

1. What is the purpose of the study?

Carotid arteries are the blood vessels that carry blood to the brain. There is one on each side of the neck and each can be easily felt. As with other blood vessels in the body these can become diseased and gradually 'furr up' called a plaque. The narrowing caused by this can result in a stroke if parts of the furred up blood vessel break off and travel into the brain. Appendices

We do not know why some people develop this disease and others do not. We do know however, that it is more common after the age of 55 and men are more commonly affected. Having a close relative who has had a stroke also increases the risk. High blood pressure, high cholesterol and Diabetes are the other risk factors known to affect the carotid artery. We think that age related changes in the carotid tissues makes them prone for cholesterol deposits and atherosclerosis. In addition we know that genetics play a role in the development of carotid plaques and may contribute to plaque instability.

One of the main problems with diseased carotid arteries is that two people may have a similar degree of narrowing in the blood vessels but be at different risk of having a stroke. We refer to those patients who are at risk of developing a stroke as having an unstable plaque whereas those who are not at risk of stroke are said to have a stable plaque.

When the narrowing in the carotid arteries reaches a certain level (with or without symptoms) we perform operation to clear the block. Because there are risks associated with this surgery we would like to operate only if we knew that the patient was at risk of having a stroke. Presently we cannot tell which patients are at risk by any means other than what has happened to the patient in the past, with mini-strokes or even major strokes being the only indicators of unstable plaques. If we could identify what is causing a plaque to become unstable we may be able to identify a blood test that could allow us to determine which plaques are unstable and which are not. This would then allow us to target surgery for those patients who would benefit most.

2. Why have I been invited?

You have been invited to participate in this study because you have been diagnosed by your doctor as having carotid artery disease.

3. What will be involved if I take part in the study?

We will ask you few questions regarding your general health and personal habits like smoking and alcohol. Then we would like to take a small amount of blood sample (15-20ml) from a vein in your arm once only, which can usually be taken along with one of the blood samples that the doctors caring for you on the ward will need or during your operation whilst you are asleep. We would also like to take a urine sample at the same time. We would also like to take a further blood and urine sample when you come back to be followed up and seen in the out-patients clinic at intervals of 6 weeks, 12 weeks and 6 months. From this we can analyse circulating chemicals in the blood and urine and also genetic differences between different participants in the study. As new potential genes that cause carotid artery disease are discovered all the time the sample taken from you may be used in future studies of these newer genes.

We would also like to take the area of disease from the blood vessel that is normally thrown away at the time of the operation and examine this in the laboratory. This may involve growing some of the cells from the diseased are in the laboratory to see how they respond to various drug treatments. This will have no effect on your operation or recovery. At the end of the study all samples and cells grown from them will be discarded. During the course of your admission you will have routine ultrasound imaging (pictures/motion pictures) taken of your diseased blood vessel. We would also like to take a look at these images, and use new computer software to analyse special features seen.

4. Will the information obtained in the study be confidential?

All details recorded in the study will be in the strictest confidence. No details that could be used to identify you will be used in any records relating to the study. All samples taken will be analysed anonymously and the information or samples taken will not be available to anyone other than the researchers conducting the study.

5. What if I am harmed by the study?

Medical research is covered for mishaps in the same way as for patients undergoing treatment on the NHS ie compensation is only available if negligence occurs.

6. What happens if I do not wish to participate in this study or wish to withdraw from the study?

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so without justifying your decision and your future treatment will not be affected.

7. What will happen to the results of the research study?

We will analyse the results of the research and disseminated the knowledge through journals and presentations in conferences. We will anonymise the data so that you will not be identified in any of the reports. The results of your blood sample will not be revealed to you at any stage of the research. Consent Form Version 2.5 Date 24/06/2010

Study Number

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: An investigation into carotid plaque instability

Name of Researcher: Mr. Matthew J Bown MD FRCS

- 1. I confirm that I have read and understand the information sheet dated 24/06/2010, *version 2.5*, for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
- 2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.
- 3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals involved in the research, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
- 4. I agree to take part in the above study.

Name of Patient

Date

Signature

Name of Person Taking consent

Date

Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Consent Form Version 2.5 Date 24/06/2010

Please initial

1	_	_	_	_	_

	 	-

		- 1
		- 1
		- 1
		- 1
		- 1

Appendix III

Solutions

1% Bovine Serum Albumin

- Bovine Serum Albumin 1g
- PBS 100ml

0.1% DEPC Water

- 0.1% Diethylpyrocarbonate (DEPC) to
- Distilled water 1000ml
- Mix well and let set at room temperature for 1 hour
- Autoclave

Eosin

- Eosin 2.5g
- Distilled water 495ml
- Glacial acetic acid 0.5ml

10% Formalin

- Formaldehyde, 37-40% 100 ml/L
- Distilled or deionized water 900 ml/L
- Sodium phosphate, monobasic 4.0 g/L
- Sodium phosphate, dibasic (anhydrous) 6.5 g/L

Mayers Haemaoxylin

- Aluminium Potassium Sulphate 50g
- Distilled Water 1000ml
- Haematoxylin 1g
- Sodium iodate 0.2g
- Glacial Acetic Acid 20ml

1% Normal Donkey Serum

- Normal Donkey Serum 1g
- PBS 100ml

Phosphate Buffered Saline (PBS) (pH 7.4)

- Potassium dihydrogen orthophosphate 0.24g
- Disodium hydrogen orthophosphate 1.44g
- Sodium chloride 8g
- Potassium chloride 0.2g
- Distilled water 800-1000ml

0.01% Sodium Azide in PBS

- Sodium Azide 10mg
- PBS 100ml

Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)

- Tri-sodium citrate (dihydrate) 2.94 g
- Distilled water 1000 ml
- Adjust pH to 6.0 with 1N HCl
- Tween 20 0.5ml

0.3% Triton X-100

- PBS 1L
- Triton 3ml

Appendix IV

Protocols

Extraction of Serum from Whole Blood

Sample Collection

Whole blood should be drawn into pyrogen free tubes containing no anticoagulant using a venepuncture vacuum system.

Sample Processing

All handlings of the sample should be done in a biohazard safety cabinet while wearing gloves. Store the serum at -80° C

Procedure

- Allow tubes to stand for 45 minutes at room temperature
- Spin the blood at roughly 2500 rpm for 15 minutes at room temperature in a centrifuge
- Remove the tubes from the centrifuge, and in a clean and safe environment, open the tubes to access the serum located at the top of the specimen
- Using a sterile transfer pipet or a 1000 µl pipettor, carefully transfer 2x 1.5 ml of the serum into appropriately labelled cryovial tubes without disturbing or collecting any of the pelleted cells
- As soon as possible, snap freeze the cryovials in liquid nitrogen, where they can then be stored or store at -80° C

Preparation of RNase-free water

- Measure water into RNase-free glass bottles
- Add 1 ml of 0.1% (v/v) diethylpyrocarbonate (DEPC) to 1000 ml water.
- Let stand overnight
- Autoclave

Extraction of RNA from tissue using Trizol® Reagent

Always use extra care and precautions to avoid contamination with RNAse and DNA contamination. Advise use of RNAse Zap and washing all equipment with DEPC (RNAse free water). Always wear gloves, which should be changed regularly. Work in a biohazard safety cabinet set aside for RNA work.

Tissue Preparation

- Cut 50-100mg of tissue into small pieces.
- Using a mortar and pestle, grind the tissue in liquid nitrogen. Transfer the tissue to a Dupont tube containing Trizol.

Homogenisation of tissue

- Add 1 ml of TRIzol to every 50-100 mg of tissue.
- Homogenize the samples using a sterile power homogeniser.

