Do retinal microvascular abnormalities shed light on the pathophysiology of lacunar stroke?

Fergus Neil Doubal

BSc (Hons), MB ChB, MRCP

Doctor of Philosophy

University of Edinburgh

2010

Declaration

I declare that this thesis was composed by me and that the work contained therein is my own, except where explicitly stated otherwise in the text.

The work within this thesis has not been submitted for any other degree or professional qualification.

Fergus Neil Doubal
Edinburgh, 2010

Publications directly related to results presented in this thesis

FN Doubal, MS Dennis JM Wardlaw. Characteristics of patients with minor ischaemic strokes and negative MRI: a cross-sectional study. *J Neurol Neurosurg Psychiatry 2010 Jun 27th [e-pub ahead of print]*

SF Stevenson, **FN Doubal**, K Shuler, JM Wardlaw. A systematic review of dynamic cerebral and peripheral endothelial function in lacunar stroke versus controls. *Stroke* 2010;41(6):e434-42.

FN Doubal, PE Hokke, JM Wardlaw. Retinal microvascular abnormalities and stroke: a systematic review. *J Neurol Neurosurg Psychiatry* 2009;80(2):158-65.

FN Doubal, B Dhillon, MS Dennis, JM Wardlaw. Retinopathy in ischemic stroke. subtypes. *Stroke* 2009;40(2):389-93.

FN Doubal, TJ MacGillivray, PE Hokke, B Dhillon, MS Dennis, JM Wardlaw. Differences in retinal vessels support a distinct vasculopathy causing lacunar stroke. *Neurology* 2009;72(20):1773-8.

FN Doubal, R de Haan, TJ MacGillivray, PE Hokke, B Dhillon, MS Dennis, JM Wardlaw. Retinal vascular geometry is associated with cerebral small vessel disease. *Int J Stroke* 2010;5:434-9.

FN Doubal, TJ MacGillivray, N Patton, B Dhillon, MS Dennis, JM Wardlaw. Fractal analysis of retinal vessels suggests that a distinct vasculopathy causes lacunar stroke *Neurology* 2010;74:1102-7.

FN Doubal, AMJ MacLullich, KJ Ferguson, MS Dennis, JM Wardlaw. Enlarged perivascular spaces on MRI support a distinct lacunar stroke arteriopathy. *Stroke* 2010;41:450-4.

Publications arising from the patients recruited from this thesis but where the results are not directly reported in this thesis

GM Potter, **FN Doubal**, C Jackson, C Sudlow, M Dennis, J Wardlaw. Associations of clinical stroke misclassification ('clinical-imaging dissociation') in acute ischemic stroke. *Cerebrovascular Diseases* 2010;29:395-402

GM Potter, **FN Doubal**, CA Jackson, FM Chappell, CLM Sudlow, JM Wardlaw. Counting cavitating lacunes underestimates the burden of lacunar infarction. *Stroke* 2010;41(2):267-72.

JM Wardlaw, **F Doubal**, P Armitage, F Chappell, T Carpenter, S Muñoz Maniega, A Farrall, C Sudlow, M Dennis, B Dhillon. Lacunar stroke is associated with diffuse blood-brain barrier dysfunction. *Ann Neurol* 2009;65(2):194-202.

C Cordonnier C, GM Potter, CA Jackson, **F Doubal**, S Keir, CL Sudlow CL, JM Wardlaw, R Al-Shahi Salman. Improving interrater agreement about brain microbleeds: development of the Brain Observer MicroBleed Scale (BOMBS). *Stroke* 2009;40(1):94

RI Lindley; Multi-Centre Retinal Stroke Study Collaborative Group, Retinal microvascular signs: a key to understanding the underlying pathophysiology of different stroke subtypes? *Int J Stroke* 2008;3:297-305.

JM Wardlaw, A Farrall, PA Armitage, T Carpenter, F Chappell, **F Doubal**, D Chowdhury, V Cvoro, MS Dennis. Changes in background blood-brain barrier integrity between lacunar and cortical ischemic stroke subtypes. *Stroke* 2008 Apr;39(4):1327-32.

TJ Macgillivray, N Patton, **FN Doubal**, C Graham, JM Wardlaw. Fractal analysis of the retinal vascular network in fundus images. *Conf Proc IEEE Eng Med Biol Soc* 2007;2007:6456-9.

Manuscripts submitted and in preparation

GM Potter, **FN Doubal**, C Jackson, MS Dennis, C Sudlow, JM Wardlaw. White matter lesions identified by magnetic resonance imaging are not related to ipsilateral carotid artery stenosis so are unlikely to be embolic. Submitted to *Neurology.*

TJ MacGillivray, **FN Doubal**, N Patton, AP Rovira, E Trucco, B Dhillon, IJ Deary, JM Wardlaw. Evaluating monofractal and multifractal analysis of the human retinal vasculature. Submitted to *Medical Engineering Physics.*

JM Wardlaw, F Chappell, **FN Doubal**, E Eadie, K Shuler, V Cvoro. Intracranial large artery stenosis is not associated with lacunar stroke.

FN Doubal, A Rumley, GDO Lowe, MS Dennis, JM Wardlaw. Coagulation, fibrinolysis and inflammation in ischaemic stroke subtypes.

Acknowledgements

I am extremely grateful to the patients (and their supportive families) who took part in this study, willingly returned for investigations, provided encouragement and showed considerable humbling interest in this work and the results.

This project was no means a single handed effort and succeeded because of the enthusiastic input from many collaborators and advisers. I thank my clinical and radiological colleagues who consistently referred suitable patients and the radiographers at the SFC Brain Imaging Centre who booked and performed all MRI brain imaging. The Wellcome Trust Clinical Research Facility, Western General Hospital housed the retinal camera. Professor Bal Dhillon provided ophthalmological expertise and training for retinal photograph assessment. Professor Martin Dennis, who had initially inspired me to undertake a PhD, provided practical clinical help combined with wise academic counsel.

I am particularly indebted to Dr Tom MacGillivray who developed the software used in the assessment of retinal features. I hope he enjoyed our discussions as much as I did – he was a patient teacher of complex physics. I was fortunate to supervise two enthusiastic and tireless Dutch medical students - Petra Hokke assisted with the systematic review of retinal microvascular abnormalities in stroke and helped develop vessel width measurement techniques and Rosemarie de Haan assisted with retinal geometrical measurements. I was also aided considerably by two medical students from the University of Edinburgh, Susan Stevenson and Fergal Marlborough who helped with the systematic reviews of endothelial dysfunction in lacunar stroke.

I would also like to thank the statisticians Francesca Chappell, Cat Graham and Steff Lewis for their helpful advice.

The Division of Clinical Neurosciences, Western General Hospital, Edinburgh was a fantastic academic environment in which to study and this project benefited immensely from the comments of fellow research fellows, scientists and professors who challenged and encouraged in equal measure. I am grateful to the Wellcome Trust who funded my clinical research training fellowship

My main thanks are to Professor Joanna Wardlaw who has given her time generously, been a super source of support and provided expert guidance. She has taught me the value of multi-disciplinary research and of proper planning and implementation of ideas. She has given much wise advice and is a continuing source of inspiration.

Final thanks go to my family - my wife Emma for her love and patience (living with a thesis writer is not easy) and my beautiful daughters Eleanor and Poppy.

Abstract

Background. Lacunar strokes account for 25% of all ischaemic stroke but the exact nature of the causative cerebral small vessel abnormality remains unknown. Pathological studies are technically difficult and brain imaging cannot adequately characterise the cerebral small vessels. The retinal blood vessels are of similar size and physiology to the cerebral small vessels and may act as a surrogate marker for these cerebral small vessels. We therefore investigated retinal microvascular abnormalities in lacunar stroke.

Methods. We performed a systematic review of retinal microvascular abnormalities in lacunar stroke to clarify associations and identify where further research was required. We then established a cohort of patients presenting with lacunar stroke with cortical stroke controls to investigate differences in retinal microvascular abnormalities between stroke subtypes. All patients had MRI brain at presentation and digital retinal photography of both eyes. We investigated the prevalence of retinopathy (hard and soft exudates or haemorrhages/microaneurysms), focal arteriolar narrowing and arteriovenous nicking . We developed, validated and used novel semi-automated techniques for measuring retinal arteriolar and venular widths, retinal arteriolar geometry (branching co-efficients (change in arteriolar cross sectional area across a bifurcation) and branching angles) and fractal dimensions (reflecting branching complexity) of the vasculature. We also assessed MRI parameters in lacunar stroke. We used multivariable analysis to correct for baseline imbalances in vascular risk factors.

Results. From the systematic review we demonstrated that retinal microvascular abnormalities are associated with incident and prevalent stroke but that in general, strokes were inadequately characterised and there were no data regarding retinal microvascular abnormalities in ischaemic stroke subtypes. We recruited 253 patients, 129 lacunar strokes and 124 cortical strokes, mean age 68 years. We found no difference in the prevalence of retinopathy, arteriovenous nicking, focal arteriolar narrowing or arteriolar widths between lacunar and cortical stroke subtypes. We found that venules were wider in lacunar stroke. We found no differences in arteriolar branching co-efficients or arteriolar branching angles between lacunar and cortical strokes but found that deep white matter white matter hyperintensities on MRI were associated with increased branching co-efficients and periventricular white matter hyperintensities associated with decreased branching co-efficients. We found that the fractal dimension of the vascular tree was decreased in lacunar stroke. Furthermore we found that enlarged perivascular spaces on MRI are associated with lacunar stroke and white matter disease.

Conclusions. We have clearly demonstrated that retinal microvascular abnormalities differ between lacunar and cortical stroke suggesting that a distinct small vessel vasculopathy may cause lacunar stroke. We have also identified MR markers of lacunar stroke. These results suggest that venular disease (a hitherto under-researched area) may play a role in the pathophysiology of lacunar stroke. Retinal microvascular abnormalities can act as markers for cerebral small vessel disease. We plan collaborative analyses with colleagues who have performed similar studies to further assess retinal abnormalities in lacunar stroke.

List of Abbreviations

ACA – Anterior Cerebral Artery

AF – Atrial fibrillation

AVN – Arterio-venous nicking (or nipping)

ARIC – Atherosclerosis Risk in Communities (study)

AVR – Arterio-venous Ratio

Ausdiab – Australian Diabetes (study)

BC – Branching Coefficient

BDES – Beaver Dam Eye Study

BMES – Blue Mountain Eye Study

CADASIL – Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and leukoencephalopathy

CHS – Cardiovascular Health Study

CI – Confidence Interval

CRAE – Central Retinal Artery Equivalent

CRP – C Reactive Protein

CRVE – Central Retinal Vein Equivalent

CT – Computed Tomography

Dbox – Monofractal dimension (measured with box counting method)

DWI – Diffusion Weighted Imaging

ECG - Electrocardiogram

ECST – European Carotid Surgery Trial

EPVS – Enlarged Perivascular Spaces

FAN – Focal Arteriolar Narrowing

FLAIR – Fluid Attenuated Axial Inversion Recovery

GRE – Gradient Echo

HR – Hazards Ratio

ICAM – Intracellular Adhesion Molecule

IHD – Ischaemic Heart Disease

IL-6 – Interleukin 6

LACS – Lacunar Stroke Syndrome

LVH – Left Ventricular Hypertrophy

MCA – Middle Cerebral Artery

MRI – Magnetic Resonance Imaging

NASCET – North American Symptomatic Carotid Endarterectomy Trial

NHS – National Health Service

NIHSS – National Institutes for Heath Stroke Scale

OCSP – Oxfordshire Community Stroke Project

OR – Odds Ratio

OXVASC – Oxfordshire Vascular (study)

PACS – Partial Anterior Circulation Stroke

PAI – Plasminogen Activator Inhibitor

PCA – Posterior Cerebral Artery

POCS – Posterior Circulation Stroke

PVD – Peripheral Vascular Disease

RAO – Retinal Arteriolar Occlusion

RR – Relative Risk

RS – Rotterdam Study

RVO – Retinal Vein Occlusion

SD – Standard Deviation

sRR – Summary Risk Ratio

TACS – Total Anterior Circulation Stroke

TIA – Transient Ischaemic Attack

TOAST – Trial of Org 10172 in Acute Stroke Treatment

TPA – Tissue Plasminogen Activator

UK – United Kingdom

US – United States

vWF – von Willebrand's Factor

WGH – Western General Hospital, Edinburgh, UK

WHO – World Health Organisation

WMH – White Matter Hyperintensities

Table of Contents

Chapter 1. Introduction and aims of thesis. Aetiology of lacunar stroke and homology between retinal and cerebral microvasculature.

1.1 Synopsis of thesis

Lacunar strokes account for 25% of ischaemic stroke but the nature of the underlying small vessel vasculopathy is uncertain. This thesis studies retinal vessel abnormalities as surrogate markers of cerebral vessel disease to gain novel insights into this vasculopathy aiming to guide further work into the causes and eventual management of lacunar stroke.

1.2 Importance of stroke

Stroke is common. The World Health Organisation estimates that 15 million stroke and 5 million stroke deaths occur annually worldwide (WHO Collaborative Group 2004). Stroke is the second most common cause of death worldwide behind ischaemic heart disease (Murray and Lopez 1997b). In the United States of America it is predicted that as many as 800,000 subjects will suffer a stroke in 2010 (Lloyd-Jones *et al.* 2010). Furthermore, stroke is the leading neurological cause of disability in adults (Murray and Lopez 1997a). The incidence of stroke increases with age and by 2030, assuming that the population continues to age as at present, stroke is predicted to become the 4th leading cause of disability in Westernized nations (Lopez *et al.* 2006). The burden of stroke is also significant and increasing in low and middle income countries (Strong *et al.* 2007). Stroke therefore carries a significant worldwide economic burden (Dewey *et al.* 2001).

1.3 Stroke subtypes

Stroke is defined by the World Health Organisation as a clinical syndrome of sudden onset of focal cerebral deficit lasting more than 24 hours or leading to death with a presumed vascular cause (Aho *et al.* 1980). Approximately 80% of strokes are ischaemic, caused by occlusion or disease in one or more arteries interrupting blood supply to the brain, 15% are caused by intracerebral haemorrhage and 5% are caused by subarachnoid haemorrhage (Warlow *et al.* 2003). Ischaemic strokes are further sub classified: 50% are caused by large artery atheroma, 20% by cardio-embolism and 25% by small vessel disease (lacunar stroke) The remaining 5% are caused by rare diseases (Sudlow and Warlow 1997).

There are different methods of subdividing ischaemic stroke. Primarily these are based upon clinical findings coupled with, in the first instance, appropriately timed brain imaging and refined in some cases by the results of further investigations.

1.3.1 The OCSP classification

The Oxfordshire Community Stroke Project classification (OCSP) was developed in epidemiological studies and divides stroke primarily on clinical findings according to the likely arterial territories involved. The four categories are total anterior circulation stroke (TACS); partial anterior circulation stroke (PACS); lacunar stroke (LACS) and posterior circulation stroke (POCS) (Bamford *et al.* 1991). The different subdivisions reflect the size of the underlying infarct and carry different prognoses. The OCSP clinical classification can be performed without investigations and is therefore applicable in most clinical situations.

1.3.2 The TOAST classification

The TOAST criteria for subdivision of ischaemic stroke were derived from a clinical trial and ascribe the most likely aetiological cause for the stroke using the results of investigations such as brain imaging and imaging to identify potential embolic

sources (Adams, Jr. *et al.* 1993). The TOAST categories are large-artery atherosclerosis (embolus or thrombosis), cardio-embolism (high-risk or medium-risk), small-vessel occlusion (lacune), stroke of other determined cause, stroke of undetermined cause, two or more causes identified, negative evaluation or incomplete evaluation. The TOAST classification certainly uses more information in assigning stroke subtype than the OCSP classification but suffers from using risk factors to assign categories so may bias epidemiological studies (eg the presence of hypertension and diabetes increases the chances of a patient having a lacunar stroke). It may be difficult to always assign a putative aetiology for stroke in many cases either because there are competing causes (eg concurrent carotid stenosis and atrial fibrillation) or because no causes are found due to incomplete or even despite intensive investigation. The proportion of patients in this "stroke of undetermined cause" category varies widely between studies which has led to questioning of the validity of this classification scheme (Landau and Nassief 2005).

1.3.3 Definition of lacunar stroke

Irrespective of which classification system is used, lacunar stroke accounts for approximately 25% of ischaemic stroke yet there has been widespread confusion and variation in the terminology used to define lacunar stroke and in distinguishing the clinical lacunar stroke syndrome from its associated features seen on brain imaging (Wardlaw 2008). A lacunar stroke can be defined as a "stroke with symptoms and signs characteristic of a small deep cerebral infarct" (Bamford *et al.* 1991). This distinct constellation of neurological deficits, characteristic of a small deep infarct, reflects lesions located where fibres are tightly packed and where relatively small lesions can cause disproportionately large clinical deficits. This can be further refined as a lacunar ischaemic stroke if appropriately timed brain imaging excludes a haemorrhage or other non vascular cause. The brain imaging may show the recent infarct, further strengthening the classification of a lacunar stroke although even within radiological definitions of lacunar infarct there is a startling lack of homogeneity with disagreement regarding the location and size (as well has how to

differentiate old lacunes from other abnormalities such as enlarged perivascular spaces) (Potter and Wardlaw 2010).

The suggested size of lacunes (3-20mm) was derived from the original pathological studies by Fisher (Fisher 1965) and subsequent studies have either adopted this upper size limit of 20mm (Longstreth, Jr. *et al.* 1998) or used a revised upper limit of 15 mm as suggested by the TOAST criteria (Adams, Jr. *et al.* 1993). There is consternation however that this lower limit of 15mm may misclassify infarcts larger than 15mm which are still nonetheless caused by small vessel disease and hence should be lacunar infarcts (Kang *et al.* 2003a). This upper size limit is an arbitrary figure and studies have shown that lesions greater than this may have a small vessel aetiology and also that smaller lesions may have a cardio-embolic or large artery atheromatous cause (Cho *et al.* 2007). It is likely that there is a continuum of decreasing proportions of lesions being caused by small vessel disease as the size of the subcortical lesion increases (Bang *et al.* 2007). The corollary is that as the lesion size increases the proportion caused by cardio-embolism and large artery atheroma increases and large striato-capsular infarcts behave in a similar fashion to cortical infarcts (Boiten and Lodder 1992).

The term "lacune" was a purely descriptive term, attributed to Dechambre, originally referring to the small holes seen deep in the brain on pathological specimens (Dechambre A 1838). This terminology did not imply causation and lacunes were initially subdivided into 3 categories : Type I small areas of cerebral infarction, Type II – cystic scars suggestive of old small haemorrhages and Type III – enlarged perivascular spaces not associated with infarction (Poirier and Derouesne 1985). The term lacune should be reserved for pathologically or radiologically observed holes (Gouw *et al.* 2008; Ikram *et al.* 2006b) but the cause and significance of these "holes" is uncertain. It is not clear what proportion of these holes were associated with clinically significant neurological deficits and indeed lacunes are found in patients without a clinical history of stroke (Wardlaw 2008).

Several studies have used serial MRI scans to identify and assess predictors of incident "lacunar infarcts" (Ikram *et al.* 2006b; Longstreth, Jr. *et al.* 1998) which do not have clinical correlates. Again, the significance of these new holes in the brain is uncertain and any associations found using this "stroke" outcome may not extrapolate to understanding the cause of lacunar ischaemic stroke. Furthermore, not all lacunar infarcts, even those associated with lacunar stroke symptoms will cavitate and it can be difficult to distinguish these old non-cavitating infarcts from white matter hyperintensities which do not have focal clinical correlates (Potter *et al.* 2010). Thus studies which assess the number of radiological lacunes of presumed ischaemic origin without clinical correlates may miss true lacunar strokes where the lesion has not cavitated (or even where there was no lesion present) or counting lacunar infarcts which were not strokes. At best this will lead to a mis-estimation of the true incidence of lacunar stroke and at worst will lead to spurious associations with lacunar stroke. Recent population based studies have, however used the term "infarct-like lesions" which reflects abnormalities visible on MRI without implying causation (Scher *et al.* 2009). Careful use of terminology so that "lacune" represents an observed hole in the brain and "lacunar infarct" represents a hole in the brain with appropriately timed symptoms suggestive of a stroke is recommended.

1.4 The lacunar hypothesis

Although lacunae were initially described over 170 years ago it is only within the last half century that we have begun to understand their importance and aetiology. The meticulous pathological dissections by CM Fisher in the 1950s and 60s prompted further work leading to the "lacunar hypothesis" (Bamford and Warlow 1988). This suggested that firstly deep small cerebral infarctions were a distinct subset of ischaemic stroke which could be identified clinically by certain patterns of neurological deficit and secondly that these infarcts were caused by disease of lenticulostriate perforating arteries leading to ischaemia in the distribution of the artery involved. Although these infarcts were small (3-20 mm diameter) they can cause severe clinical deficits as they tend occur deep in the brain where there are high concentrations of nerve fibres coursing from the cortex to the spinal cord.

1.4.1 Lacunar stroke syndromes

There are five classical lacunar stroke syndromes which represent a constellation of symptoms which are usually caused by small deep infarcts (Bamford *et al.* 1991): pure motor stroke, pure sensory stroke, sensorimotor stroke (although in practice distinguishing between these is difficult as many patients with motor stroke will have sensory symptoms which are often short lived), clumsy hand dysarthria syndrome and ataxic hemiparetic syndrome. These 5 categories encompass most cases of lacunar strokes but there is also a large number of atypical syndromes caused by lacunar infarcts which typically account for 7% of all lacunar strokes (Arboix *et al.* 2006).

1.5 Clinical significance of lacunar stroke

From the UK population based OCSP, the one year case fatality rate for lacunar strokes (10%) was lower than that for TACS (60%) or PACS (20%) (Bamford *et al.* 1991). This one year case fatality rate was confirmed in the Italian population based L'Aquila study which found a one year case fatality rate post lacunar stroke of 13%. During this first year of follow up post index stroke, the rate of recurrent stroke was lower for lacunar than non-lacunar stroke (3% v 5%) but from the second year the rate of recurrent stroke were similar (Sacco *et al.* 2006). Lacunar stroke has a low immediate (30 day) case fatality rate of 2% compared with 12% for atherothrombotic and 14% for cardio-embolic stroke (de Jong *et al.* 2003). Lacunar strokes have similar long term case fatality rates to large artery stroke as a Dutch study using the TOAST criteria with a median follow up time of 15 years in patients who had survived for at least 30 days found that the case fatality rate for lacunar strokes was 76% and large artery atheroma 79% although patients with cardio-embolic stroke had a significantly higher rate of 87% (Staals *et al.* 2008).

Mortality rates are a blunt tool with which to assess the clinical significance of stroke. Disability is of paramount importance to patients, carers and health service providers. In a follow up study of 196 patients with lacunar stroke, 32% were disabled (modified Rankin score 0-2) at 16 months and 4% had died (Micheli *et al.* 2008). In addition to its effect on mortality, lacunar stroke is also associated with other clinical markers of cerebral small vessel disease such as cognitive decline and depression as well as radiological markers of small vessel disease such as white matter hyperintensities and microhaemorrhages (Wardlaw *et al.* 2006). A pathological study found that in 72 brains of patients without evidence of Alzheimer's Disease or cortical infarction, increased number of lacunes identified in the basal ganglia was associated with decreased cognitive function (Gold *et al.* 2005). This finding, albeit using "lacunes" identified on MRI, was replicated in the recent LADIS study which found that in a cross sectional study, the number of lacunes present in non-disabled patients was associated with decreased cognition (Benisty *et al.* 2009). Decreased cognitive performance is common post lacunar stroke with a reported incidence at three months post stroke of 52% (Mok *et al.* 2004) and at one month of 75% (Rasquin *et al.* 2007). Neither of these studies however used a non-lacunar stroke control group so it is not clear if this cognitive impairment is due to lacunar stroke or having had any stroke. The Nun Study reported in 1997 that pathological evidence of lacunar infarcts increased the chance of neurofibrillary tangles resulting in dementia (Snowdon *et al.* 1997) but this study did not correlate post mortem lacunes with life time stroke symptoms. One study which did compare post stroke cognitive function between lacunar and non-lacunar subtypes found no differences between subtypes but was underpowered for this analysis (Srikanth *et al.* 2004).

1.6 Aetiology of lacunar stroke

Despite having had the better part of two centuries to study the causes of lacunar stroke, the exact nature of the underlying small vessel vasculopathy is not known. Most cortical (or non lacunar) ischaemic strokes are caused by cardio-embolism or large artery atherothromboembolism. There is evidence that these mechanisms can certainly cause a proportion of lacunar stroke: small numbers (6%) of emboli have

been proven in monkey studies to enter the deep penetrating arteries (Macdonald *et al.* 1995), and studies have linked lacunar stroke with cardio-embolic and larger artery atherothromboembolic phenomena (Cho *et al.* 2007), but attributing aetiology to stroke is often an imprecise art and whilst cardioembolism and large artery atherothromboembolism can cause lacunar infarcts they are considered to account for only 10-15% of lacunar infarcts (Wardlaw 2005). Clearly there must be an alternative vasculopathy that perhaps may account for up to 80-85% of lacunar strokes.

1.6.1 Why is the aetiology of lacunar stroke poorly understood?

Lacunar stroke has a low immediate (30 day) case fatality rate of 2% compared with 12% for atherothrombotic and 14% for cardio-embolic stroke (de Jong *et al.* 2003). This, coupled with recent declining autopsy rates over the last 40 years (Burton and Underwood 2007) has resulted in a paucity of brain specimens with well characterised clinically apparent lacunar infarctions available for analysis. In addition to this, further difficulties are caused by the small size of lacunes and it is technically difficult and time consuming to dissect and analyse these lesions and their vascular supply.

Although the field is rapidly progressing and with a powerful 7 tesla MRI scanner the lenticulostriate arterioles can be visualised (Kang *et al.* 2009), current brain imaging techniques do not have adequate resolution to characterise the cerebral small vessel abnormalities. There have been no suitable animal models of lacunar stroke developed although perhaps the spontaneously hypertensive stroke prone rat may provide useful information (Bailey *et al.* 2009).

1.6.2 Lacunar stroke is difficult to diagnose

Clinical studies demonstrate that lacunar stroke is difficult to diagnose (Lindgren *et al.* 2000) without appropriately timed brain imaging – using clinical criteria alone

will result in approximately a 20% misclassification of lacunar as cortical stroke and vice versa (Mead *et al.* 2000). This misclassification will have diluted the findings of studies which have assessed risk factors and the epidemiology of lacunar ischaemic stroke (Jackson and Sudlow 2005). The 20% mismatch between clinical syndrome and actual lesion site may be explained by the close proximity of the lacune to part of the cortex and may explain the perceived association of some lacunar strokes with potential embolic sources (see below). DWI imaging at the time of the stroke can accurately distinguish stroke subtypes but other imaging, such as CT and T2, T1 or FLAIR MRI, are too non-specific (if the patient has several holes already, one cannot distinguish the new from old infarcts).

1.7 There may exist two different subtypes of lacunar stroke

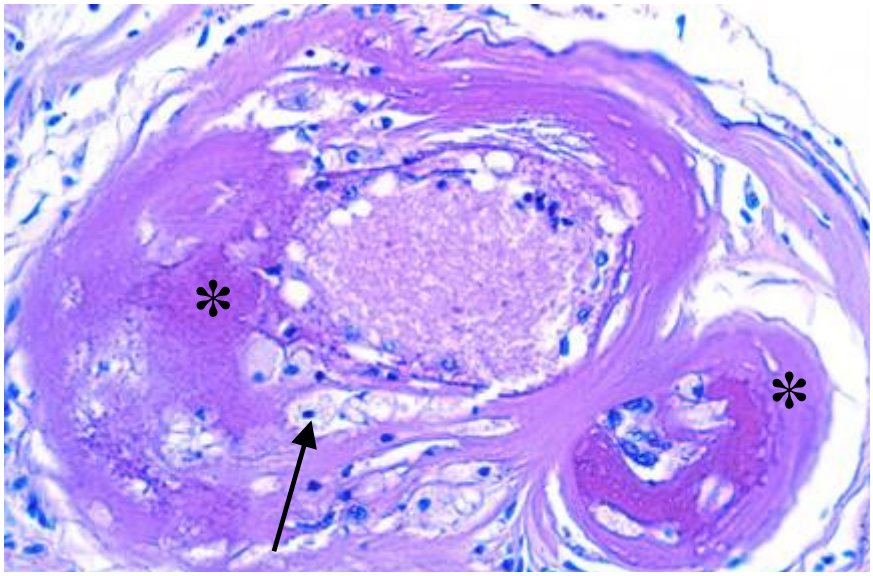
In addition to the difficulties of identifying and distinguishing lacunar from non-lacunar stroke, the study of lacunar stroke causation has been further confounded by the gradual realisation that there may be at least two different types of lacunar stroke (Boiten *et al.* 1993; Markus 2008). Type I consists of an isolated lacunar infarct and type II when there is a lacunar infarct with concurrent white matter disease in the form of other silent lacunar infarcts or leukoaraiosis on CT or white matter hyperintensities on MR. The original hypothesis was generated by an observed increased prevalence in hypertension in Type II compared to Type I (Boiten *et al.* 1993) and follow up data from a Dutch cohort of patients who were recruited between 1987 and 1992 when CT was used to diagnose and subtype lacunar stroke. Patients with type I "isolated" lacunar stroke had a lower 15 year mortality than type II "multiple silent lesion" lacunar strokes (Staals *et al.* 2008). Risk factor analysis from a separate group confirms that patients with Type II lacunar infarcts have a higher prevalence of hypertension and lower prevalence of hypercholesterolaemia and concomitant ischemic heart disease than those with "isolated" Type I (Khan *et al.* 2007). Studies of serum markers of endothelial activation (or function) have demonstrated different levels of activation in Type I and Type II lacunar stroke (Hassan *et al.* 2003b; Knottnerus *et al.* 2010). The two subtypes may have differing

aetiologies with atherosclerosis causing Type I "isolated" lacunar stroke and a diffuse arteriopathy causing type II lacunar stroke. Interestingly, in the original pathology papers, Fisher intimated that the larger lacunes might have been caused by atheroma and the smaller ones by a diffuse arteriopathy (Fisher 1982).

1.8 Pathological studies of the small vessel abnormality

Much of our knowledge arises from the important and painstaking pathological studies of lacunar stroke, lacunes and the small arteries by C Miller Fisher in the middle of the 20th century. Fisher initially described the pathological appearance of 50 lacunes. He found that in 40 of these 50 lacunes (mostly asymptomatic) there was narrowing and in some cases occlusion of the perforating artery by segmental disorganisation and thickening of the vessel wall. This process was later termed lipohyalinosis (reflecting the fact that the lipid containing macrophages within it stained readily for fat). This process tended to occur in the smaller arteries (40 – 200 microns in diameter). Lipohyalinosis is characterised in the acute phase by fibrinoid necrosis and later by arterial wall disorganisation and collagenous sclerosis and is associated with areas of focal dilatation (see figure 1.1). Fisher found that in patients with symptomatic lacunes the lacunes tended to be larger with larger diseased arteries (200-800 microns in diameter). The disease causing the lacune in these slightly larger arteries was microatheroma with plaques of foam cells (lipid laden macrophages) with or without overlying thrombosis. Fisher's results offer an intriguing insight into the pathology behind lacunar stroke but there are caveats. Many of the subjects had severe hypertension (a condition less prevalent today) and not all of the lacunes were correlated with lacunar stroke syndromes and in those that were, the lesions were of varying age. Furthermore many of the lacunes studied came from one patient, further decreasing the generalisability of these findings.

Fig 1.1 Pathological cross-section of a lenticulostriate artery with lipohyalinosis, showing an asymmetric, disorganised arterial wall with fibrinoid material (*) and mural foam cells (arrow)



Reproduced with permission from John Wiley and Sons (pub) from Lammie GA, Hypertensive Cerebral Small Vessel Disease, *Brain Pathology* 2002:12:358-370.

Subsequent pathological studies, which have also tended to include small number of patients, have confirmed the presence of intimal thickening, narrowing and hyalinisation in association with lacunar strokes (Arboix *et al.* 1996; Challa *et al.* 1990; Ogata 1999). A further study of 70 brains with evidence of small vessel disease found that the main pathological finding was of concentric hyaline wall thickening. The authors did not find significant evidence of fibrinoid necrosis (but these were not patients with clinical lacunar stroke) (Lammie *et al.* 1997).

It is not clear whether the described hyalinosis and thickening of the vessel wall (especially the intima) is the cause or a reaction to a previous insult. The following discussion reviews the evidence for and against both conventional and novel lacunar stroke aetiologies.

1.9 Possible causes of lacunar stroke.

Lacunar stroke is associated with other manifestations of cerebral small vessel disease such as white matter lesions and microhaemorrhages (Wardlaw *et al.* 2006). Both lacunar stroke and WML are conventionally considered to be "ischaemic" but the cause of the ischaemia is debated. Although traditional ischaemic stroke mechanisms such as embolism from the heart or carotid atheroma can cause lacunar stroke, they are not common and would not necessarily explain the small vessel pathological changes (originally described as segmental arteriolar disorganisation and latterly as lipohyalinosis or fibrinoid necrosis) described previously. Intracranial microatheroma might explain the small vessel appearance and was initially described by Fisher but the evidence that the vessel changes are associated with other evidence of atheroma is limited. There is increasing evidence that endothelial dysfunction is associated with small vessel disease (Markus 2008) and this could explain the small vessel changes. Again the underlying cause of the endothelial dysfunction is unclear but thickening of the arterioles and capillaries (and possibly even veins) could result in a loss of cerebral autoregulation but again it is unclear how failure of autoregulation might create ischaemic damage. What causes the endothelial malfunction in the first place is uncertain – there is increasing evidence that progression of neurological deficit in lacunar stroke are associated with increased serum markers of inflammation (Castellanos *et al.* 2002) which may damage the endothelium with consequent vessel wall and brain changes. But could the endothelial dysfunction be the primary problem and release inflammatory markers into peripheral blood? Alternative hypotheses for which there is also increasing hard evidence include altered permeability of the blood brain barrier which could lead to microvessel wall thickening and perivascular damage (Wardlaw *et al.* 2009). This could arise from endothelial malfunction from inflammation.

1.9.1 Does embolism cause lacunar strokes?

In the original clinicopathological studies by Fisher he suggested that in the absence of atheroma and with the normal appearance of the perforating arteries, embolism may cause lacunes (Fisher 1969). Emboli may arise from the heart or from atheroma in the aorta or the extra or intra-cranial arteries. Animal studies in rats have demonstrated that small numbers of particles can enter and lodge in the lenticulostriate arteries post non-occlusive thrombus in the carotid artery (Futrell *et al.* 1989), a finding replicated in monkeys (Macdonald *et al.* 1995). Extrapolating this to humans is difficult however as the rat brain differs in several key respects, notably in the much smaller proportion of white matter compared to human brain. Additionally, in putative animals of lacunar stroke, most emboli caused cortical rather then lacunar stroke (Bailey *et al.* 2009). Proving that emboli *can* occur does not prove that they *are* the usual cause of lacunar stroke.

Clinical researchers have used two main strategies to investigate the association between emboli and lacunar infarct. The first approach demonstrates the co-existence of embolic sources in patients with lacunar stroke (from which determining cause and effect is difficult) and the second assumes - probably erroneously - that if multiple diffusion weighted imaging positive lesions are present on MRI at the time of the lacunar stroke the most likely aetiology is embolism.

Emboli may arise from a stenosed internal carotid artery: Tejada et al found that ipsilateral stenosis was more common than contralateral stenosis in 135 patients with an isolated lacunar infarct on CT (Tejada *et al.* 2003). Patients with lacunar stroke and an associated carotid stenosis had less severe stenosis than patients with non-lacunar stroke yet still benefitted from carotid endarterectomy (although the benefits were not as large as for non-lacunar strokes) in the North American Carotid Endarterectomy Trial (Inzitari *et al.* 2000). The European Carotid Surgery Trial was not powered to look at the beneficial effects of endarterectomy in stroke subtypes although patients in this selected group had less severe stenosis than non-lacunar strokes (Boiten *et al.* 1996). Interestingly the results from the NASCET and ECST

trial have been used to argue both for (benefit with endarterectomy) and against (lacunar stroke associated with less severe stenosis) embolism causing lacunar stroke. One study of 259 patients showed conclusively that the distribution of severe stenosis was the same in the ipsilateral and contralateral internal carotid arteries in first ever lacunar stroke suggesting that the stenosis may be an innocent bystander. Most previous studies either did not compare symptomatic and asymptomatic stenoses or had referral bias – eg those in the NASCET and ECST trials all had a significant carotid artery stenosis as one of the inclusion criteria (Mead *et al.* 2002). It has tentatively been suggested that emboli may arise from the aortic arch but the only relevant study compared 62 lacunar stroke patients with non-stroke controls and is therefore unable to attribute the presence of aortic arch atheroma either to lacunar stroke or stroke in general (Kazui *et al.* 2000).