Phase Separation

- Incubate samples for 5 minutes at room temperature
- Add 0.2 ml of chloroform to each tube
- Cap each tube
- Shake samples vigorously by hand for 15 seconds
- Incubate samples for 5 minutes at room temperature
- Centrifuge samples for 15 minutes at 12,000 x g at 4°C

RNA Precipitation

- Transfer the upper aqueous phase to a fresh tube
- Add 0.5 ml of isopropyl alcohol to precipitate RNA. Incubate the samples at room temperature for 5 minutes and centrifuge at 12,000 x g for 10 minutes at 4°C
- Transfer the sample in a new tube
- Incubate for 5-10 minutes at room temperature
- Centrifuge for 10 minutes at 12,000 x g at 4°C. The RNA will form a pellet on the side or bottom of the tube

RNA Wash

- Discard the supernatant
- Wash pellet with 1 ml 75% ethanol.
- Mix sample by vortexing. The RNA pellet may float.
- Centrifuge at 12000 x g for 5 minutes at 4°C. If this RNA will be used for RT-PCR repeat wash twice.

Re-suspending the RNA

- Remove supernatant
- Air dry the pellet for 5-10 minutes. Do not completely dry out the pellet
- Dissolve pellet in 30 to 60 µl RNase free water

Determination of RNA Concentration and Purity

- Take 2 to 5 µl RNA sample from the original stock, diluted with 998 or 995 µl RNase free water in a 1.5 ml microcentrifuge tube. This will give you 500 or 200 time dilution of the RNA sample.
- Pipet 1 ml RNAse free water in a clean cuvette and read absorbance as blank.
- Pipet the diluted RNA sample in to a clean cuvette and read absorbance at 260 nm and 280 nm.
- Use the formula below to determine RNA Concentration of the original sample:

[RNA $\mu g/\mu l$]= A₂₆₀ x 33 x dilution factor / 1000

• To determine the purity of the RNA sample, calculate ratio of A260/A280. Ratios between 1.7 to 2 represent good RNA)

Qualitative and Quantitative Assessment of RNA using Agilent Bioanalyzer

Materials

- RNA samples (up to 12 per chip)
- RNA 6000 ladder (Ambion # 7152)

Bioanalyzer RNA Nano Kit (Agilent # 5064-8229) Includes chips and reagents

RNAse Zap

DEPC water

Instrumentation

Agilent 2100 Bioanalyzer

Heat block (e.g. VWR standard)

Microcentrifuge (e.g. IEC Micromax RF)

Chip Priming Station (Agilent #5065-4401)

Vortexer (e.g. IKA model MS1)

Vortex Mixer Adapter (Agilent #5022-2190)

Reagent preparation

Filtered Gel (warm reagents to room temperature before use 15 to 20 mins)

- Add 400µl of RNA Gel Matrix onto a filter column.
- Spin for 10 minutes at 1500xg.
- Remove column, and date tube.
- Expires 4 weeks when stored at 4°C.

Gel-Dye Mix (warm reagents to room temperature before use)

- In a new tube, add 1µl of Dye to 65µl of Filtered Gel. (or 2µl Dye to 130µl Filtered Gel)
- Vortex tube to mix. Date tube
- Cover with tin foil when stored
- Spin for 10 minutes at max speed prior to each use.
- Expires 1 week when stored at 4°C

Ladder

- Denature an aliquot of ladder for 10 minutes at 70°C.
- Snap cool on ice

Samples

- Denature 2 minutes at 70°C
- Snap cool on ice
- •

Procedure

Preparing the Bioanalyzer

- Electrode Cleaner chips: add 350µl DEPC water to one & 350µl RNAse Zap to another.
- Place Zap filled chip in the Bioanalyzer to soak for 30 sec to 1 minute.
- Place Water filled chip in the Bioanalyzer to soak for 30 sec to 1 minute.
- Leave the lid open for 10 sec to allow the electrode to dry.

Loading the Chip

- Prepare the priming station for use (clean gasket, tight syringe, position silver clip).
- Pull the syringe to the 1ml mark.
- Place the chip in the priming station.

- Pipette 9µl of Gel-Dye mix into the well marked with G in a black circle
- Snap the priming station lid closed until you hear it click
- Push the plunger of the syringe down to 0.2 ml until it fits under the silver stopper
- Wait exactly 30s
- Squeeze the sliver stopper to release the plunger and let it rise to a complete
- Stop, above or equal to 0.7 ml
- Gently lift the priming station lid
- Recommend: Inspect the back of the chip for any air bubbles before proceeding
- Air bubbles may appear in the area between the wells
- Pipette 9µl of Gel-Dye mix into the other two G wells
- Pipette 5µl of the Nano Marker into every sample well and ladder well
- Add 1 µl of sample into each well
- Empty wells should be brought up to 6µl using 1µl RNAse-free water
- Pipette 1µl of the ladder into the ladder well
- Place the chip in the vortexer
- Set the dial to zero
- Turn the power on
- Turn the dial to 2200 rpm, such that the chip appears almost stationary
- Vortex the chip for 1 minute at about 2200 rpm

Running the Chip

- Open the Expert software
- Open the lid, and you should see an open lid on the screen
- Place the chip in the Bioanalyzer and gently close the lid
- You should see a chip appear on the screen
- Choose the Assay you wish to run: (Assays \rightarrow RNA \rightarrow Eukaryotic Total RNA Nano)
- Start
- Enter the number of samples you are running
- Click Start.
- Taskbar status
- Script will download (approx. 1 minute)

- Warming (4 seconds)
- Focusing; monitor for 10 seconds
- Assay will return to previous states as the run proceeds
- If no errors after 10 sec., the run may be left unattended
- Assay time for a full chip is 15 minutes
- If errors occur, the run will abort and indicate the error in the upper right corner
- Check that you have done all steps correctly
- Assay Progression
- The ladder will proceed first followed by well 1, 2, 3, 4, etc.
- Traces will appear in real time on the screen.
- Click "Data" on the left to view the traces that have completed.
- Click "Data" on the left to enter the sample names.

Clean Up

- Remove the chip from the Bioanalyzer promptly and discard.
- Clean as for "Preparing the Bioanalyzer"

Whole-Genome Gene Expression DASL® Assay

Make SUR

This process reverse transcribes sufficient RNA from each individual sample to be used once in the Whole-Genome DASL Assay.

Time:

- Hands on 15 minutes
- 10 minute incubation
- 1 hour incubation

Consumables:

- MCS Reagent (Illumina)
- RNA Samples
- 96-well 0.2ml skirted microplate

Preparation:

- Preheat the heat sealer.
- Preheat a heat block to 42°C.
- Thaw the MCS tube to room temperature. Vortex to mix, and then pour the tube into a new, non-sterile, disposable reservoir.
- Apply a SUR barcode label to a new 96-well microplate.

Steps:

- Normalize intact RNA samples to 20–100ng/µl (or partially degraded RNA samples to 40 - 200ng/µl) with DEPC-treated H2O.
- [Add 5 μ l MCS to each well of columns 1, 2, and 3 of the SUR plate.
- Quickly add 5 µl normalized RNA sample to each well of columns 1, 2, and 3 of the SUR plate. Change tips between RNA sample dispenses.
- Seal the SUR plate with a microplate heat seal.

- Vortex the sealed plate at 2300 rpm for 20 seconds.
- Pulse centrifuge to 250g for 1 minute.
- Incubate the SUR plate at room temperature at least 10minutes (up to 1hour).
- Place the SUR plate on the preheated 42°C heat block and close the lid to reduce condensation on the plate seal. Incubate at 42°C for 1 hour.
- Pulse centrifuge the SUR plate to 250 xg for 1 minute to remove condensation from the walls of each well.
- Do one of the following
- Immediately set a heat block to 70°C and proceed to *Make ASE*. Start thawing the DAP and OB1 reagents
- If you do not plan to proceed immediately to *Make ASE Plate*, then store the sealed SUR plate after the 42°C incubation up to four hours at 2 to 8°C or up to 24 hours at 15 to -25°C. Hands-on time: ~15 minutes Incubation time: One 10-minute incubation, one 1-hour incubation

Make MUR

This process reverse-transcribes sufficient RNA from each individual sample to be used at least *six times* in the Whole-Genome DASL Assay.

Time:

- Hands on 15 minutes
- 10 minute incubation
- 1 hour incubation

Consumables:

- MCM Reagent (Illumina)
- RNA Samples
- 96-well 0.2ml skirted microplate

Preparation:

• Preheat the heat sealer.

- Preheat a heat block to 42°C.
- Thaw the MCM reagent tube to room temperature. Vortex to mix. Pour the MCM tube into a new, non-sterile, disposable reservoir.
- Apply a MUR barcode label to a new 96-well microplate.

Steps:

- Normalize intact RNA samples to 20–100 ng/µl (or partially degraded RNA samples to 40 - 200 ng/µl) with DEPC-treated H2O.
- Add 40 μ l MCM to each well of columns 1, 2, and 3 of the MUR plate.
- Quickly add 40 µl normalized RNA sample to each well of columns 1, 2, and 3 of the MUR plate. Change tips between RNA sample dispenses.
- Seal the MUR plate with a microplate heat seal.
- Pulse centrifuge to 250 xg for 1 minute.
- Incubate the MUR plate at room temperature for at least 10 minutes (up to 1 hour).
- Place the MUR plate on the preheated heat block and close the lid to reduce condensation on the plate seal. Incubate the plate at 42°C for 1 hour.
- Pulse centrifuge the sealed MUR plate to 250 xg for 1 minute to remove condensation from the walls of each well.
- Do one of the following:
- Immediately set a heat block to 70°C and proceed to *Make ASE*. Start thawing the DAP and OB1 reagents.
- If you do not plan to proceed immediately to *Make ASE*, then store the sealed MUR plate after the 42°C incubation up to four hours at 2 to 8°C or up to 24 hours at -15 to -25°C. Hands-on time: ~15 minutes Incubation time: One 10 minute incubation, one 1hour incubation

Make ASE

This process combines the biotinylated cDNAs with Assay-specific oligos (ASOs), hybridization reagents, and paramagnetic particles in an Assay Specific Extension (ASE) plate. The plate is then placed in a heat block and the ASOs for each sequence target of interest are allowed to anneal to the biotinylated cDNA samples. The cDNA is simultaneously captured by paramagnetic particles. The resulting ASE plate is ready for the extension and ligation of the hybridized oligos on the bound cDNAs. Time:

- Hands on 30 minutes
- 14-20 hours incubation

Consumables:

- OB1 Reagent (Illumina)
- DAP Reagent (Illumina)
- 96-well 0.2ml skirted microplate

Preparation:

- Preheat the heat sealer.
- Preheat the heat block to 70°C.
- If the SUR or MUR plate was stored at -15 to -25°C overnight, thaw it to room temperature and then pulse-centrifuge to 250 xg for 1 minute.
- Thaw the DAP reagent tube to room temperature and vortex the contents to mix completely, then pulse centrifuge to collect the contents at the bottom of the tube. Pour the entire contents of the tube into a sterile reservoir.
- Thaw the OB1 tube to room temperature. Vortex to completely re-suspend the solution. Pour the entire contents of the OB1 tube into a sterile reservoir. Do not centrifuge the OB1 tube.
- Apply an ASE barcode label to a new 96-well microplate.

Steps:

- Add 10 μ l DAP to each well of columns 1, 2, and 3 of the ASE plate.
- Add 30 μ l OB1 to each well of columns 1, 2, and 3 of the ASE plate.
- Carefully remove the heat seal from the SUR or MUR plate.
- Transfer 10 µl biotinylated cDNA from each occupied well of the SUR or MUR plate to the corresponding well of the ASE plate.
- Heat-seal the ASE plate with a microplate heat sealer.
- Pulse centrifuge the ASE plate to 250 xg for 1 minute.

- Vortex the ASE plate at 1600 rpm for 1 minute or until all beads are completely resuspended.
- Place the sealed ASE plate on the preheated 70°C heat block and close the lid. Handson time: ~30 minutes Incubation time: 14–20 hours
- Immediately change the set temperature of the heat block to 30°C. Leave the ASE plate in the heat block for 14–20 hours while it cools to 30°C.
- Proceed to *Add MEL*.

Add MEL

After the oligos are hybridized to the cDNA, mis-hybridized and excess oligos are washed away. Next, an extension and ligation master mix (consisting of extension and ligation enzymes) is added to each cDNA sample. The extension and ligation reaction occurs at 45°C.

Time:

- Hands on 45 minutes
- 15 minutes incubation

Consumables:

- AM1 Reagent (Illumina)
- UB1 Reagent (Illumina)
- MEL Reagent (Illumina)

Preparation:

- Thaw the MEL tube to room temperature. Pour the tube into a sterile reservoir right before using it.
- Remove the AM1 bottle from the refrigerator and leave it at room temperature for 10 minutes. Pour 11 ml AM1 into a second sterile reservoir. Add 10 ml for each additional plate.
- Remove the UB1 bottle from the refrigerator. Pour 11 ml UB1 into a third sterile reservoir.

• Remove the IP1 and SCM tubes from the freezer and let them thaw.

Steps:

AM1 Washes

- Remove the ASE plate from the heat block and reset the heat block to 45°C.
- Centrifuge the ASE plate to 250 xg.
- Immediately place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.
- Carefully remove the heat seal from the ASE plate
- Using an 8-channel pipette with new tips, remove all the liquid (~50 μl) from the occupied wells and discard it. Leave the beads in the wells.
- With the ASE plate on the raised-bar magnetic plate, use an 8-channel pipette with new tips to add 50 µl AM1 to each occupied well of the ASE plate.
- Seal the ASE plate with clear adhesive film.
- Vortex the ASE plate at 1600 rpm for 20 seconds or until all beads are resuspended.
- Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes or until the beads are completely captured.
- Using the same 8-channel pipette with the same tips, remove all AM1 reagent from each occupied well. Leave the beads in the wells.
- Hands-on time: ~45 minutes Incubation time: 15 minutes
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UB1 Washes

- Remove the ASE plate from the raised-bar magnetic plate.
- Using an 8-channel pipette with new tips, add 50 μl UB1 to each occupied well of the ASE plate.
- Place the ASE plate onto the raised-bar magnetic plate for approximately 2 minutes or until the beads are completely captured.
- Using the same 8-channel pipette with the same tips, remove all UB1 reagent from each occupied well. Leave the beads in the wells.
- Repeat steps 1 through 4 once.

Add MEL

- Using an 8-channel pipette with new tips, add 37 μl MEL to each occupied well of the ASE plate.
- Seal the plate with clear adhesive film.
- Vortex the plate at 1600 rpm for 1 minute to resuspend the beads.
- Incubate the ASE plate on the preheated 45°C heat block for exactly 15 minutes. During the incubation, perform the *Make PCR* process.

Make PCR

This process adds the DNA Polymerase and Uracil DNA Glycosylase to the SCM master mix for PCR. It creates a 24-sample plate for the Inoc PCR process.

Time:

• Hands on time 15 minutes

Consumables:

- DNA Polymerase
- Uracil DNA Glycosylase
- SCM Reagent (Illumina)
- 96-well 0.2ml skirted microplate

Preparation:

- Apply a PCR barcode label to a new microplate.
- Invert the thawed SCM tube 10 times to mix.

Steps

- Add 64 µl Illumina-recommended DNA Polymerase to the SCM tube.
- Add 50 µl Uracil DNA glycosylase to the SCM tube.