A small study looked at 27 patients with lacunar infarction and atrial fibrillation and compared them with patients with lacunar infarction in sinus rhythm and concluded that cardio-embolism can cause lacunar infarction (Jung *et al.* 2001). Small vessel disease can occur in the presence of atrial fibrillation illustrating the difficulties and false precision often associated with assigning stroke aetiologies.

Up to a third of patients with lacunar stroke will have concurrent acute DWI lesions either in the subcortex or cortex (Chowdhury *et al.* 2004; Norrving 2008). These have been interpreted as indicating proximal embolism in some studies which have subsequently investigated these patients seeking possible sources of embolism. One such study (although the authors do not state whether the concurrent lesions were lacunar or not) found that in 43% of lacunar stroke patient with multiple DWI lesions there was another identifiable stroke aetiology other than small vessel disease but this is also true of other stroke subtypes. This finding was repeated in a separate study of 73 patients with lacunar stroke where the presence of multiple lesions (not necessarily subcortical) was associated with non small vessel disease aetiologies (Wessels *et al.* 2005). A study which did present details about where these concurrent lesions were located (mostly subcortical) suggested that the presence of these lesions was associated with an identifiable source of embolism (Ay *et al.*

1999b). There are limitations to these studies in that whilst atrial fibrillation or mural thrombus is obviously a major source of embolism, the proportions of patients with embolic sources was perhaps artificially inflated by the inclusions of soft embolic sources eg it is not clear what embolic risk mild aortic stenosis or LVH confer. The results from other studies are inconsistent in that multiple DWI lesions in lacunar stroke were not associated with sources of embolism (Chowdhury *et al.* 2004) nor were microemboli detected with trans cranial Doppler in patients with lacunar stroke as they were with cortical stroke patients (Kaposzta *et al.* 1999).

In summary, embolic phenomena can and may cause a small proportion of lacunar stroke (although the presence of multiple infarcts does not necessarily imply embolism but rather a mechanism acting at different locations simultaneously (Moustafa *et al.* 2010)).

1.9.2 Does atheroma cause lacunar strokes?

Since the initial descriptions by Fisher of microatheroma causing disease in the larger small cerebral arteries there has been little further evidence for this, reflecting the paucity of pathological studies. It is conceivable that macroatheroma may cross the origin of the lenticulostriate arteries thus blocking the smaller vessel, however it is more difficult to demonstrate atheroma within the small vessel.

Intracranial stenosis (thought to be more prevalent in Asian populations) has been shown to be associated with a subset of lacunar stroke but it is not clear whether these strokes were caused by emboli or lenticulostriate ostial occlusion or should be taken as evidence for atheroma (Bang *et al.* 2004). Carotid intima medial thickness can be used as a marker for atheroma but has been found to be increased in non lacunar stroke compared to lacunar stroke (Cupini *et al.* 2002; Fulton *et al.* 2006).

1.9.3 Risk factor analysis

It was initially thought that lacunar stroke was associated with diabetes and hypertension and indeed the TOAST classification used these risk factors to diagnose lacunar stroke. A systematic review and meta-analysis however challenged this, finding that diabetes was not associated with lacunar stroke subtype and that hypertension was only weakly associated with lacunar compared with non-lacunar stroke subtype (Relative Risk 1.11 95% CI 1.04 to 1.19) when risk factor free definitions of lacunar stroke were used (Jackson and Sudlow 2005). Reflecting the embolic and atheromatous aetiology behind non-lacunar stroke, this review found that atrial fibrillation and carotid stenosis were associated with non-lacunar rather than lacunar stroke.

1.9.4 Animal models of lacunar stroke

A recent comprehensive systematic review of potential animal models of lacunar stroke found that most animal models either produced lacunar size infarcts in the cortex or that the small artery disease causing lacunar stroke was not adequately modelled (Bailey *et al.* 2009). An exception to this was the spontaneously hypertensive stroke prone rat which begins to develop hypertension at about 6 weeks which is established at 12 weeks. In the brain, changes suggestive of blood barrier breakdown with perivascular oedema related white matter lesions appear before subsequent arteriolar damage and clinically apparent strokes. The arterioles have not been well characterised but older rats (16-24 weeks) show fibrinoid necrosis, lipohyalinosis and occasional thrombotic occlusion superimposed on a disrupted arteriolar wall as a late secondary phenomenon but long after the brain damage has occurred. If the same changes occur in humans this might explain why Fisher found the occasional occluded arteriole on a background of small vessel brain disease.

1.9.5 Does increased blood brain barrier permeability cause lacunar stroke?

There is mounting evidence apart from the SHSRP model of lacunar stroke that increased blood brain barrier permeability may cause lacunar stroke. The blood brain barrier is a selectively permeable interface that regulates entry and egress of substances between plasma and the brain (Abbott 2000; Abbott *et al.* 2006). This is controlled by the cerebral vascular endothelium which has tight junctions between cells and this is maintained by glial cells (astrocytes) which together with the neurons and endothelial cells form the neurovascular unit. Blood brain barrier permeability (measured with a variety of techniques) increases with normal ageing and is associated with cognitive impairment and white matter disease (Farrall and Wardlaw 2007; Starr *et al.* 2003). Lacunar stroke is associated with white matter disease and cerebral microhaemorrhages (Cordonnier *et al.* 2007; Wardlaw *et al.* 2006). Fisher demonstrated findings compatible with increased blood brain barrier leak (areas of focal haemorrhage through the disorganised vessel wall) and recent pathological findings have demonstrated perivascular oedema (with no vessel occlusion) in association with lacunes (Lammie *et al.* 1998; Ma and Olsson 1997).

Permeability MRI, where cerebral accumulation of contrast is measured over 30 minutes provides direct evidence that increased blood brain barrier may cause lacunar stroke. In 97 patients, 51 of whom had lacunar stroke and 46 cortical stroke, patients with lacunar stroke had increased accumulation of contrast representing generalised increased blood brain barrier permeability compared to cortical stroke (Wardlaw *et al.* 2009) confirming the results of a previous pilot study (Wardlaw *et al.* 2008). Increased blood brain barrier permeability may cause lacunar stroke and endothelial dysfunction has been implicated as a cause of this increased permeability (Markus 2008).

1.11 Endothelial dysfunction in lacunar stroke

Endothelial dysfunction is a nebulous term that lacks an exact definition but has been interpreted and assessed in a myriad of different methods. Endothelial dysfunction, perhaps more accurately termed endothelial activation and most commonly investigated as a precursor to atherosclerosis, can be assessed by measuring serum biomarkers or the dynamic endothelium dependent performance of blood vessels (Landmesser *et al.* 2004). The functions of the endothelium are wide ranging but encompass maintaining vascular tone, haemostasis and the expression of adhesion molecules which influence inflammatory pathways (Knottnerus *et al.* 2009). Endothelial activation represents a change from a quiescent state of the endothelium to one which involves the host response (Deanfield *et al.* 2007) which can lead to raised serum biomarkers of endothelial dysfunction.

1.12 Serum biomarkers of endothelial dysfunction in lacunar stroke: a systematic review.

1.12.1 Introduction

Endothelial dysfunction may lead to lacunar stroke yet the published literature produces conflicting results and there is a wide range of serum markers that have been measured. We performed a systematic review to ascertain whether serum markers of endothelial dysfunction are associated with lacunar stroke. We were interested primarily in whether serum markers were raised in lacunar ischaemic stroke compared to ischaemic non-lacunar stroke. From an initial search of the literature we chose the following markers to represent endothelial dysfunction: tissue plasminogen activator(t-PA), von Willebrand's Factor (vWF), Intracellular Cell Adhesion Molecule (ICAM), Vascular Cell Adhesion Molecule (VCAM), plasminogen activator inhibitor (PAI), P & E selectin, fibrinogen, C-reactive protein (CRP), homocysteine, Interleukin-6 (IL-6) and D-dimer.

1.12.2 Methods

We searched Medline (from January 1966) and Embase (from January1980) on 5th August 2009 for studies which assessed serum markers of endothelial function in lacunar stroke with a control group consisting of ideally non-lacunar stroke but including non-stroke control groups (see appendix 1 for the search strategy). We checked references in review and primary papers and hand-searched the journal *Stroke.*

We included papers in humans which were presented in full and in a language we were able to easily translate. We excluded those which dealt with leukaraoisis or white matter disease solely. Two reviewers (FD, Fergal Marlborough) reviewed all titles and extracted data. A third reviewer (Joanna Wardlaw) adjudicated in any disagreements.

1.12.3 Results

The initial search generated 1190 titles/abstracts from which we reviewed the full text of 40 papers. 8 papers were rejected as we were unable to translate these papers and a further 15 were not relevant after full text review leaving 17 papers that were included in this review. Of these 17 papers, most papers used dual control groups of both non-lacunar stroke and non-stroke controls. Ten papers compared lacunar stroke with non-lacunar stroke (Ageno *et al.* 2002; Kataoka *et al.* 2000; Khan *et al.* 2008; Kilpatrick *et al.* 1993; Kozuka *et al.* 2002; Licata *et al.* 2009; Nakase *et al.* 2008; Ohira *et al.* 2006; Parnetti *et al.* 2004; Takano *et al.* 1992). 13 papers compared lacunar stroke patients with non-stroke controls (Ageno *et al.* 2002; Hassan *et al.* 2003a; Hassan *et al.* 2003b; Iso *et al.* 2004; Khan *et al.* 2008; Kozuka *et al.* 2002; Licata *et al.* 2009; Lindgren *et al.* 1996; Ohira *et al.* 2006; Parnetti *et al.* 2004; Salobir *et al.* 2002; Salobir and Sabovic 2004; Takano *et al.* 1992).

In general, 14 papers mentioned which stroke classification system was used to delineate lacunar stroke and 15 papers stipulated which method of brain scanning was used (CT or MR). 14 papers provided a definition for lacunar stroke.

In general serum markers of endothelial dysfunction were higher in lacunar stroke patients compared to non-stroke controls although there was heterogeneity between these results and between different serum biomarkers. These results are detailed in the table below.

Table 1.1 showing serum markers of endothelial function in lacunar versus non stroke controls.

Study	Lacunar group N(age)	Non-stroke Controls N(age)	Endothelial marker	Lacunar marker level	Control marker level	P value
Ageno 2003	31	63 (75.4)	D-dimer	0.7±0.1g/mL	0.5 ±0.1 µg/mL	ns
Hassan 2003	47 (62)	50 (66)	ICAM -1	**454.23ng/mL**	341.90ng/mL	P<0.001
Hassan 2004	172 (67±10)	172 (66±10)	Homocyst	**13.61 µmol/L**	12.01µmol/L	P=0.04
Iso 2004	75 (64.9)	225 (65)	Homocyst	9.2 µmol/L	8.9 µmol/L	ns
Kataoka 2000	58 (68±13)	32 (66±12)	Fibrinogen	306 mg/dL	300 mg/dL	ns
			D-dimer	0.6µg/mL	0.5µg/mL	ns
Khan 2008	144	179 (65)	Homocyst	**13.6 µmol/L**	11.8 µmol/L	P<0.001
Kozuka 2002	27 (69.0)	86 (60.0)	vWF	**165%**	132.5%.	P<0.05
			P selectin	**41.2ng/mL**	24.6 ng/mL .	P<0.05
			E selectin	**53.9 ng/mL**	38.4 ng/mL .	P=0.038
Licata 2009	46 (66.5)	123 (69)	vWF	6 ng/mL	4 ng/mL	ns
			IL-6	4 pg/mL	9 pg/mL	ns
Lindgren 1996	33 (69)	77 (66)	tPA antigen	11µg/L	6µg/L	ns
			PAI antigen	22µg/L	8µg/L	ns
Ohira 2006	105 (56.2)	13957 (54)	vWF	**136%**	117%	P<0.01
			fibrinogen	**326mg/dL**	302 mg/dL	P<0.01
Parnetti 2004	50	152 (69±13)	Homocyst	**13.9 µmol/L**	8.1 µmol/L	P<0.001
Salobir 2002*	16(37)	47 (38)	tPA antigen	**6.9ng/mL**	5.3ng/mL	P<0.05
			tPA activity	0.52 IU/mL	0.54 IU/mL	ns
			PAI antigen	10.6ng/mL	8.8ng/mL	ns
			PAI activity	11.7 IU/mL	8.5 IU/mL	ns
			Fibrinogen	**2.6±0.4 g/L**	2.3±0.4 g/L	P<0.05
			D-dimer	57 ng/L	59 ng/L	ns
Salobir 2003*	16 (42)	52 (41)	tPA	0.5 IU/mL	0.5 IU/mL	ns
			PAI-1	9.5 IU/mL	6.7 IU/mL	ns
			Fibrinogen	**2.4g/**L	2.3 g/L.	p<0.05
			D-dimer	57ng/mL	59ng/mL	ns
Salobir 2004*	16 (42)	47 (41)	IL-6	1.6pg/mL	1.4pg/mL	ns
Takano 1992	23 (65)	20 (61)	D-dimer	115±15ng/mL	82±9 ng/mL	ns

The numbers in bold represent the significantly higher values. ICAM = intra cellular adhesion molecule, vWF = vonWillebrand's Factor, IL-6 = inter-leukin-6, tPA = tissue plasminogen antigen, PAI-1 = plasminogen activator inhibitor. The Salobir studies marked with * may represent duplicate publication. Ns = non significant.

In lacunar versus non-lacunar stroke 7 different biomarkers were compared in 8 studies. In general there were higher levels of serum biomarkers in non-lacunar stroke compared with lacunar stroke. These results are presented in table 1.2.

Table 1.2 showing serum markers of endothelial function in lacunar versus non-lacunar stroke controls. Please see footnote.

Study	**Lacunar stroke group N(age)**	**Non-lacunar stroke Controls N(age)**	**Endothelial marker**	**Lacunar marker level**	**Non-lacunar marker level**	**P value**
Ageno 2002	31	34	D-dimer	0.7±0.1 µg/mL	**1.3±0.2µg/mL**	P=0.01
Kataoka 2000	58	41	Fibrinogen	306 µg/ml	**412 µg/ml**	P<0.001
	(68±13)	(66±14)	D-dimer	0.6 ng/mL	1.7 ng/mL	ns
Khan 2008	144	40 (65.4)	Homocyst	13.6 µmol/L	11.6µmol/L	P<0.001
Kozuka 2002	27 (69.0)	16 (65.0)	vWF	165%	176.5%	ns
			P selectin	41.2 ng/ml	48.9 ng/ml	ns
			E selectin	53.9 ng/ml	44.3 ng/ml	ns
Licata 2009	46 (66.5)	50 (75)	IL-6	4 pg/mL	**8 pg/mL**	P=0.003
Ohira 2006	105 (56.2)	326 (57.3)	vWF	136%	132%	ns
			fibrinogen	326 mg/dL	321 mg/dL	ns
Parnetti 2004	50	43	Homocyst	13.9 µmol/L	**17.8 µmol/L**	P=0.046
Takano 1992*	23 (65)	10 (64.9)	D-dimer	115±15 ng/mL	171±29 ng/mL	ns

Footnote for table 1.2. The numbers in bold represent the significantly higher values. vWF = vonWillebrand's Factor, IL-6 = inter-leukin-6, PAI-1 = plasminogen activator inhibitor. Ns = non significant. *Data are mean and standard deviation apart for Takano where data are mean and standard error.

1.12.4 Conclusions

It is likely that the higher levels of most serum biomarkers in non-lacunar stroke compared with lacunar stroke reflects the higher degree of vascular damage in especially cardio-embolic but also large artery atherothromboembolic stroke compared to the relatively small volumes of the parenchymal lesion present in lacunar stroke. The results from this systematic review illustrate the importance of choosing the correct control group as lacunar strokes have higher levels of serum markers of endothelial dysfunction when compared to non-stroke controls (it is likely that having had a stroke of any subtype will raise serum markers) but lower levels of serum endothelial when compared to non-lacunar stroke control groups. There is a relative paucity of studies comparing serum markers of endothelial dysfunction in ischaemic stroke subtypes and significant heterogeneity amongst those published. The endothelial dysfunction evident may be subsequent to having had any stroke or represent the consequences of exposure to common risk factors for stroke such as hypertension, diabetes or cigarette smoking.

1.13 Dynamic measures of endothelial dysfunction in lacunar stroke: a systematic review.

1.13.1 Introduction

Endothelial function can be measured by assessing the endothelial dependent (and primarily nitric oxide dependent) vascular response to an insult (Tousoulis *et al.* 2005). This can be measured peripherally (by measuring the flow mediated dilatation to cuff inflation in a peripheral artery such as the brachial artery) or in the cerebral circulation by assessing blood flow response to vascular insults such as induced hypercapnia, infusion of acetazolamide or L-arginine. We therefore systematically assessed published studies which tested dynamic endothelial function in patients with lacunar stroke in the cerebral or peripheral circulation.

1.13.2 Methods

We searched Medline and Embase (from Jan 1995) on 15th February 2008 using Ovid and a carefully designed search strategy (see appendix 2) for studies which assessed dynamic endothelial function in patients with lacunar stroke versus cortical ischaemic stroke or non-stroke controls. We checked references in review and primary papers and hand-searched the journal *Stroke.* Two reviewers extracted data (FD, Susan Stevenson); a third resolved disagreements (Joanna Wardlaw). We were able to combine individual study results by calculating standardised mean difference in vascular reactivity, +/- 95% confidence intervals (SMD, 95%CI) between groups.

1.13.3 Results

The initial search generated 1135 title/abstracts from which we obtained the full text for 27 papers. Three papers were excluded as we were unable to translate them (Russian and Slovakian) and a further nine papers failed to meet the inclusion criteria.

We therefore identified 15 relevant publications (885 patients) reporting on vascular reactivity in the cerebral or peripheral arterial circulations. Twelve papers assessed endothelial function in the cerebral circulation (Chamorro *et al.* 1996; Cupini *et al.* 2001; de Leeuw *et al.* 2003; Gur *et al.* 2007; Hund-Georgiadis *et al.* 2003; Immink *et al.* 2005; Maeda *et al.* 1993; Mochizuki *et al.* 1997; Molina *et al.* 1999; Panczel *et al.* 1999; Pretnar-Oblak *et al.* 2006c; Pretnar-Oblak *et al.* 2006b). Four papers assessed brachial artery flow-mediated dilatation, expressed as the percentage change in arterial diameter (Chen *et al.* 2006; Pretnar-Oblak *et al.* 2006c; Pretnar-Oblak *et al.* 2006a; Pretnar-Oblak *et al.* 2006b).

Several techniques were used to assess cerebral endothelial function, including the vascular response to hypercapnia, infusion of acetazolamide or L-arginine, expressing the response as a percentage increase in mean arterial blood velocity in the MCA or basilar artery. Change in blood oxygen level dependent signal during

hypercapnia detected using functional MRI, percent increase in regional cerebral blood flow in response to hypercapnia using stable Xenon CT and "dynamic autoregulation" (the ability to restore cerebral blood flow following sudden changes in perfusion pressure) were also used. Most studies were small with large standard deviations on the measures of reactivity. Thirteen studies used age matched normal controls, two used age and risk factor matched controls, 5 papers compared lacunar stroke patients with cortical stroke, and four compared patients with more than one lacunar infarct.

Cerebrovascular reactivity (n=534) was reduced in lacunar stroke patients compared with age-matched normal (SMD -0.94, 95% CI, -1.17, -0.70, P<0.00001), but not age + risk factor-matched controls (SMD 0.08, 95% CI -0.36, 0.53) or cortical strokes (SMD -0.29, 95% CI -0.69, 0.11); forearm flow mediated dilatation (n=312) was reduced compared with age-matched normal controls (SMD -1.16, -1.52, -0.80) and age + risk factor-matched controls (SMD -0.65, 95% CI-1.10, -0.19), but not cortical strokes (SMD -0.23, 95% CI -0.55, 0.08). See figure 1.2.

Figure 1.2. Forest plot showing standardised mean difference in cerebrovascular reactivity in patients with lacunar ischaemic stroke vs. age-matched, age+risk factor matched and cortical ischaemic stroke controls and in patients with lacunar ischaemic stroke with vs. without multiple silent lacunar infarcts. Squares represent the ratio of the lacunar response divided by the control response with the solid black lines representing the 95% CI. The diamond represents the summary result.

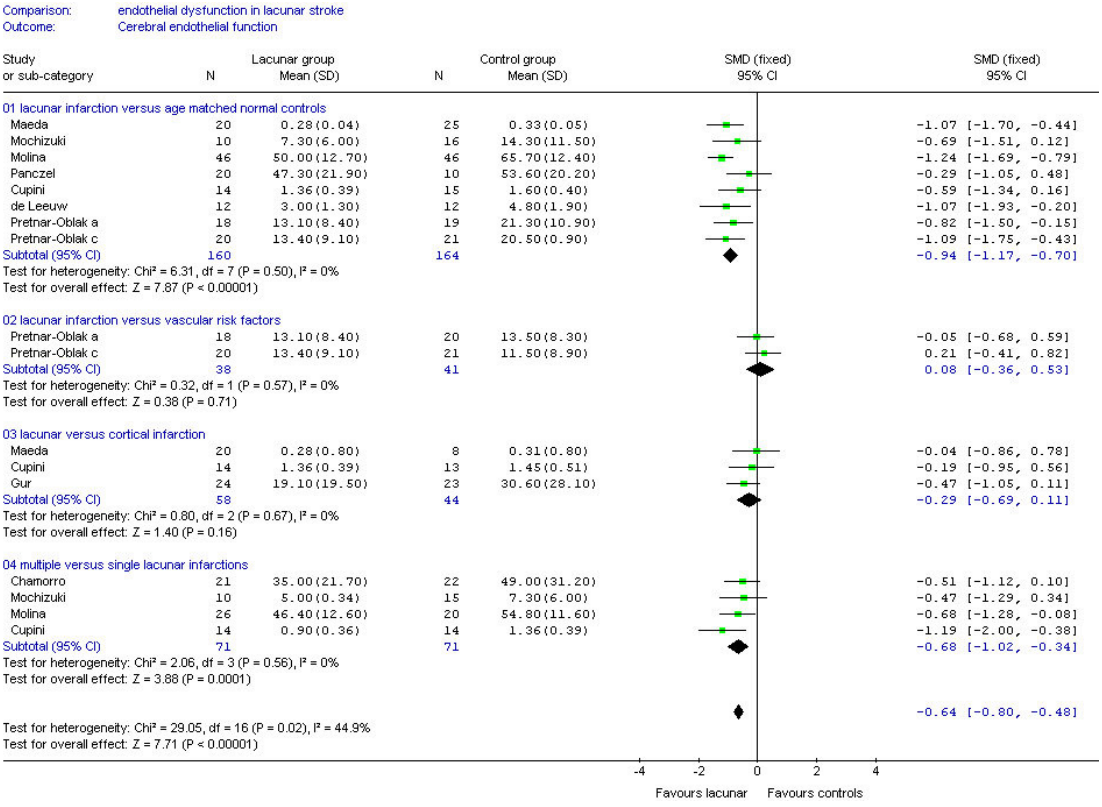
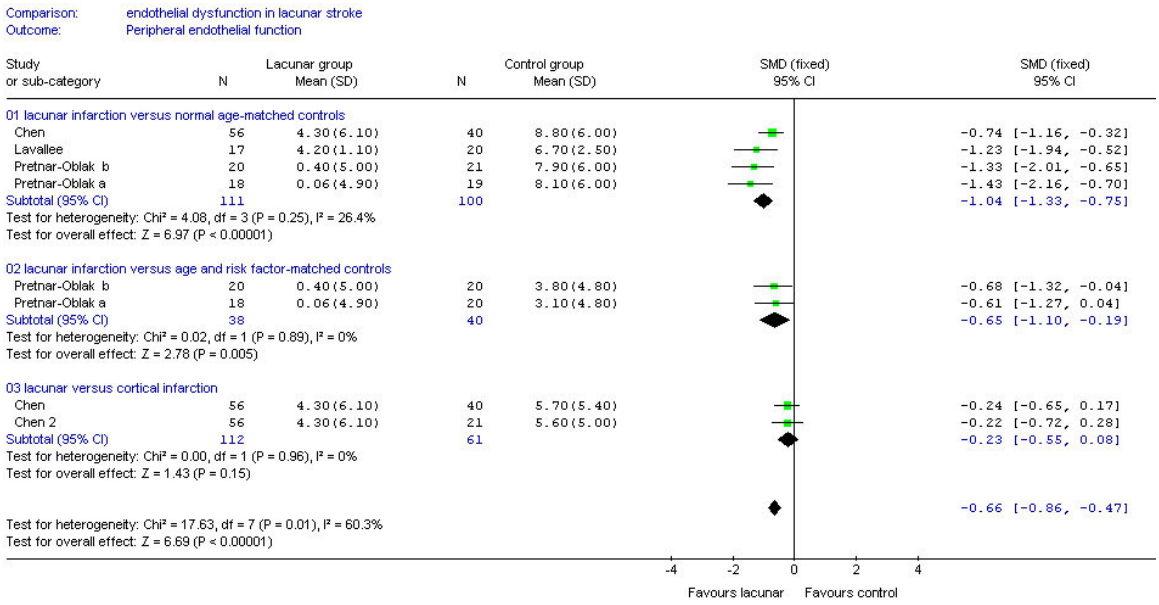


Figure 1.3 Forest plot of standardised mean difference in peripheral endothelial function in patients with lacunar ischaemic stroke vs. age-matched, age and risk factor matched and cortical ischaemic stroke controls.



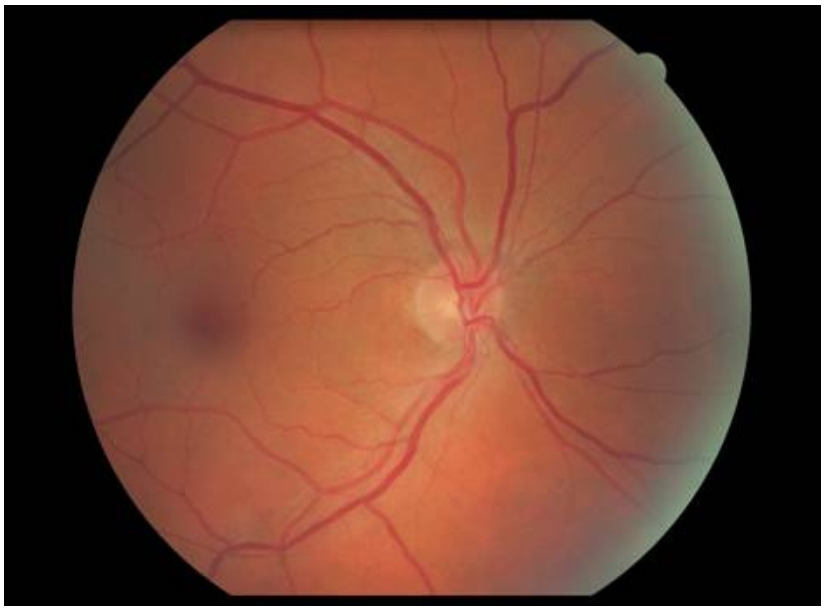
1.13.4 Conclusions.

From this systematic review we have shown that lacunar stroke patients have impaired endothelial function compared with normal controls but this is not so clear-cut when compared with patients with vascular risk factors or cortical stroke. Again these results reiterate the importance of careful control group selection and suggest that endothelial dysfunction may simply be a consequence of exposure to vascular risk factors. We would recommend further, much larger studies with cortical stroke controls to exclude the possibility that modest endothelial dysfunction is associated specifically with lacunar stroke.

1.14 Alternative methods for studying lacunar stroke

Despite many efforts over the last 50 years and despite a multifaceted approach to understanding the aetiology of lacunar stroke, little progress has been made (Davis and Donnan 2004). Differences in risk factor analysis, survival data and complications as well as associations with other manifestations of cerebral small vessel disease suggest firstly that lacunar stroke may have a distinct aetiology compared to larger artery and cardio-embolic stroke and secondly that lacunar strokes may not be isolated phenomena and may be one of the cerebral manifestations of a systemic vasculopathy, an idea gaining credence (Thompson and Hakim 2009). The study of vascular beds other than those in the brain e.g. the retina may shed light on the pathophysiology of lacunar stroke.

Fig 1.4 Digital Retinal photograph of a fundus with no macroscopic abnormalities



1.14.1 Retinal and cerebral vessels: comparative anatomy

The retinal arterioles and cerebral arterioles causing small vessel disease share many characteristics. They are embryologically similar as they both arise from the third

branchial arch of the developing foetus at the same developmental stage. They are of similar size: central retinal arteries have been estimated to be approximately 150 microns in diameter with subsequent branches smaller than this (Ikram *et al.* 2006b) and the cerebral small arterioles range from 50 – 800 microns in diameter (Fisher 1982). Although retinal collaterals have been described as a response to severe sclerotic hypertensive retinopathy (Ball and Henkind 1967), the arterioles tend to be end arterioles with few anastomotic connections. Similarly the lenticulostriate arterioles are end arteries with again few anastomotic connections. Both retinal and cerebral arterioles have tight junctions between endothelial cells which are attached to a basement membrane with surrounding smooth muscle cells (Patton *et al.* 2005).

At a capillary level the retinal capillaries are slightly smaller (5-6 microns) than their cerebral counterparts and both are lined with a single layer of endothelial cells with tight junctional intercellular complexes which are composed of proteins including claudins, junctional adhesion molecules and occludins (Patton *et al.* 2005). There is a surrounding basement membrane in both into which pericytes are embedded. These mesenchymal cells provide structural integrity to the capillary (much like the smooth muscle does to the arteriole) and have contractile properties. There is a higher density of pericytes in the retina compared to the cerebrum (Frank *et al.* 1990). Both retinal and cerebral capillaries are surrounded by the perivascular end feet (Abbott *et al.* 2006) of astrocytes.

1.14.2 Retinal and cerebral vessels: comparative physiology

The retinal and cerebral microcirculations both have a barrier function which regulates the transport and exchange of particles from the blood to the retina/brain (Bernacki *et al.* 2008; Kaur *et al.* 2008). This barrier has a physical (the presence of tight junctions between the endothelial cells) and a chemical component with transport proteins regulating the transport of cells, proteins and molecules (Patton *et al.* 2005). The surrounding astrocytes play a role in the maintenance of this barrier (Abbott *et al.* 2006).

1.14.3 Retinal and cerebral vessels: comparative pathology

In an unselected autopsy series in India of 43 patients who did not have clinical evidence of atherosclerosis, the authors report arterioslcerosis in the retinal arteries consisting of fibrosis and hyalanization with thickening of the vessel wall and subsequent narrowing of the lumen. These changes were mirrored in the small vessels of the brain (Patwardhan *et al.* 1970) and are confirmed in another autopsy series which correlated retinal arteriosclerosis (fibrinoid degeneration and hyalo-fibrinoid thickening of the arterioles) with cerebral arteriosclerosis (Goto *et al.* 1975).

1.14.4 Retinal photography

The retinal vasculature can be directly visualised and although direct ophthalmoscopy by a human operator with a fundoscope is prone to error the retina can be easily photographed (Patton *et al.* 2006b). Initial retinal cameras were analogue and there was a delay until the film was developed and the quality of the photograph known. Recently the development of digital cameras has revolutionised the quality of retinal photographs as the image quality can be assessed immediately and if poor, discarded and repeated.

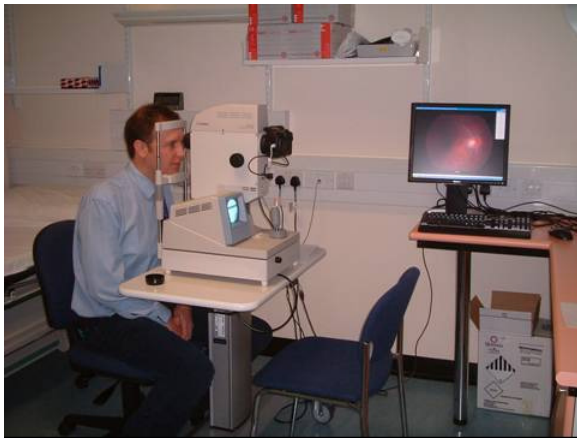


Fig 1.5 Digital Retinal camera. The subject is the author of this thesis. Note the subject needs to maintain head posture and keep eyes still.

The training for retinal photographers is straightforward and retinal photography is feasible in large population based studies with relatively low rates of ungradeable quality photographs (Ikram *et al.* 2006a; Wong *et al.* 2001). Even in a technically demanding cohort of patients with acute stroke the rate of ungradeable photographs was only approximately 10% (Lindley *et al.* 2009).

1.14.5 Techniques for assessing retinal vessels

There are many different techniques for assessing static retinal photographs ranging from quantitative assessment of focal retinal changes such as retinopathy to semi automated assessment of quantitative parameters such as vessel widths to fully automated assessment such as fractal analysis. I will concentrate on these different methods of retinal analysis in the appropriate chapters of this thesis. There are retinal imaging techniques that can capture dynamic changes in the retinal microvasculature ie record vessel widths changes over a short period of time (Liew *et al.* 2008c) but these are not dealt with in this thesis.

1.14.6 Associations between retinal microvascular abnormalities and systemic disease in humans

There are well established associations between retinal microvascular abnormalities (especially decreased arteriolar width, focal arteriolar narrowing and arteriovenous nicking) and hypertension (DellaCroce and Vitale 2008; Wong and Mitchell 2007). Retinal microvascular abnormalities have been used in the clinical assessment of patients with hypertension to establish end-organ damage and hence give an indication to duration of hypertension both in the routine assessment of hypertension (Wong and Mitchell 2004) and in the acute phase of malignant hypertension. A recent systematic review assessed whether routine fundoscopy was useful in the assessment of a patients with hypertension and concluded that fundoscopy did not confer additional benefit over measuring the blood pressure (van den Born *et al.*

2005). It was not clear if this referred to direct fundoscopy by a clinician or retinal photography, important as direct fundoscopy has a low inter and intra rater reliability for the detection and characterisation of retinal lesions (Dimmitt *et al.* 1989). Retinal microvascular abnormalities can predict future risk of both hypertension and other cardiovascular disease (Wong and McIntosh 2005b) although the clinical significance of this above measurement and correction of modifiable traditional risk factors is uncertain.

Diabetic retinopathy secondary to thickening of the basement membrane with subsequent increased permeability of the blood retinal barrier (Bernacki *et al.* 2008) is a common complication of diabetes reflecting glycaemic control and duration of disease and can be an independent predictor of future cardiovascular disease (Liew and Wang 2007).

In this thesis I will present details of the associations between retinal microvascular abnormalities and stroke. Retinal microvascular abnormalities have also been linked with clinical features of small vessel disease in other vascular beds including impaired cognition (Ding *et al.* 2008; Patton *et al.* 2007; Wong *et al.* 2002b), the presence of cerebral white matter lesions on MRI (Qiu *et al.* 2009; Wong *et al.* 2002a) and renal disease (Ikram *et al.* 2008).

1.14.7 Applications for retinal photography

There are three main applications for retinal photography. The first is as a diagnostic aid to guide immediate therapy such as screening for diabetic retinopathy or identifying retinal artery or venule occlusion (Patton *et al.* 2006b). The second application is as a predictor of future disease whether ocular or systemic. Retinal features have been identified as independent predictors of future cardiovascular events (Wong 2004) although the additional clinical benefit over measuring conventional risk factors is uncertain. Thirdly and of most relevance to this thesis the retinal vessels may act as a marker to aid understanding of the pathophysiology of cerebral vascular disease.

1.15 Aims and objectives of this thesis

The aim of this thesis is to use retinal microvascular abnormalities as a surrogate marker of cerebral small vessel disease in combination with detailed MR brain imaging to gain insights into the hitherto unknown aetiology of lacunar stroke. The main hypothesis is that retinal microvascular abnormalities will be more prevalent in lacunar stroke patients compared with cortical stoke patients.

The specific questions I will address are:

1. To what extent are published retinal microvascular abnormalities associated with stroke and ischaemic subtypes?
2. Can MRI changes in lacunar stroke provide more information regarding the underlying aetiology of lacunar stroke?
3. Does the prevalence of retinopathy (thought to reflect blood-retinal barrier breakdown) differ between lacunar and non-lacunar stroke?
4. Do retinal vessel widths differ in lacunar and non-lacunar stroke?
5. Does retinal vessel geometry differ in lacunar and non-lacunar stroke?
6. Does fractal analysis of the retinal vasculature differ between lacunar and non-lacunar strokes?

To do this, this thesis addresses these questions from several angles: a systematic review of retinal vascular and retinopathic changes in patients with stroke and those who subsequently develop stroke, new data collection of a cohort of very carefully characterised (clinically and radiographically) patients with retinal imaging to examine retinopathic and retinal-vascular associations with lacunar and cortical stroke, detailed analysis of retinal vascular branching patterns including development of new software to assess branching co-efficients, angles and fractal dimensions of the vascular tree and a detailed analysis of associations between retinal and brain imaging features with particular reference to small vessel versus large vessel changes.