- Invert the tube several times to mix the contents.
- Using an 8-channel pipette, add 30 µl of the SCM mixture to each well of columns 1,
 2, and 3 of the PCR plate.
- Seal the PCR plate with clear adhesive film.
- As soon as the 15 minute ASE plate incubation is complete, proceed immediately to Inoc PCR. Hands-on time: ~15 minutes

Inoc PCR

This process uses the template formed during the extension and ligation process in a PCR reaction. This PCR reaction uses two universal primers. One is labeled with a fluorescent dye and the other is biotinylated. The biotinylated primer captures the PCR product and allows the strand containing the fluorescent signal to be eluted.

Time:

• Hands on time 30 minutes

Consumables:

- UB1 Reagent (Illumina)
- IP1 Reagent (Illumina)

Preparation:

- Pour 6 ml UB1 into a sterile reservoir.
- Pour the entire contents of the IP1 tube into a second sterile reservoir

Steps:

Remove Supernatant

- Remove the ASE plate from the heat block
- Reset the heat block to 95°C
- Place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured

Using an 8-channel pipette, remove and discard the supernatant (~50 μl) from all occupied wells of the ASE plate. Leave the beads in the wells

UB1 Wash

- Leaving the plate on the magnet and using an 8-channel pipette with new tips, add 50 µl UB1 to each occupied well of the ASE plate.
- Leave the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.
- Remove and discard the supernatant (~50 μl) from all occupied wells of the ASE plate. Leave the beads in the wells.

Add IP1

- Using an 8-channel pipette with new tips, add 35µl IP1 to each occupied well of the ASE plate
- Seal the plate with clear adhesive film
- Vortex at 1800 rpm for 1 minute or until all beads are resuspended
- Place the plate on the preheated 95°C heat block for 1 minute
- Place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured
- Using an 8-channel pipette with new tips, transfer 30 µl supernatant from each occupied well of the ASE plate to the corresponding well of the PCR plate. Pipette the PCR plate wells up and down 3–4 times
- Change tips between column dispenses. Hands-on time: ~30 minutes
- Seal the PCR plate with the appropriate PCR plate-sealing film for your thermal cycler
- Pulse centrifuge the plate to 250 xg for 1 minute
- Immediately transfer the PCR plate to the thermal cycler
- Proceed to Cycle PCR
- This concludes the Pre-PCR processes for the Whole-Genome Gene Expression DASL Assay.

Cycle PCR

This process thermal cycles the PCR plate to fluorescently label and amplify the templates generated in the pre-PCR process.

Time: Cycle time ~2 hours 45 minutes

Steps:

- Place the sealed plate into the thermal cycler and run the thermal cycler program shown in this table.
- Do one of the following:
- Proceed immediately to *Bind PCR*.
- Seal and store the PCR plate at -15 to -25°C.

	Temperature	Time
	37°C	10 minutes
	95°C	3 minutes
V24	95°C	35 seconds
	56°C	35 seconds
X34	72°C	2 minutes
	72°C	10 minutes
	4°C	5 minutes

Bind PCR

In this step, the double-stranded PCR products are immobilized by binding the biotinylated strand to paramagnetic particles. The solution is transferred to a filter plate and incubated at room temperature so that the PCR product may bind to the paramagnetic particles.

Time:

- Hands on time 20 minutes
- Incubation time: 1 hour

Appendices

Consumables:

- MPB Reagent (Illumina)
- Filter plate with lid

Preparation:

- Vortex the MPB tube several times or until the beads are completely resuspended. Pour the MPB tube into a sterile reservoir.
- Write the PCR plate barcode number in the space provided on the filter plate label. Apply the filter plate label to the top of the filter plate next to column 12.

Steps:

- Pulse centrifuge the PCR plate to 250 xg for 1 minute.
- Place new tips onto a 5–50 µl multichannel pipette and transfer 20 µl resuspended MPB from the reservoir into each occupied well of the PCR plate.
- Place new tips on an 8-channel pipette and set it to 85μ l. Place the PCR and filter plates next to each other with the A1 wells in the upper left corner.
- Pipette the solution in the PCR plate up and down several times to mix the beads with the PCR product. Transfer the mixed solution from each occupied well of the PCR plate into the corresponding well of the filter plate. Change pipette tips between column dispenses.
- Cover the filter plate with the filter plate lid.
- Store at room temperature, protected from light, for 1 hour.
- Proceed to Make INT. Hands-on time: ~20 minutes Incubation time: 1 hour

Make INT

In this step, the single-stranded fluor-labeled PCR product from the filter plate is washed and then eluted into an intermediate (INT) plate.

Time:

• Hands on time 20 minutes

Appendices

Consumables:

- 0.1N NaOH
- UB2 Reagent (Illumina)
- MH1 Reagent (Illumina)
- 96-well V bottom plate
- 96-well 0.2ml skirted microplate
- Filter plate adaptor

Preparation:

- Apply a INT barcode label to a new 96-well 0.2 ml skirted microplate.
- Using a serological pipette, transfer 10 ml UB2 into a sterile reservoir.
- Pour 5 ml 0.1N NaOH into a second sterile reservoir.
- Pour the contents of an MH1 tube into a third sterile reservoir.

Steps:

- Place the filter plate adapter on an empty, unlabeled 96-well V-bottom plate (waste plate).
- Place the filter plate containing the bound PCR products onto the filter plate adapter.
- Centrifuge to 1000 xg for 5 minutes at 25°C.
- Using an 8-channel pipette with new tips, add 50 µl UB2 to each well of columns 1, 2, and 3 of the filter plate. Dispense slowly to avoid disturbing the beads.
- Replace the filter plate lid.
- Centrifuge to 1000 xg for 5 minutes at 25°C.
- Using an 8-channel pipette with new tips, add 30 µl MH1 to each well of columns 1,
 2, and 3 of the INT plate.
- Replace the waste plate with the INT plate. Orient the INT plate so that well A1 of the filter plate matches well A1 of the INT plate.
- Using an 8-channel pipette with new tips, add 30 µl 0.1N NaOH to each occupied well of the filter plate.
- Replace the filter plate lid. Hands-on time: ~20 minutes

- Centrifuge immediately to 1000 xg for 5 minutes at 25°C. At the end, no beads should be visible in the wells of the INT plate.
- Discard the filter plate.
- Gently mix the contents of the INT plate by moving it from side to side without splashing.
- Seal the INT plate with a 96-well cap mat. Store the plate in the dark until ready to proceed with precipitation of samples.
- Do one of the following:
- Proceed to Precipitate and Wash INT.
- If you do not plan to use the INT plate immediately in the protocol, store it at -15 to 25°C for up to 24hours.

Precipitate and Wash INT

In this step the single-stranded product from the INT plate is precipitated, washed and resuspended. The product from this plate is hybridized to the BeadChip. Item Quantity Storage Supplied By PS1 reagent 3.2 ml per INT plate 2 to 8°C Illumina 2-propanol Bottle Room temperature User 70% EtOH Bottle Room temperature User MH1 300 µl per INT plate Room temperature Illumina HYB reagent 1.2 ml per INT plate -15 to -25°C Illumina

Time:

• Hands on time 1 hour

Consumables:

- PS1 Reagent (Illumina)
- 2-propanolol
- 70% EtOH
- MH1 (Illumina)
- HYB Reagent (Illumina)

Preparation:

- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- Preheat the Illumina Hybridization Oven to 58°C. Allow 30 minutes for it to equilibrate.
- Place the HYB tube in the 58°C oven for 10 minutes to dissolve any salts that may have precipitated in storage. If any salts remain undissolved, incubate at 58°C for another 10 minutes. Cool to room temperature and mix thoroughly before using.
- Preheat a heat block to 65°C and allow the temperature to stabilize.
- Vortex PS1 bottle then transfer 3.2 ml into a sterile reservoir.
- Pour 10 ml 2-propanol into a second sterile reservoir.
- Pour 20 ml 70%EtOH into a third sterile reservoir.
- In a sterile 15 ml centrifuge tube combine 300 µl MH1, 300 µl nuclease-free water and 1.2 ml HYB. Mix well by vortexing, followed by pulse centrifugation.
- Pour the MH1/water/HYB mix into a fourth sterile reservoir.