The study was part of a large programme of work addressing different aspects of potential mechanisms of vascular disease causing lacunar stroke and cerebral small vessel disease. As such this thesis focuses on the retinal findings but benefited from the substantial pilot work, additional funding and ongoing assessments of other factors (blood markers, carotid and intracranial doppler imaging, detailed brain imaging, blood brain barrier permeability imaging, experimental work and other systematic reviews) that made up the different components of this programme.

Finally the retinal component was designed to complement a contemporaneous study of retinal features in stroke being conducted in Australia and Singapore (Principal Investigator Prof Richard Lindley, University of Sydney) which was anticipated to have a larger sample size but less detailed brain imaging and hence more noisy stroke phenotyping compared with the present study and we ultimately intend to merge the two databases to perform a large individual patient based meta analysis.

Chapter 2. Edinburgh Mild Stroke Study: aims, design and methodology.

2.1 Aims of chapter

In this chapter I describe the background, aims and methods used in the Mild Stroke Study.

2.2 Overview of the Edinburgh Mild Stroke Study

The Mild Stroke Study aimed to elucidate the aetiology of lacunar stroke by comparing retinal, blood markers, large artery disease, risk factors and brain MRI based parameters between patients with lacunar stroke and a control group of patients with cortical stroke. The Mild Stroke Study was part of a large programme of work to address gaps in knowledge concerning mechanisms of lacunar stroke. In addition to the diagnostic MR and retinal imaging, we collected serum biomarkers of coagulation, fibrinolysis and inflammation, performed transcranial doppler studies and a subset of the patients were studied with blood brain barrier permeability contrast enhanced MRI scans. This thesis focuses on the retinal and brain findings.

2.3 Contributors to the Mild Stroke Study

Prof Joanna Wardlaw developed the hypothesis for causes of lacunar stroke which was then tested as part of an extended programme of work which benefitted from several different sources of funding. Prof Wardlaw was my PhD supervisor and all work that I completed on the Mild Stroke Study was under her direct supervision. A component of this programme was to examine retinal features in lacunar stroke which is the focus of this thesis. I, with support from my supervisors and sponsor Peter Sandercock, obtained funding from the Wellcome Trust for my clinical research training fellowship which also funded purchase of the retinal camera. The fellowship application and subsequent development of study protocols benefitted

considerably from previous grants and pilot work in this area by Prof Wardlaw with patient recruitment by research fellows Drs Vera Cvoro, Debashish Chowdhury and Kristin Haga. I liaised with the Ethics Committee regarding modifications to the original approval which had been submitted by Prof Wardlaw. I adapted the main study data collection forms and designed the retinal data collection forms, recruited all patients (other than 8 which were recruited by another research fellow), developed the database (with input from David Perry, head of IT in the Division of Clinical Neurosciences), entered the data and performed all of the statistical analysis (with input and advice from statisticians Cat Graham, Francesca Chappell and Steff Lewis). I co-ordinated the booking of most of the MRI scans all of which were performed by the radiographers in the SFC Brain Imaging Research Centre for Scotland (SBIRC) which is part of the Scottish Imaging Network, A Platform for Scientific Excellence (SINAPSE) collaboration.

I took or directly supervised the taking of all retinal photographs to ensure adequate quality in the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh. I bought and established the retinal camera in the Wellcome Trust Research Facility and trained other photographers with close collaboration with our colleagues in Australia (Prof Richard Lindley) with whom we intend to pool data. I graded all retinal photographs for the presence of retinopathy, arteriovenous nicking and focal arteriolar narrowing. Prof Bal Dhillon, ophthalmologist, graded any questionable lesions. Dr Tom MacGillivray (Senior Image Analyst), in conjunction with me, developed the custom written software with which the retinal photographs were analysed for retinal vessel widths. I graded all retinal images for vessel widths using the semi-automated software. Rosemarie den Haan (a medical student from the University of Amsterdam) helped us develop the software for assessing retinal geometry and subsequent grading of the retinal photographs. Dr MacGillivray processed the retinal photographs to assess their fractal properties. Prof Martin Dennis supervised the clinical aspect of the study and provided help with clinical sub-typing of stroke. University of Edinburgh medical students Susan Stevenson and Fergal Marlborough helped collect data for the systematic reviews on endothelial dysfunction in lacunar stroke. University of Amsterdam medical student Petra

Hokke helped collect data for the systematic review of retinal microvascular abnormalities in stroke and helped pilot early versions of the retinal widths measuring software. Prof Wardlaw obtained the funding for the overarching main study which included funding for the brain scans, all computational analysis and for statistical advice. Prof Wardlaw coded all of the brain scans and supervised the whole project.

2.4 Design of the Mild Stroke Study

The Mild Stroke Study was a prospective observational cross-sectional study.

2.5 Ethical approval

The Mild Stroke Study received ethical approval from the Lothian Regional Ethics Committee and all patients provided written informed consent (ethics committee reference number LREC/2002/8/64). There were several subsequent amendments to address minor study modifications and to keep abreast of regulatory changes. See appendices 3 and 4 for details of the Patient Information Leaflet and the Consent Form.

2.6 Setting

The Mild Stroke Study recruited patients who presented with symptoms suggestive of mild ischaemic stroke (defined as lacunar or mild cortical stroke) to the Western General Hospital in Edinburgh, UK. The Western General Hospital is a tertiary university teaching hospital with a specialist acute stroke service (16 bedded stroke unit, 22 bedded rehabilitation ward) and 24 hour stroke thrombolysis program. The population that the hospital serves is a largely urban population of about 250,000.

During the recruitment period of the Mild Stroke Study patients who had symptoms suggestive of a stroke would have been either referred by their general practitioner (family physician) to the thrice weekly neurovascular clinic or to the medical

receiving unit if their needs were such that in-patient admission might be warranted. Patients who called for an emergency ambulance would either have been taken to the acute receiving unit of the Western General Hospital or the Accident and Emergency Department of the other main hospital in Edinburgh, the Royal Infirmary of Edinburgh.

2.7 Time course of study

Pilot work began in 2003 to test the mechanisms of patient recruitment, the brain imaging methods, the collection of demographic and other patient variables and the retinal photography. The patients described in this thesis were recruited from April 2005 to January 2008.

2.8 Inclusion criteria

We recruited patients at presentation to the hospital with definite lacunar or mild cortical ischaemic stroke as defined in the Oxfordshire Community Stroke Project (Bamford *et al.* 1991) and diagnosed by an experienced stroke physician. We included patients presenting up to three months after the onset of their symptoms and used diagnostic MRI to diagnose the recent stroke lesion so as to have the best chances of being able to accurately subtype ischaemic stroke. All patients were able to give informed written consent and understand the written patient information sheet (see appendices 3 and 4) which was explained in detail by me to each patient and their relatives/carers if present and relevant.

We defined a lacunar stroke syndrome as one causing one of the 5 classical lacunar syndromes: pure motor stroke involving either weakness of the face and arm, arm and leg or all three, pure hemi sensory stroke involving either sensory loss of the face and arm, arm and leg or all three, sensorimotor stroke as weakness and sensory loss in the face and arm, arm and leg or all three, ataxic hemiparesis or clumsy hand dysarthria syndrome. We defined a mild cortical stroke syndrome as; weakness or sensory loss affecting one limb (or just face) or dysphasia/neglect/inattention or

homonymous hemianopia or any motor weakness and dysphasia/neglect/inattention, dysphasia/neglect/inattention and homonymous hemianopia equivalent to a partial anterior circulation stroke in the OCSP classification (Bamford *et al.* 1991).

We used a control group of patients with cortical ischemic stroke to control for having a stroke, risk factor profiles, and secondary stroke prevention medications (as opposed to normal age-matched controls, which would not have controlled for any of these factors). The vascular risk factor profiles of patients with lacunar and cortical stroke are similar (Jackson and Sudlow 2005) other than for carotid stenosis and atrial fibrillation. We used mild cortical stroke patients and not those with severe cortical strokes as retinal photography is impractical in patients with severe stroke (Lindley *et al.* 2009) and the mechanisms behind severe cortical stroke are represented in patients with mild cortical stroke.

2.9 Exclusion criteria

We excluded patients presenting with symptoms or brain imaging findings that were inconsistent with a diagnosis of definite stroke. We excluded patients with contraindications to MR e.g. cardiac pacemakers, metal implants elsewhere in the body and screened with orbital X-rays patients with previous metal fragment injuries to the eyes. We excluded patients who were unable to lie flat or who did not fit into the scanner as they were too wide. We excluded patients whose subsequent MR scan showed a haemorrhage causing their stroke syndrome or in whom the MR scan showed an alternative pathology causing their symptoms e.g. tumour. We performed permeability MRI in some patients which involved injecting intra-venous gadolinium contrast and therefore we excluded patients with impaired renal function (estimated glomerular filtration rate <50 ml/min) to avoid nephrogenic systemic fibrosis. We excluded patients who had complex lesions on MRI scanning such as gliosis secondary to trauma prohibiting reliable sub-typing into lacunar or cortical stroke. We excluded patients who were medically unstable (as lying flat for a MRI for 20 minutes might have exacerbated their condition and compliance with retinal photography is reduced in medically unstable patients).

2.10 Methods of patient recruitment

I identified potential patients in several different ways. All stroke physicians and neurologists who were seeing acute stroke patients in the Western General Hospital were aware of the study and contacted me directly by phone or via letter if they thought a patient was suitable patient. I reviewed on a daily basis the stroke unit and acute receiving wards and all neurovascular clinic lists were reviewed searching for suitable patients. Prof Wardlaw identified potentially suitable patients by screening CT/MR and carotid doppler requests. During the pilot phases of the trial patients were recruited by Drs Vera Cvoro and Debashish Chowdhury. For patients described in this thesis, Dr Kristin Haga recruited the first 8 patients in the study. I recruited all subsequent 245 patients.

2.11 Clinical assessment and data collection

I clinically assessed all potential patients at presentation to the hospital with stroke and collected data on a specifically designed and piloted data collection sheet (see appendix 5). I performed a detailed history and clinical examination to ascertain current symptoms, past medical history and current neurological and cardio vascular impairments. I collected data on the date and time of current symptoms, the date that the patient was clinically assessed and if the symptoms were persisting or if they had resolved how long they had lasted. I recorded the side of the body which was affected (if appropriate). I recorded whether there was weakness and if so whether the face, arm or leg was affected. I recorded whether there was sensory loss and if so whether the face, arm or leg was affected. I recorded the presence of the following; dysphasia, neglect, visual disturbance, posterior circulation symptoms. I recorded whether the patient was left or right handed and assessed NIHSS score both at the time of the clinical assessment and as there were some delays to when patients were seen after their stroke, I also recorded estimated worst NIHSS. I recorded gender of the participant and their date of birth and age at stroke onset.

I recorded presence of hypertension (either previous history of physician/general practitioner diagnosed hypertension or patient on treatment for hypertension), diabetes (either previous history of physician/general practitioner diagnosed Type I or Type II diabetes or on treatment for diabetes – oral hypoglycaemic agents or insulin) and if it was diagnosed at the time of presentation, peripheral vascular disease (either surgeon/physician/general practitioner diagnosed history of or current symptoms of intermittent claudication), ischaemic heart disease subdivided into previous angina, myocardial infarct, coronary angioplasty, coronary artery bypass grafting (either physician/general practitioner diagnosed history of angina or myocardial infarct or previous coronary angioplasty or coronary artery bypass grafting), Transient Ischaemic Attack (TIA) (either physician or GP diagnosed history of), stroke (either physician/GP diagnosed history of) for which I collected details of side of stroke, type of stroke (haemorrhagic/infarct) and location (lacunar/ischaemic) if known. I also recorded if there was a history of atrial fibrillation (physician or general practitioner diagnosed history of), hyperlipidaemia (physician/general practitioner diagnosed history of), left ventricular systolic dysfunction (physician/GP diagnosed or echocardiographic evidence of) and structural heart disease (physician/GP diagnosed history of significant valve lesions, known patent foramen ovale).

I collected data on cigarette/tobacco smoking status (defined as current or ex smoker less than 12 months, ex smoker greater than 12 months or never), current alcohol use measured in unit per week and whether the participant previously drank excessive amount of alcohol and whether the patient was independent in activities of daily living prior to the stroke. I recorded the presence of a family history of stroke in a first degree relative. I recorded a single blood pressure at presentation in either arm, normally in the sitting position unless the patient was unwell and recumbent.

We obtained electrocardiograms on all participants and recorded whether the participant was in sinus rhythm or the presence of atrial fibrillation and the presence of left ventricular hypertrophy on ECG. All patients had carotid arteries assessed and we recorded which method (doppler ultrasound in the majority or magnetic

resonance angiography in a few) and the percentage internal carotid artery stenosis measured using the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria. If a participant had an echocardiogram we recorded any relevant abnormalities that might have been a potential source of embolism. We recorded random serum glucose and total serum cholesterol taken at the time of initial assessment and measured as part of the normal clinical stroke protocol in the local laboratories in the Western General Hospital.

We recorded the use of the following medications or medication types at the time of the stroke: aspirin, dipyridamole, warfarin, diuretic, angiotensin converting enzyme (ACE) inhibitor/angiotensin II receptor blocker (ARB), beta blocker, other antihypertensive medication, oral hypoglycaemic agents, insulin, statin or other cholesterol lowering medication.

2.12 MRI procedures

Patients had diagnostic MR brain imaging on a research dedicated 1.5T MR brain scanner (GE Signa LX EchoSpeed scanner, Milwaukee, WI). The sequences were optimized for diagnostic imaging in stroke after extensive testing. Regular quality assurance tests of all imaging methods were performed. The sequences obtained were T1, T2, Gradient Echo, Diffusion Weighted (DWI) and Fluid Attenuated Axial Inversion Recovery (FLAIR). The table below show technical data for each sequence. Sets of axial DW-echo planar images (sensitization levels b=0 and 1000 s/mm^2) were collected with diffusion gradients applied sequentially along 6 non-collinear directions (5 acquisitions including baseline T2WI-EP image and 6 diffusion-weighted EP images per slice position) with 5mm slice thickness, 1mm slice gap, 128 x 128 image matrix and 24 x 24 field of view (FOV). Other MR parameters are shown in the table below. The total scan time was approximately 20 minutes.

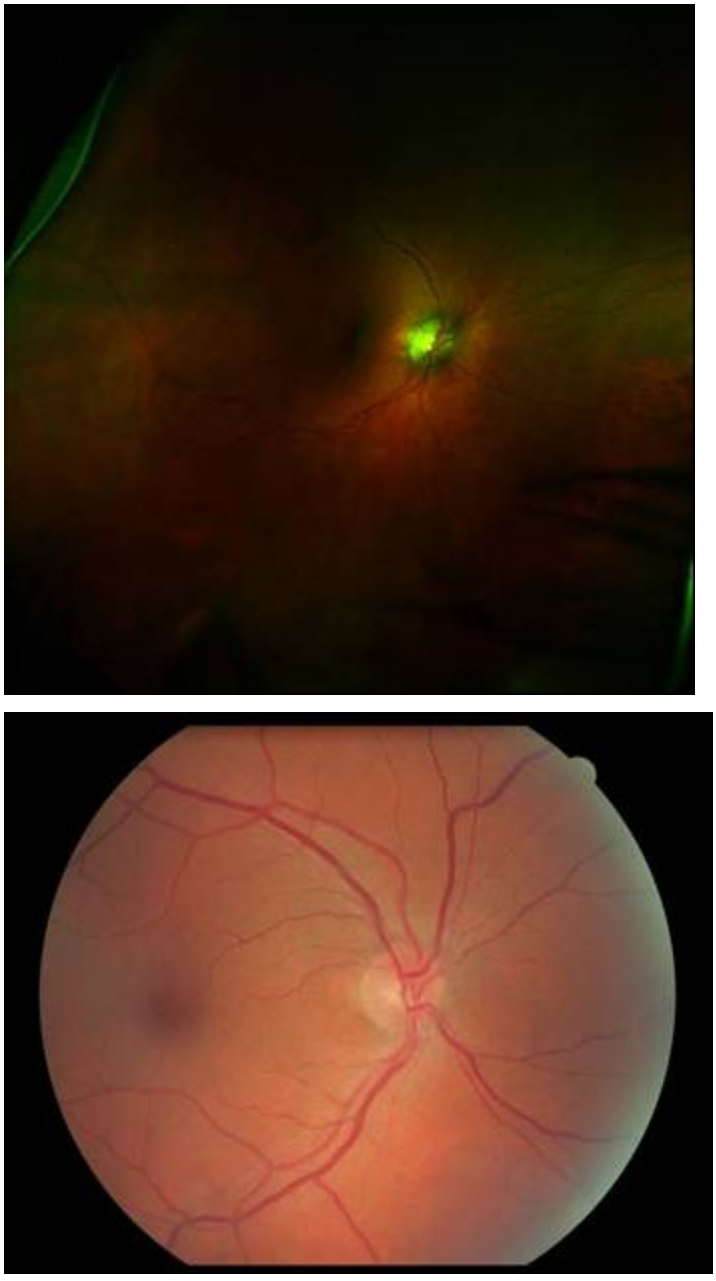
Table 2.1. Technical details of MR sequences performed

Sequence	TE	TR	Flip angle	Slice thickness	Slice gap	Matrix	FOV
T2	102	6300		5mm	1.5mm	256x256	24x24
T1	min	450		5mm	1.5mm		
GRE/T2*	15	625	20^{o}	5mm	1.5mm	256x192	24x24
FLAIR	140	9000		5mm	1mm		

2.13 Retinal photography

During the pilot phase of this study patients either had their retinal photographs taken on a conventional 45 degree non mydriatic retinal camera (Topcon, Topcon Medical Instruments Inc.) or a widefield Optos P200, a 200 degree scanning laser ophthalmic camera (Optos plc). We found that the Optos widefield camera did not produce images of suitable quality for automated assessment or identification of subtle signs of retinopathy so used a conventional retinal camera for the remainder of the study.

Fig 2.1 Example of an Optos 200 degree (on the top) and an example of a Canon DRGi 45 degree retinal photograph (below) illustrating superior image quality of the Canon DRGi.



The first 8 patients described in this thesis had their photographs taken at the Princess Alexandra Eye Pavilion, Edinburgh by trained medical photographers using a Topcon non mydriatic digital retinal camera. The remaining patients were photographed by me in the Wellcome Trust Clinical Research Facility in the Western

General Hospital with a Canon CR-DGi non-mydriatic 45 degree field of view retinal camera (Canon USA Inc.) with a Canon 20D body. We purchased and established this retinal camera specifically for this study. We were influenced in the choice of this camera by expert advice from our collaborators in Australia (under the instruction of Profs Paul Mitchell and Richard Lindley and A/Prof Jie Jin Wang) who were undertaking a similar study of retinal changes in acute stroke as we were keen to ensure that retinal images were taken with identical cameras and software as we plan to merge study databases to increase power and generalizability.

Images were saved on a dedicated Dell PC which was continuously connected to the retinal camera. All images were captured and manipulated with Digital Healthcare Optomize software (Digital Healthcare, Cambridge, UK). Images were anonymised and stored on a secure server which was backed up daily, on the password protected hard drive of the retinal camera dedicated PC and on a removable hard drive which was kept in a locked filing cabinet in a different building.

After piloting use of the camera without eye drops we decided to use pupil dilatation. We used 1% tropicamide eye drops (2 drops per eye) with a 15 minute wait to dilate the pupil if necessary and if there were no contra-indications (history of glaucoma or if the patient declined eye drops). We screened all patients for glaucoma before administering tropicamide and provided information to the patient about who to contact if they experienced any eye discomfort in the few hours after eye drop administration in the form of a leaflet. If the patient was a car driver and had returned to driving after their stroke we asked the patient not to drive a car for 4 hours after administration of the eye drops. I took photographs of six fields per eye – centred on the optic disc, centred on the macular, lateral to the macula, nasal to the optic disc, upper arcade and lower arcade of each eye for each patient. If I was unable to take all fields I recorded the reason why.

2.14 MRI assessment

All MRI scans for each patient were read by a single experienced consultant neuroradiologist (Joanna Wardlaw) using a data collection sheet designed specifically for this study (see appendix 6). We collected the date and time of and sequences performed. We recorded the presence of a clinically relevant acute lesion to the recent stroke symptoms and whether this was present on DWI and/or FLAIR/T2 weighted sequences. We recorded which side of the brain the lesion was present.

We defined a lacunar infarct as a small round lesion less than 20 mm in diameter in a location typical for and suggestive of infarction secondary to disease in a small perforating artery – normally one of the lenticulostriate branches of the middle cerebral arteries. Lacunar infarcts were differentiated from white matter lesions by their pattern, shape and presence of lesions on different MRI sequences – lacunar infarcts tend to be larger, more likely to be unilateral and single lesions and are more likely to be present on DWI images than white matter lesions. Lacunar infarcts are distinguished from enlarged perivascular spaces (EPVS) as they do not follow characteristic linear patterns that EPVS do either on adjacent slices in the basal ganglia or on single slices in the centrum semiovale. If the lesion was a lacunar infarct we recorded the location from the following categories; internal capsule, internal border zone, centrum semiovale, thalamus, lentiform nucleus, brainstem and the size of the lesion (in millimetres) and whether it extended to the cortical margin.

If the lesion was cortical we classified its location as one of the following; small temporal, small parietal, basal ganglia, subcortical, anterior half of the peripheral Middle cerebral artery territory, posterior half of the peripheral MCA, anterior anterior cerebral artery, posterior ACA, anterior posterior cerebral artery, posterior PCA, anterior border zone, posterior border zone. We recorded the presence and number of old stroke lesions and recorded for each lesion the side (left/right), location (lacunar/cortical/posterior circulation) and type (infarct/haemorrhage).

We counted the number of cerebral microhaemorrhages present (defined as small punctuate round smooth lesions with signal drop out representing haemosiderin deposition on GRE/T2* sequences). We assessed periventricular and deep white matter hyperintensities with the Fazekas scale (none, mild, moderate, severe) for the left and right hemispheres separately and with an overall score for both hemispheres combined (Fazekas *et al.* 1987). We also rated white matter lesions with the De Leeuw method which counts the number of lesions of certain diameters (and thus enabling an estimate of white matter lesion volume)(de Leeuw *et al.* 1998). The diameter strata are <3mm, 3-10mm and >10mm for the left and right hemispheres and overall for both hemispheres combined. We rated enlarged perivascular spaces on a previously published scale (MacLullich *et al.* 2004) from 0-3 (see table) in both the basal ganglia and centrum semiovale for both the left and right hemispheres separately and then combined into an overall score. In the scale EPVS were defined as punctuate round high intensity signal changes on consecutive T2 slices in the basal ganglia and characteristic linear shaped hyperintensities in the centrum semiovale. This scale was developed within the department and counts the number of EPVS in the slice with the largest number of EPVS before categorizing he number as detailed below. This has limitations as we only used one grader and have not yet analysed inter-rater reliability. Furthermore there may be misclassification when the number of EPVS is on the boundaries of the categories eg around 10 or 20. We coded deep and superficial cerebral atrophy as none, mild, moderate and severe compared to a standard age matched template (Farrell *et al.* 2008).

Table 2.2 .EPVS scoring scheme

EPVS score	Number of EPVS
0	0
1	1-10
2	11-20
3	21-40
4	>40

2.15 Retinal photograph assessment

All retinal photographs were assessed by me blinded to the patients' clinical details. Photographs were identified with study identification numbers. I was trained under the supervision of Prof Bal Dhillon and the Lothian Diabetic Retinopathy Screening Service to assess retinal photographs for retinopathy and cross checked with standard retinopathy examples from Prof Paul Mitchell in Sydney. I also performed site visits to Singapore and Australia to ensure conformity with our sister retinal stroke study. We collaborated extensively to develop appropriate retinal assessment tools. Details of how retinal photographs were graded for specific features will be presented in detail in the separate results chapters.

2.16 Sub-typing of ischaemic stroke

For each patient I assigned a clinical classification of lacunar or cortical stroke with previously defined criteria based upon the Oxfordshire Community Stroke Project classification (Bamford *et al.* 1991). Cases were discussed, and stroke diagnosed and sub-typed at a weekly multi disciplinary stroke meeting with consultant stroke physicians, neurologists and neuroradiologists which helped to ensure sub-typing accuracy. Where the clinical differed from the radiological following discussion the imaging classification was used. A neuroradiologist coded diagnostic MRI scans blind to clinical details to assign an imaging classification of cortical or lacunar and the two clinical classification and the imaging classification were combined. A recent infarct was defined as a hyperintense area on diffusion imaging (with corresponding reduced signal on the apparent diffusion coefficient image) with or without increased signal on fluid-attenuated inversion recovery or T2-weighted imaging in a distribution compatible with an arterial territory. Lacunar infarcts were in the cerebral hemispheric white matter, basal ganglia, or brainstem and <2 cm diameter if recent (lesions >2 cm were classed as striatocapsular/cortical because they have large artery disease causes). If there was a discrepancy between the two classifications then we used the imaging classification to assign a final classification

as this is a more reliable method than using clinical assessment alone in assigning stroke subtype (Mead *et al.* 2000).

2.17 Database details

Data regarding demographics, clinical details, diagnostic MRI, retinal and Transcranial Doppler findings were manually recorded by me onto specially designed data collection forms which were then entered by me into the specifically designed database. The database was designed to be expandable to include additional data (eg on follow up information) and to match with data derived from other sources such as information from blood brain barrier imaging or blood biomarker results.

The Mild Stroke Study had a bespoke database management system written (by David Perry) to help manage the secure collation, storage, data keying (punching) and encoding of its data. This was written using Microsoft SQL Server 2000 and a web based front end. To use the system, the user first provided their username and password. Each patient had up to 5 data collection forms that were completed by hand and then keyed into the database. The system was tasklist driven which helped manage the workflow to ensure that all data was collected for all registered patients. As data were entered into the system, they were validated and responses were encoded as per the Data Dictionary. This ensured that similar responses were always recorded with the same values; eg M for Male, F for Female. Changes to the data are automatically audited by use of data triggers applied to the SQL database tables. This allowed us to know who changed what and when. The data was stored on centrally managed servers that were protected against data loss.

The database was specifically designed to allow future collaboration and combination of data with collaborators in Australia (Lindley and Multi-Centre Retinal Stroke Study Collaborative Group 2008). Data from the database were

retrieved with Excel . The database had several internal validity data checks and I checked a random sample of data entries to ensure adequate data quality and keying.

2.18 Statistical analysis

I performed all analyses using Minitab statistical software (Version 14; Minitab Inc). Further details are given in the specific results chapters.

Chapter 3: Retinal microvascular abnormalities and stroke – a systematic review.

3.1 Aims of chapter

In this chapter I present the results of the systematic review and meta-analysis of the associations between retinal microvascular abnormalities and incident and prevalent stroke and between ischaemic stroke subtypes. Please note that the formatting differs in this chapter due to the large number of references cited.

3.2 Introduction

As discussed in chapter 1, the exact aetiology of lacunar stroke is unclear (Wardlaw 2005). Possible causes include atherothromboembolism, intracranial large artery stenosis, intrinsic microvascular atheroma, or endothelial dysfunction manifesting either through ischaemia or failure of the blood brain barrier (Wardlaw *et al.* 2003).

The cerebral small vessels that cause lacunar stroke share many characteristics with retinal vessels (Patton *et al.* 2005), as they are developmentally related during embryogenesis and of similar diameter. Retinal vessels are visibly affected by known risk factors for stroke such as diabetes and hypertension (Wong 2004). Therefore, determining any association between retinal microvascular abnormalities and stroke may improve understanding of the nature of the cerebral small vessel changes underlying lacunar ischaemic stroke and aid cerebrovascular and cardiovascular risk profiling.

Several studies have examined a possible link between stroke and the retina, including large epidemiological studies in community dwelling populations and small detailed studies of patients with specific diseases attending tertiary hospitals.

The presence and strength of any reported associations between retinal vascular abnormalities and stroke vary considerably possibly reflecting the differing populations, methodologies, definitions of disease and abnormalities sought.

We therefore performed a systematic review to clarify associations between a range of retinal microvascular abnormalities and any stroke and with specific subtypes of both stroke and ischaemic stroke. We also determined where more information was needed from new studies.

3.3 Methods

3.3.1 Search strategy

We searched Ovid Medline (from 1st Jan 1950) and Embase (from 1st Jan 1980) to 5th October 2007 for papers investigating associations between retinal microvascular abnormalities and stroke (defined as stroke/transient ischaemic attack/asymptomatic brain infarct identified on imaging). A full list of the search terms used for Medline can be found in appendix 8. The electronic search was supplemented by the authors' personal files, hand searching the bibliographies of papers selected from the electronic search, checking references of review articles and by surveillance of key journals from April 2005 to October 2007 – (Neurology, Lancet, Brain, Ophthalmology, Stroke, British Journal of Ophthalmology, British Medical Journal and Retina).

I conducted the search, reviewed all titles and extracted data from all of the papers. A second reviewer (Petra Hokke, a Dutch medical student whom I was supervising on a 6 month research placement) independently reviewed all titles and extracted data from all of the papers. A third reviewer (Joanna Wardlaw) assessed a sample of papers for inclusion, extracted data and adjudicated in any disagreements.

3.3.2 Inclusion criteria for papers

We included studies published in full which assessed the association between retinal microvascular abnormalities and stroke (as defined above) in adult humans.

3.3.3 Exclusion criteria for papers

We excluded studies published only as abstracts or presented in conferences without full subsequent publication, review papers, papers dealing with specific single gene related disorders such as CADASIL, papers studying only macular degeneration and duplicate publications. We excluded papers dealing with retinal microvascular abnormalities and only cerebral white matter disease (leukoaraiosis) seen on brain imaging.

3.3.4 Definitions of stroke and retinal outcomes

Stroke is defined by the World Health Organisation as a focal sudden neurological deficit lasting greater than 24 hours of presumed vascular aetiology with brain imaging excluding other causes (Hatano 1976). For the purposes of this review we defined "stroke" as either a clinically diagnosed stroke or transient ischaemic attack (with or without cerebral imaging) or a cerebral infarct identified on brain imaging without definite associated clinical features being documented. We defined "incident stroke" as strokes which occurred after the patient/subject had been enrolled in the study and "prevalent stroke" as strokes which had preceded patient enrolment in the study.

"Retinal microvascular abnormalities" included retinopathy (microaneuryms, soft and hard exudates, haemorrhages), retinal arteriolar and venular width measurements (including arterio-venous ratios - AVR), focal arteriolar narrowing (FAN), arterio-venous nicking (AVN)), retinal artery (RAO) or vein occlusion (RVO), retinal artery emboli and alterations in retinal vascular geometry (angles or vessel tortuosity).

3.3.5 Paper assessment

We collected data on a form designed and piloted specifically for this review. We recorded study population details (age, selection criteria, co-morbidities), study design and assessed definitions of stroke and retinal outcomes.

We assigned the reference standard for retinal assessment as high quality retinal photographs of both eyes (preferably multi field) and blinded assessment of the retinal appearance with a standardised and externally validated tool. We collected data on which retinal features were measured, methods of retinal assessment, use of mydriatic drugs, number of fields imaged per eye and which eyes were photographed, type of camera and field of view of the camera.

The reference standard for stroke diagnosis was assessment of the patient by a stroke specialist at the time of the stroke with appropriate cerebral imaging to differentiate haemorrhagic from ischaemic stroke and stroke mimic and, if ischaemic, to identify whether lacunar or non-lacunar ischaemic stroke. We considered the following methods of stroke diagnosis to be less reliable: patient questionnaires, retrospective case note analysis, death certificates and review of centrally held health records. We assessed studies for the method of subject selection, the presence of a suitable control group, sub-typing of stroke and blinding of assessment of images/patients. We extracted data on numbers with stroke or transient ischaemic attack (TIA) and different retinal features in as much detail as possible, any calculated odds ratios, relative risks or hazards ratios and whether these were unadjusted or adjusted for shared risk factors such as hypertension or diabetes. When counting total numbers of patients and stroke events that contributed to each analysis we were careful to count patients from studies contributing more than one paper only once to avoid unnecessary bias. We did not have the resources to contact authors of studies to ask regarding missing data or individual patient data.

3.3.6 Statistical analysis

We summarised the numbers of subjects in the different population samples, with or without various retinal abnormalities and stroke. Stroke outcomes were rare (incidence less than 10%) and odds ratios, hazards ratios and relative risk were considered to be equivalent. We converted all ratios to summary risk ratios and prepared Forrest Plots using Revman (Review Manager Software Version 5, Cochrane, Oxford, UK) and Excel (version 2003; Microsoft Inc, Redmond, Wash.). We used a random effects generic inverse variance method to plot summary risk ratios and 95% confidence intervals (CI) using a logarithmic scale from the adjusted ratios and 95% CI. We used adjusted ratios in preference to unadjusted to best account for differences in risk factors between populations. We tested for associations between stroke and pre-defined retinal microvascular abnormalities and then separately examined associations in important predefined subgroups such as incident or prevalent stroke, ischaemic versus haemorrhagic stroke and ischaemic stroke subtypes. We tested for heterogeneity between study results with the Chi square test for heterogeneity with an alpha level for significance set at p=0.05. When heterogeneity was detected we investigated the methodology and definitions of variables used in the heterogeneous studies to identify possible causes.

3.4 Results

3.4.1 Included studies

The initial search produced 4461 titles and abstracts, subsequent screening of which yielded 52 potentially relevant papers describing probable retinal microvascular abnormalities and stroke for which we assessed the full texts. Of the 52, 15 were excluded (13 were not relevant, and 2 were in Japanese and not easily translated), leaving 37 papers which met the inclusion criteria. Figure 2.1 shows the selection process. Table 3.1 documents the main findings of the included papers.

Figure 3.1. Flow diagram representing selection for papers included in systematic review.