Steps:

- Remove the seal from the INT plate.
- Add 30 μ l PS1 reagent to each well of the INT plate.
- Using a multichannel pipette, thoroughly mix the contents by pipetting the solution up and down several times until the solution is uniformly blue.
- Add 90 μ l 2-propanol to each well of the INT plate. Hands-on: ~1 hour
- Using a multichannel pipette, thoroughly mix the contents by pipetting the solution up and down several times until the solution is uniformly blue.
- Seal the INT plate with clear adhesive film.
- Centrifuge the plate to 3000 xg at 2to8°C for 20minutes.
- Remove the INT plate seal and decant the supernatant by inverting the INT plate and smacking it down onto an absorbent pad.
- Tap the inverted plate onto the pad to blot excess supernatant.
- Add 150 µl 70% EtOH to each well of the INT plate.

- Using a multichannel pipette, thoroughly wash the blue pellet in 70% EtOH by pipetting up and down several times.
- Seal the INT plate with clear adhesive film.
- Centrifuge the plate to 3000 xg at 2 to 8°C for 10minutes.
- Remove the INT plate seal and decant the supernatant by inverting the INT plate and smacking it down onto an absorbent pad.
- Tap the inverted plate onto the pad to blot excess supernatant.
- Place the INT plate in the preheated heat block and close the lid.
- Incubate the INT plate at 65°C for 5minutes or until the residual EtOH has evaporated.
- [Add 15 µl of the MH1/water/HYB mix to each well of the INT plate.
- Seal the INT plate with clear adhesive film.
- Pulse centrifuge the plate to 250 xg.
- Using a multichannel pipet, thoroughly dissolve the pellets by pipetting the solution up and down several times.
- Seal the INT plate with a 96-well cap mat. Store the plate in the dark until ready to dispense sample onto a BeadChip.
- Do one of the following:
- Proceed to *Hyb BeadChip*.
- If you do not plan to use the INT plate immediately in the protocol, store it at --15 to 25°C for up to 24 hours.

Hyb BeadChip

In this process the BeadChips are hybridized using the Hyb Chamber. After the Hyb Chamber has been assembled, the samples are ready for hybridization. The BeadChip is hybridized overnight in the Illumina Hybridization Oven at 58°C. Item Quantity Storage Supplied By HCB reagent Tube -15 to -25°C Illumina Hyb Chamber 1 per 4 BeadChips Illumina BeadChips (8x1) 3 per 24 samples Illumina

Time:

- Hands on time: 30 minutes
- Incubation time: 14-20 Hours

Appendices

Consumables:

- HCB Reagent (Illumina)
- Hyb Chamber
- Bead chips (8x3)

Preparation:

- In the Sentrix_ID column of the Sample Sheet, enter the BeadChip ID for each BeadChip section. page 26
- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- Preheat the Illumina Hybridization Oven to 58°C.
- Place the HCB tube in the 58°C oven for 10 minutes to dissolve any salts that may have precipitated in storage. If any salts remain undissolved, incubate at 58°C for another 10 minutes. Cool to room temperature and mix thoroughly before using.
- If the INT plate has been frozen, thaw it completely at room temperature in a lightprotected drawer and then pulse centrifuge it to 250 xg for 1 minute.

Steps

- Place the following items on the bench top : BeadChip Hyb Chamber (1 per 4 BeadChips); BeadChip Hyb Chamber gasket (1 per Hyb Chamber); BeadChip Hyb Chamber inserts (4 per Hyb Chamber)
- Place the Hyb Chamber Gasket into the Hyb Chamber.
- Match the wider edge of the Hyb Chamber gasket to the barcode-ridge side of the Hyb Chamber.
- Lay the gasket into the Hyb Chamber, and then press it down all around.
- Make sure the Hyb Chamber gasket is properly seated.
- Add 200 µl HCB into the eight humidifying buffer reservoirs in the Hyb Chamber.
 Only fill the reservoirs of sections that will contain BeadChips.
- Close and lock the BeadChip Hyb Chamber lid.

 Leave the closed Hyb Chamber on the bench at room temperature (22°C) until the BeadChips are loaded with DNA sample. Hands-on time: ~30 minutes Incubation time: 14–20 hours

Prepare BeadChips for Hybridization

- Remove all the BeadChips from their packages.
- Place each BeadChip in a Hyb Chamber Insert, orienting the barcode end so that it matches the barcode symbol on the Hyb Chamber Insert.

Load Sample

Using a single-channel precision pipette, add 15 μ l sample onto the center of each inlet port .

- Visually inspect all sections. Ensure sample covers all of the sections of the stripe. Record any sections that are not covered. Some residual sample may still remain in the inlet port. This is normal.
- Open the Hyb Chamber.
- Load 4 Hyb Chamber Inserts containing sample-laden BeadChips into each Hyb Chamber.
- Position the barcode end over the ridges indicated on the Hyb Chamber and ensure the inserts are securely seated.

Hybridize BeadChips

- Close and lock the BeadChip Hyb Chamber lid.
- Place the Hyb Chamber into the 58°C Illumina Hybridization Oven.
- Start the rocker by turning on the switch just above the power switch (optional).
- Incubate for 16 hours at 58°C.
- In preparation for the next day's washes, prepare 1X High-Temp Wash buffer from the 10X stock by adding 50 ml 10x High-Temp Wash buffer to 450 ml nuclease-free water.
- Place the Hybex Waterbath insert into the Hybex Heating Base.
- Add 500 ml prepared 1X High-Temp Wash buffer to the Hybex Waterbath insert.
- Set the Hybex Heating Base temperature to 55°C.
- Close the Hybex Heating Base lid and leave the High Temp Wash buffer to warm overnight.

• Proceed to *Wash BeadChip* the next day.

Wash BeadChip

In this process, prepare for the wash steps by removing the BeadChips from the overnight hybridization. Remove the BeadChip cover seals and then wash the BeadChips. Item Quantity Storage Supplied By E1BC Buffer Tube -15 to -25°C Illumina HTW Bottle Room temperature Illumina 100% EtOH Bottle Room temperature User PB1 Bottle Room temperature Illumina XC4 Bottle -15 to -25°C Illumina

Time:

- Hands on time: 30 minutes
- Incubation time: Two 5 minute washes, one 10 minute wash, one 1-hour incubation

Consumables:

- E1BC Buffer (Illumina)
- HTW (Illumina)
- 100% EtOH
- PB1 (Illumina)
- XC4 (Illumina)

Preparation:

- In preparation for the Coat BeadChip protocol, follow these steps to resuspend the XC4 reagent:
- Remove the XC4 bottle from the freezer and thaw.
- Add 335 ml 100% EtOH to the XC4 bottle. The final volume will be 350 ml.
- Each XC4 bottle contains enough to process up to 24 BeadChips.
- Re-cap the bottle, shake vigorously for 15 seconds, and place on a rocker for 30–40 minutes to resuspend. Place the bottle on the side opposite to the frozen pellet if possible.

- After 30-40 minutes, shake the bottle vigorously by hand to ensure all XC4 is in suspension and none is still coating the container. If coating is visible, vortex at 1625 rpm until the XC4 is in complete suspension.
- Once resuspended, use XC4 at room temperature. You can store it at 2 to 8°C overnight. Keep the XC4 in the bottle in which it was shipped until ready for use.
- Add 6 ml E1BC buffer to 2L RNase-free water to make the Wash E1BC solution.
- Place 1 L of diluted Wash E1BC buffer in a Pyrex No. 3140 beaker. Hands-on: 30 minutes Incubation: Two 5 minute washes, one 10 minute wash, one 1-hour incubation

Steps:

Seal Removal

- Remove the Hyb Chamber from the oven and place it on the lab bench. Disassemble the chamber.
- Using powder-free gloved hands, remove all BeadChips from the Hyb Chamber and submerge them face up at the bottom of the beaker.
- Using powder-free gloved hands, remove the cover seal from the first BeadChip. Ensure that the entire BeadChip remains submerged during removal.
- Using tweezers or powder-free gloved hands, transfer the BeadChip to the slide rack submerged in the dish containing 250 ml Wash E1BC solution.
- Repeat steps 3 and 4 for all BeadChips from the same Hyb Chamber.