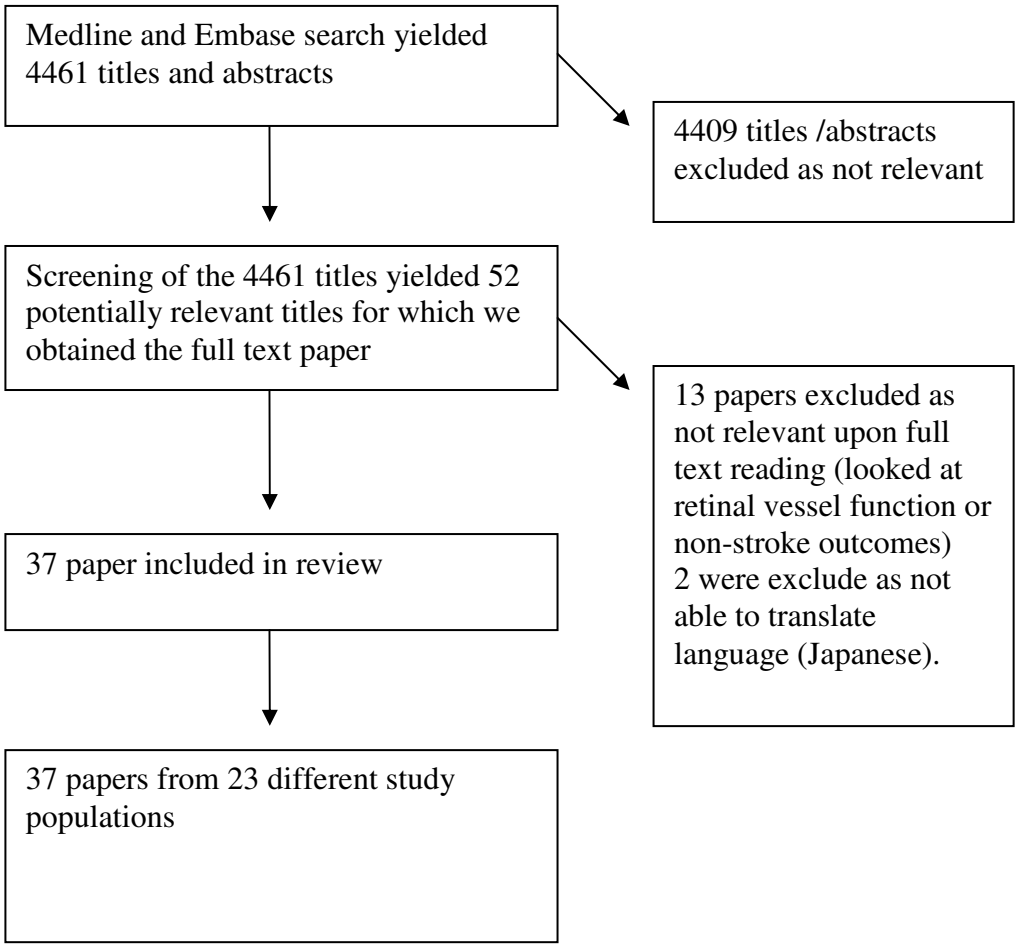


Table 3.1 Descriptions of included studies arranged by stroke outcome. *Ausdiab –the australian diabetes, obesity and lifestyle study, CHS – cardiovascular health study, BMES – blue mountains eye study, ARIC – atherosclerosis risk in communities study, WESDR – wisconsin epidemiological study of diabetic retinopathy, BDES – beaver dam eye study, ROTT – the rotterdam study, RVO – retinal vein occlusion, RAO – retinal arteriolar occlusion, AVR – arteriovenous ratio, AVN – arterio-venous nicking, FAN – focal arteriolar narrowing, inc = increased, dec = decreased, art = arteriolar, ven = venular. ? = not given in paper and unable to calculate from data. Link? = presence of significant association between retinal feature and stroke outcome (continued overleaf).*

Study	Origin	Size (n)	Retinal Feature	Stroke outcome	Link ?	Stroke (n)
Prevalent Stroke						
Abu El Asrar 2002		648	Retinopathy	Prevalent ischaemic stroke	Yes	8
Petitti 1995		2124	Retinopathy	Prevalent ischaemic stroke	Yes	56
Luijckx 1998		59	Arteriosclerosis	Prevalent ischaemic stroke	No	59
Hayreh 2001		1090	RVO	Prevalent stroke	Yes	43
Mitchell 1996	BMES	3654	RVO	Prevalent stroke	Yes	?
Klein 2000	ARIC	8772	AVR	Prevalent stroke	No	?
			AVN	Prevalent stroke	No	
			FAN	Prevalent stroke	No	
Wong 2005	AUSDIAB	1027	Retinopathy	Prevalent stroke	Yes	42
Wong 2003	CHS	2050	Retinopathy	Prevalent stroke	Yes	133
Kwon 2006		550	Retinopathy	Prevalent MRI infarct	Yes	61
Inoue 1996		361	Retinopathy	Prevalent MRI infarct	Yes	101
Kobayashi 1997		933	Arteriosclerosis	Prevalent MRI infarct	Yes	99
Longstreth 2007	CHS	1717	AVR	Prevalent MRI infarct	Yes	496
			AVN	Prevalent MRI infarct	Yes	
			FAN	Prevalent MRI infarct	No	
			Retinopathy	Prevalent MRI infarct	No	
Cooper 2006	ARIC	1684	AVR	Prevalent MRI infarct	Yes	183
			FAN	Prevalent MRI infarct	Yes	
			Haemorrhage	Prevalent MRI infarct	Yes	
			Soft exudates	Prevalent MRI infarct	No	
			Microaneurysms	Prevalent MRI infarct	Yes	
			AVN	Prevalent MRI infarct	Yes	
Ueda 2002		185	RVO/RAO	Prevalent MRI infarct	Not clear	?
Kwa 2002		179	Arteriosclerosis	Prevalent MRI infarct	No	?
			Art tortuosity	Prevalent MRI infarct	No	
Ikeda 1994		318	Retinopathy	Prevalent CT infarcts	Yes	56

Incident Stroke						
Cheung 2007	ARIC	1617	Retinopathy	Incident ischaemic stroke	Yes	75
Bruno 1995		140	Retinal emboli	Incident ischaemic stroke	Yes	19
Wong 2001	ARIC	10358	Retinopathy	Incident ischaemic stroke	Yes	113
			AVN	Incident ischaemic stroke	Yes	
			FAN	Incident ischaemic stroke	No	
			AVR	Incident ischaemic stroke	No	
Ikram Neurol. 2006a	ROTT	6780	Inc ven caliber	Incident ischaemic stroke	Yes	411
			Dec art caliber	Incident ischaemic stroke	Yes	
Wong 2006	CHS	1992	Inc ven caliber	Incident stroke	Yes	113
			Dec art caliber	Incident stroke	No	
Howard 1987		85	RAO	Incident stroke	Yes	22
Klein 2004	WESDR	996	Retinopathy	Incident stroke	Yes	59
Cohen 2003		950	Retinopathy	Incident stroke	Yes	41
Kim 2002		365	Retinopathy	Incident stroke	No	?
Fuller 2001		4753	Retinopathy	Incident stroke	Yes	293
Wong 2002	ARIC	1684	Retinopathy	Incident stroke	Yes	32
Mitchell 2005	BMES	3583	Retinopathy	Incident stroke/TIA	Yes	132
			FAN	Incident stroke/TIA	Yes	
			AVN	Incident stroke/TIA	No	
			AVR	Incident stroke/TIA	No	
Ikram Brain 2006b	ROTT	490	Inc ven caliber	Incident MRI infarct	Yes	33
			Dec art caliber	Incident MRI infarct	No	
Klein WESDR 1999b	WESDR	2366	Retinopathy	Incident death with stroke	Yes	274
Hirai 2007	BDES	4284	Retinopathy	Incident death with stroke	No	?
Klein BDES 1999a	BDES	4856	Retinal embolus	Incident death with stroke	Yes	97
Wang 2006	BMES/ BDES	8580	Retinal embolus	Incident death with stroke	Yes	344
Witt 2006	BDES	684	Dec art caliber	Incident death with stroke	No	28
			Tortuosity	Incident death with stroke	No	154
Klein 2003	BDES	4926	Bifurcation angle	Incident death with stroke	No	
			Retinal emboli	Incident death with stroke	Yes	
Cugati 2007	BDES/ BMES	8580	RVO	Incident death with stroke	No	341
Tsaloumas 2000		95	RVO	Incident death with stroke	No	18

3.4.2 Description of included studies

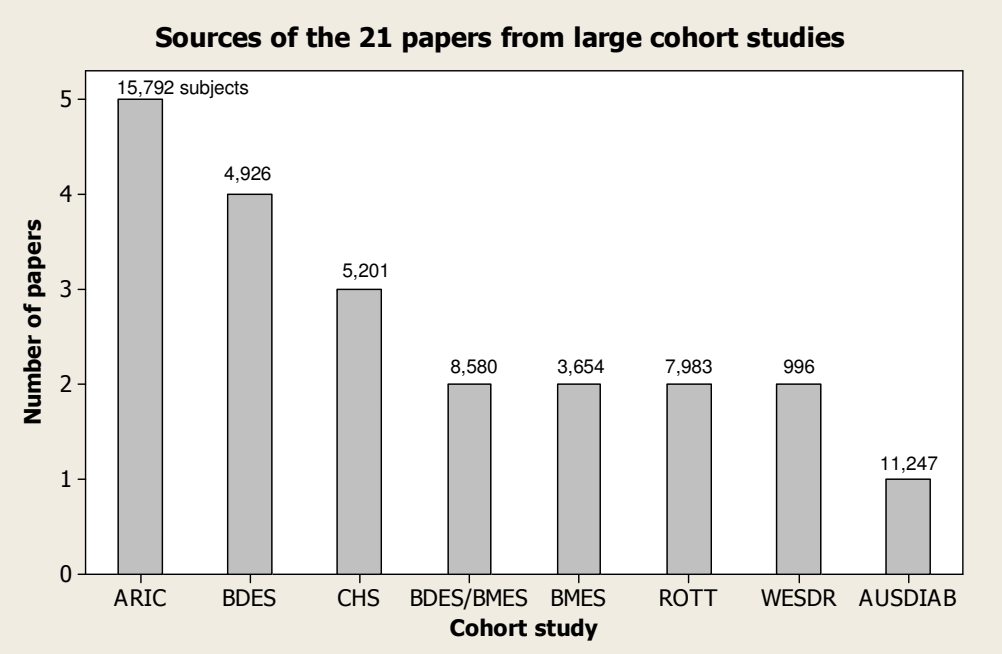
Of the 37 papers, 21 originated from large population-based cohort studies (table 3.2 and fig 3.1) (Cheung *et al.* 2007c; Cooper *et al.* 2006; Cugati *et al.* 2007; Hirai *et al.* 2007; Ikram *et al.* 2006a; Ikram *et al.* 2006b; Klein *et al.* 2004; Klein *et al.* 2003; Klein *et al.* 1999b; Klein *et al.* 1999a; Klein *et al.* 2000; Longstreth, Jr. *et al.* 2007; Mitchell *et al.* 1996; Mitchell *et al.* 2005; Wang *et al.* 2006; Witt *et al.* 2006; Wong *et al.* 2003; Wong *et al.* 2005a; Wong *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2002a). **Of the remaining 16 papers, 14 originated from hospital based studies** (Abu El-Asrar *et al.* 2001; Bruno *et al.* 1995; Hayreh *et al.* 2001; Howard and Russell 1987; Ikeda *et al.* 1994; Inoue *et al.* 1996; Kim *et al.* 2002; Kobayashi *et al.* 1997; Kwa *et al.* 2002; Kwon *et al.* 2007; Luijckx *et al.* 1998; Petitti and Bhatt 1995; Tsaloumas *et al.* 2000; Ueda *et al.* 2002), **one from within a trial of blood pressure reduction** (Cohen *et al.* 2003) **and one from a World Health Organization (WHO) study of follow up in diabetic patients** (Fuller *et al.* 2001). **The papers were from the US, UK, Australia, Japan, Korea, the Netherlands, Saudi Arabia and Greece, Switzerland, Poland, Germany, Croatia, Hong Kong and Cuba.**

Table 3.2 *Background information of population based studies contributing to the review*

Study	Acronym	Size	Age (yrs)	Description
Atherosclerosis Risk in Communities Study	ARIC	15,792	45-64	Longitudinal US population based study of cardiovascular disease
Beaver Dam Eye Study	BDES	4.926	43-84	Longitudinal US population based study of eye disease and medical health
Cardiovascular Health Study	CHS	5,201	>65	Longitudinal US population based study of coronary heart disease and stroke
Rotterdam Study	RS	7,983	>55	Longitudinal Dutch population based study of chronic diseases
Blue Mountains Eye Study	BMES	3,654	>49	Longitudinal Australian population based study of vision , common eye diseases and health outcomes
Wisconsin Epidemiologic Study of Diabetic Retinopathy	WESDR	996	all	Longitudinal US study of patients receiving treatment for type I diabetes
Ausdiab Study	-	11,247	>25	Cross sectional Australian population based survey

The 37 papers include 62,975 subjects. The mean age of the patients in the 31/37 papers which provided this information was 62.5 years (SD 7.7). Amongst the 62,975 subjects, 1,900 had a clinically evident stroke (total of incident and prevalent stroke) and 993 had infarction seen on brain imaging with no stated clinical correlate. We were unable to extract the number of strokes from 6/37 papers as the raw numbers were not given and any odds ratios presented were already corrected for certain confounders (Hirai *et al.* 2007; Kim *et al.* 2002; Klein *et al.* 2000; Kwa *et al.* 2002; Mitchell *et al.* 1996; Ueda *et al.* 2002). **The number of strokes per paper varied from 8-496** (Abu El-Asrar *et al.* 2001; Longstreth, Jr. *et al.* 2007).

Figure 3.2 *Number of papers each cohort study contributed to the review (ARIC – Atherosclerosis Risk in Communities Study, BDES – Beaver Dam Eye Study, CHS – Cardiovascular Health Study, BMES – Blue Mountains Eye Study, ROTT – The Rotterdam Study, WESDR – Wisconsin Epidemiological Study of Diabetic Retinopathy, AUSDIAB –The Australian Diabetes, Obesity and Lifestyle Study - BDES/BMES relates to papers which combined these study populations.) The numbers on top of each column represent the number of subjects in each cohort study.*



3.4.2 Ascertainment of stroke

Twenty one papers examined incident stroke (Bruno *et al.* 1995; Cheung *et al.* 2007c; Cohen *et al.* 2003; Cugati *et al.* 2007; Fuller *et al.* 2001; Hirai *et al.* 2007; Howard and Russell 1987; Ikram *et al.* 2006a; Ikram *et al.* 2006b; Kim *et al.* 2002; Klein *et al.* 2004; Klein *et al.* 2003; Klein *et al.* 1999b; Klein *et al.* 1999a; Mitchell *et al.* 2005; Tsaloumas *et al.* 2000; Wang *et al.* 2006; Witt *et al.* 2006; Wong *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2002a) (total population 34,894; 1442 (4.1%) strokes during follow up). **Fifteen papers examined prevalent stroke** (Abu El-Asrar *et al.* 2001; Cooper *et al.* 2006; Hayreh *et al.* 2001; Ikeda *et al.* 1994; Inoue *et al.* 1996; Klein *et al.* 2000; Kobayashi *et al.* 1997; Kwa *et al.* 2002;

Kwon *et al.* 2007; Luijckx *et al.* 1998; Mitchell *et al.* 1996; Petitti and Bhatt 1995; Ueda *et al.* 2002; Wong *et al.* 2003; Wong *et al.* 2005a) **(total population 21,950; 501 (2.2%) had prior stroke) and one paper examined both prevalent and incident stroke** (Longstreth, Jr. *et al.* 2007).

The definition of stroke varied between the papers. 30/37 papers defined "stroke" in the index paper or related publications (Abu El-Asrar *et al.* 2001; Bruno *et al.* 1995; Cheung *et al.* 2007c; Cooper *et al.* 2006; Cugati *et al.* 2007; Hayreh *et al.* 2001; Hirai *et al.* 2007; Ikram *et al.* 2006a; Ikram *et al.* 2006b; Inoue *et al.* 1996; Kim *et al.* 2002; Klein *et al.* 2004; Klein *et al.* 2003; Klein *et al.* 1999b; Klein *et al.* 1999a; Klein *et al.* 2000; Kobayashi *et al.* 1997; Kwa *et al.* 2002; Kwon *et al.* 2007; Longstreth, Jr. *et al.* 2007; Luijckx *et al.* 1998; Mitchell *et al.* 2005; Petitti and Bhatt 1995; Ueda *et al.* 2002; Wang *et al.* 2006; Witt *et al.* 2006; Wong *et al.* 2003; Wong *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2002a). **Nine of the 37 papers used asymptomatic infarcts seen on brain imaging** (Cooper *et al.* 2006; Ikeda *et al.* 1994; Ikram *et al.* 2006a; Ikram *et al.* 2006b; Inoue *et al.* 1996; Kobayashi *et al.* 1997; Kwa *et al.* 2002; Kwon *et al.* 2007; Longstreth, Jr. *et al.* 2007; Ueda *et al.* 2002). **The other 28 papers assessed some measure of clinical stroke of which only 9/28 papers sub-typed stroke into haemorrhagic or ischaemic classifications** (Abu El-Asrar *et al.* 2001; Bruno *et al.* 1995; Cheung *et al.* 2007c; Ikram *et al.* 2006a; Klein *et al.* 2000; Luijckx *et al.* 1998; Petitti and Bhatt 1995; Wong *et al.* 2001; Wong *et al.* 2002a). **Of these 9 papers, 6 further sub-typed ischaemic strokes** (Bruno *et al.* 1995; Cheung *et al.* 2007c; Klein *et al.* 2000; Luijckx *et al.* 1998; Wong *et al.* 2001; Wong *et al.* 2002a) **but only one paper reported retinal microvascular abnormality differences between the ischaemic stroke sub-groups** (Luijckx *et al.* 1998).

The methods of stroke identification varied widely. Two papers used the reference standard of specialist examination at the time of stroke (Bruno *et al.* 1995; Luijckx *et al.* 1998); **twelve papers used clinical assessment but not necessarily at the time of the stroke by study investigators** (Abu El-Asrar *et al.* 2001; Cohen *et al.* 2003; Fuller *et al.* 2001; Hayreh *et al.* 2001; Kim *et al.* 2002;

Klein *et al.* 2004; Klein *et al.* 2000; Mitchell *et al.* 1996; Mitchell *et al.* 2005; Petitti and Bhatt 1995; Wong *et al.* 2003; Wong *et al.* 2005a); **fourteen papers used phone interviews, review of case notes or death certificates** (Cheung *et al.* 2007c; Cugati *et al.* 2007; Hirai *et al.* 2007; Howard and Russell 1987; Ikram *et al.* 2006a; Klein *et al.* 2003; Klein *et al.* 1999b; Klein *et al.* 1999a; Tsaloumas *et al.* 2000; Wang *et al.* 2006; Witt *et al.* 2006; Wong *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2002a); **and 9 papers used brain imaging without clinical correlation** (Cooper *et al.* 2006; Ikeda *et al.* 1994; Ikram *et al.* 2006b; Inoue *et al.* 1996; Kobayashi *et al.* 1997; Kwa *et al.* 2002; Kwon *et al.* 2007; Longstreth, Jr. *et al.* 2007; Ueda *et al.* 2002).

3.4.3 Ascertainment of retinal microvascular abnormalities

In general the retinal assessment techniques were well described and performed. 27/37 performed retinal photography (Bruno *et al.* 1995; Cheung *et al.* 2007c; Cohen *et al.* 2003; Cooper *et al.* 2006; Cugati *et al.* 2007; Hirai *et al.* 2007; Ikram *et al.* 2006a; Ikram *et al.* 2006b; Klein *et al.* 2004; Klein *et al.* 2003; Klein *et al.* 1999b; Klein *et al.* 1999a; Klein *et al.* 2000; Kobayashi *et al.* 1997; Kwa *et al.* 2002; Kwon *et al.* 2007; Longstreth, Jr. *et al.* 2007; Luijckx *et al.* 1998; Mitchell *et al.* 1996; Mitchell *et al.* 2005; Wang *et al.* 2006; Witt *et al.* 2006; Wong *et al.* 2003; Wong *et al.* 2005a; Wong *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2002a), **6 direct ophthalmoscopy** (Abu El-Asrar *et al.* 2001; Fuller *et al.* 2001; Hayreh *et al.* 2001; Howard and Russell 1987; Ikeda *et al.* 1994; Tsaloumas *et al.* 2000), 2 used hospital records (Kim *et al.* 2002; Petitti and Bhatt 1995), **and 2 did not record method of retinal assessment** (Inoue *et al.* 1996; Ueda *et al.* 2002). **22/37 papers used retinal abnormality classifications that had been validated either internally or externally** (Cheung *et al.* 2007c; Cooper *et al.* 2006; Cugati *et al.* 2007; Hirai *et al.* 2007; Ikram *et al.* 2006a; Ikram *et al.* 2006b; Klein *et al.* 2004; Klein *et al.* 2003; Klein *et al.* 1999b; Klein *et al.* 1999a; Klein *et al.* 2000; Kwon *et al.* 2007; Longstreth, Jr. *et al.* 2007; Mitchell *et al.* 1996; Mitchell *et al.* 2005; Wang *et al.* 2006; Witt *et al.* 2006; Wong *et al.* 2003; Wong *et al.* 2005a;

Wong *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2002a). **Of the 27 papers which used retinal photography the number of fields used and the angle of the field of view varied: 4 papers gave no information regarding which retinal fields were taken** (Bruno *et al.* 1995; Kobayashi *et al.* 1997; Kwon *et al.* 2007; Luijckx *et al.* 1998), **15 took one field centred on the optic disc and macula** (Cooper *et al.* 2006; Cugati *et al.* 2007; Hirai *et al.* 2007; Ikram *et al.* 2006a; Ikram *et al.* 2006b; Klein *et al.* 2003; Klein *et al.* 1999a; Klein *et al.* 2000; Kwa *et al.* 2002; Longstreth, Jr. *et al.* 2007; Witt *et al.* 2006; Wong *et al.* 2003; Wong *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2002a) **and 8 took more than one field** (Cheung *et al.* 2007c; Cohen *et al.* 2003; Klein *et al.* 2004; Klein *et al.* 1999b; Mitchell *et al.* 1996; Mitchell *et al.* 2005; Wang *et al.* 2006; Wong *et al.* 2005a). **8/27 papers gave no information regarding the angle of the field of view used** (Bruno *et al.* 1995; Cohen *et al.* 2003; Klein *et al.* 2004; Klein *et al.* 1999b; Kobayashi *et al.* 1997; Kwon *et al.* 2007; Luijckx *et al.* 1998; Wong *et al.* 2005a), **2 papers used 20° cameras** (Ikram *et al.* 2006a; Ikram *et al.* 2006b), **8 papers used 30° cameras** (Cugati *et al.* 2007; Hirai *et al.* 2007; Klein *et al.* 2003; Klein *et al.* 1999a; Mitchell *et al.* 1996; Mitchell *et al.* 2005; Wang *et al.* 2006; Witt *et al.* 2006), **8 papers used 45° cameras** (Cheung *et al.* 2007c; Cooper *et al.* 2006; Klein *et al.* 2000; Longstreth, Jr. *et al.* 2007; Wong *et al.* 2003; Wong *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2002a) **and one paper used a 50° camera** (Kwa *et al.* 2002).

The retinal features that were assessed varied greatly, the most common being retinopathy (19/37 papers) defined as any of the following – microaneurysms, haemorrhages, or exudates (Abu El-Asrar *et al.* 2001; Cheung *et al.* 2007c; Cohen *et al.* 2003; Cooper *et al.* 2006; Fuller *et al.* 2001; Hirai *et al.* 2007; Ikeda *et al.* 1994; Inoue *et al.* 1996; Kim *et al.* 2002; Klein *et al.* 2004; Klein *et al.* 1999b; Kwon *et al.* 2007; Longstreth, Jr. *et al.* 2007; Mitchell *et al.* 2005; Petitti and Bhatt 1995; Wong *et al.* 2003; Wong *et al.* 2005a; Wong *et al.* 2001; Wong *et al.* 2002a). **Two papers from the same population based study divided retinopathy into its constituent parts and presented separate associations for these constituent parts** (Cooper *et al.* 2006; Longstreth, Jr. *et al.* 2007). **Other papers used retinal artery occlusion/emboli (6/37 papers)** (Bruno *et al.* 1995; Howard

and Russell 1987; Klein *et al.* 2003; Klein *et al.* 1999a; Ueda *et al.* 2002; Wang *et al.* 2006), **retinal vein occlusion (5/37 papers)** (Cugati *et al.* 2007; Hayreh *et al.* 2001; Mitchell *et al.* 1996; Tsaloumas *et al.* 2000; Ueda *et al.* 2002), **vessel calibre of either the venules or arterioles, or generalized or focal narrowing (9/37 papers)** (Cooper *et al.* 2006; Ikram *et al.* 2006a; Ikram *et al.* 2006b; Klein *et al.* 2000; Longstreth, Jr. *et al.* 2007; Mitchell *et al.* 2005; Witt *et al.* 2006; Wong *et al.* 2001; Wong *et al.* 2006), **arteriovenular nicking** (5/37 papers) (Cooper *et al.* 2006; Klein *et al.* 2000; Longstreth, Jr. *et al.* 2007; Mitchell *et al.* 2005; Wong *et al.* 2001), **retinal geometry (2/37 papers)** (Kwa *et al.* 2002; Witt *et al.* 2006) **or retinal artery sclerosis (3/37 papers)** (Kobayashi *et al.* 1997; Kwa *et al.* 2002; Luijckx *et al.* 1998).

3.4.4 Association between retinal microvascular abnormalities and stroke

We were able to extract data and combine risk ratios for 24 papers (total study population 39,376, 1696 stroke (figs 3.2 and 3.3).

3.4.5 Retinopathy and stroke

Retinopathy was associated with incident stroke; amongst 8 papers providing data (total population 25,354; 1,019 strokes) (Cheung *et al.* 2007c; Fuller *et al.* 2001; Hirai *et al.* 2007; Klein *et al.* 2004; Klein *et al.* 1999b; Mitchell *et al.* 2005; Wong *et al.* 2001; Wong *et al.* 2002a), **the summary Risk Ratio (sRR) for incident stroke in the presence versus absence of retinopathy was 2.1 (95% CI 1.7-2.6; fig. 3.2) with no significant heterogeneity between papers (chi square p=0.46).**

Retinopathy was associated with prevalent stroke; amongst 7 papers providing data (total population 8,083; 979 strokes) (Abu El-Asrar *et al.* 2001; Cooper *et al.*

2006; Kwon *et al.* 2007; Longstreth, Jr. *et al.* 2007; Petitti and Bhatt 1995; Wong *et al.* 2003; Wong *et al.* 2005a), **the sRR for prevalent stroke in the presence versus absence of retinopathy, was 2.45 (95% CI 1.4-4.3; fig. 3.3) although with significant heterogeneity between papers (chi squared p=0.003).**

3.4.6 Arteriolar and venular widths and stroke

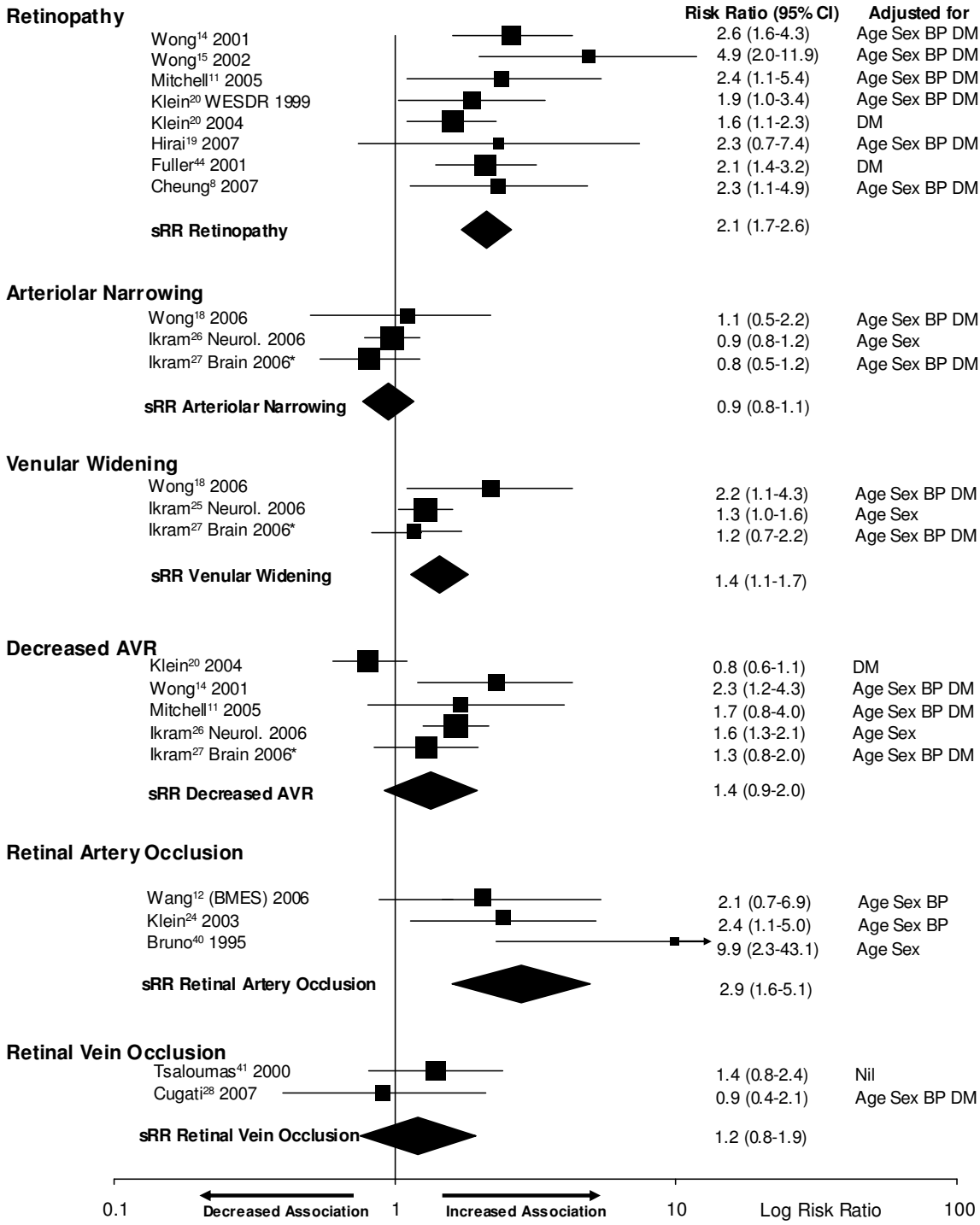
Decreased arteriolar width was not associated with incident stroke; amongst 3 papers providing data (total population 9262; 557 strokes) (Ikram *et al.* 2006a; Ikram *et al.* 2006b; Wong *et al.* 2006), **decreasing arteriolar width was not associated with incident stroke – sRR 0.9 (95% CI 0.8 to 1.1; fig. 3.2) with no significant heterogeneity between papers (chi squared p=0.67)**

Increased venular width was associated with incident stroke; amongst the same three papers providing data for this analysis (total population 9262; 557 strokes) (Ikram *et al.* 2006a; Ikram *et al.* 2006b; Wong *et al.* 2006), **venular widening was associated with incident stroke - sRR 1.4 (95% CI 1.1 to 1.7; fig. 3.2) with no significant heterogeneity between papers (chi squared p=0.36). No papers assessed arteriolar or venular widths and prevalent stroke.**

Five papers investigated the association between the arteriovenous ratio and incident stroke (Ikram *et al.* 2006a; Ikram *et al.* 2006b; Klein *et al.* 2004; Mitchell *et al.* 2005; Wong *et al.* 2001) **(total population 21,717;715 strokes) and found that decreased AVR was not associated with incident stroke with a sRR 1.4 (0.9 to 2.0) but with significant clinical heterogeneity between result (p<0.001).**

Three papers investigated the association between decreased AVR and prevalent stroke (Cooper *et al.* 2006; Longstreth, Jr. *et al.* 2007; Wong *et al.* 2003), **(total population 3734; strokes 679) and found that decreased AVR was associated with prevalent stroke with a sRR of 1.2 (1.1 to 1.3).**

***Fig 3.3**. Summary risk ratios (sRR) showing associations of incident stroke and different microvascular abnormalities. The size of the square denotes weight attributed to each paper and the horizontal lines represent 95% CI. The diamond represents sRR with the width representing the 95% CI. Arrowhead denotes that the upper CI is not marked on the chart. Right hand column indicates key adjustments. BP = blood pressure, DM = Diabetes Mellitus. *denotes studies which used only radiological diagnosis of infarct. BMES = Blue Mountains Eye Study.*

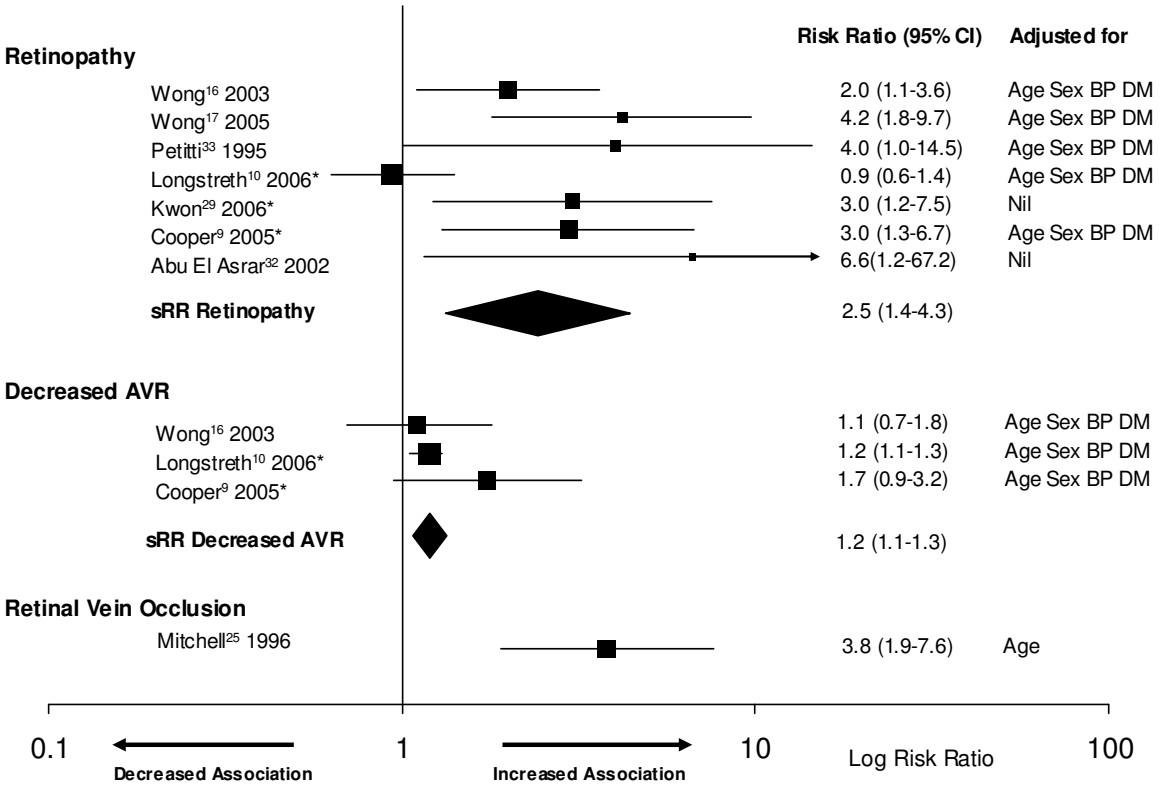


Six papers assessed the relationship between AVR and incident stroke and we could extract data from 5 papers (21,717 subjects, 748 strokes) (Ikram *et al.* 2006a; Ikram *et al.* 2006b; Klein *et al.* 2004; Mitchell *et al.* 2005; Wong *et al.* 2001). **Decreased AVR was not associated with incident stroke (sRR 1.4, 95% CI 0.9-2.0; fig 3.2) although there was significant heterogeneity between these papers (chi squared p=0.002). Three further papers found a significant association between decreased AVR and prevalent stroke (5451 subjects, 812 strokes**) (Cooper *et al.* 2006; Longstreth, Jr. *et al.* 2007; Wong *et al.* 2003) **with sRR 1.2 (95% CI 1.1-1.3; fig. 3.3) with no significant heterogeneity between papers (chi squared p=0.47) but another paper from which we could not extract data reported no association between AVR and prevalent stroke** (Klein *et al.* 2000).

3.4.7 Retinal arteriolar emboli or arteriolar occlusion and stroke

Six papers assessed retinal emboli and incident stroke (Bruno *et al.* 1995; Howard and Russell 1987; Klein *et al.* 2003; Klein *et al.* 1999a; Ueda *et al.* 2002; Wang *et al.* 2006) **from which we could extract data from 4 papers** (Bruno *et al.* 1995; Klein *et al.* 2003; Klein *et al.* 1999a; Wang *et al.* 2006) **however Klein 1999** (Klein *et al.* 1999a) **presents 5 year follow up data from the BDES and Klein 2003** (Klein *et al.* 2003) **presents 10 year follow up from the same patients and thus when combining studies we excluded Klein 1999. Furthermore Wang 2006** (Wang *et al.* 2006) **presented a pooled analysis of data from the BDES and the BMES. As the data from the BDES is included in Klein 2003 we present in this analysis the data presented in Wang 2006 from the BMES (to avoid double counting) therefore a total of 8720 subjects and 266 strokes gave a sRR of 2.9 (95% CI 1.6-5.1; fig. 1) with no significant heterogeneity between papers (chi squared p=0.21). One paper assessed retinal artery occlusion and prevalent stroke but we were unable to extract data from this paper** (Ueda *et al.* 2002).

Fig 3.3 *Summary risk ratios (sRR) showing associations of prevalent stroke and different microvascular abnormalities. The size of the square denotes weight attributed to each paper and the horizontal lines represent 95% CI. The diamond represents sRR with the width representing the 95% CI. Arrowhead denotes that the upper CI is not marked on the chart. Right hand column indicates key adjustments. Risk ratios for Abu El Asrar calculated directly from raw data. BP = blood pressure, DM = Diabetes Mellitus. *denotes studies which used only radiological diagnosis of infarct. BMES = Blue Mountains Eye Study*



3.4.8 Retinal vein occlusion and stroke

Two papers that investigated the association between RVO and incident stroke (9168 subjects; 359 strokes) found no association - sRR 0.9 (95% CI 0.4-2.0; fig. 3.2) (Cugati *et al.* 2007; Tsaloumas *et al.* 2000) **with no significant heterogeneity between papers (chi squared p=0.37). A further two papers studied RVO and prevalent stroke (4744 subjects, 43 strokes) - the one paper (3654 subjects) from which we could extract data found RVO was associated with prevalent strokes (sRR 3.8, 95% CI 1.9-7.6; fig. 3.3)** (Mitchell *et al.* 1996).

3.4.9 Retinal microvascular abnormalities in ischaemic versus haemorrhagic stroke

No papers directly compared and reported differences between retinal microvascular abnormalities in haemorrhagic versus ischaemic stroke.

3.4.10 Retinal microvascular abnormalities and large artery versus small artery stroke

Only one pilot study (Luijckx *et al.* 1998) **directly compared retinal microvascular abnormalities in ischaemic stroke subtypes. The diagnosis of stroke and sub-typing was based on clinical and radiological features at the time of the stroke. This small study (59 patients in total) found that there were similarly high levels of mild retinal arteriosclerosis (defined as altered central arteriolar light reflex) in lacunar stroke (prevalence 92%) and cortical stroke (prevalence 80%).**

3.4.11 Retinal microvascular abnormalities and transient ischaemic attacks

No papers solely examined retinal disease and TIA. Two papers that included TIAs in their stroke outcomes did not present separate data for TIA (Abu El-Asrar *et al.* 2001; Mitchell *et al.* 2005).

3.5 Discussion

This review has demonstrated an association between retinal microvascular abnormalities and any stroke despite large variations in study design, stroke outcomes used and a lack of detail in diagnosing both the presence and subtype of stroke. It has shown that the presence of retinopathy is associated with incident stroke and prevalent stroke. Furthermore, incident stroke was associated with retinal artery embolism, venular widening and decreased AVR but not with arteriolar narrowing. There were no data on arteriolar narrowing or venular widening and prevalent stroke and decreased AVR was not associated with prevalent stroke. Thus there were some inconsistencies in results, which combined with the heterogeneity between studies for some analyses, means that the data on different types of retinal microvascular abnormality and stroke should be viewed cautiously. No published studies have adequately compared retinal microvascular abnormalities in haemorrhagic versus ischaemic stroke or between different ischaemic stroke subtypes therefore we are unable to answer with this systematic review whether retinal microvascular abnormalities may shed light on the pathophysiology of small vessel disease.

Others have reviewed the associations between retinal microvascular abnormalities and cerebrovascular disease (Baker *et al.* 2008; Sharrett 2007; Wong and McIntosh 2005a) but these have been largely narrative reviews based upon results of large epidemiological studies. They did not employ a systematic search strategy or

combine result in meta-analysis. This is the first systematic review and meta-analysis of retinal changes in stroke.

Population–based studies contributed the majority of papers and therefore the total number of subjects was large (62,975 subjects) but in healthy and relatively young populations (mean age 62.5 years, younger than the mean age of stroke onset in the UK of 72 years) stroke is rare making it difficult to show differences between exposure groups. The rareness of strokes is reflected in wide 95% CI (Wong *et al.* 2002a), the heterogeneity between studies for some associations and the occasional need to combine cohorts to demonstrate associations in some primary publications (Wang *et al.* 2006) indicating that the data on specific retinal features and stroke should be viewed with caution. Although the general association between any retinal microvascular abnormalities and stroke may be robust, the details of the associations between different retinal microvascular features and stroke are inconsistent and may be less reliable. Case control studies can be used when the outcome of interest is rare but studies in this review used the general population as controls (Hayreh *et al.* 2001; Howard and Russell 1987; Tsaloumas *et al.* 2000) or MRI scans of patients who self presented to hospital for general health checks (Kobayashi *et al.* 1997). We were unable to perform any sub analysis by age group but it is likely that older populations and populations where all patients have had a stroke will have more retinal abnormalities thereby increasing the chance of finding associations between retinal changes and stroke subtypes.

A significant limitation of the literature is that each population base study has contributed several papers to this review where data from individual patients is published more than once. This can lead to duplication of publication and it can be difficult from the information presented to differentiate between patient cohorts within studies. Although we tried to minimize this we cannot therefore exclude the possibility that data may have been double counted. We did perform a sensitivity analysis using only one paper from each study and found that the size or direction of any of the results presented in Figs 2.2 and 2.3 did not change.

The overall results of the review are promising in that for most analyses, the heterogeneity between studies is low. However there are inconsistencies. For example, it is odd that decreased AVR should be associated with prevalent stroke but not incident stroke. This may be due to the significant heterogeneity in the results from the incident stroke papers (perhaps resulting from Klein 2004 (Klein *et al.* 2004) using patients with Type I diabetes as their cohort who were younger than the patients in the other studies) possibly causing a type II statistical error for the association between decreased AVR and incident stroke or alternatively recall bias affecting the detection of prevalent stroke. Although there were a similar number of strokes, there were many more subjects in the analysis of incident stroke than of prevalent stroke and AVR. Similarly there is significant heterogeneity between the studies for retinopathy and prevalent stroke but not retinopathy and incident stroke – this may reflect the wide variety of methods used to identify and diagnose prevalent stroke as the number of strokes in the two analyses were similar. Some heterogeneity in the association between retinopathy and prevalent stroke may have resulted from Longstreth (Longstreth, Jr. *et al.* 2007) investigating associations with MRI defined infarcts in the Cardiovascular Health Study which involved an older age group (>65years) than the others in the subgroup investigating retinopathy and prevalent stroke. Although we have not demonstrated much heterogeneity it should be noted that with small numbers of outcomes the power to detect heterogeneity is low and our calculations may have missed a small degree of heterogeneity.

Stroke is difficult to diagnose. Ideally the patient should be assessed by a suitably trained stroke expert at the time of the stroke and the diagnosis made with clinical features and appropriately timed brain imaging. Many of the incident stroke studies were population based and it is almost impossible for study investigators to assess every possible stroke. Accordingly, the diagnosis of stroke was made at either regular review when patients were asked if they had had a stroke in the preceding follow-up period and medical records reviewed, or patients were asked to report possible strokes to the investigators. Alternatively discharge summaries or even death certificates were used to diagnose stroke. Although these techniques allow for the assessment of large numbers of subjects they come at the price of reduced

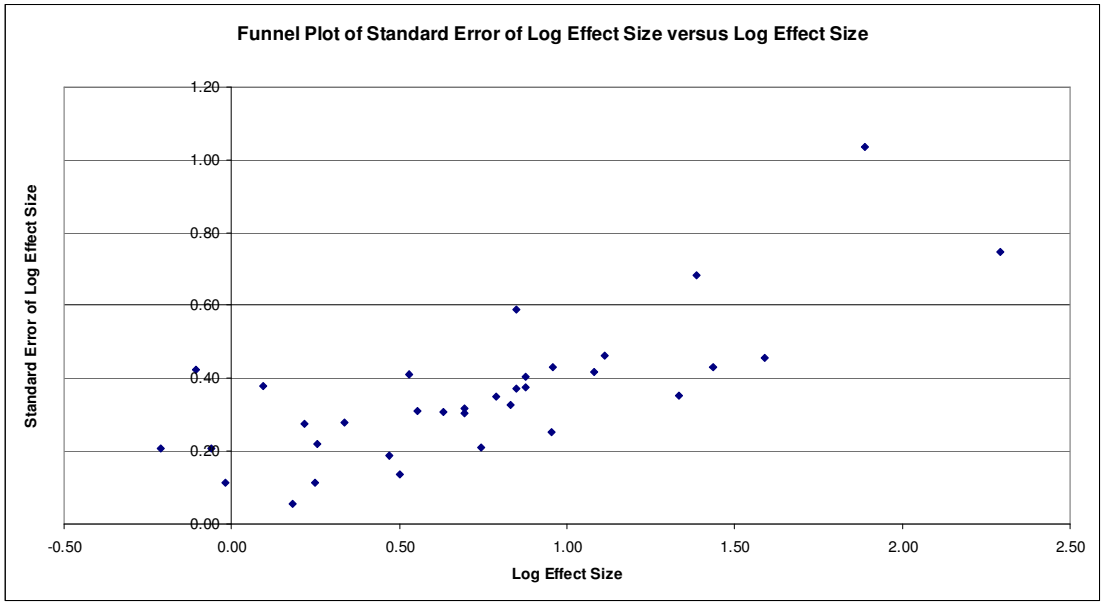
accuracy (Leibson *et al.* 1994; Leppala *et al.* 1999; Piriyawat *et al.* 2002) as discharge summaries and death certificates are often completed by inexperienced junior medical staff. To ascertain whether a patient had a history of stroke, many studies asked the patient and searched medical records. Although prone to recall bias, this approach is deemed acceptable for assessing prevalent stroke epidemiologically, but may be inadequate for detailed studies of pathophysiology. We have grouped together studies investigating certain retinal features and stroke outcomes but there were varying methods of both retinal assessment and more pertinently stroke assessment. We have tried to minimize this variation but it is a limitation of the results of this review that this variation exists.

For this review we used a clearly specified wide definition of stroke to encompass the range of definitions used in the papers. Whilst the vast majority of papers did not use the reference standard for stroke diagnosis (indeed very few did) we considered that there was sufficient clinical homogeneity to permit grouping of the outcome of "stroke" and clearly discuss the limitation that this imposes on our conclusions. In fact, we highlight the heterogeneity as well as the lack of clarity and reliability of the diagnosis of "stroke" used in these papers as one of the main problems affecting this area of research and a major area for methodological improvement in future studies. If we had excluded those which did not conform to the gold standard there would have been virtually no data, and the exercise would have been unhelpful in flagging the fact that better characterisation of the cerebral disorder is needed to match the very detailed classification and characterisation of the retinal one. We note the heterogeneity present in these studies and urge caution in the over-interpretation of the pooled estimates.

In order to assess for small study bias we plotted a Funnel plot (Fig 3.4) of the logarithm of the effect size and standard error of the effect size and found that there was a paucity of small negative studies. Furthermore the larger studies tended to have smaller effect sizes detailing the association between retinal microvascular abnormalities and stroke. One of the possible causes of this small study bias is

publication bias and it is likely that publication bias has affected the results of this review.

Figure 3.4 Funnel plot of standard error of log effect size versus log effect size showing small study bias and a paucity of negative small studies.



Stroke is a heterogeneous disorder. The two main pathophysiological divisions are haemorrhagic and ischaemic stroke and ischaemic stroke is further sub-typed into lacunar (or small vessel disease) and cortical (large artery atheroma and cardiac embolism subtypes). Furthermore, whilst stroke subtypes share many vascular risk factors, their underlying pathophysiology vary reflecting different disease processes. Any study aiming to understand the pathophysiology of stroke, or even to assess novel risk factors for stroke, should sub divide stroke into ischaemic and haemorrhage and preferably further subtype ischaemic stroke. This requires careful clinical assessment and appropriate brain imaging. In the ARIC study, strokes were sub-typed according to their likely cause based on clinical and imaging criteria where available – haemorrhagic or ischaemic with ischaemic strokes further sub-typed into the important subdivisions of thrombotic or embolic stroke but not lacunar versus

cortical. However, due to the small number of strokes (and especially haemorrhagic strokes) (Wong *et al.* 2001; Wong *et al.* 2002a) direct comparisons between stroke subtypes were not reported. The Rotterdam Study investigated cerebral infarction (but brain imaging was only available for 68% of patients(Ikram *et al.* 2006a)). Other papers sub-typed stroke but did not compare retinal microvascular abnormalities between the different stroke subtypes but only between any stroke and non-stroke controls (Bruno *et al.* 1995; Petitti and Bhatt 1995). One study that did compare retinal microvascular abnormalities in lacunar and cortical stroke directly and had adequate diagnosis and sub-typing (Luijckx *et al.* 1998), was underpowered (n=60), and used subjective measures of retinal arteriolar sclerosis (altered central light reflex).

MRI used appropriately is a powerful tool for diagnosing and sub-typing stroke but in the absence of clinical data about neurological deficits, the abnormal areas seen on imaging are difficult to interpret, especially as they are common in older populations (Vernooij *et al.* 2007). Lacunar infarcts can have considerable overlap with white matter lesions. We excluded studies dealing solely with white matter lesions but some of the included studies may have misclassified white matter lesions as lacunes and vice versa thus introducing a small bias. Without detailed diffusion weighted MR imaging, or careful CT assessment at the time of any symptoms, it is not clear which, if any, lacunes may have represented previous clinically evident stroke. As such, stroke remains a clinical diagnosis and associations between retinal features and lacunes seen on MRI may be less reliable in aiding to understand the pathophysiology of stroke subtypes.

A few earlier studies used direct ophthalmoscopy to record retinal microvascular abnormalities. Apart from this, the quality of retinal assessment used in most papers was high. Almost all of the later studies and certainly all of the large population-based cohort studies used retinal photography with grading by trained analysts using internally and externally validated techniques blinded to other clinical information.

The strength of the associations between retinal microvascular abnormalities and stroke is encouraging, especially given the consistency between studies. This suggests that retinal examination offers an excellent way to study non-invasively the effects of common vascular risk factors on small vessels and possibly for gaining better understanding of the pathophysiological processes involved in cerebral small vessel disease. The lack of studies which directly compare retinal microvascular abnormalities and stroke ischaemic subtypes is therefore disappointing and further studies carefully characterising stroke subtype with comprehensive assessment at the time of the stroke are required if retinal microvascular abnormalities are to shed light on the pathophysiology of lacunar stroke. The paucity of strokes, lack of detailed stroke assessment and heterogeneity between studies for some associations mean that the data on individual retinal features and stroke should be viewed with caution and underpins the need for more large, stroke-focussed, robust studies.

The results from this review highlighting the lack of knowledge about retinal appearances and ischaemic stroke subtypes provide justification for the Edinburgh Mild Stroke Study which, amongst other factors, investigated retinal microvascular abnormalities in ischaemic stroke subtype. Details of this study are presented subsequently.

Chapter 4. Baseline demographics and MRI based results from the Mild Stroke Study.

4.1 Aims of chapter

This chapter details the baseline characteristics of the study population.

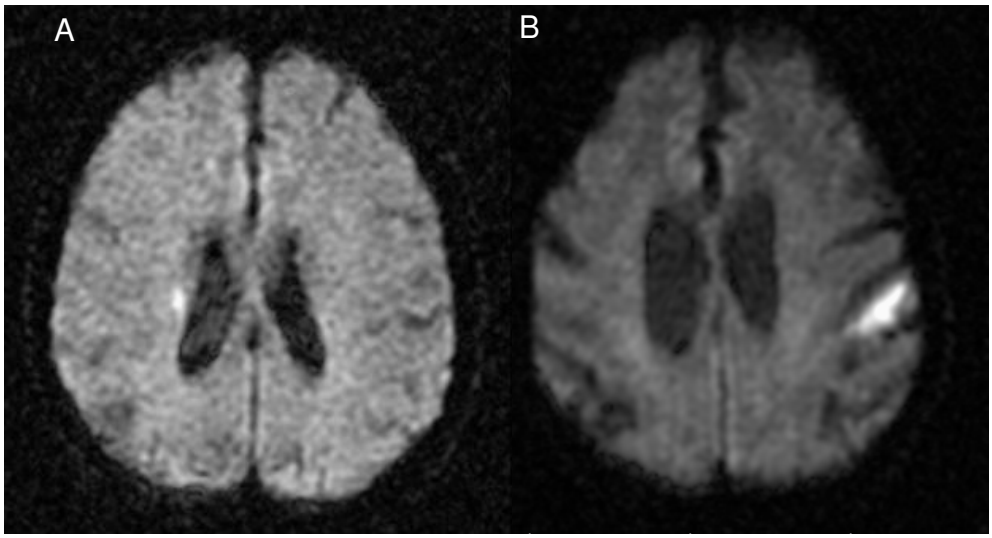
4.2 Introduction

4.1.1 Diagnosis of stroke and rate of negative MRI

Accurate diagnosis and sub-typing of ischaemic stroke is essential to investigate the pathophysiology of stroke sub-types. There is however no gold standard method for diagnosing stroke. The World Health Organisation (WHO) defines stroke as "rapidly developing clinical signs of focal (at times global) disturbance of cerebral function, lasting more than 24 hours or leading to death with no apparent cause other than that of vascular origin" (Aho *et al.* 1980). In the Mild Stroke Study we used the reference standard of stroke as a stroke diagnosis based upon the expert opinion of an experienced stroke physician with a MRI scan including DWI sequences at the time of presentation to hospital with subsequent confirmation by panel discussion in possession of all available data (the panel consisted of at least a stroke physician, a vascular neurologist and a neuroradiologist). We felt that in the absence of a gold standard this was the most robust definition available. We did not however test the reliability of this consensus meeting in diagnosing stroke and it is conceivable that as the personnel in the panel were not fixed there may have been some inter meeting variation in stroke diagnosis. The diagnosis of ischaemic stroke was confirmed by either a MRI scan showing an acute stroke ischaemic lesion or a scan not showing an alternative explanation for the presenting symptoms eg haemorrhage, brain tumour or demyelination.

DWI imaging to identify brain ischaemia is a relatively recent development (Moseley *et al.* 1990) but it is widely used in the acute setting and by some centres as part of a MR sequence with perfusion scanning to identify tissue at risk of infarction and to guide thrombolysis (Albers 2001). MRI (including DWI) is recommended in UK guidelines as the brain imaging of choice in mild stroke (Department of Health *et al.* 2007; Scottish Intercollegiate Guidelines Network 2008). MRI involving DWI is more sensitive and specific at diagnosing acute ischaemic stroke than both conventional MRI (without DWI) and CT (Chalela *et al.* 2007; Fiebach *et al.* 2002; Lansberg *et al.* 2000a; Lansberg *et al.* 2000b; Lutsep *et al.* 1997; Mullins *et al.* 2002; Urbach *et al.* 2000). Abnormal hyperintense DWI signals can persist for at least several months post stroke (Eastwood *et al.* 2003; Geijer *et al.* 2001; Schulz *et al.* 2007) but can produce false negative results both very early and late after stroke onset (Ay *et al.* 1999a; Oppenheim *et al.* 2000). False negative DWI lesions occur especially in patients presenting with minor stroke (Schulz *et al.* 2004; Sylaja *et al.* 2008) but most studies investigating rates of negative DWI and associated features have included both strokes and TIAs in their cohorts (Schulz *et al.* 2004; Sylaja *et al.* 2008) using presence of stroke as an explanatory variable for the presence of a negative scan. We therefore investigated the rate and associated features of negative DWI in patients presenting with mild stroke as one of our preliminary analyses.

Fig. 4.1 DWI showing increased signal right semiovale lacunar infarct (image A) and left hyperintense cortical infarct (image B).



4.2.2 Subtyping of ischaemic stroke

We sub-typed stroke based on radiological and clinical grounds into lacunar or cortical stroke. Where the two differed we sub-typed based on the radiological findings in preference to the clinical symptoms as cortical strokes can be misdiagnosed clinically as lacunar syndromes and vice versa (Mead *et al.* 2000; Seifert *et al.* 2005). Furthermore where there is discrepancy between the classifications, patients tend to behave like, and have similar prognosis to the type of lesion seen on imaging, ie a patient with a cortical syndrome and a lacunar ischaemic stroke lesion will have the prognosis of a lacunar stroke patient. Accurate sub-typing is important both clinically for guiding patient management and indicating prognosis but also within research to ensure that any sub-type specific treatments are patient relevant and to ensure accurate epidemiological trials. It is therefore important to fully understand which features may lead to mismatch between clinical and radiological sub-typing. We investigated in this cohort how many patients had their clinical stroke sub-type altered by imaging and which features might predict this and present these results in this chapter, hypothesising that the location and size of the infarct may lead to clinical-radiological mismatch.

4.2.3 Statistical methods

All statistical analyses were performed in Excel (Microsoft Inc,CA, US) and Minitab (version 14, Minitab Inc, PA, USA). In comparing baseline differences between ischaemic stroke sub-types and where the data were normally distributed we used a 2 sample t test (age), where the data was not normally distributed (eg NIH, time to scan) we compared the two groups with a Mann-whitney U test and finally when the variable had a binary nature we used differences in proportions. Significance levels were set at p=0.05 and where possible we reported both the size and direction of the effect rather than just a p value. We used multivariable binary logistic regression to assess predictors of both negative DWI/MRI and the presence of clinico-radiological mismatch.

4.3 Results

4.3.1 Mild Stroke Study recruitment

The Mild Stroke Study recruited patients presenting to the acute stroke services (which covers the inpatient stroke unit, out patient neurovascular clinics and the acute medical receiving unit) of the Western General Hospital (WGH) in Edinburgh, United Kingdom between April 2005 and January 2008. From internal audit data (not published) the WGH saw approximately 550 patients a year with possible (diagnosis of stroke one of many possible diagnoses but not the most likely), probable (diagnosis of stroke is the most likely amongst a list of possible diagnoses) or definite stroke (almost no uncertainty that stroke is the most likely diagnosis).

We recruited 253 patients. Thirty four patients did not have retinal photographs of whom 20 declined further participation in the study after having had the initial MRI scan, we were unable to contact 10 patients to arrange the retinal photography (despite multiple telephone calls and letters inviting the patients to return) and there were technical difficulties photographing 4 patients. The exact numbers of patients included in each analysis are detailed in the relevant result chapters.

4.3.2 Characteristics of study participants

Of the 253 patients, the mean age was 68.1 years with Standard Deviation (SD) 11.6. There were 165 males (65%) and the median National Institute for Health Stroke Scale (NIHSS) was 2 (interquartile range 2-3). We found that age was normally distributed and therefore used parametric tests to assess associations with age. NIH and time to imaging were not normally distributed and therefore we used non-parametric tests. All other variables were tested with differences in proportions. These baseline characteristics are detailed in table 4.1

***Table 4.1**, Baseline characteristics of the study group. SD – standard deviation, NIHSS – National Institute for Health Stroke Scale, IQR – Inter quartile range, AF – atrial fibrillation, TIA – Transient Ischaemic Attack, IHD – Ischaemic Heart Disease, ACE – angiotensin converting enzyme, ARB – angiotensin receptor blocker,*

Characteristic	Value
n	253
Mean age (SD) years	68.1 (11.6)
Median NIHSS (IQR range)	2 (2-3)
Male (n) (%)	165 (65%)
AF	22 (9%)
Carotid Stenosis >50%	19 (8%)
History TIA	32 (13%)
History Stroke	23 (9%)
History IHD	51 (20%)
History Hypertension	154 (61%)
History Diabetes Mellitus	36 (14%)
History Peripheral Vascular Disease	11 (4%)
Current smoker	77 (30%)
Antiplatelet at time of stroke	132 (52%)
Statin at time of stroke	92 (36%)
ACE Inh/ARB at time of stroke	68 (27%)
Diuretic	68 (27%)
Beta blocker	62 (24%)
Other antihypertensive	48 (19%)
Median alcohol use per week (units)	1 (0-10)
Median time from stroke onset to scan	12 (IQR 5-27) days

4.3.3 Lacunar –Cortical Ischaemic Stroke Baseline Characteristics

Patient characteristics by final ischaemic stroke subtype are detailed in table 4.2.

Table 4.2 *Patient characteristics by stroke sub-type.*

Characteristic	Lacunar Stroke	Cortical Stroke	Difference and significance
n	129	124	
Mean age (SD) years	66.3 (11.6)	70.0 (11.5)	3.6 (0.8, 6.5) **p=0.01**
Median NIHSS (IQR range)	3	2	**P<0.001**
Male (n) (%)	77 (60%)	88 (70%)	10% (-1%, 22%) P=0.06
AF	6 (4.6%)	16 (12.90%)	8% (1, 15) **p=0.02**
Carotid Stenosis >50% (NASCET)	5 (3.88%)	14 (11.29%)	7% (1, 13) **p=0.02**
History TIA	19 (14.96%)	13 (10.48%)	4% (-4, 12) p=0.29
History prev Stroke	9 (6.98%)	14 (11.38%)	4% (-3, 12) p=0.23
History IHD	17 (13.18%)	34 (27.42%)	14% (4, 24) **p=0.004**
History Hypertension	84 (65.32%)	70 (56.59%)	9% (-3, 20) p=0.15
History Diabetes Mellitus	22 (17.05%)	14 (11.29%)	6% (-3, 14) p=0.19
History Peripheral Vascular Disease	5 (3.88%)	6 (4.84%)	1% (-4, 6) p=0.71
Current smoker	50 (39%)	27 (22%)	17% (6, 28) **p=0.003**
Antiplatelet at time of stroke	63 (48.84%)	69 (55.65%)	7% (-5, 19) p=0.28
Statin at time of stroke	45 (34%)	47 (37%)	3% (-8, 15) p=0.69
ACE Inh/ARB at time of stroke	35 (27.13%)	33 (26.61%)	1% (-10, 11) p=0.93
Diuretic	34 (26.32%)	34 (27.42%)	1% (-10, 12) p=0.85
Other antihypertensive	29 (22.48%)	19 (15.32%)	7% (-2, 17) p=0.14
Median alcohol use per week (units)	1	1	P=0.77
Beta Blocker at time of stroke	20 (15.50%)	42 (33.87%)	18% (8, 29) **p=0.001**

The cortical patients were significantly older with a lower NIHSS score and rate of currently smoking, higher rates of AF, carotid stenosis >50% NASCET, Ischaemic Heart Disease, and Beta blocker use than lacunar patients. Of the other features, although there were baseline differences, none of these reached conventional levels of significance (p=0.05).

4.3.4 Rates of positive and negative DWI/FLAIR/T2

DWI showed an acute relevant stroke lesion in 165/253 (65%) patients leaving 88 (35%) patients with no relevant hyperintense DWI lesion. For the total study population the median time between onset of symptoms and scan was 12 days (IQR 5-27). Table 4.3 shows the characteristics of the two groups. With univariable regression the group with positive DWI had a higher NIHSS, shorter time between stroke and scan and were more likely to be male. There were small differences that did not reach statistical significance in age, percentage with lacunar stroke, proportion with persisting weakness and proportion with motor weakness between the group with positive DWI and the group with negative DWI.

Table 4.3 *Characteristics of patients with positive and negative DWI scans with univariable analyses.*

Characteristic	Positive DWI	Negative DWI	Difference
N	165	88	
Age	68.8 (11.5)	66.81 (12.0)	2.0 yrs (-1.1, 5.1) p=0.20
Lacunar stroke	49.09	54.55	5.4% (-7.4, 18.3) p=0.41
Median NIHSS	2 (2-3)	2 (1-3)	**P=0.007**
Time to imaging (days)	9.5 (3.5-20.6)	21.5 (10.5-35.0)	9.5 days (5.9, 13.9) **p<0.0001**
Symptoms persist	87.42	80.23	7.1% (-2.7, 17.1) p=0.15
Motor symptoms	88.34	82.95	5.4% (-3.8, 14.7) p=0.26
Male gender	71.52	54.02	17.4% (5.0, 30.0) **p=0.006**

With multivariable binary logistic regression using presence of a DWI lesion as the dependent variable and correcting for all of the explanatory variables in the table above, male sex, increased NIHSS score and decreased time to scan were all independently associated with increased likelihood of a positive DWI lesion. These results are detailed in Table 4.4.

Table 4.4 *Multivariable predictors of positive DWI scans. All OR are corrected for the presence of the other explanatory variable in the table*

Variable	OR	95% CI	P value
Age (per year increase)	1.02	1.00, 1.05	0.071
Male sex	2.2	1.22, 3.96	**0.008**
Lacunar sub-type	0.77	0.43, 1.40	0.400
NIHSS	1.47	1.11, 1.95	**0.007**
Presence of persisting symptoms	1.48	0.71, 3.10	0.300
Presence of motor weakness	1.14	0.49, 2.66	0.764
Time from stroke onset to scan (per day)	0.98	0.97, 0.99	**0.006**

There were 88/253 patients without an acute DWI lesion visible however 21 of these 88 patients had acute lesions present on T2/FLAIR sequences meaning that there were 67/253 patients who had no evidence of a recent stroke lesion on any sequence of MRI. With univariable analysis, having a positive MRI scan (ie DWI/FLAIR or T2) for the acute lesion was associated with increasing age, decreased time between stroke and brain imaging, the presence of persisting symptoms, and male gender (see Table 4.5).

Table 4.5. *Characteristics of patients with positive and negative MRI scans (T2, FLAIR and DWI sequences) and univariable associations.*

Characteristic	**Positive Scan**	**Negative Scan**	**p value for difference**
N	186	67	
Age (per year increase)	69.3 (11.2)	64.64 (12.2)	**p=0.007**
Lacunar stroke n (%)	91 pts	38 pts	p=0.271
Median NIH	2 (2-3)	2 (2-3)	P=0.120
Time to imaging (days)	11 (4-23)	20 (10-32)	**p=0.003**
Symptoms persisting	87.22%	78.46%	p=0.123
Motor symptoms	85.33%	89.55%	p=0.354
Male gender	70.27%	52.24%	**p=0.010**

With multivariable binary logistic regression with presence of a positive scan as the dependent variable and correcting for all the explanatory variables present in table 4.6, increasing age, male sex and increased NIH were all associated independently with the presence of a positive MRI scan. Time to imaging, the presence of a motor weakness and time from stroke onset to imaging were not associated with the presence of a positive scan.

Table 4.6. *Multivariable predictors of positive MRI scans. All OR are corrected for the presence of the other explanatory variable in the table*

Variable	OR	95% CI	p
Age (per year increase)	1.04	1.01, 1.07	**0.002**
Male sex	2.10	1.13, 3.90	**0.018**
Lacunar sub-type	0.80	0.43, 1.51	0.496
NIHSS	1.50	1.11, 2.03	**0.009**
Presence of persisting symptoms	1.64	0.75, 3.57	0.211
Presence of motor weakness	0.44	0.16, 1.23	0.118
Time from stroke onset to scan	0.99	0.98, 1.01	0.241

4.3.5 Sub-typing misclassification of ischaemic stroke subtypes

Of the 253 patients, 126 presented with a clinical diagnosis of lacunar stroke syndrome and 127 with a clinical diagnosis of cortical stroke syndrome. Overall 41/254 (16.1%) patients had their initial clinical classification changed by imaging eg patient presenting with symptoms suggestive of a lacunar stroke with a cortical infarct on imaging and vice versa. Of the 126 with a lacunar stroke syndrome, 20 had their sub-typing changed to cortical ischaemic stroke and of the 127 with a cortical stroke syndrome, 21 had their classification changed to lacunar ischaemic syndrome.

Table 4.7 *Univariable predictors of presence of classification change.*

Variable	**Classification Change Present**	**No Classification Change Present**	**P value for difference**
n	41	212	
Mean Age (yrs)	70.7 (SD 10.3)	67.6 (SD 11.8)	p=0.09
Median NIHSS	3	2	P=0.19*
Median Time to assessment (days)	9	14	P=0.08*
Motor weakness	40 (98%)	177 (84%)	p=0.023**
Lacunar Stroke sub-type	21 (16%)	20 (16%)	p=0.97

* Mann-Whitney U Test

** Fishers exact test

Table 4.8 *Multivariable predictors of the presence of a classification change*

Variable	OR	95% CI	P value
Age (per year increase)	1.03	1.00 to 1.07	0.04
NIHSS	1.19	0.93 to 1.53	0.16
Time to assessment	0.99	0.98 to 1.01	0.52
Motor weakness	6.54	0.84 to 50.97	0.07

With multivariable regression, only increasing age was independently associated with clinical-radiological mismatch. In lacunar strokes we further investigated whether the location of the infarct led to increased clinical –radiological mismatch. There were 129 patients with a final classification of lacunar ischaemic stroke. Of the 129 patients, 92 had acute stroke lesions seen on MRI scan. Of the 92 patients, 72/92 had presented with a clinical lacunar stroke classification but 20/92 had presented with symptoms suggestive of a cortical stroke. The lacunar infarct seen on MRI extended to the cortical margin in 8/20 patients who had presented with a cortical syndrome but had a final diagnosis of lacunar stroke (clinical radiological mismatch) and the lacunar infarct extended to the cortical margin in 10/72 patients who presented with lacunar syndrome and a final classification of lacunar stroke. Patients with a final clinico-radiological classification of lacunar stroke who presented initially with a cortical syndrome were more likely to have lesions extending to the cortical margin than patients with a final diagnosis of lacunar stroke who had presented with a lacunar stroke syndrome (Chi square statistic 6.7, p=0.009) but the small number of outcomes only permits univariable analysis.

In a similar analysis, patients with a final classification of lacunar stroke who presented initially with a cortical classification were more likely to have lesions in the centrum semiovale than patients with a final diagnosis of lacunar stroke who had presented with a lacunar stroke syndrome (Chi square statistic 4.4, p=0.036).

Furthermore, amongst the 92 patients with an acute lacunar infarct on MRI, lesion size was associated with clinical radiological classification mismatch. Patient with clinical radiological mismatch had a mean lesion size of 11.25 mm (SD 3.86mm) and patients with no clinical radiological mismatch had a mean lesion size of 9.1 mm (SD 3.5mm). The difference is 2.1 mm (95% CI 0.2-4.2) p=0.03 (Students t test). Presence of a clinical history of stroke (Mismatch history of stroke prevalence 7.3%, no mismatch history of stroke prevalence 9.4%, difference = 2.1%, (95% CI -6.7% to 11.1%) p=0.63) or the presence of old infarcts on MRI scan (mismatch prevalence of old stroke lesions 34.1%, no mismatch prevalence of old stroke lesions 37.7%, difference = 3.5%, (95% CI -12.3% to 19.5%), p=0.66) were not associated with the presence of mismatch with patients presenting with a final classification of lacunar stroke.

4.4 Discussion

4.4.1 Baseline characteristics and comparison with other cohorts.

The patients in this cohort had a mean age of 68 (SD 11) years, median NIHSS 3 and 65% were male. The mean age of stroke in our cohort is lower than the mean age at stroke onset in the UK which reflects firstly the recruitment of patients with minor stroke and secondly we recruited patients who were able to participate in a research study (that involved MRI scanning and retinal photography) and recruitment bias will have selected a younger more able and willing to participate cohort. The average age for hospital based cohorts investigating risk factors for stroke sub-types however is also lower than the mean age of stroke onset in the UK of 72 years at 66 years (Jackson and Sudlow 2005) which is similar to our cohort.

How do these results compare with other studies? The patients in this study had a considerable number of risk factors for and burden of pre-existing vascular disease and it is useful to compare these rates with other studies from the United Kingdom. OXVASC was a community based study of 91 000 participants cared for by study

general practitioners in Oxfordshire, UK investigating incident major vascular events in any arterial territory (mainly stroke/TIA, coronary and peripheral artery disease) (Rothwell *et al.* 2005). Within the OXVASC population the researchers piloted a scheme for rapid access to a neurovascular clinic for patients requiring out patient assessment and care for possible stroke/TIA (Rothwell *et al.* 2007). The main focus of this study was to assess whether a complex intervention of rapid access and instigation of early secondary prevention would influence outcome but the baseline characteristics of patients presenting with stroke/TIA to the outpatients clinic in this population based study are similar to the Mild Stroke Study with a prevalence of hypertension of 59% (MSS 61%), diabetes mellitus 12% (MSS 14%), peripheral vascular disease 8% (MSS 5%), prior antiplatelet use 46% (MSS 52%) and prior statin use 32% (MSS 36%). The authors do not present mean age or data on IHD combined as we do. Other studies have presented similar proportions of patients with hypertension, diabetes and IHD to the MSS (Sandercock *et al.* 1989). The population based south London Registry has reported vascular risk factors similar to the MSS for patients presenting with first ischaemic stroke (despite representing an ethnically diverse population compared with the MSS where 2 out of 254 patients were of South Asian extraction rather then Caucasian) with prevalences of hypertension 66%, diabetes mellitus 20%, AF 15% and current smoking 32% (Heuschmann *et al.* 2008). Although these studies represent slightly different cohorts it is encouraging that they report similar prevalences of risk factors to the MSS study which increases the generalisability and likely reproducibility of the results from the Mild Stroke Study.

4.4.2 Baseline characteristics by ischaemic stroke subtype

The patients in the cohort were evenly split between lacunar and cortical stroke. The lacunar patients were significantly younger, with a higher NIHSS and with a lower prevalence of Ischaemic Heart Disease, Atrial Fibrillation and symptomatic carotid stenosis>50% NASCET than the patients with cortical stroke. This is consistent with a large artery disease and cardio embolic causes for cortical stroke. The prevalences of hypertension, diabetes, peripheral vascular disease and previous stroke and TIA

did not differ significantly between the two groups. Prior antiplatelet use, prior statin use, prior ACE Inhibitor/Angiotensin II receptor antagonists use, prior diuretic and other antihypertensive use did not differ between the two groups. Beta blocker use in the cortical stroke group was significantly higher than in the lacunar stroke group reflecting the higher prevalence of ischaemic heart disease in the cortical group.

How do these risk factor differences compare with other populations? It is difficult to generalise from other studies of risk factors for ischaemic stroke sub-types as different studies used different classification systems which produced varying results. Some studies used the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria (Adams, Jr. *et al.* 1993) which was developed for use in a multicentre clinical trial (Grau *et al.* 2001; Saposnik *et al.* 2004). This classification assigns stroke sub-types based on presumptive causation from the clinical findings, risk factor status and brain, heart and vessel imaging results of; large artery atherosclerosis, cardio-embolic, lacunar, undetermined aetiology and other determined aetiology (effectively the rarer causes of stroke). The main flaw with this classification system is that it uses risk factor status to assign sub-type classification eg a history of diabetes or hypertension supports the diagnosis of lacunar stroke. Unsurprisingly use of this definition leads to classification bias and these studies report associations between ischaemic stroke sub-types and risk factors that are biased. Other studies have used a classification of ischaemic stroke based upon the clinical features of the stroke syndrome (often the Oxfordshire Community Stroke Project classification (Bamford *et al.* 1991) altered by brain imaging results that refine the clinical classification to define the location of the infarct. These methods are not biased by risk factor status and infer less about the cause of the stroke than the TOAST method. Other studies have used a purely imaging based method of assessing stroke sub-type with no clinical correlates to the infarcts seen on imaging (Moulin *et al.* 2000) but this approach is limited as it is not certain what these "silent" holes/infarcts in the brain represent.