High Temp Wash

- Using the slide rack handle, transfer the rack into the Hybex Waterbath insert containing High-Temp Wash buffer.
- Close the Hybex lid.
- Incubate static for 10 minutes with the Hybex lid closed.

First Room-Temp Wash

- After the 10 minute incubation in High-Temp Wash buffer is complete, immediately transfer the slide rack back into a dish containing 250 ml fresh Wash E1BC buffer.
- Using the slide rack handle, plunge the rack in and out of the solution 5 to 10 times.

- Set the orbital shaker to medium-low.
- Place the dish on the orbital shaker and shake at room temperature for 5 minutes.
 Shake at as high a speed as possible without allowing the solution to splash out of the dish.

Ethanol Wash

- Transfer the rack to a clean dish containing 250 ml fresh 100% Ethanol.
- Using the slide rack handle, plunge the rack in and out of the solution 5 to 10 times.
- Place the dish on the orbital shaker and shake at room temperature for 10 minutes.

Prepare Wash Dishes and Tube Racks

- Take the utmost care to minimize the chance of lint or dust entering the wash dishes, which could transfer to the BeadChips.
- In preparation for XC4 BeadChip coating, wash tube racks and wash dishes thoroughly before and after use. Rinse with DI water.
- Place Kimwipes in three layers on the lab bench. Place a tube rack on top of the Kimwipe layers. Do not place on absorbent lab diapers.
- Prepare an additional clean tube rack. Allow one rack per 8 BeadChips.

Coat BeadChip

- Lay out the following equipment on the lab bench: 1 staining rack;1 vacuum desiccators;1 tube rack; Self-locking tweezers; Large Kimwipes; Vacuum hose
- Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until completely dissolved.
- Set up two top-loading wash dishes, labeled as shown in.
- To indicate the fill volume before filling wash dishes with PB1 and XC4, pour 310 ml water into the wash dishes and mark the water level on the side. Empty the water from the wash dish.
- Pour 310 ml PB1 into the wash dish labeled "PB1."
- Submerge the unloaded staining rack into the wash dish with the locking arms and tab facing *you*.

- Let the staining rack sit in the wash dish. Quickly transfer each BeadChip from the EtOH wash to the staining rack while it is submerged in PB1.
- Place the BeadChips in the staining rack while it is submerged in PB1. Put four BeadChips above the staining rack handle and four below.
- Move the staining rack up and down 10 times, breaking the surface of the PB1.
- Allow the BeadChips to soak for an additional 5 minutes.
- Pour 310 ml XC4 into the dish labeled "XC4," and cover the dish.
- Place the bottle with excess XC4 in a readily available location for topping off the XC4 wash dish during the coating procedure.
- Remove the staining rack from the dish containing PB1 and place it directly into the wash dish containing XC4. The barcode labels on the BeadChips must face *away* from you, while the locking arms on the handle face towards you, for proper handling and coating.
- Move the staining rack up and down 10 times, breaking the surface of the XC4.
- Allow the BeadChips to soak for an additional 5 minutes.
- Prepare a clean tube rack for the staining rack by placing two folded Kimwipes under the tube rack.
- Prepare one additional tube rack per 8 BeadChips that fits the internal dimensions of the vacuum desiccator.
- Remove the staining rack in one smooth, rapid motion and place it directly on the prepared tube rack, making sure the barcodes face up and the locking arms and tab face down.
- To ensure uniform coating, place the staining rack on the center of the tube rack, avoiding the raised edges. For the top four BeadChips, working top to bottom: Continuing to hold the staining rack handle, carefully grip each BeadChip at its barcode end with self-locking tweezers. Place the BeadChip on a tube rack with the barcode facing up and towards you.
- Holding the top of the staining rack in position, grasp the handle between your thumb and forefinger. Push the tab up with your thumb and push the handle away from you to unlock it. Pull up the handle and remove.
- Remove the remaining BeadChips to the tube rack with six BeadChips on top of the rack and two BeadChips on the bottom. The barcode ends should be towards you, and the BeadChips should be completely horizontal.

- To prevent wicking and uneven drying, do not allow the BeadChips to rest on the edge of the tube rack or to touch each other while drying.
- Place the tube rack in the vacuum desiccator. Each desiccator can hold one tube rack (8 BeadChips).
- Ensure the vacuum valve is seated tightly and securely.
- Remove the red plug from the three-way valve before applying vacuum pressure.
- Start the vacuum, using at least 508 mm Hg (0.68 bar).
- To ensure that the desiccator is properly sealed, gently lift the lid of the vacuum desiccator. It should not lift off the desiccator base.
- Dry under vacuum for 50–55 minutes.
- Release the vacuum by turning the handle very slowly.
- Store the desiccator with the red valve plug in the desiccators' three-way valve Remove the red plug from the three-way valve before applying vacuum pressure.
- Touch the borders of the chips (*do not touch the stripes*) to ensure that the etched, barcoded side of the BeadChips are dry to the touch.
- If the underside feels tacky, manually clean the underside of the BeadChip to remove any excess XC4.
- Clean the Hyb Chambers:
- Discard unused reagents in accordance with facility standards.
- Proceed to Image BeadChip on iScan System or Image BeadChip on the BeadArray Reader.

Image BeadChip on iScan System

The iScan Reader uses a laser to excite the fluor of the single-base extension product on the beads of the BeadChip sections. Light emissions from these fluors are then recorded in high-resolution images of the BeadChip sections. Data from these images are analyzed to determine SNP genotypes using Illumina's GenomeStudio Gene Expression Module.

Time:

• 8-12 minutes per bead chip

Preparation:

On the lab tracking form, record the following for each BeadChip: 1. Scanner ID;
 2.Scan date

Steps:

• Turn on the iScan Reader, boot up the iScan PC, and start the iScan Control Software application.

Starting Up the iScan System

- Turn on the iScan Reader and the attached PC.
- Let the iScan Reader warm up for at least 5 minutes.
- For each BeadChip, copy the mini-CD provided with the BeadChip into the Decode folder. The folder name should be the BeadChip barcode.
- Double-click the iScan Control Software icon on the desktop.
- Set the LIMS dropdown list to None and enter your Windows user name.
- Click Start.

Loading BeadChips and Starting the Scan

- Load the BeadChips into their carrier and place the carrier into the iScan Reader tray. Click Next.
- The Type column should say "BeadChip 8x1" and the Scan Setting should say "Direct Hyb".
- If the Scan Setting field beside each BeadChip does not say " Direct Hyb", click Settings. The Scan Settings File window appears.
- Select "Direct Hyb" and click Open.
- If you want to change the image format (*.jpg or *.tif), click the Menu button and select Tools | Options. The Options dialog box appears.
- JPEG files let you review the image of the scanned array sections, but you cannot extract bead intensity data.
- TIFF files let you review the scanned images and extract bead intensity data. The file size is much larger than *.jpg.
- Click the Scan Settings tab.

- Select "Direct Hyb" in the left pane. The scan settings appear in the right pane.
- Click the down arrow beside Image Format, and select Tiff. Click OK.
- Make sure that the input and output paths are correct.
- If you do not want to scan certain sections of a BeadChip, click the barcode to display an image of the corresponding BeadChip.Click any BeadChip section to remove it from the scan.
- If you want to remove an entire BeadChip from the scan, delete the barcode from the Setup window.
- To begin scanning the BeadChips, click Scan.
- At the end of the scan, a Review window appears. If any stripes failed to scan successfully, click Rescan to automatically rescan all failed areas.
- When you finish reviewing the data, click Done to return to the Start window.