A recent systematic review (Jackson and Sudlow 2005) found 28 studies which investigated risk factor profiles in lacunar versus non lacunar stroke. This review

reports a variation in associations between risk factors and ischaemic stroke sub-type which may be in part be due to the classification system used. The studies which used a risk factor free classification system found that the presence of diabetes was not associated with lacunar stroke sub-type (pooled relative risk 0.95; 95% CI 0.83 to 1.09) and that the presence of hypertension was weakly associated with lacunar stroke sub-type (pooled RR 1.11; 95% CI, 1.04 to 1.19). In both cases the pooled relative risk was higher when stroke was sub-typed with risk factor dependent classification systems. These results are consistent with the results from the MSS in not showing a strong association between diabetes and hypertension and lacunar stroke. In keeping with the results of the MSS this review also reports that atrial fibrillation is more common in non-lacunar stroke than lacunar stroke - RR for association with lacunar stroke 0.51; 95% CI 0.42 to 0.62 and that ipsilateral carotid stenosis is also more common in non-lacunar stroke (RR 0.35; 95% CI 0.03 to 0.25) both consistent with atherothromboembolic causes for cortical stroke.

In our cohort we also found that patients with a lacunar stroke had a higher NIHSS score than patients with cortical stroke. Conventionally, patients with lacunar stroke tend to have milder symptoms and lower NIHSS scores than patients with non-lacunar stroke (Adams, Jr. *et al.* 1999), but we should emphasize that in this study we excluded severe cortical stroke and that the NIHSS (which was originally designed as a research tool to assess differences in intervention groups) may have been influenced by ischaemic stroke classification eg a patient with a lacunar stroke and a weak arm and leg will have a higher NIHSS score than a patient with a cortical stroke and a monoparesis.

The baseline characteristics of this cohort are similar to the characteristics of previously published cohorts.

4.4.3 Rates of negative DWI and associated features

We found that in patients presenting with mild stroke the rate of false negative DWI scans was 35%. Decreased severity of stroke (NIHSS), increased time between

stroke and scan and female gender were associated with the presence of a negative DWI scan (independently of age, presence of persisting symptoms, presence of motor weakness and stroke sub-type). The rate of negative MRI scan (patients with no infarct visible on T2/FLAIR or DWI) was lower at 26% and with multivariable analysis younger age, decreased stroke severity and female gender were associated with a negative MRI scan (independently of time between stroke and scan, presence of persisting symptoms and presence of motor weakness).

This study has several strengths: it is to the best of our knowledge the largest study to investigate the rate and associations of negative DWI solely in minor stroke patients (previous studies included TIA patients), it was a prospective study and all patients had a definite diagnosis of stroke made by an experienced stroke physician with MRI at the time of presentation and the diagnosis of stroke made by a panel of experts. The study was representative of patients who present to the hospital with minor strokes as the clinical deficit is often minimal and therefore there is often a delay between onset of symptoms and the patients seeking clinical attention. We used a dedicated research scanner with standardised sequences and protocols to ensure conformity of scanning and all the scans were coded by an experienced neuroradiologist using a tool based upon a previously validated grading scheme (Wardlaw and Sellar 1994). All patients were carefully characterised and we were able to investigate associations adjusted for key possible confounders.

There are however some weaknesses in the study. Despite aiming to limit it by only including patients where a senior physician felt that there had been a definite stroke and discussing cases at a weekly multidisciplinary meeting to ensure consensus opinion it is inevitable that there will have been a small number of patients in the negative DWI group who did not have a stroke. Other studies have confirmed a diagnosis of stroke in patients with initially negative DWI scans with abnormalities on subsequent follow up scan (Oppenheim *et al.* 2000) but this approach misses patients who never have a relevant lesion. This is a cross sectional study and as such we can only report associations without implying causality. The presence of DWI lesion in longitudinal studies post stroke demonstrates high inter individual

variability (Schulz *et al.* 2007) and therefore we are unable to make any comments regarding how long DWI lesions will remain positive on a patient to patient basis other than to assess the median differences between the positive and negative DWI groups. It has been reported that asymptomatic DWI lesions appear after ischaemic stroke (Kang *et al.* 2003b) and it is possible that the DWI lesions we saw did not correspond to the presenting symptoms but a subsequent event. We sought to minimise this by only reporting a DWI as positive if the lesion was in an arterial territory relevant to the presenting symptoms and using the most recent symptoms that the patients reported to sub-type stroke and estimate time between symptom onset and scan (although most patients only had one episode of symptoms).

The patients for this study were selected according to willingness to take part in a research study and also for suitability for undergoing MRI procedures. This will have inevitably led to selection bias but previous research from this centre has shown that as many as 85% of patients admitted with acute stroke are able and willing to undergo MRI in the acute setting (Hand *et al.* 2005). Patients who decline MRI scans tend to be older with more medical problems but it is reassuring to see that the mean age of our patients (68.1 years) was similar to hospital based estimates of mean age of stroke onset (65-70) (Jackson and Sudlow 2005).

How do our results compare with other studies? No other studies have investigated the rate and associations with false negative DWI scans in patients presenting solely with minor stroke although several have studied TIA and minor stroke together (Schulz *et al.* 2004; Sylaja *et al.* 2008). It is possible to draw simple comparisons between our cohort and certain univariable analyses from these papers but the more useful multivariable analyses were performed for the total study population of patients presenting with TIA and stroke combined. Our rate of negative DWI in patients presenting to hospital with minor stroke was 35%. This is much higher then previous quoted rates of 5.8% in patients scanned within 48 hours of stroke onset where the negative scans were associated with scanning within 24 hours of stroke onset (Oppenheim *et al.* 2000) and 6% when DWI MRI was used in the emergency room setting (Mullins *et al.* 2002) although in a similar emergency room setting the

rate of negative DWI in patients with a final diagnosis of stroke was 17% (Chalela *et al.* 2007).

If, however, we compare our results to other patient cohorts presenting with less severe stroke symptoms and imaged at presentation in the subacute setting the results are remarkably similar. A recent study investigated DWI MRI at presentation in patients presenting with TIA or minor stroke to an outpatients clinic at least 2 weeks after the onset of symptoms (Schulz *et al.* 2003). This study recruited 101 patients of whom 51 had had a minor stroke. The rate of negative DWI was 43% and the median time to scan 21 days (IQR17-28). With univariable analysis they found that there was no effect of time to scanning on delay (although time was dichotomized to 2-4 weeks versus >4 weeks perhaps losing statistical power to detect small differences and the sample size was small at 51) but that persistent symptoms and signs predicted a positive DWI. A further study from the same authors which recruited patients in the same time period as the first study (although it is not clear whether they are different patients) with TIA or minor stroke at presentation to an outpatients study where the median time to scan was 17 days (IQR10-23)(Schulz *et al.* 2004). The study recruited 300 patients of whom 164 were minor strokes. Amongst the minor strokes the rate of negative DWI was 30% and with univariable analysis persistence of symptoms/signs were still present (uncorrected for age, NIHSS score and other vascular risk factors). It is impossible to extract data from the multivariable regression correcting for age, sex and diagnosis. A further study from a different group assessing patients presenting with recent TIA/minor stroke found that all of the patients with negative DWI and stroke had lacunar or brainstem infarcts. Despite stating that 26 patients with stroke had false negative scans the study does not however report how many of the 401 patients had TIA or stroke and without a denominator it is therefore impossible to determine the rate of negative DWI scans in minor stroke (Sylaja *et al.* 2008).

Decreased NIHSS in our study was associated with both negative DWI and MRI scans for relevant ischaemia. This finding is replicated elsewhere (Chalela *et al.* 2007; Schulz *et al.* 2004) – it seems logical that small infarcts are less likely to be

picked up on imaging than larger infarcts. Furthermore, although infarct size has been shown in a small longitudinal study not to predict persistence of DWI lesions (Schulz *et al.* 2007) it is possible that small infarcts may resolve quicker than larger infarcts and then not be seen if there is a delay to imaging.

Increased time to imaging was associated with negative DWI scans although not negative MRI scans. This confirms other findings (Schulz *et al.* 2004) and is likely to reflect the fact that DWI lesions which reflect areas of impaired water diffusion largely secondary to ischaemia, subsequent cell swelling and then death (Gass *et al.* 2004) can resolve after time. Although the DWI may have returned to normal there are often persisting abnormalities seen on T2 and FLAIR weighted images and we have demonstrated that T2/FLAIR identifies more infarcts in patients presenting subacutely with minor stroke.

We found that female gender was associated with both negative DWI and negative MRI scans. Other studies have reported similar rates (Schulz *et al.* 2004) in male and females and the reason for this discrepancy is not clear. Perhaps other studies did not have large enough sample sizes to demonstrate this small effect of perhaps the result was due to residual confounding due to baseline differences in age and vascular risk factors and stroke severity not fully corrected for by our regression model. Despite aiming not to include stroke mimics it is notable that migraine and functional disease may be associated with female gender and this may have biased our sample.

In keeping with some studies (Schulz *et al.* 2004) we did not find an association between lacunar/cortical sub-types and negative DWI although another study stated that all of the patients with stroke and negative DWI had either brainstem or lacunar infarcts (Sylaja *et al.* 2008).

We have shown that in a population presenting with minor stroke there is a high rate of negative DWI which is associated with younger age, decreased stroke severity, increased time to scan and female gender. This is of importance to both clinicians

and researchers in that the results will guide clinicians as to when to expect a negative DWI in a patient with minor stroke and should inform them not to exclude the diagnosis in the presence of a negative scan. We included patients with negative DWI if the panel consensus was such that the diagnosis remained a stroke we felt that this was the most robust method for diagnosing stroke. It is a potential limitation of this thesis that inevitably, and despite our best efforts, some patients may have been included who had not had a stroke. Many studies of stroke only include patients in which there is DWI evidence of ischaemia however this approach will exclude a significant proportion of patients presenting with minor stroke leading to significant selection bias. It is this population (along with TIA patients) without significant residual neurological deficit and high risk of early recurrent stroke (Giles and Rothwell 2007) that research should be concentrating on to identify and treat risk factors for subsequent disabling stroke.

4.4.4 Subtyping of ischaemic stroke

We sub-typed stroke using the OCSP classification refined by the results of appropriate MRI imaging to produce a final classification. This method produces a sub-typing of stroke that conveys both information regarding outcome and likely pathophysiology. As mentioned in the previous section there are other classification schemes largely based on the TOAST criteria but these systems have 2 main flaws; firstly the sub-typing is risk factor dependent leading to classification bias in risk factor analysis and secondly in practice there are a large number of strokes which fall into the undetermined category (estimates range from 8-41% of strokes (Jackson and Sudlow 2005) which will lead to loss of data and heterogeneity between different studies. A significant advantage of using clinical features and brain imaging to assign stroke sub-type is that the large proportion of undetermined stroke is avoided as almost every stroke can be sub-typed. There are however problems associated with this as diagnosing stroke and assigning a clinical sub-type can be difficult (Allder *et al.* 1999) and there is a significant proportion of patients in whom there is a mismatch between the clinical and the radiological sub-type eg a patient presenting

with a lacunar syndrome has a cortical lesion on their scan and vice versa (Mead *et al.* 2000).

We found that 41/254 (16%) of patients had their clinical classification changed by the imaging classification and that with multivariable analysis increasing age was independently associated with the presence of mismatch but that clinical classification, NIHSS, time to assessment and presence of motor weakness were not associated with the presence of mismatch. We further investigated the 129 patients with a final diagnosis of lacunar stroke of whom 21 had a presented with cortical syndromes and found that both increased size of the lacunar infarct on MRI and if it extended to the cortical margin were associated with mismatch.

How does this compare to other studies? There have been no other large (>100 patients) studies that have used MRI to accurately identify the recent infarct in all patients. A large Dutch cohort of patients presenting with either first ever deep (lacunar) or territorial (cortical) acute infarcts was investigated with CT at presentation (Lodder *et al.* 1994). Of 350 patients, 42 (12%) had clinical-radiological mismatch. They further investigated which features may predict the presence of mismatch and found that in patients with deep (lacunar) infarct on scan the patients with mismatch (i.e. who had presented with a syndrome suggestive of a territorial infarct) were more likely to have increased severity of stroke, a cardio-embolic source, concurrent leukoaraoisis and asymptomatic infarcts. In patients with a territorial (cortical) infarct on scan the patients who had mismatch (i.e. who had presented with a syndrome suggestive of a deep infarcts) were more likely to have had a cardio-embolic source for their stroke or to have presented with a pure motor stroke. Other studies have reported that in a cohort of 441 patients with a final diagnosis of cortical stroke and 265 patients with a final diagnosis of lacunar stroke mismatch was present in 13% and that neither side of brain or time from scan to brain imaging (dichotomized to less than or greater than 48 hours) predicted the presence of mismatch (Mead *et al.* 2000).

No other studies have investigate whether the size of the lesion or lesion location (particularly whether the lacune adjoins the cortical margin) predict the presence of mismatch. We found that in patients with final diagnosis of lacunar infarction those patients with mismatch (i.e. who had presented with a cortical syndrome) had larger infarcts than those with no mismatch. This finding does not have a direct correlate in other studies but in a study of patients presenting with subcortical infarcts who were intensively characterised with MRI at presentation and angiography it was found that increased size of the subcortical infarction was associated with an increased chance of finding a non small vessel disease cause for the stroke such as cardio-embolism or large artery disease (Bang *et al.* 2007). It should be noted however that the mean size of subcortical infarct of the small vessel disease group was 15mm (SD 7 mm) and in the cardio-embolism/large artery group of subcortical infarctions was 20 (SD 11mm). In our study with a maximum cut off of 20 mm for lacunar infarction half of this second group would have been classified as cortical infarctions.

The finding that lacunar infarctions extending to the cortical margin are associated with mismatch is novel. Although lacunar infarcts are often small they often cause a neurological deficit disproportionate to their size largely due to the fact they occur in areas of the brain where the neurons are tightly packed. If a lacunar infarct is situated more peripherally then the nerve fibres will be less tightly packed and a similarly sized but more peripherally located infarct may produce a smaller neurological deficit for example affect a single limb which would lead to a cortical syndrome. We did not find an association between time to assessment and misclassification in keeping with previous studies albeit over a shorter time period (Mead *et al.* 2000). We had included this variable as we felt that with increased time to assessment and with mild stroke with possible full recovery at the time of assessment there might be recall bias in terms of patients recounting what their maximal deficit had been. This did not appear to have been the case. Previous studies using CT found that the presence of asymptomatic infarcts was associated with the presence of mismatch (Lodder *et al.* 1994). We did not find that this is the case probably because MRI scans read by an experienced neuroradiologist will permit assessment of old and recent infarcts with greater accuracy than CT scanning.

Ascribing an aetiology to all ischaemic strokes is difficult as the pathophysiology of stroke is complex. Not all lacunar infarcts are caused by small vessel disease either based on clinical/risk factor analysis (Ay *et al.* 1999b; Baumgartner *et al.* 2003; Micheli *et al.* 2008; Seifert *et al.* 2005) or with pathological studies which have shown small lacune like lesions involving deep cortical layers and arcuate fibres without small vessel disease (De Reuck *et al.* 2004). We have however shown that a substantial proportion of patients are misclassified using purely clinical methods and that the location of the infarct can affect this misclassification. Judicious use of neuroimaging, preferably MR can lessen the prevalence of clinical radiological mismatch in a population and future studies investigating differences between ischaemic stroke sub-types should be aware of and ensure that they carefully sub-type stroke to avoid misclassification bias.

4.5 Chapter summary

This chapter has demonstrated that the patients in the Mild Stroke Study are similar to other patients in hospital based studies and have similar risk factor profiles. We have described rates of clinical-radiological dissociation in sub-typing of minor ischaemic stroke (16%) and demonstrated features which will predict mismatch, largely whether lacunar infarcts extend to the cortical margin, the size of the lacunar infarcts and increasing age of the patient. Finally we have demonstrated that up to 35% of DWI scans in patients presenting sub-acutely with mild stroke are negative and that this is independently associated with decreased stroke severity, younger age, increased time between onset of stroke symptoms and female gender which will have important implications both for clinicians treating and scientists researching minor stroke.

Chapter 5: Retinopathy in Ischaemic Stroke Subtypes

5.1 Aims of chapter

In this chapter we test the hypothesis that lacunar stroke is caused by increased blood brain barrier permeability by comparing the prevalence of retinopathy (a marker of blood retinal barrier breakdown) in lacunar versus cortical stroke subtypes.

5.2 Introduction

As detailed in previous chapters the exact aetiology of lacunar stroke remains unknown (Wardlaw 2005). There is mounting evidence that disordered small vessel endothelium or blood-brain barrier dysfunction may contribute to the aetiology of cerebral small vessel disease (Rosenberg 2009; Topakian *et al.* 2008; Wardlaw *et al.* 2008).

The blood-retinal barrier is analogous to the blood-brain barrier (Patton *et al.* 2005). Animals studies suggest that in ageing rats with behavioural signs of cognitive dysfunction (one of the clinical manifestations of cerebral small vessel disease) there is evidence of blood retinal barrier breakdown compared to cognitively intact younger rats (Chan-Ling *et al.* 2007). We have shown in Chapter 3 of this thesis that large population studies demonstrate associations between retinopathy (defined as the presence of hard or soft exudates, haemorrhage or microaneurysms) and previous stroke as well as future stroke risk (Doubal *et al.* 2009). Retinopathy is associated with increased permeability of the blood-retinal barrier (Krogsaa *et al.* 1981) and we therefore hypothesized that there would be higher rates of retinopathy in patients with acute ischaemic lacunar stroke compared to acute ischaemic cortical stroke controls where the mechanism is largely atherothromboembolic if lacunar strokes are caused by increased blood brain barrier permeability.

5.3 Methods

We recruited patients prospectively with acute clinical lacunar or mild cortical ischaemic stroke. The exact details of patient recruitment for this study are given in Chapter 2 of this thesis but in brief we included patients who presented with mild cortical or lacunar ischaemic stroke.

All patients were examined by an experienced stroke physician and classified initially into lacunar or cortical stroke clinical syndromes according to the Oxfordshire Community Stroke Project classification (Bamford *et al.* 1991). Patients had diagnostic cerebral MRI (including diffusion-weighted imaging, DWI and GRE) at presentation to identify the site of the recent infarct and exclude haemorrhage.

All patients had six field retinal photography (centred on the disc, macula, lateral macula, nasal to the disc, upper arcade and lower arcade) of the left and right eyes, with 1% tropicamide eye drops where possible, using a Canon CR-DGi digital retinal camera (Canon USA Inc.).

5.3.1 MRI Image analysis

All MRI scans were coded by an experienced neuroradiologist (JW) for the presence, location and size of the recent symptomatic infarct and any old infarcts or haemorrhages.

5.3.2 Retinal image analysis

I graded each retinal photograph masked to clinical details for retinopathy (defined as haemorrhage, microaneurysms or hard or soft exudates see figures 5.1, 5.2, 5.3 and 5.4 for examples) as absent, questionably present or present according to an established, validated proforma (Mitchell *et al.* 2005). Retinopathy was deemed present if any of the multiple retinal photographs for each patient showed a

retinopathic lesion (although in practice it was rare for there to be isolated retinopathic lesions). I was trained under the supervision of Professor Bal Dhillon (BD) with test images and with ophthalmologists performing regular diabetic screening. All lesions identified were checked by a consultant ophthalmologist (BD). If an image was missing or I was unable to grade an image I recorded this and the reason why. All images were graded on a standard flatscreen monitor under consistent lighting conditions. The intra-rater repeatability Kappa score for the presence versus absence of retinopathy (for 30 randomly chosen cases in this study performed 1 month apart) was 0.84 (excellent). As I was the only rater for this study I was unable to perform inter-rater reliability scores. This technique was based upon a retinopathy grading scale (microaneurysms and haemorrhages) with a published inter-rater Kappa score of 0.9 (Yu *et al.* 1998).

5.3.3 Statistical analysis

I used 2 sample t-tests, Fishers Exact Test and Chi Square tests for association to investigate baseline characteristics between the lacunar and cortical groups and associations with retinopathy and multiple binary logistic regression to assess multivariable effects of explanatory variables including assessing for significant interactions. All analyses were performed with Minitab software (version 14, Minitab Inc, PA, USA). Limited sample size calculation based on existing literature on retinal findings in stroke suggested that 197 patients would be needed to detect a difference of prevalence in retinopathy of 10% with 80% power at the 0.05 significance level.

Fig. 5.1 Enlarged section of retinal photograph showing retinal haemorrhages (arrow)

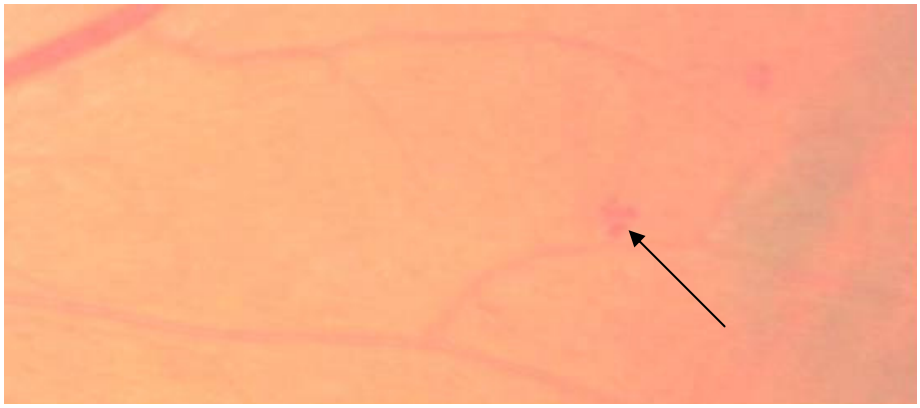


Fig. 5.2 Retinal photograph showing multiple soft exudates (white cotton wool spots)

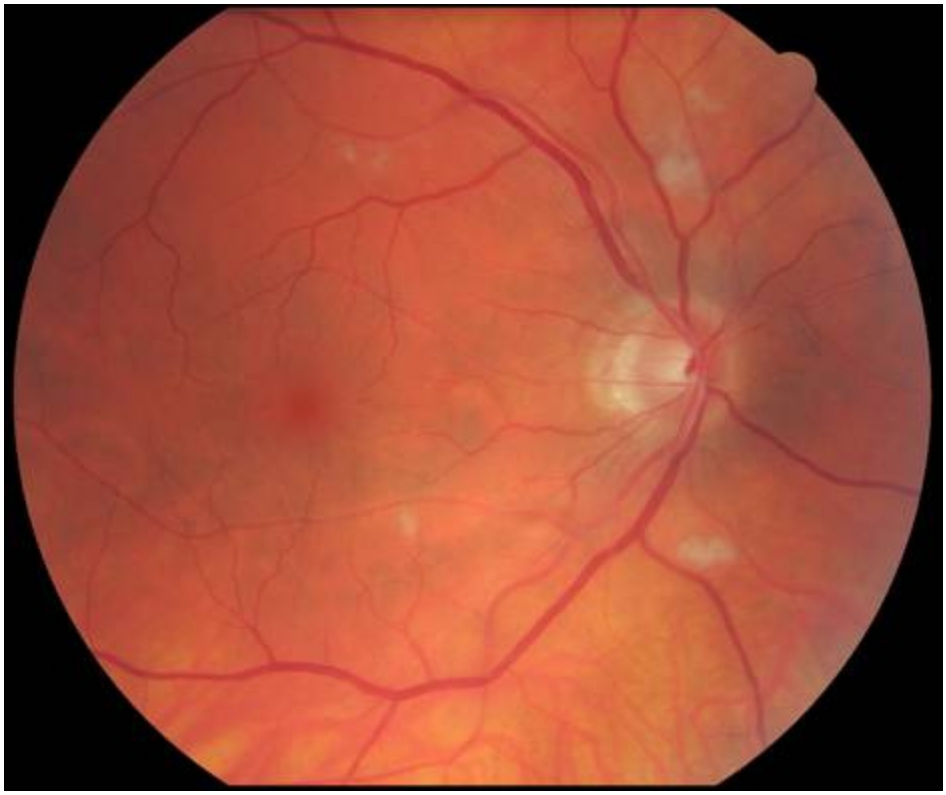
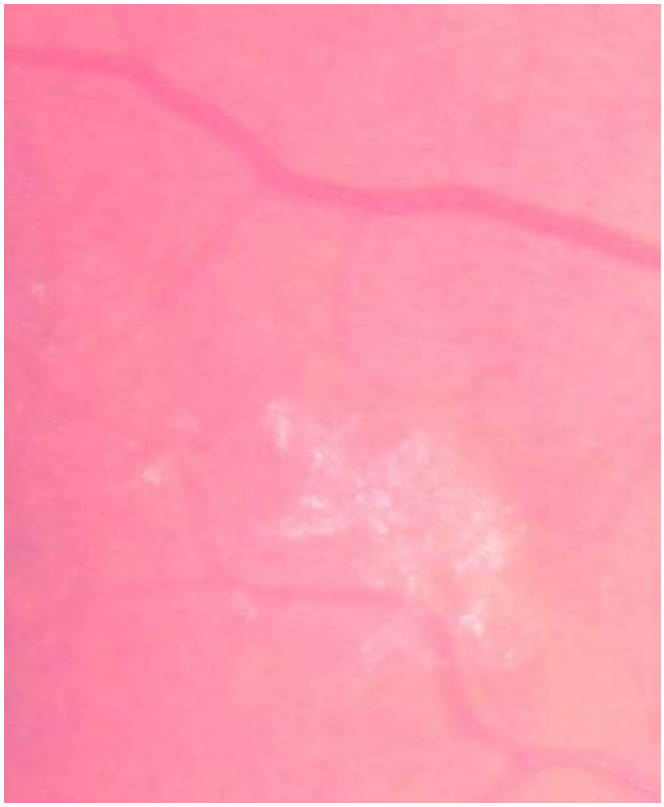


Fig. 5.3 Enlarged section of retinal photograph showing two dot like red microaneurysms (lower right quadrant of photograph)

Fig. 5.4 Enlarged section of retinal photograph showing yellow/white hard exudates.



5.4 Results

We recruited 220 patients of whom 6 were excluded with photographs of inadequate quality to assess retinopathy (due to cataract, poor compliance or inadequate dilatation) leaving 214 patients for analysis. The mean age was 68.4 years (SD 11.6 years), 62% were male, and the median NIHSS was 2. There were 109 patients with acute cortical stroke and 105 with acute lacunar stroke. Acute stroke lesions were seen on MRI (DWI and/or T2/FLAIR based on signal characteristics and lack of focal atrophy) in 159/214 (74%) patients of whom 144/214 (67%) of the cohort had DWI positive lesions (the rate reflecting the small nature of the lesions and occasional delays to scanning). Consistent with previous studies (Mead *et al.* 2000) 38/214 (18%) patients had their stroke subtype classification changed by MRI findings from cortical to lacunar or vice versa. 73/214 patients had old infarcts on imaging – in 16 the old infarct was of a different subtype to the acute lesion and 4 had both old cortical and lacunar infarcts.

The baseline characteristics of the lacunar and cortical groups are shown in Table 5.1. The cortical patients were older than the lacunar stroke patients (mean 70.6 years v 66.3 years, 2 sample t test estimate of difference 4.25 95% CI 1.15-7.34 yrs p=0.007) with higher NIHSS (median 3 v 2 Mann-Whitney U test $p<0.01$) and in keeping with cortical strokes being caused by large artery disease and cardio-embolism had higher rates of atrial fibrillation (14% v 4% p=0.009) and ischaemic heart disease (29% v 13% p=0.004). There were no significant differences between the two groups in gender or rates of hypertension, diabetes or PVD.

Table 5.1. Baseline characteristics of lacunar and cortical stroke subtypes. Statistical test used – Mean age (2 sample t test), median NIHSS (Mann-Whitney U test), PVD and AF (Fishers Exact Test), all others Chi square test for association.

Characteristic	**Lacunar Stroke**	**Cortical Stroke**	**P value for difference**
n	105	109	-
Age years (SD)	66.3 (11.6)	70.6 (11.4)	**0.007**
Male n (%)	60 (57%)	74 (68%)	0.10
Median NIHSS	3	2	**<0.01**
Atrial Fibrillation n (%)	4 (4%)	15 (14%)	**0.01**
Past Medical History of:			
Diabetes n (%)	18 (17%)	11 (10%)	0.13
Ischaemic heart disease n (%)	14 (13%)	32 (29%)	**0.004**
Peripheral vascular disease n (%)	5 (5%)	5 (5%)	0.95
Hypertension n (%)	59 (56%)	62 (66%)	0.14
TIA n (%)	16 (15%)	13 (12%)	0.46
Stroke n (%)	9 (9%)	12 (11%)	0.55
Old infarct on MRI n (%)	35 (33%)	38 (35%)	0.81

Of 214 patients, 40 patients (18.7%) had retinopathy present: 19/105 patients (18%) with lacunar stroke and 21/109 patients (19%) with cortical stroke had retinopathy (Chi squared statistic 0.48, p=0.8) See figure 5.4. There was no difference between the rate of retinopathy in stroke subtypes. Table 5.2 shows individual components of retinopathy by stroke subtype. There were no significant differences between individual components of retinopathy and stroke subtype. (Figure 5.5)

Figure 5.4 Bar chart showing proportions of patients with retinopathy by stroke subtype

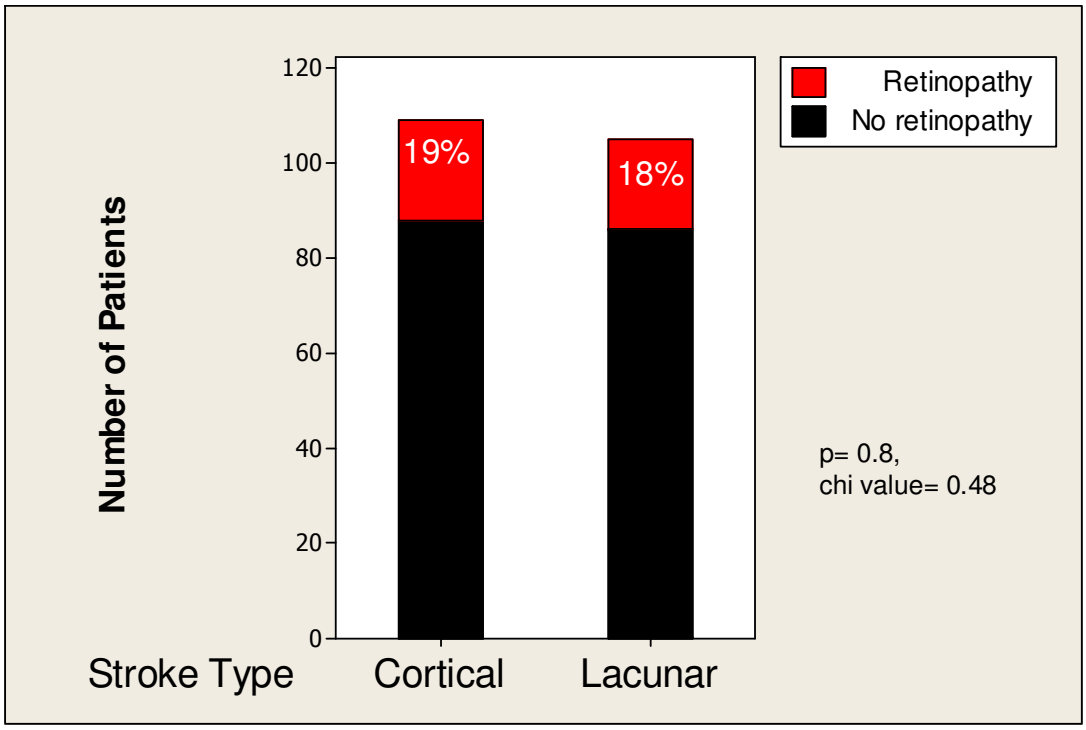
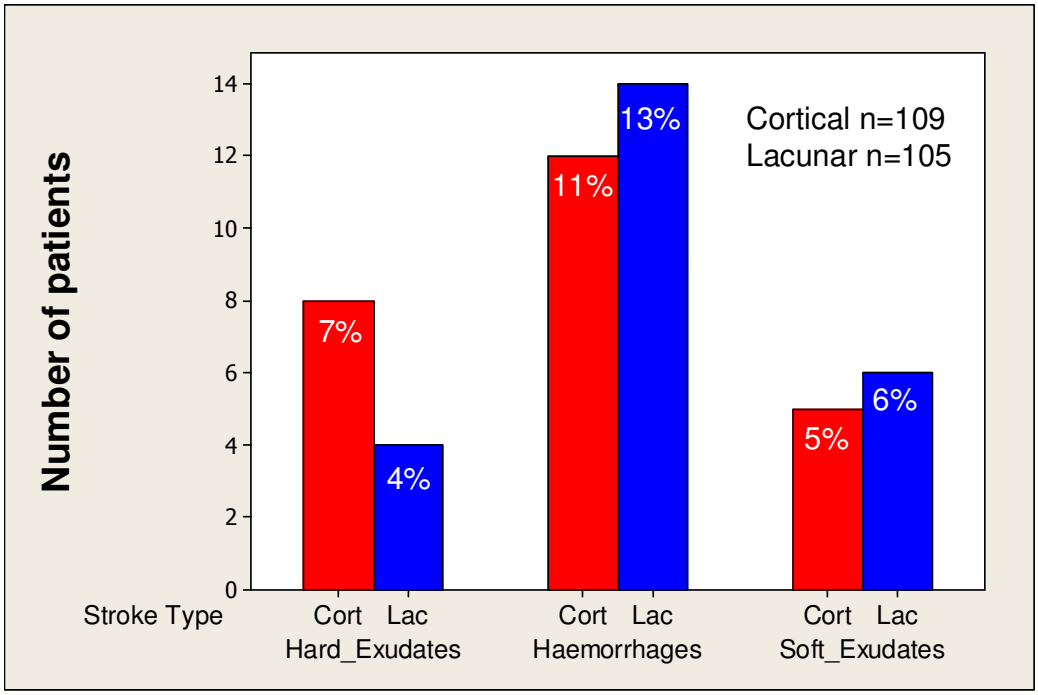


Table 5.2. Number of patients with individual components of retinopathy by stroke subtype. There are no significant differences in proportions with retinopathy features between stroke subtypes.

Characteristic	**Lacunar Stroke**	**Cortical Stroke**	**% Difference (95% CI)**	**P value**
n	105	109		
Hard Exudates (%)	4 (4%)	8 (7%)	3.5% (-2.6%, 9.6%)	0.26
Soft Exudates (%)	6 (6%)	5 (5%)	1.1% (-4.8%, 7.0%)	0.71
Haemorrhage/ microaneurysm(%)	14 (13%)	12 (11%)	2.3% (-6.4%, 11.0%)	0.60

Figure 5.5 Chart of number of patients with Retinopathy subtypes types by ischaemic stroke subtypes. Cort = cortical, lac = lacunar



With univariable analysis the presence of diabetes was associated with retinopathy (Chi square statistic 4.8, p=0.028) but not age, NIHSS, hypertension, IHD, PVD, previous TIA/Stroke or AF. After correcting for mild baseline differences in age, hypertension and diabetes with multivariable binary logistic regression, there was no association between ischaemic stroke subtype and presence of retinopathy (OR 0.76 – 95% CI 0.37-1.57 p=0.46) see Table 5.3. The only variable independently associated with the presence of retinopathy after correction for age, stroke subtype and hypertension was diabetes (OR 3.02 -95% CI 1.24-7.34 p= 0.01).

To examine possible effect modifiers, we assessed all two way interactions (between age, hypertension, diabetes and stroke subtype) and sequentially removed those interactions contributing least to the model where their effect was non significant. After this process the only significant interaction that remained was between hypertension and age in predicting retinopathy (OR for a combined age multiplied by

presence of hypertension explanatory variable of 0.94 95% CI 0.88 -1.00 p=0.04) when modelled with age, hypertension, diabetes and stroke subtype, but this did not significantly alter the associations shown in Table 5.3.

Table 5.3. Multivariable analysis of associations with retinopathy for all 214 patients. All OR are corrected for the other variables in the table.