Image BeadChip on the BeadArray Reader

The Illumina BeadArray Reader uses a laser to excite the fluor of the hybridized singlestranded product on the beads of the BeadChip sections. Light emissions from these fluors are then recorded in high-resolution images of the BeadChip sections. Data from these images are analyzed using Illumina's GenomeStudio Gene Expression Module.

Time: 1–2 hours warm-up for the BeadArray Reader (first use of the day only) 52 minutes to scan each BeadChip using BeadScan 3.2 FastScan settings

Preparation:

On the lab tracking form, record the following for each BeadChip: 1.Scanner ID;
 2.Scan date

Steps:

- Open the BeadScan software.
- Log in and click Scan.
- From the Docking Fixture listbox, select BeadChip.
- Check the Data Repository path and the Decode Map path in the Settings area.

- Copy the decode map (*.dmap) files for each BeadChip from the BeadChip CD to the Decode Map path directory.
- For each BeadChip: Place the BeadChip into the BeadArray Reader tray. If either the Sentrix Type or Scan Settings are not correct, click Browse (...) to open the Select Scan Settings dialog box. Select Direct Hyb and click Select.
- Make sure that the BeadChips are properly seated in the BeadArray Reader tray.
- Click Scan.

Scanning Process

BeadScan begins the BeadArray Reader Tilt and Align processes:

Once the Tilt and Align processes are complete, the Scan process begins. Hover over any of the green dots in the close up image to see the relative intensity and the XY position. The red value should be at or close to zero, because this is a one-colour assay. As the BeadArray Reader scans, the front panel blue Scanning indicator lights flash in sequence. When the BeadArray Reader finishes scanning, a green message screen appears if the scan was successful, or a red message if it completed with any warnings.

If Scan is Successful

- Click OK on the Scan Completed message to view the next screen.
- Click Done in the Review pane
- When the application returns to the Welcome screen, click Open Tray. The BeadArray Reader tray, loaded with the scanned BeadChips, will eject.
- Remove the BeadChips from the tray.
- Do one of the following: If you have more BeadChips to scan, repeat the scanning process. If this is the last use of the day: Wipe the BeadArray Reader tray with a lint-free, absorbent towel. Pay particular attention to the tray edges where reagent may have wicked out. Close the tray. Turn the power switch at the back of the scanner to the OFF position. Shut down the BeadArray Reader BeadScan software. To exit, right-click near the Illumina logo and click Exit.

If Scan is not successful

• Re-scan the array. If the scanner was unable to locate the alignment fiducials (focus points), you may need to clean the edges of the BeadChip before re-scanning.

High Capacity cDNA Reverse Transcription Kits Protocol (Applied Biosystems). Conversion RNA to cDNA

Good Laboratory Practice:

- PCR assays require special laboratory practices to avoid false positive amplifications.
- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation)
- Use clean gloves when preparing samples for PCR amplification. Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas, dedicated equipment, and supplies for: 1. Sample preparation and PCR setup; 2. PCR amplification and post-PCR analysis
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes and reaction plates carefully.
- Do not splash or spray PCR samples.
- Keep reactions and components sealed as much as possible.
- Clean lab benches and equipment periodically with freshly diluted 10% chlorine bleach solution.

Materials and Equipment:

- 10× RT Buffer
- $10 \times \text{RT}$ Random Primers
- 25× dNTP Mix (100 mM)
- MultiScribe[™] Reverse Transcriptase, 50 U/µL
- RNase Inhibitor
- Thermal Cycler with 96-Well Aluminium Sample Block Module
- Centrifuge with 96-well adapter Major laboratory supplier
- Microcentrifuge
- Vortexer
- 96-Well Reaction Plate
- 8-tube strip Applied Biosystems
- Clear adhesive film
- Optical Caps, 8 Caps/Strip

- Nuclease-free H2O
- Pipette tips, aerosol-resistant

Preparation:

Prepare the $2 \times RT$ master mix using the kit components before preparing the reaction plate. Prepare the RT master mix on ice.

To prepare the 2 × RT master mix (per 20- μ L reaction):

Component	Volume Reaction (µl)
10X RT Buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe [™] Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H20	3.2
Total per Reaction	10.0

Prepare cDNA RT Reactions:

- Pipette 10 μL of 2× RT master mix into each well of a 96-well reaction plate or individual tube.
- Pipette 10 μ L of RNA sample into each well, pipetting up and down two times to mix.
- Seal the plates or tubes
- Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
- Place the plate or tubes on ice until you are ready to load the thermal cycler.

Perform Reverse Transcription:

- Program the thermal cycler as per table below
- Set the reaction volume to $20 \ \mu L$.

- Load the reactions into the thermal cycler.
- Start the reverse transcription run.
- Store long-term at -15° C to -25° C

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 minutes	120 minutes	5 minutes	x

Real-Time PCR Amplification using Taqman Probes

Recommended principles for PCR practices:

- Wear a clean lab coat and clean gloves when preparing samples for PCR amplification
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for: 1. Sample preparation; 2. PCR setup; 3. PCR amplification

Equipment, Consumables & Reagents:

- The TaqMan Gene Expression Master Mix contains: AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), Uracil-DNA glycosylase, dNTPs with dUTP, ROX™ Passive Reference, Optimized buffer components
- Custom designed 384-well plate with genes of interest and housekeeping genes
- RNase-free, sterile-filtered water
- Centrifuge with plate adapter
- Disposable gloves
- Microcentrifuge
- Pipet tips, aerosol resistant
- Pipettors: Multichannel
- Polypropylene tubes
- Vortexer
- Light Cycler (Roche) Real Time PCR system

Procedure:

Prepare the reverse transcription master mix:

Component	Volume (µl/reaction)
TaqMan Gene Expression Master Mix $(2 \times)$	10.0
TaqMan Gene Expression Assay (20×)	1.0
cDNA template + H2O	9.0
Total Volume	20.0

Prepare the PCR Plate:

- Prepare the reaction mix for each sample as above
- Calculate the volume of each component of the PCR reaction mix by multiplying the volume of each component by the number of replicates for each sample. Include excess volume for the loss that occurs during reagent transfers.
- Use 10 to 100 ng of cDNA per replicate.
- Cap the tube(s).
- Vortex the tube(s) briefly to mix the solutions.
- Centrifuge the tube(s) briefly to spin down the contents and eliminate any air bubbles from the solutions.
- Transfer the 20µl reaction mixture to each well of the 384-well optical plate
- Cover the plate with a MicroAmp[™] Optical Adhesive Film
- Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions

Running the PCR Plate:

- Place reaction plate in the light cycler
- Run according to table below

Stop	UDG Incubation	AmpliTaq Gold, UP Enzyme Activation	PCR	
Step	HOLD	HOLD	Cycle (40 Cycles)	
			Denature	Anneal/Extend
Time	2 minutes	10 minutes	15 seconds	1 minute
Temperature	50 °C	95 °C	95 °C	60 °C

- Set reaction volume to 20µl
- Run the plate

Analysing Results:

The general process for analyzing the data from gene expression assay involves:

- Viewing the amplification plots for the entire plate
- Setting the baseline and threshold values to determine the threshold cycles (CT) for the amplification curves
- Using the relative standard curve method or the comparative CT method to analyze your data

Immunohistochemistry Protocols

Heat Induced Antigen Retrieval (Microwave Method)

When using this method, it is possible for the buffer to boil over, and a large amount of the retrieval buffer will evaporate. Be sure to watch the buffer level of the slide vessel, and add more buffer if necessary. Do not allow the slides to dry out.

Materials and reagents:

- Domestic Microwave (850 W)
- Microwaveable vessel with slide rack to hold approximately 400-500 ml or Coplin jar
- Antigen retrieval buffer (Sodium citrate pH 6.0)

Method:

- De-paraffinise and rehydrate the sections as above.
- Add the antigen retrieval buffer to the microwaveable vessel
- Remove the slides from the tap water and place them in the microwaveable vessel. Place the vessel inside the microwave. Set to full power and wait until the solution comes to the boil. Boil for 20 minutes from this point.
- When 20 minutes has elapsed, remove the vessel and run cold tap water into it for 10 minutes. Use care with hot solution.
- Continue with the immunohistochemical staining protocol.

Note: The decision to use a microwave at 20 minutes using sodium citrate buffer was made after a series of control experiments using different antigen retrieval methods with basic, acidic and neutral buffers with various incubation periods.

R&D Systems Protocol for Chromogenic Staining of Paraffin-embedded Sections

Warning:

• Gloves and safety glasses should be worn and all steps performed inside a fume hood. DAB is a hazardous material

Consumables and Reagents Required:

- Wash Buffer: 1X PBS ((0.137 M NaCl, 0.05 M NaH₂PO₄, pH 7.4)
- Incubation Buffer: Incubation Buffer: 1% bovine serum albumin, 1% normal donkey serum, 0.3% Triton® X-100, and 0.01% sodium azide in PBS
- Primary Antibodies
- Cell and Tissue Staining Kits: Kits include Biotinylated Secondary Antibodies, Serum Blocking Reagent, Peroxidase Blocking Reagent, Avidin Blocking Reagent, Biotin Blocking Reagent, High Sensitivity Streptavidin-HRP Conjugate (HSS-HRP), and Chromogen Solution. Kits are available with chromogenic substrates 3,3' Diaminobenzidine (DAB, brown precipitate).
- DAB Enhancer (Catalog # CTS010)
- Hematoxylin Counterstain
- Aqueous Mounting Medium (Catalog # CTS011)
- Gelatin-coated Slides (Protocol for Gelatin-coated Slides for Histological Tissue Sections)
- Coverslips

Procedure

- Tissue must be rehydrated before commencing staining protocol.
- Immerse the slides in xylene (mixed isomers) 2 times for 10 minutes each.
- Immerse the slides in 100% alcohol 2 times for 10 minutes each.
- Immerse the slides in 95% alcohol for 5 minutes.
- Immerse the slides in 70% alcohol for 5 minutes.
- Immerse the slides in 50% alcohol for 5 minutes.

- Rinse the slides with deionized H₂O.
- Rehydrate the slides with wash buffer for 10 minutes. Drain the excess wash buffer.
- Surround the tissue with a hydrophobic barrier using a barrier pen.
- To quench endogenous peroxidase activity, incubate the sample with 1-3 drops peroxidase blocking reagent (3% H₂O₂ in water or methanol) for 5-15 minutes.
- Rinse the sample, then gently wash in wash buffer for 5 minutes.
- To reduce non-specific hydrophobic interactions between the primary antibodies and the tissue, block the section with 1-3 drops of serum blocking reagent for 15 minutes. Drain the slides and wipe away any excess blocking reagent before proceeding to the next step. Do not rinse.
- To block binding to endogenous biotin, incubate the sample with 1-3 drops of avidin blocking reagent for 15 minutes. Rinse the sample with wash buffer, drain slides, and wipe away any excess wash buffer.
- To block subsequent binding to the avidin applied in step 6, incubate the sample with 1-3 drops of biotin blocking reagent for 15 minutes. Rinse with wash buffer, drain the slides, and wipe away any excess wash buffer.
- Incubate the sample with primary antibodies in Incubation Buffer overnight at 2-8 °C
- Include a negative control using the incubation buffer with no primary antibody to identify non-specific staining of the secondary reagents. Rinse the sample with wash buffer. Wash 3 times with wash buffer for 5 minutes each, and drain the slides.
- Incubate the sample with 1-3 drops of biotinylated secondary antibodies for 30-60 minutes, adjusting the incubation time depending on the thickness of the section (approximately 30 minutes for 5-10 μm thick sections and 60 minutes for 10-20 μm thick sections).
- Rinse with wash buffer 3 times for 15 minutes each and drain the slides.
- Incubate the sample with 1-3 drops of High Sensitivity Streptavidin-HRP conjugate (HSS-HRP) for 30 minutes.
- Rinse and wash 3 times in wash buffer for 2 minutes each.
- Calculate the required working volume of DAB Chromogen Solution given that 100-200 µL is required to cover the entire tissue section on a single slide.
- Add 1-5 drops of DAB Chromogen Solution to cover the entire tissue section and incubate for 3-20 minutes.

- Monitor the intensity of the tissue staining under a light microscope. Coloured precipitate will localize to the sites of antigen expression as the chromogenic substrate is converted by HRP enzyme into insoluble end product.
- Rinse the sample with wash buffer 3 times for 10 minutes each.
- Rinse in deionized H₂O and drain the slides.
- Using haematoxylin perform nuclear counterstaining for better visualization of the tissue morphology.
- Cover stained tissue with a coverslip of an appropriate size, place slides vertically on filter paper or a towel to drain excess mounting medium, and allow them to dry.
- Visualize staining of tissue under a microscope using a bright-field illumination.

Human ELISA Kit (Abcam)

Equipment, Reagents and Consumables:

- Microplate: 96 wells (12 strips x 8 wells) coated with anti-human CCL19/anti-human CTSG
- Wash Buffer Concentrate (20x): 25 ml of 20x concentrated solution.
- Standards (Item C): 2 vials, recombinant human CCL19/CTSG
- Assay Diluent (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (serum) diluent.
- Detection Antibody CCL19/CTSG (Item F): 2 vials of biotinylated anti-human CCL19/CTSG (each vial is enough to assay half microplate.
- HRP-Streptavidin concentrate (Item G): 200 µl of 400x concentrated HRP-conjugated streptavidin.
- TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
- Stop Solution (Item I): 8 ml of 0.2 M sulphuric acid.
- 1 Microplate reader capable of measuring absorbance at 450nm.
- Precision pipettes to deliver 2 µl to 1 ml volumes.
- Adjustable 1-25 ml pipettes for reagent preparation.
- 100 ml and 1 litre graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- 8 Tubes to prepare standard or sample dilutions.

Preparation:

- Bring all reagents and samples to room temperature (18 25°C) before use.
- Sample dilution: If samples need to be diluted, Assay Diluent (Item E) is used for dilution of serum.
- Assay Diluent (Item E) should be diluted 5-fold with deionized or distilled water before use.

- Preparation of standard: Briefly spin the vial of Item C. Add 400µl 1x Assay Diluent (Item E) into Item C vial to prepare a 50 ng/ml standard. Dissolve the powder thoroughly by a gentle mix. Add 10µl Macrophage Inflammatory Protein 3 beta standard from the vial of Item C, into a tube with 990 µl Assay Diluent Buffer to prepare a 500 pg/ml standard solution. Pipette 300 µl 1x Assay Diluent into each tube. Use the 500 pg/ml standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Gently vortex to mix. 1x Assay Diluent Buffer serves as the zero standard (0 pg/ml).
- If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.
- Briefly spin the Detection Antibody vial (Item F) before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent and used in step 4 of part 7 Assay Method.
- Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 400-fold with 1x Assay Diluent.

Procedure:

- Bring all reagents and samples to room temperature (18 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
- Add 100 µl of each standard (see Preparation of Reagents step 4) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C with gentle shaking.
- Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel pipette or autowasher.
 Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

- Add 100 µl of 1x prepared biotinylated antibody (see Preparation of Reagents step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
- Discard the solution. Repeat the wash as in step 3.
- Add 100 µl of prepared Streptavidin solution (see Preparation of Reagents step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking.
- Discard the solution. Repeat the wash as in step 3.
- Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
- Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately

Data Analysis:

• Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points

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