Variable	**Multivariable OR for association with retinopathy**	**95% CI**	**P value**
Lacunar stroke subtype	0.76	0.37-1.57	0.46
Age	0.98	0.95-1.01	0.25
Hypertension	0.82	0.80-1.75	0.61
Diabetes	3.02	1.24-7.34	0.01

To ensure accurate ischaemic stroke classification, we performed a subgroup analysis excluding patients who had an old lesion of a different type to the acute stroke classification e.g. a patient presenting with an acute lacunar stroke who had an old cortical lesion. Multivariable regression correcting for age, diabetes and hypertension in these 194 patients did not change the size, direction and significance of the results in Table 5.3.

5.5 Discussion

We have not demonstrated an association between ischaemic stroke subtype and the presence of any retinopathy. In this study the only variable which was associated with retinopathy on multivariable analyses was diabetes.

The strengths of the study are that stroke was diagnosed by an expert at the time of the stroke, all patients had diagnostic MRI at presentation to permit accurate diagnosis and subtyping, I used a pre-specified clinical and imaging based hierarchy to classify stroke subtype and our sample size exceeded our original calculated estimate that would be required to detect a 10% difference between stroke subtypes. We can therefore be reasonably confident that we have not missed an important difference in retinopathy prevalence simply through inadequate study design. Furthermore all patients had retinal photography performed at the time of the stroke and no previous studies have compared retinopathy in ischaemic stroke subtypes.

The limitations of this study are that the cross-sectional design means that we can only report on associations between retinopathy and stroke without implying causality or delineating temporal associations. There were modest imbalances in baseline variables which may not have been completely corrected with multivariable analyses. Although I recruited beyond the number indicated in our sample size calculation which, although based upon the best figures available, may not have been accurate, more patients would be needed to show differences in overall prevalence of less than 10%, or in specific subtypes of retinopathy. Whilst we sought to minimize any misclassification of retinopathy by having an experienced ophthalmologist reviewing all questionable retinopathy lesions it is nonetheless a weakness of this study that I was the sole rater for retinopathy. Ideally there would have been another rater with which to compare results but this was not feasible with the funding available for this study.

Fluorescein angiography may have demonstrated subclinical differences in microvascular perfusion between stroke subtypes, however is more invasive (with a significant risk of anaphylaxis) than colour retinal photography, and therefore would have restricted recruitment, would not have been deemed ethical and therefore not feasible in this study. The study sample size was perhaps limited by exclusion of patients with severe strokes (equivalent to total anterior circulation strokes) and the need for patients to have a definite diagnosis of stroke rather than a possible diagnosis of stroke. However retinal photography would have been difficult in severely unwell patients (the subject needs to sit up, hold their head still and follow

commands) and the atherothromboembolic disease mechanisms present in severe cortical strokes will be represented in the included milder cortical strokes.

We subtyped acute ischaemic stroke into lacunar and cortical stroke using the OCSP classification modified by the results of the brain MRI, and therefore unbiased by using risk factors to classify stroke (eg as in the TOAST classification) (Jackson and Sudlow 2005). This study focused on patients with acute clinically proven strokes, however some patients had old lesions on their MRI scans that may or may not have represented clinical strokes (most appeared not to – we did not ascertain a past history of clinically-evident stroke in 65/73 of patients with old infarcts). We repeated the analysis excluding the few patients who had an old lesion type that differed from the acute ischaemic type and this sensitivity analysis did not change the main results shown in Table 5.3. It is a limitation of this study that we included 20 patients who had an old infarct on brain imaging that may have represented a different ischaemic stroke phenotype to the acute event ie acute lacunar stroke with an old cortical infarct. It is not however clear that these old infarct appearing lesions were in fact strokes (indeed the vast majority were not clearly associated with clinical deficits) and therefore probable that this may not have diluted the results significantly. Ideally we would have recruited "pure" lacunar and cortical phenotypes without old infarcts in brain imaging but this would have severely limited recruitment and hence generalisability. It is also reassuring that the sensitivity analysis performed excluding the patients with a different old stroke phenotype did not change the results significantly.

We demonstrated a prevalence of retinopathy of 19% in patients with mild ischaemic stroke and mean age 67 yrs. This is slightly higher than population-based prevalences of 7.0% (ARIC) (Wong *et al.* 2001) and 8.3% (CHS) (Wong *et al.* 2003) probably reflecting the fact that the patients in the present study had higher rates of vascular disease than community-dwelling healthy subjects. The prevalence in the present study was slightly lower than in diabetic patients with ischaemic stroke of 33% (ARIC) (Cheung *et al.* 2007c), but reassuringly similar to that in patients in CHS who had a history of ischaemic stroke, 19% of whom had retinopathy (Wong *et al.* 2003).

How does our study relate to previous information on retinopathy and stroke? In the large population-based Atherosclerosis Risk in Communities (ARIC) study, the presence of any retinopathy predicted future ischaemic stroke during 3.5 years follow up with a relative risk (RR) of 2.58 (95% CI 1.59-4.20) corrected for diabetes, hypertension, age and other vascular risk factors (Wong *et al.* 2001). A similar finding is reported for diabetic subjects with a longer follow up from the same study (Cheung *et al.* 2007c). Furthermore, baseline retinopathy in the Blue Mountains Eye Study was reported as being significantly associated with future risk of stroke and TIA with a RR of 1.7 (95% CI 1.0-2.8) (Mitchell *et al.* 2005). Although demonstrating a link between retinopathy and future stroke in general, none of these studies reported or compared associations with different subtypes of ischaemic stroke. The evidence for an association between retinopathy and history of stroke is less compelling (perhaps reflecting the inherent difficulties in obtaining accurate medical histories and differing methods of defining prevalent stroke) – the Cardiovascular Health Study (CHS) reported that retinopathy was associated with a clinical history of stroke (OR 2.0 95% CI 1.1-3.6)(Wong *et al.* 2003) and MRI defined cerebral infarction of unspecified subtype (OR 1.18 95% CI 1.05-1.34) (Longstreth, Jr. *et al.* 2007). At the time of initial publication there were no other studies which had investigated retinopathy in ischaemic stroke subtypes.

Our collaborators, The Multi Centre Retinal Stroke Study have since published details of associations between retinal microvascular abnormalities and stroke subtypes (Baker *et al.* 2010). This paper investigated retinal changes in deep intracranial haemorrhage (n=51) compared with both lacunar ischaemic (n=93) and non-lacunar ischaemic stroke (n=486) and not between ischaemic stroke subtypes. It is possible to calculate from the result that the prevalence of retinopathy in this cohort was 32% - higher than the rate of 19% that we found. This difference may partly be explained by the higher rate of diabetes (22% compared with 15%). Incidentally, the prevalence of retinopathy for lacunar strokes in the MCRS cohort was 35% and for non-lacunar stroke 33%. The authors do not present statistics regarding these prevalences (where multivariable analysis would be required) but there would not appear to be a difference certainly with univariable analysis.

Has retinopathy been associated with other clinical markers of brain disease? A systematic review found that retinopathy was associated with decreased cognition but that this association was tempered by heterogeneity between studies and variable study quality (Ding *et al.* 2008). Studies with improved quality published after this review have shown that in the ARIC study, the presence of retinopathy predicted subsequent cognitive decline (Lesage *et al.* 2009). In a cross sectional study from the BMES, retinopathy in subjects without hypertension (n=48) and after multivariable correction for age, sex, diabetes, smoking, education level, systolic blood pressure and history of cardiovascular disease (total 8 variables with 48 outcomes of interest) was associated with the presence of severe cognitive impairment (mini mental state exam <23) with an odds ratio of 1.7 (95% CI 1.0-3.2, p=0.03) (Liew *et al.* 2009). This is a borderline result in a subgroup analysis that just reaches statistical significance in a regression model that may have been over-fitted with variables and so should therefore be treated with caution.

Has retinopathy been associated with other brain imaging markers of brain disease? In a well designed genetic Icelandic study of 4,176 subjects, retinopathy lesions were not associated with white matter lesion load on MRI scans (Qiu *et al.* 2009). This lack of an association between retinopathy and white matter hyperintensities on MRI had been suggested in the Cardiovascular Health Study (Longstreth, Jr. *et al.* 2007) yet findings from the ARIC study suggest that patients with retinopathy have a higher prevalence of white matter lesions (after adjustment for vascular risk factors (Wong *et al.* 2002a). These conflicting results demonstrate the heterogeneity apparent between studies.

What do these results mean? The aim of the present study was to investigate whether there were higher rates of retinopathy in patients with lacunar stroke as this might reflect blood-retinal barrier breakdown. There is growing evidence that cerebral small vessel disease may be associated with dysfunctional blood-brain barrier (Rosenberg 2009; Topakian *et al.* 2008; Wardlaw *et al.* 2009) and retinal imaging offers an excellent non-invasive method of studying blood vessels that are similar to cerebral vessels. That the rates of retinopathy do not differ between acute ischaemic

stroke subtypes may be due to several reasons other than our hypothesis being incorrect.

Firstly the simple presence of retinopathy may be too blunt a tool to measure what are likely to be subtle differences between ischaemic stroke subtypes. In diabetic retinopathy the constituent features of retinopathy appear to have a common precursor in that one of the first stages in the development of diabetic retinopathy is subtle breakdown of the blood-retinal barrier (Qaum *et al.* 2001) (visible on techniques like fluorescein angiography). It is not, however clear what the classic retinopathy changes represent or the final pathways causing them (McLeod 2005; Schmidt 2008; Stanford 2004). In the present study we have looked at any retinopathy (haemorrhage, exudates and microaneurysms) but perhaps only different retinopathic components are associated with blood-retinal barrier leak to varying degrees whilst others reflect other pathological processes. Certainly cotton wool spots are considered to represent focal areas of ischaemia as a late development of the accumulation of axoplasmic constituents (McLeod 2005). Although we have reported the findings for interest and perhaps to generate hypotheses, this study was not powered to identify differences in individual retinopathy features, only the presence of any retinopathy.

Secondly, although very similar, it may be that retinal vessels do not behave exactly like cerebral vessels and the changes are too subtle to be identified without retinal fluorescein angiography. Microvascular changes in the brain and the retina may not move in parallel – it is possible that in these patients (with a low stroke severity) the retinal changes may have not yet developed and perhaps studying patients with more severe stroke may have revealed subtype-specific retinopathic differences. Alternatively, it may be that retinopathy is a marker of vascular risk and is co-associated with stroke but not stroke subtypes, as lacunar and cortical strokes have similar hypertension and diabetes risk profiles (Jackson and Sudlow 2005).

Future research should concentrate on carefully sub-typing stroke and investigating the use of retinopathy as a marker of risk factor effects on end organs in the individual and how this relates to any future stroke risk.

Chapter 6: Retinal vessel width and morphology in ischaemic stroke subtypes.

6.1 Aims of Chapter

In this chapter we describe detailed morphological differences including retinal arteriolar and venular widths, focal arteriolar narrowing and arteriovenous nicking in retinal vessels in ischaemic stroke subtypes aiming to elucidate the underlying vasculopathy in lacunar stroke.

6.2 Introduction

We have demonstrated in chapter 3 of this thesis and others have subsequently confirmed that retinal microvascular abnormalities are associated with stroke: (Baker *et al.* 2008) retinal vessel widths predict future risk of stroke (McGeechan *et al.* 2009a) and may be associated with a previous history of stroke (Cooper *et al.* 2006; Longstreth, Jr. *et al.* 2007). Arteriovenous nicking (AVN) and focal arteriolar narrowing (FAN) are associated with infarcts on MR brain scanning (Cooper *et al.* 2006; Longstreth, Jr. *et al.* 2007). However, although some studies suggest that there are associations between retinal changes and lacunar stroke, there is little information about whether retinal appearances truly differ in ischemic stroke subtypes. This is because these studies either did not subtype ischemic stroke (Ikram *et al.* 2006a) or only investigated asymptomatic lacunes seen on MRI scanning which are of uncertain relevance to clinical stroke (Ikram *et al.* 2006b; Wardlaw 2008). Therefore we studied retinal arteriolar and venular widths, focal arteriolar narrowing (FAN) and arteriovenous nicking (AVN) in patients presenting with acute lacunar and cortical ischemic stroke to test the hypothesis that the retinal small vessels would be morphologically different in patients with lacunar stroke compared to patients with large artery cortical atherothromboembolic stroke and that the nature of these abnormalities may shed light on the pathophysiology underlying lacunar stroke.

6.3 Methods

We prospectively recruited consecutive patients with clinical lacunar or mild cortical stroke seen at our hospital stroke service, aiming to recruit all relevant patients as consecutively as possible as detailed in Chapter 3 of this thesis.

All patients had six field retinal photography (centred on the disc, macula, lateral macula, nasal to the disc, upper arcade and lower arcade) of the left and right eyes, with 1% tropicamide eye drops where necessary, using a Canon CR-DGi digital retinal camera (Canon USA Inc.).

6.3.1 MRI Analysis

All MRI scans were coded for the presence, location and size of the recent infarct and any old infarcts or haemorrhages.

6.3.2 Retinal image analysis for vessel widths

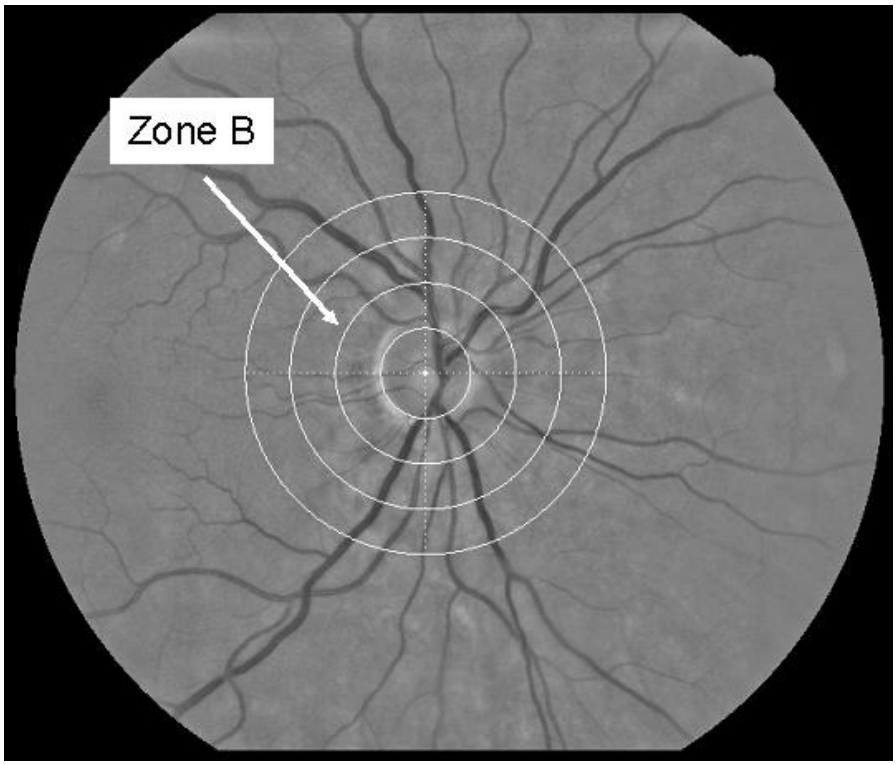
All retinal images were analyzed by a grader (FD) who was blinded to clinical and brain imaging features. Each colour retinal image was stored within OptoMize® (Digital Healthcare, Cambridge, UK) software as Tagged Image File Format files. Left and right eye vessel widths are highly correlated (Wong *et al.* 2004) and we randomly chose one image centred on the optic disc from one eye from each patient. For measurement of Arterio-Venular Ratios (AVR), Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE), images were analyzed using a custom written validated image analysis program (within MatLab: The MathWorks, Natick, MA).

Processing was performed on the green channel of the colour fundus images as this typically exhibits the greatest contrast between vessel and background. Fundus images are often non-uniformly illuminated and exhibit local luminosity and contrast variability. This may have an adverse affect on the measurement of vessel diameters

and so each image was corrected by first estimating background illumination using a median filter with a mask of size greater than the expected maximum diameter of vessels (200 pixels x 200 pixels) and then calculating correction coefficients by dividing the maximum grey-level value in the median filtered image by the grey-level value of each pixel in the filtered image. The green channel was then multiplied by the correction coefficients. Finally, contrast stretching was performed whereby the range of gray levels in the corrected image is stretched to make full use of all possible values, i.e. 0 to 255.

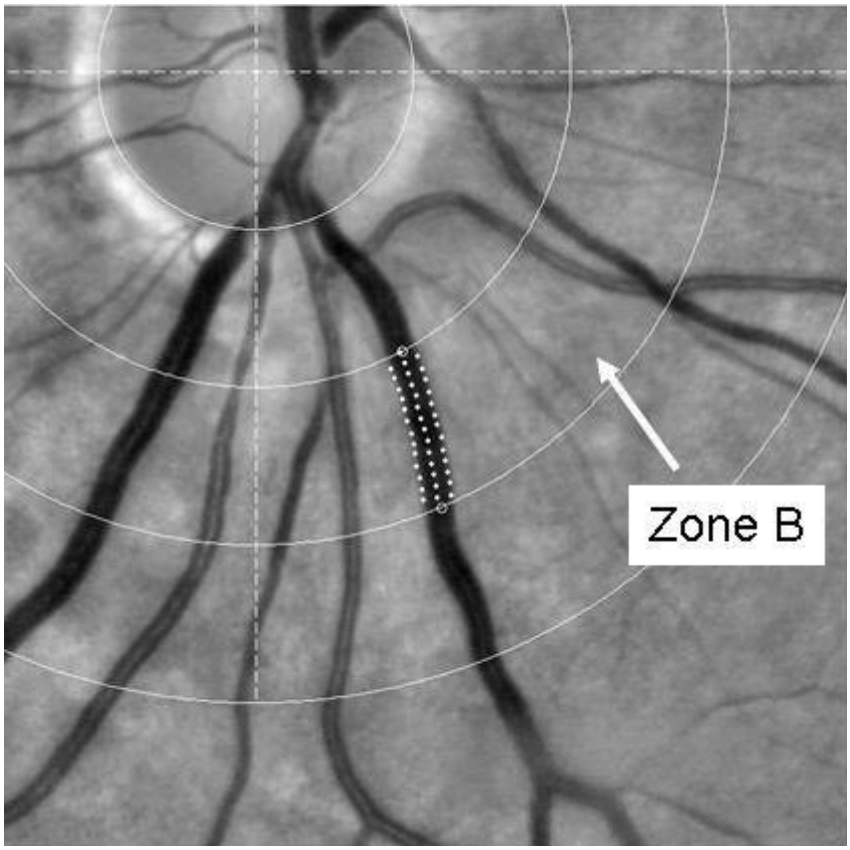
After image processing as described above, the software identified the optic disc and drew a circle delineating the disc. After the disc was identified, the position was checked by the grader. The software drew circles at half a disc diameter and a full disc diameter concentrically out from the centre of the disc (Fig 6.1).

Figure 6.1. Retinal photograph post processing with circles centred on the disc at half disc diameter distance apart superimposed and Zone B where the six largest arteries and venules were measured.



If the software was unable to locate the disc then the grader marked the centre of the disc and the outer border and the software fitted a circle based on these 2 reference points. The grader then identified the six largest arterioles and venules passing through the circular area between the circle drawn at ½ a disc diameter from the edge of the optic disc and the circle drawn at 1 disc diameter from the edge of the optic disc (Zone B shown on figures 6.1 and 6.2) and on each vessel identified 2 points between which the software tracked, measuring the vessel profile at set intervals (either 5 pixels or 10 pixels decided by the operator).

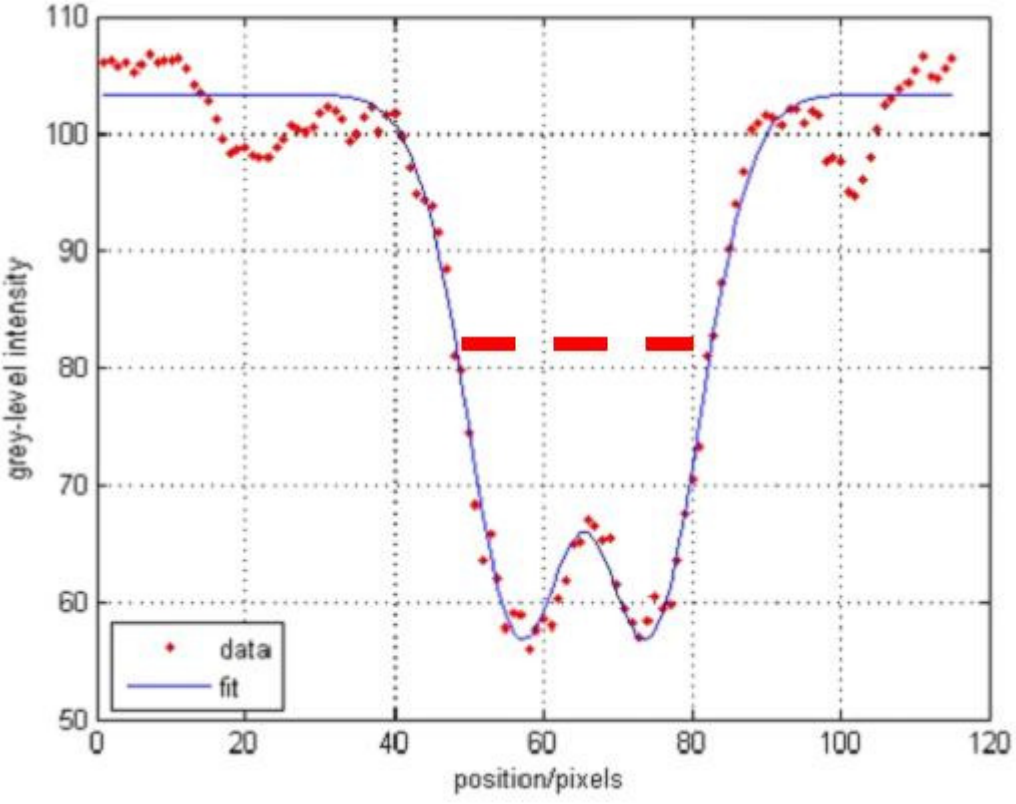
Figure 6.2 showing vessel tracking of a venule in doughnut shaped zone B



The software measured the vessel profile with microdensitometry and fitted the profile to a double Gaussian curve (figure 6.3). Each profile was either accepted or rejected by the grader as being a good fit for the vessel curve (we accepted a r correlation co-efficient of >0.7). The vessel width was calculated for each profile as

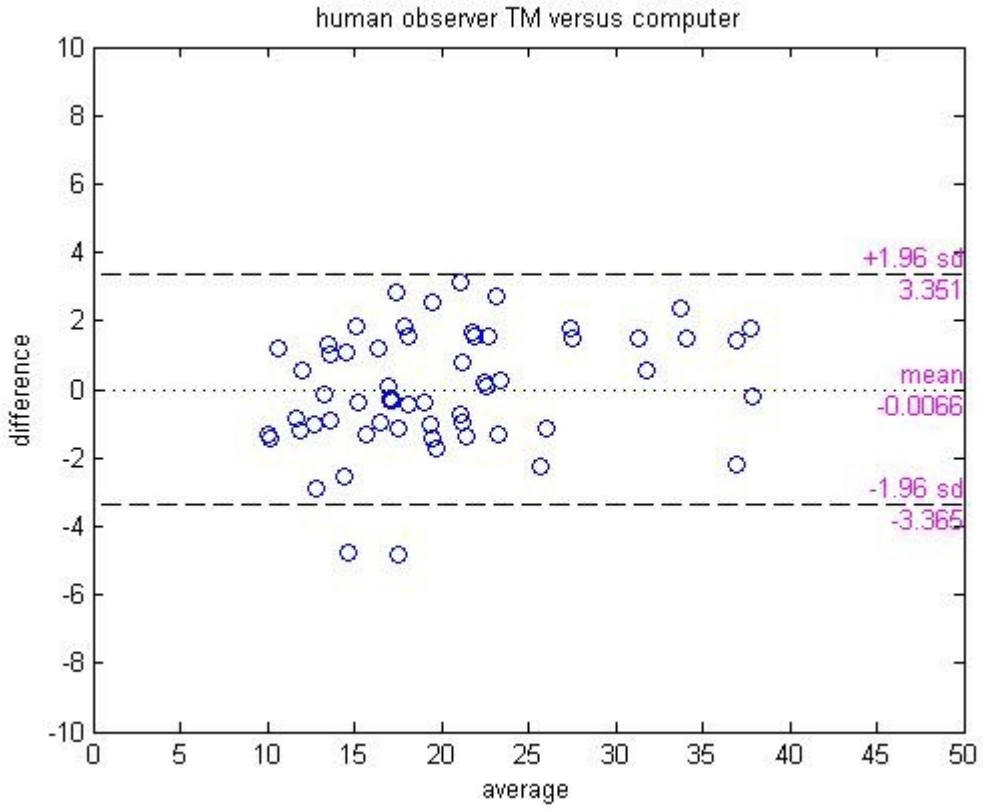
the width of the intensity profiles at half the height of the intensity profile peak (Newsom *et al.* 1992).

Figure 6.3 Vessel profile: Plot of pixel intensity along a transect across a vessel with superimposed Gaussian Line. The width is taken as the half-height width measure and represented by the dashed line.



We validated this process with Bland Altman plots comparing software performance to best human measurement (with a calliper on images that had been enlarged so that the vessel measured approx 20-30 mm) and found no evidence of systematic bias and a mean difference between human and software measurements for 50 randomly chosen vessels of only 0.006 pixels (95% CI -3.3 to 3.3 pixels). See figure 6.4.

Figure 6.4. Bland Altman plot of difference between human observer derived width measurements (in pixels) and computer derived widths measurements against width of vessel showing mean difference between the two of 0.0066 pixels and most differences falling between +/- 3.3 pixels. There is no systematic bias evident.



We combined arteriolar widths to produce a Central Retinal Arteriolar Equivalent (CRAE) for each eye using the following formula (Patton *et al.* 2006a) which adjusts Knudston's formula (Knudtson *et al.* 2003) to take into account the fact that the branching co-efficient varies with asymmetry of the widths of the daughter vessels. We combined the largest artery with the 6th largest artery to form a summary width in the following formula where Wc = summary width, Wa = 6th largest arteriole width, Wb = largest arteriole width. The values 0.78 and 0.63 represent the regression equation which relates the asymmetry index to the branching co-efficient (Patton *et al.* 2006a)

$$W_c = \sqrt{W_a^{\,2} + W_b^{\,2}} \Big/ \left(0.78 + 0.63 \times \frac{W_a}{W_b}\right)$$

We then combined the 2nd largest with the 5th largest and the 3rd largest with the 4th largest. This left us with 3 summary widths - we combined the largest with the smallest and then this summary width with the middle value to leave us with a single summary measure (CRAE) after 5 iterations. We performed a similar analysis using the following formula to obtain a summary measure for the venules (CRVE) – where Wc = summary width, Wa = 6th largest venule width, Wb = largest venule width and 1.22 is the branching co-efficient (for calculating venular summary widths the branching co-efficient is a constant as not affected by asymmetry of the daughter venules) (Patton *et al.* 2006a)

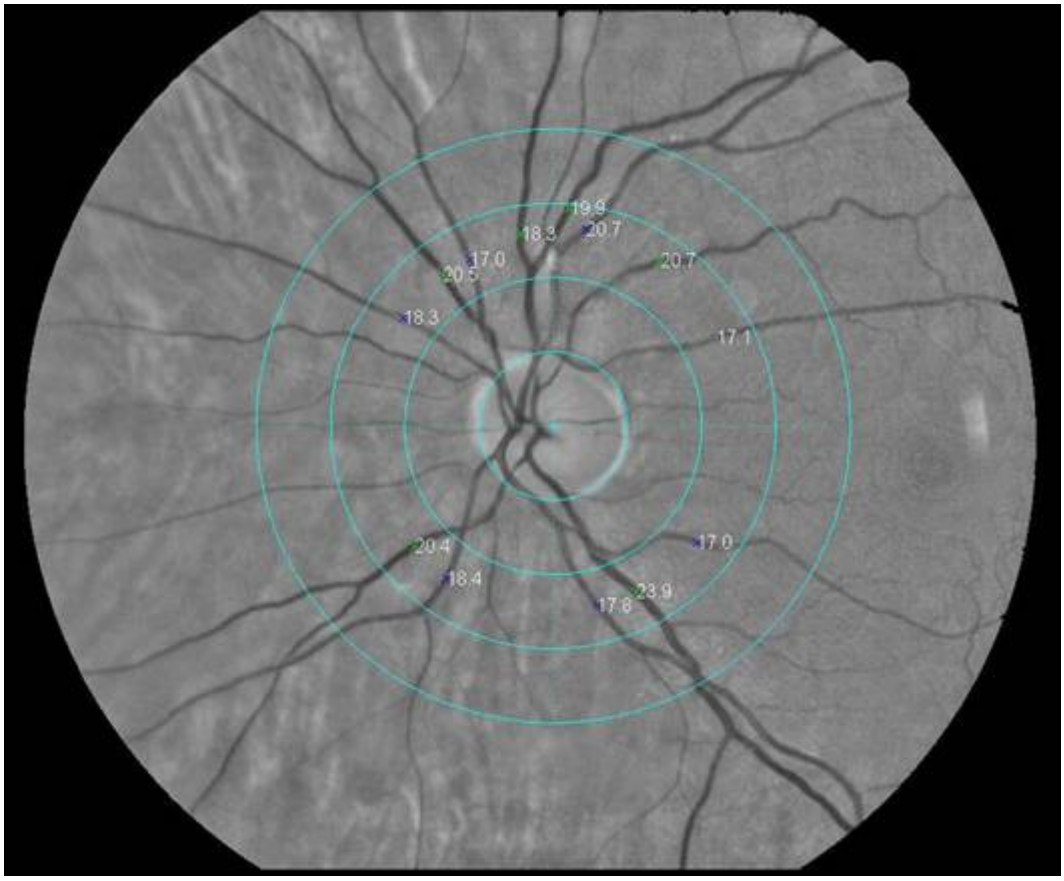
$$W_c = \sqrt{W_a^2 + W_b^2 / 1.22}$$

We calculated AVR for each eye as the ratio of CRAE divided by CRVE.

To convert the pixel measurements obtained from the image to absolute measurements (microns) we assumed that the average disc diameter was 1850 microns (Hubbard *et al.* 1999) and using our cohort mean disc diameter of 381 pixels multiplied all of our pixels measurements by 4.855 (1850/381) to convert pixel measurements to microns.

In a randomly chosen sample of 20 retinal images the intraclass correlation co-efficients for intra-rater reliability (with assessments performed 1 month apart) were excellent at 0.94 for CRAE, 0.98 for CRVE and 0.91 for AVR. Using a second grader (Tom MacGillivray) the intraclass correlation co-efficients for inter rater reliability were excellent at 0.95 for CRAE, 0.89 for CRVE and 0.90 for AVR.

Figure 6.5 Retinal photograph with 12 vessel widths (in pixels) marked. The software extracts these widths to produce CRAE and CRVE.



6.3.3 Retinal image analysis for focal arteriolar narrowing and arteriovenous nicking

Our coding for focal arteriolar narrowing and arteriovenous nicking was based upon that of the ARIC study (Hubbard *et al.* 1999). A physician (FD) specifically trained in retinal vessel assessment coded the presence (no/questionable/yes) and severity (mild, moderate, severe) of AVN. We defined AVN as a reduced and tapering width of a venule on either side of an arteriole where the arteriole crossed the venule (ignoring rare instances where venules crossed arterioles). We graded AVN mild if the venule was narrowed up to three quarters of it original diameter. We graded

AVN moderate where the venule was narrowed between three quarters and half of its original diameter. We graded AVN severe where the venule was narrowed to less than half of its original diameter.

For FAN we assessed arterioles estimated to be over 50 microns in diameter (approx one third of the diameter of a major venule at the disc margin). FAN was present if there was a focal segmental narrowing of an artery to at least two thirds of the original width with resumption of the original width distal to the area of focal narrowing. FAN was mild/moderate if the sum total length of focal narrowing in a photograph was less than 2 disc diameters and severe if greater than 2 disc diameters. All questionable lesions were graded by an ophthalmologist (BD) with a specialist interest in retinal disease. This method of assessment has intra rater kappa scores of 0.87 for AVN and 0.80 for FAN (Sherry *et al.* 2002).

Figure 6.5. Magnified section of original retinal colour photograph illustrating arteriovenous nicking.

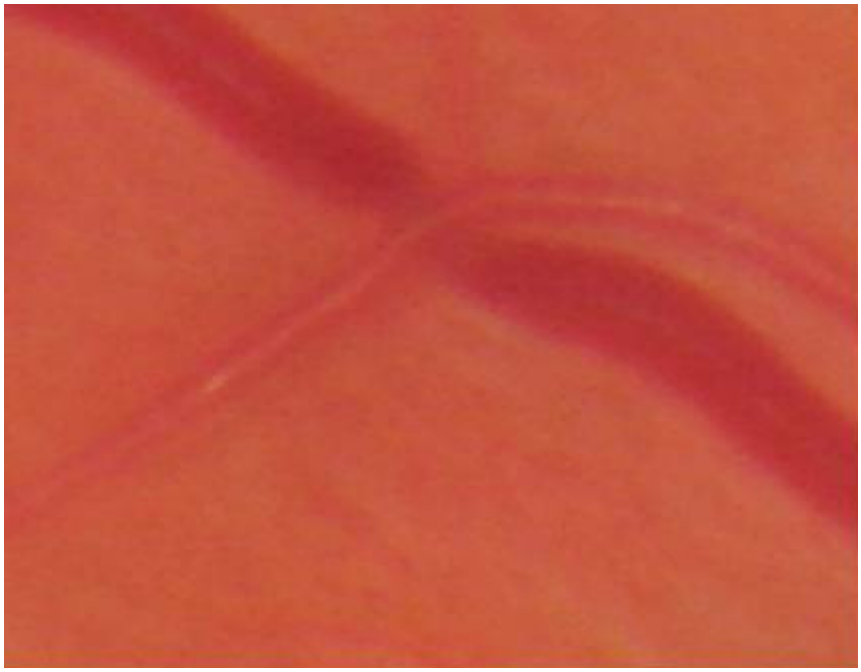
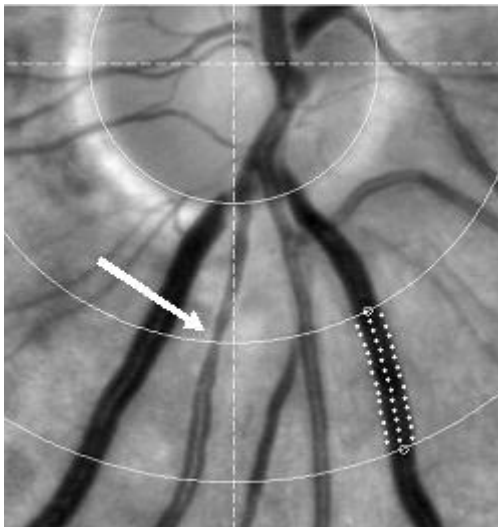


Figure 6.6. Example of focal arteriolar narrowing (white arrow).



6.3.4 Statistical analysis

I compared baseline characteristics between the lacunar and cortical stroke groups with Students t test, Chi square test for association and Fishers exact test. CRAE, CRVE and AVR were normally distributed (figures 6.7, 6.8) and I compared unadjusted CRAE, CRVE and AVR between groups with Students t-test. I performed multiple linear regression analysis with stroke subtype, age and vascular risk factors as explanatory variables and CRAE, CRVE, AVR as the dependent variable. CRAE and CRVE were co-variates and therefore modelled together. I dichotomized FAN and AVN data into present versus absent and used binary logistic regression to assess differences between stroke subtypes correcting for vascular risk factors. I used odds ratios (OR) with 95% confidence intervals (CI) to examine associations between retinal and other features. All analysis was performed with Minitab (Version 14, Minitab Inc, PA, USA). Sample size calculation based on existing literature on retinal findings in stroke suggested that 197 patients would be needed to detect a difference of prevalence of FAN or AVN of 10% with 80% power at the 0.05 significance level between stroke subtypes.

Figure 6.7. Histogram showing distribution of CRVE measured in pixels by 2 pixel measurements indicating normal distribution.

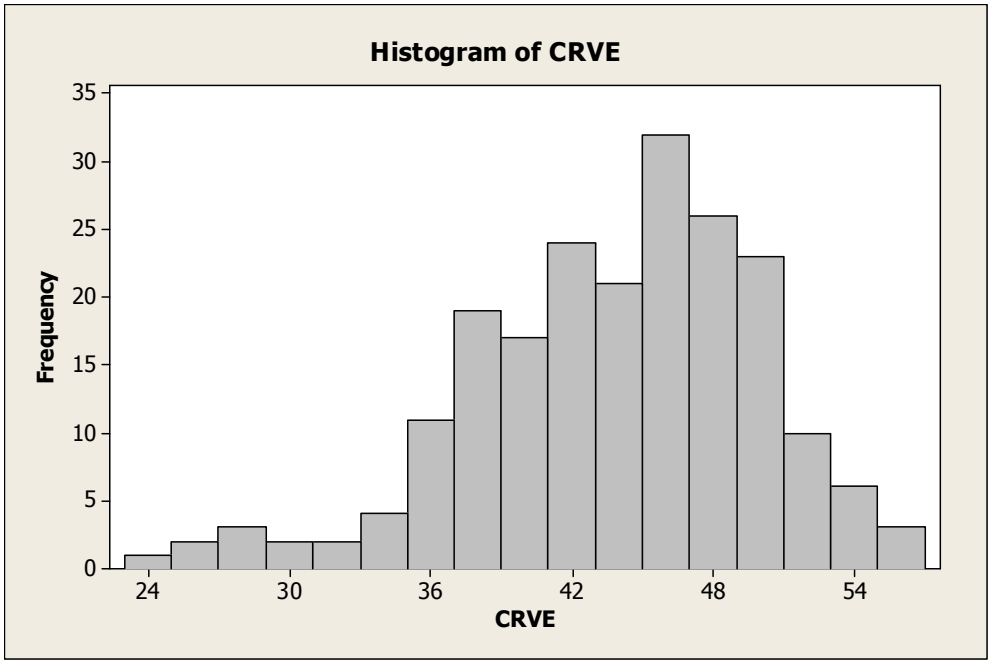
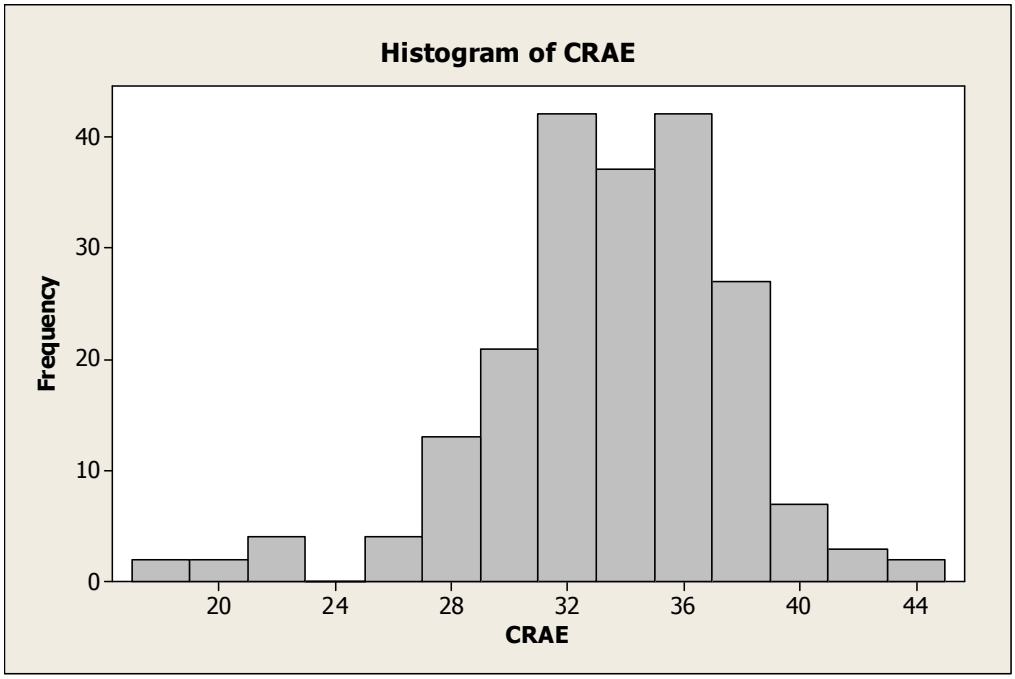


Figure 6.8. . Histogram showing distribution of CRAE measured in pixels by 2 pixel measurements indicating normal distribution.



6.4 Results

We recruited 220 patients. Eight were excluded (6 had poor quality photographs, 2 with missing photographs) leaving 212 for analysis of FAN and AVN and 206 with photographic quality permitting analysis of vessel widths. Of the 212 patients there were 105 lacunar strokes and 107 cortical strokes. The mean age was 68.1 (SD 11.5) years and median NIHSS score 2 (interquartile range 2-3) (table 6.1). The lacunar stroke patients were younger with marginally higher stroke severity and lower rates of atrial fibrillation, symptomatic carotid stenosis >50% NASCET and ischemic heart disease than cortical strokes (table 6.1).

Table 6.1 Baseline characteristics of lacunar and cortical stroke subgroups. Groups are compared with 2 sample t-test (age), Mann Whitney U test (NIHSS) and differences in proportions (all others). SD = Standard Deviation, NIHSS = National Institute for Health Stroke Scale, TIA = Transient Ischemic Attack

Characteristic	**Lacunar Stroke**	**Cortical Stroke**	**P value for difference between groups**
n	105	107	
Age years (SD)	66.2 (11.5)	70.0 (11.3)	**0.017**
Male gender n (%)	62 (59%)	73 (68%)	0.163
Median NIHSS	3	2	**<0.001**
Atrial Fibrillation n (%)	4 (4%)	14 (13%)	**0.02**
Symptomatic Carotid Stenosis >50% n (%)	4 (4%)	13 (12%)	**0.02**
Past Medical History of:			
Diabetes n (%)	19 (18%)	12 (11%)	0.15
Ischemic heart disease n (%)	14 (13%)	31 (29%)	**0.004**
Peripheral vascular disease n (%)	5 (5%)	5 (5%)	0.97
Hypertension n (%)	59 (56%)	70 (65%)	0.17
TIA n (%)	17 (16%)	11 (10%)	0.19
Stroke n (%)	8 (8%)	12 (11%)	0.52

6.4.1 Vessel widths

206 patients contributed to this analysis. For the total study population the mean CRAE was 33.47 pixels (162μ) mean CRVE was 43.87 pixels (212μ) and mean AVR 0.77. CRAE and CRVE were correlated with a Pearson's correlation coefficient of 0.66. Patients with lacunar stroke had higher CRVE (44.9 SD 5.5 pixels v 42.9 SD 6.4 pixels, p=0.01), lower AVR (0.76 SD 0.1 pixels v 0.78 SD 0.01 pixels, p=0.03) and similar CRAE to patients with cortical stroke (33.2 SD 4.4 pixels v 33.7 SD 4.3 pixels, p=0.4). Table 6.2 shows the adjusted associations with arteriolar and venular widths. Increased CRVE was significantly and independently associated with lacunar stroke subtype, lower age and increased CRAE (after correcting for the presence of diabetes, hypertension and gender). Increased CRAE was significantly and independently associated with female gender and increased CRVE (correcting for age, diabetes, hypertension and stroke subtype). Decreased AVR was significantly and independently associated with lacunar stroke subtype and younger age (correcting for diabetes, hypertension and gender).

I performed a sensitivity analysis excluding patients with a prior history of stroke and found that in 186 patients the results were similar to those presented in table 6.2 although the association between increased venular width and lacunar stroke subtype was strengthened (data not shown).

Table 6.2. Associations between vessel widths and key patient variables on multivariable linear regression showing beta co-efficient and p value for CRAE, CRVE and AVR. All analyses are corrected for the presence of the other variables in the table. CRAE = Central retinal arteriolar equivalent, CRVE = Central Retinal Venular Equivalent, AVR = arteriovenous ratio. Beta = beta coefficient

Explanatory variable	**CRAE**		**CRVE**		**AVR**	
	Beta	**p value**	**Beta**	**p value**	**Beta**	**p value**
Age	0.03	p=0.12	-0.11	<0.001*	0.002	p=0.002*
Male Gender	-1.1	p=0.02*	-0.10	0.87	-0.01	p=0.3
Lacunar stroke subtype	-0.48	p=0.29	1.33	0.03*	-0.02	p=0.047*
Diabetes	0.02	p=0.97	-0.29	0.74	0.002	p=0.8
Hypertension	-0.95	p=0.05	0.94	0.15	-0.02	p=0.06
CRAE	n/a		0.88	<0.001*	n/a	
CRVE	0.48	p<0.001*	n/a		n/a	

*significant p<0.05

6.4.2 Arteriovenous nicking and focal arteriolar narrowing

212 patients contributed to this analysis. AVN was present in 83/107 (78%) patients with cortical stroke and 85/105 (81%) patients with lacunar stroke (difference = 3%, 95% CI -7% to 14% p=0.53). FAN was present in 28/107 (26%) patients with cortical stroke and 37/105 (35%) of patients with lacunar stroke (difference = 9%, 95% CI -3% to 21%, p=0.15), distribution of FAN and AVN by stroke subtype are given in table 6.3. I performed binary logistic regression to adjust for the baseline risk factor and age imbalances between the cortical and lacunar stroke groups with FAN or AVN as the dependent variable (dichotomized to present or absent). Only hypertension independently predicted the presence of AVN; no risk factors were independently associated with FAN (Table 6.4). Excluding patients with a history of prior stroke did not alter these results.

Table 6.3 Numbers of patients with morphological abnormalities by stroke subtype. None of these differences reach statistical significance. FAN = focal arteriolar narrowing, AVN = arteriovenous nicking.

Variable		Lacunar Stroke (n=105)	Cortical Stroke (n=107)
AVN	None	20 (19%)	24 (22%)
	Mild	31 (30%)	31 (29%)
	Moderate	29 (28%)	30 (28%)
	Severe	25 (24%)	22 (21%)
FAN	None	68 (65%)	79 (74%)
	Mild	31 (30%)	22 (21%)
	Severe	6 (6%)	6 (6%)

Table 6.4 Multivariable binary logistic regression for associations with presence of FAN and AVN. FAN = focal arteriolar narrowing, AVN = arteriovenous nicking, OR = Odds ratio, CI = Confidence interval

	OR predicting presence of AVN (95% CI) and p value	OR predicting presence of FAN (95% CI) and p value
Age (yrs)	0.98 (0.95-1.01) p=0.16	0.99 (0.96-1.02) p=0.52
Male Gender	0.89 (0.43-1.82) p=0.75	1.13 (0.61-2.10) p=0.70
Diabetes	1.29 (0.45-3.68) p=0.63	0.83 (0.35-1.96) p=0.67
Hypertension	2.05 (1.00-4.19) p=0.049*	1.18 (0.62-2.25) p=0.61
Lacunar Stroke Subtype	1.18 (0.59-2.37) p=0.63	1.55 (0.84-2.84) p=0.16

*significant p<0.05

6.5 Discussion

Retinal microvessel morphology differs between lacunar and cortical ischemic stroke subtypes. Increased retinal venular diameters and decreased AVR are both independently associated with lacunar rather than cortical stroke subtype, supporting the concept of a distinct small vessel vasculopathy in cerebral small vessel disease. We have not demonstrated a strong association between retinal arteriolar diameter, focal arteriolar narrowing or arteriovenous nicking and lacunar stroke subtype. The cross-sectional design means that we can only report on associations so we do not know whether these retinal vessel differences are longstanding and predispose to the lacunar phenotype, or are acquired in later life as part of the lacunar disease. Nor can we tell whether they are causative or associative. The parity of arteriolar parameters (width, AVN and FAN) between stroke subtypes coupled with associations between hypertension and AVN and decreased arteriolar width suggest that arteriolar parameters reflect exposure to vascular risk factors. In response to these risk factors some patients may develop lacunar and others a large artery (cortical) stroke phenotype.

No previous studies have compared quantitative vessel width measurement, AVN or FAN between ischemic stroke subtypes. Previous studies did not subtype stroke and compared retinal arteriolar parameters between subjects with stroke and those without. Those that combined arteriolar and venular diameters into the dimensionless AVR produced conflicting results about associations with both future stroke and history of stroke (Ikram *et al.* 2006a; Klein *et al.* 2000; Mitchell *et al.* 2005; Wong *et al.* 2006). Changes in AVR were previously thought to reflect only arteriolar narrowing (Hubbard *et al.* 1999), known to change in response to systemic disease, but it is now known that venular diameter also varies (Ikram *et al.* 2004). Those studies which analyzed arteriolar and venular widths separately found that the increase in AVR predicting the presence of any stroke (of any subtype) compared with absence of stroke was due to venular widening rather than arteriolar narrowing (Ikram *et al.* 2006a; Ikram *et al.* 2006b; Wong *et al.* 2006).

There are also conflicting reports regarding the associations between AVN and FAN and any stroke (Cooper *et al.* 2006; Klein *et al.* 2000; Longstreth, Jr. *et al.* 2007; Mitchell *et al.* 2005; Wong *et al.* 2003). This heterogeneity may reflect inherent methodological difficulties in identifying stroke, i.e. studies which showed an association with stroke tended to use MRI-based definitions of cerebral infarct rather than clinical definitions. However it is uncertain what "holes" in the brain represent (Wardlaw 2008), and case record assessments are of limited accuracy for diagnosing stroke (Piriyawat *et al.* 2002). We were not able to find any difference in retinopathy between lacunar and large artery stroke as reported in the previous chapter of this thesis despite many previous reports of associations between retinopathy and stroke. The lack of differences in arteriolar changes between stroke subtypes in our study could be because the retinal arteriolar circulation does not accurately reflect cerebrovascular disease, because this studies may not be powered to identify these subtle, fractions of a micron (for CRAE) and subjective (for AVN/FAN), differences. Alternatively retinal features may simply reflect exposure to systemic risk factors and not be specific for small versus large artery disease.

The finding that increased venular diameter and decreased AVR are associated with lacunar compared with cortical ischemic stroke subtype is novel, and may provide useful insights into the microvascular mechanisms of lacunar stroke. Two population-based studies showed that increased venular diameter predicted future risk of any clinical stroke (Ikram *et al.* 2006a; Wong *et al.* 2006). One study found larger CRVE in subjects with lacunes seen on MRI (Ikram *et al.* 2006b), but again silent lacunes are of uncertain clinical relevance (Wardlaw 2008).

Why should venular diameters and AVR differ in lacunar stroke? Increased venular width is associated with plasma markers of inflammation ((De Jong *et al.* 2007; Klein *et al.* 2006; Wong *et al.* 2006), decreased arteriolar oxygen saturation levels (De Jong *et al.* 2008) and recently with decreased endothelial function measured with forearm plethysmography and decreased flow mediated dilatation in the brachial artery (Nguyen *et al.* 2010). Plasma markers of endothelial activation and inflammation are elevated in patients with cerebral small vessel disease compared to

age matched controls but raised inflammatory markers might simply reflect any vascular disease (Hassan *et al.* 2003b). Thus, increased venular diameter could reflect an inflammatory component in patients with lacunar stroke, but clarification of this would require direct comparisons of plasma inflammatory markers between ischemic stroke subtypes.

The strengths of the present study are the subtyping of ischemic stroke and the use of cortical stroke patients to control for potential confounding risk factors and that stroke was diagnosed by an expert with MRI at presentation. We maintained careful blinding of brain and retinal images to each other and to clinical features throughout. We used detailed retinal photos, careful training of retinal graders and validated assessment methods. CRAE and CRVE are correlated, therefore models assessing multivariable associations with either need to correct for the other (Liew *et al.* 2007a), something which previous studies of retinal arteriolar diameter have not done (Ikram *et al.* 2006a; Ikram *et al.* 2004; Ikram *et al.* 2006b; Wong *et al.* 2006). In order to minimize confounding of stroke subtyping we performed a sensitivity analysis excluding patients with a history of previous stroke which did not change the results for the total study population. A larger sample size might be needed to show differences in arteriolar diameters, FAN and AVN. We have also used a concrete definition of small vessel disease, ie clinically evident lacunar stroke with best available imaging backup rather than a purely imaging or clinical based definition or one with uncertain relevance even to stroke.

Since the first publication of the results detailed in this chapter (April 2009), our Australian collaborators from the Multicentre Retinal Stroke Study (MCRS) have published the main findings from their large cohort (n=1321) (Lindley *et al.* 2009). The detailed comparisons between our findings and those of the MCRS study are presented in chapter 10 of this thesis and are not straight forward as although the overall sample size of the MRCS study was large there were a number of different stroke subtyping classifications and these subgroup analyses were further stratified by the presence of diabetes. In general, the MCRS found that lacunar stroke was associated with widened venules and narrower arterioles although the strength of the

latter association was weaker than that for venules and the absolute differences were small. Furthermore these associations were not present consistently across the different subtyping methods (and the association with wider venules only present in those patients without diabetes).

The MCRS also reported in some of the subgroup analyses that lacunar stroke was associated with an increased prevalence of focal arteriolar narrowing and arteriovenous nicking although again the odds ratios were not large. These results are consistent with the results presented in this thesis and it is interesting to note that arterioles in lacunar stroke patients were 2 microns smaller than those in non-lacunar stroke patients. We found a similar 2 micron difference but perhaps our study was underpowered to show statistical significance with such a small absolute difference.

Chapter Seven: Retinal vascular geometry in ischaemic stroke subtypes.

7.1 Aims of chapter

In this chapter we assess retinal vascular geometry in ischaemic stroke subtypes studying the hypothesis that patients with lacunar stroke will have altered retinal vascular geometry reflecting small vessel disease compared with patients with cortical stroke.

7.2 Introduction

The exact aetiology of lacunar stroke remains uncertain but possible causes include microatheroma, microemboli, vasospasm or a diffuse arteriopathy possibly related to endothelial dysfunction and altered blood brain barrier permeability (Wardlaw *et al.* 2003). Lacunar strokes are associated with another manifestation of cerebral small vessel disease, white matter hyperintensities on MRI scans (WMH) (Wiszniewska *et al.* 2000) which are in turn associated with ageing (Gunning-Dixon and Raz 2000), cognitive impairment and dementia (Sitoh *et al.* 2004). The exact aetiology of these white matter hyperintensities however remains unknown.

We have demonstrated in chapter 3 of this thesis that retinal vascular abnormalities are associated with stroke but they also predict white matter disease presence and progression (Ikram *et al.* 2006a; Ikram *et al.* 2006b; Wong *et al.* 2002a); and we have previously shown in chapter 6 of this thesis that retinal vessel widths differ between stroke subtypes . Therefore retinal vessel abnormalities may act as markers for cerebral small vessel disease.

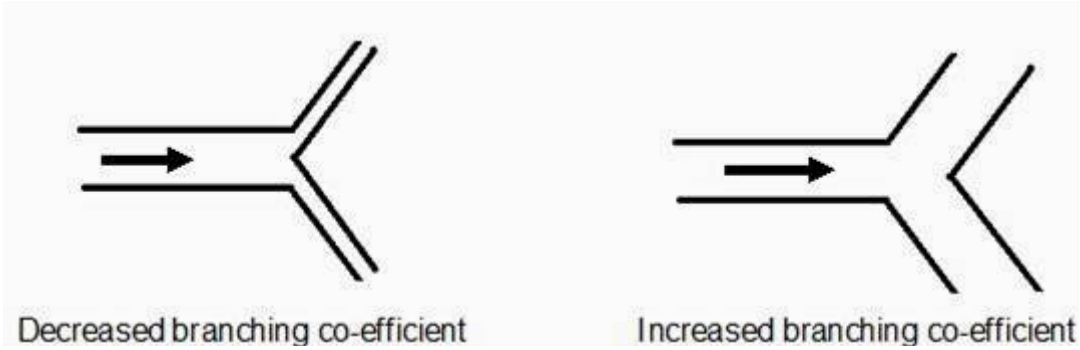
In order to fulfil its role of nutrient delivery the retinal arterial tree (a complex and highly developed structure) needs to efficiently deliver blood to tissues with minimal

power losses at bifurcations yet ensuring that blood velocity slows by the time the capillaries are reached to allow delivery of nutrients yet also minimizing total blood volume. The geometry of arterioles affects the efficiency of the arteriolar circulation in achieving these aims (Murray 1926). There are several different measures of retinal vascular geometry. In this chapter we focus on arteriolar branching co-efficients and branching angles.

The branching coefficient of an arteriolar bifurcation measures the change in cross sectional area across a bifurcation i.e. the ratio of the cross sectional area of the parent arteriole and the cross sectional areas of the two summed daughter vessels. In most arteriolar trees, with every bifurcation the total cross sectional area of the vascular tree increases slightly. Taken to extremes, in humans the cross sectional area of the aorta is much smaller than the total summed cross sectional area of the small arterioles. An increased branching co-efficient across a bifurcation represents wider daughter vessels in relation to the parent vessel and a decreased branching coefficient indicates narrower daughters in relation to the parent vessel.

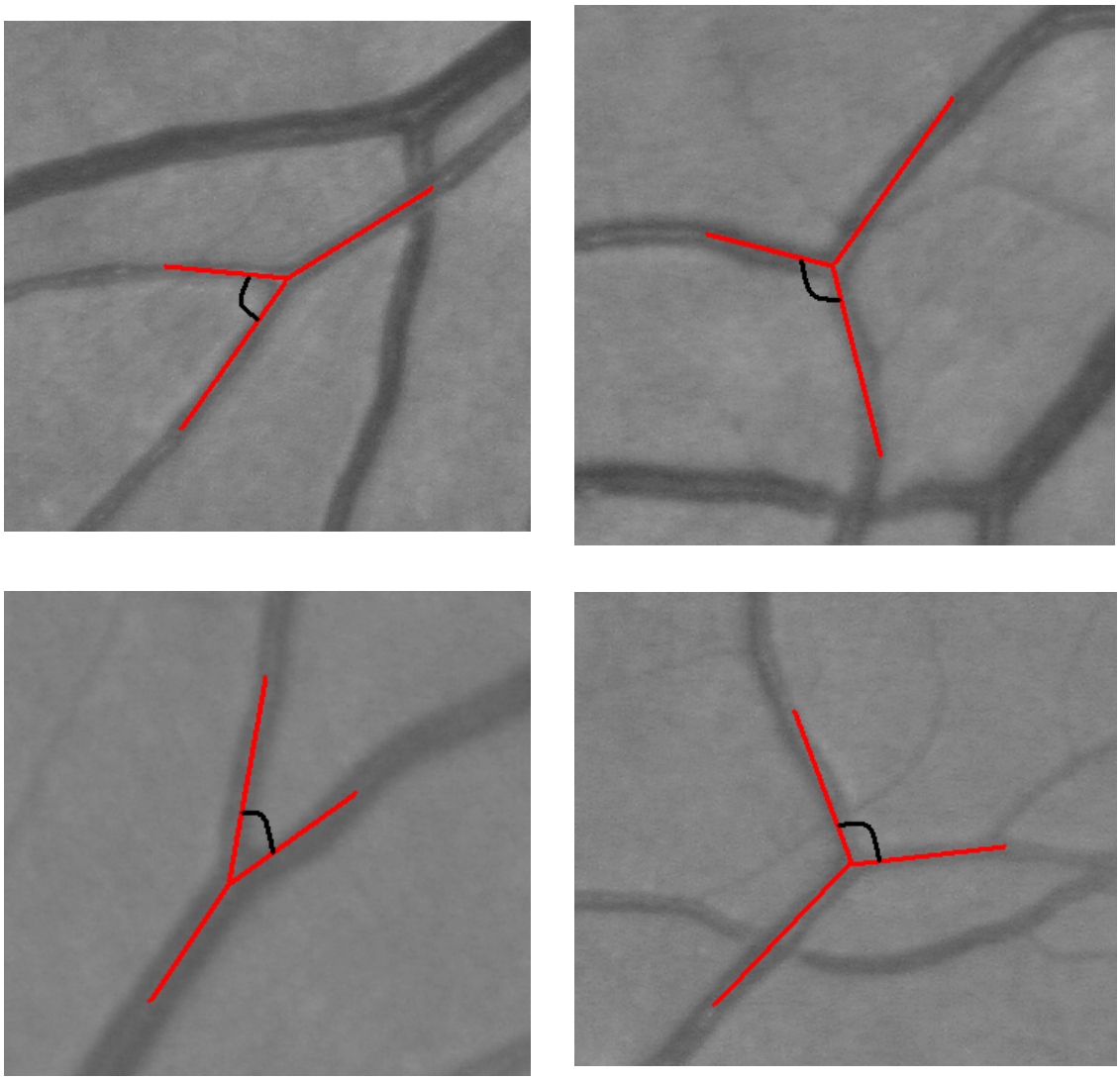
Both increased and decreased branching co-efficients may affect the energy required to deliver blood around the body and hence the efficiency of the circulatory system. A few studies suggest that this theory may hold true in biology; abnormalities in arteriolar branching co-efficient have been associated with cognitive impairment (Patton *et al.* 2007), peripheral vascular disease (Chapman *et al.* 2002) and predict death from ischaemic heart disease (IHD) (Witt *et al.* 2006) although the mechanisms behind these observed changes are not clear and methods used to measure branching co-efficients differ.

Figure 7.1. Examples of inefficient branching co-efficients where blood flow is compromised. With a decreased branching co-efficient (image on the left), blood flow is reduced across the junction resulting in poor blood delivery. With an increased branching co-efficient (image on the right), blood delivery is maximised but in order to maintain such junctions across the arteriolar tree in the human, the blood volume needed would be huge and clearly impractical. Furthermore the design of this junction would not slow down blood flow and one of the functions of the vascular tree is to slow down blood flow over each junction to ensure transfer of nutrients at the capillary level.



Retinal arteriolar branching angles represent the angle subtended by the two daughter vessels. A change in absolute angles or deviation away from a theoretical optimum branching angle may affect the efficiency of the arteriolar tree by altering blood flow across the junction and by altering the ability of the vascular network to distribute blood. A few studies have shown associations between retinal arteriolar branching angles and hypertension (Stanton *et al.* 1995) and cognitive function (Patton *et al.* 2007) but not death from stroke (Witt *et al.* 2006).

Figure 7.2. Retinal photographs illustrating retinal arteriolar branching angles (curved black lines) and demonstrating range of arteriolar branching angles in the human retina.



We hypothesised that if cerebral small vessel disease is due to an intrinsic small vessel abnormality that might impair the efficiency of nutrient delivery, then patients with cerebral small vessel disease (defined as either lacunar stroke or white matter hyperintensities on MRI) would have altered retinal arteriolar branching co-efficients

and branching angles compared with patients with large artery atheromatous stroke or patients with fewer or no white matter hyperintensities.

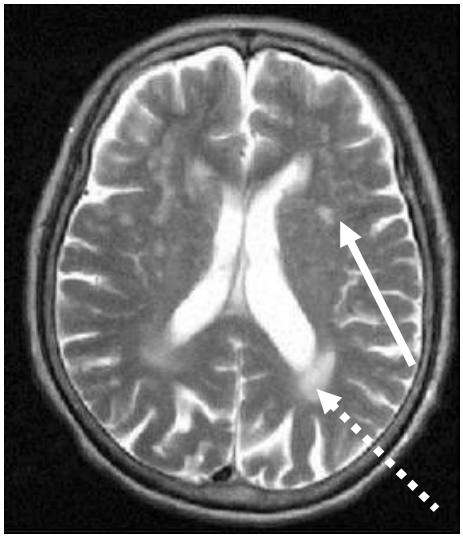
7.3 Methods

We recruited patients with lacunar or mild cortical stroke from our university hospital based stroke service as detailed in chapter 2 of this thesis. Patients had diagnostic brain MRI at presentation and six field retinal photography (centred on the disc, macula, lateral macula, nasal to the disc, upper arcade and lower arcade) of the left and right eyes, with 1% tropicamide eye drops where necessary, using a Canon CR-DGi digital retinal camera (Canon USA Inc.).

7.3.1 MRI analysis

MRI scans were coded for the presence, location and size of the recent infarct and any old infarcts or haemorrhages by a neuroradiologist blind to additional clinical details. MRI scans were also coded for deep (lesions not contiguous with the ventricles) and periventricular (lesions contiguous with the ventricles) WMH with the Fazekas scale which rates lesions in both regions from 0-3 (Fazekas *et al.* 1987). Those whose ages were outside the template range were matched to the nearest age.

Figure 7.3. T2 weighted MRI showing deep (solid arrow) and periventricular (dotted arrow) white matter hyperintensities.



7.3.2 Retinal assessment

For each patient we selected photographs centred on the optic disc for each eye. Images were analysed within custom written Matlab software (The Mathworks Inc, Natick, NA) blind to all clinical and imaging details. Colour images were converted to greyscale and processed to maximize the contrast between vessel and background.

7.3.3 Branching co-efficient assessment

A single trained grader identified the five most proximal measurable arteriolar junctions to the optic disc and used semi-automated computer software to measure the branching co-efficient of each bifurcation. The grader identified the centre point of each bifurcation and placed a cursor on the parent vessel and each daughter vessel. The software then tracked down each vessel from the centre point of each bifurcation (Figure 7.5) fitting a profile of signal intensity at right angles to the longitudinal axis

of the vessel with a Gaussian curve to determine the width of each vessel (See figure 7.6).

Figure 7.5. Measurements across a bifurcation. The software tracks down each branch from a central point to obtain width measurements from which to calculate BC. Please note in this image we have used a venule for illustration purposes.

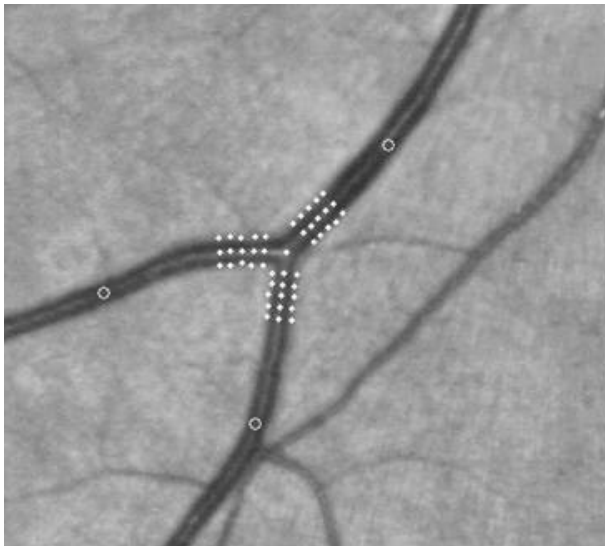
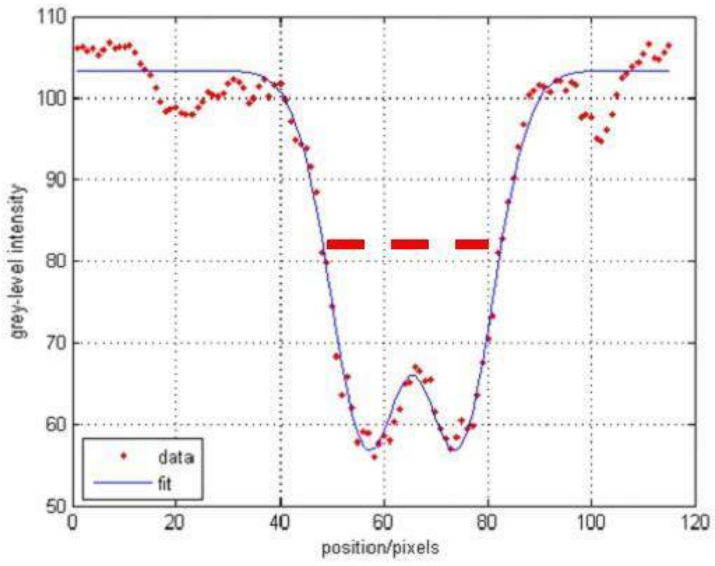


Figure 7.6. Vessel profile: Plot of pixel intensity along a transect across a vessel with superimposed Gaussian Line. The width is taken as the half-height width measure and represented by the dashed line.



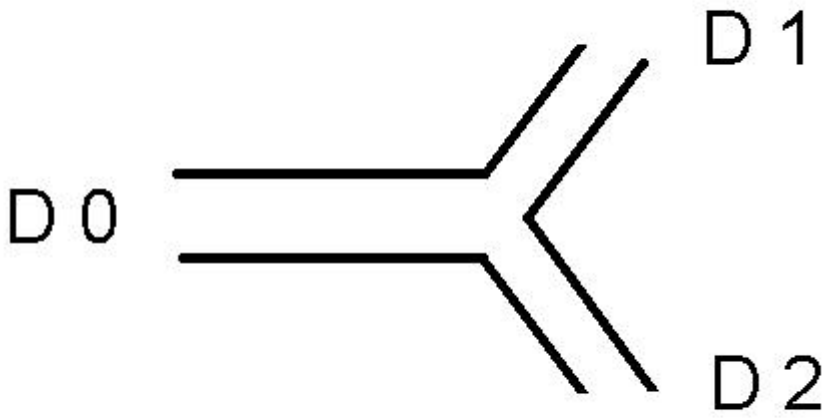
Each profile was manually inspected and rejected if the Gaussian line did not fit well (r correlation <0.7). The mean of at least 3 profiles were used to calculate the width of the vessel which was accepted by the grader if the standard deviation of the mean was less that 10% of the vessel width.

We validated this process with Bland Altman plots comparing software performance to best human measurement (with a calliper on enlarged images) and found no evidence of systematic bias and that the mean difference between human and software measurements for 50 randomly chosen vessels was 0.006 pixels (95% CI -3.3 to 3.3 pixels) see figure 6.4 in preceding chapter for further details

The widths of the parent and daughter vessels were combined to produce the branching co-efficient with the following formula illustrated in Figure 7.3: (Patton *et al.* 2007)

Figure 7.4. Illustration of branching co-efficient calculation where BC = branching coefficient, D1 = width of daughter vessel 1, D2 = width of daughter vessel 2 and D0 = width of parent branch

$$BC=D_1^2 + D_2^2 /D_0^2$$



We aimed to measure the branching co-efficients of the 5 most proximal measurable bifurcations to the optic disk but included patients in whom 3 or more were measurable in our primary analysis. In a prespecified sensitivity analysis, we also analysed patients who had at least 5 branching co-efficient measurements. We avoided assuming that branching co-efficients within each eye were normally distributed by taking the median branching co-efficient for each eye.

The within patient correlation between left and right eyes showed a Pearson's correlation co-efficient 0.53 for 32 randomly chosen patients and we randomly chose the left or the right eye from a list or randomly generated numbers eye to measure where photographical quality was suitable for both eyes. If one eye had an unsuitable photographic quality we used the other eye. The intra-rater intra class

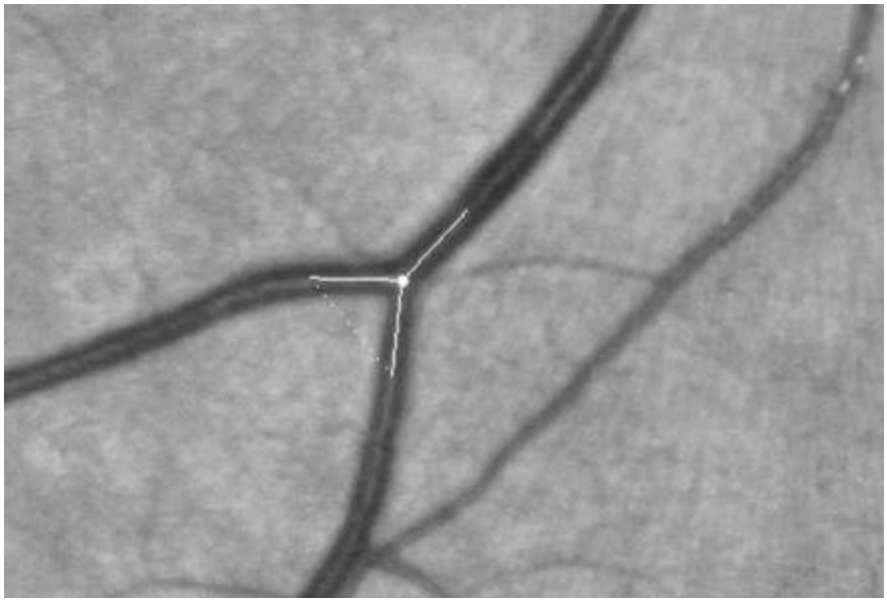
correlation co-efficient for a random sample of 10 images with analyses performed 2 weeks apart was 0.82.

Our primary aim was to investigate absolute branching co-efficients but we also assessed deviation from a theoretically calculated optimum branching coefficient of 1.26 (Murray 1926; Patton *et al.* 2007). The optimum branching co-efficient of 1.26 assumes that the daughter vessels are of equal width but when the daughter vessels have asymmetrical widths the optimum varies between 1.00 and 1.26, according to the asymmetry index which is the ratio of the smaller daughter diameter over the larger daughter diameter (Zamir 1978). We used the corrected (for asymmetry) optimum bifurcation specific branching co-efficient to calculate the deviation for each angle. We took the absolute deviation for each eye and then normalized the data with a square root transformation to permit linear regression.

7.3.4 Arteriolar branching angles

A single trained grader (Rosemarie den Haan) identified the 5 most proximal measurable junctions to the optic disc, selected the centre point of the bifurcation and the parent and daughter vessels. The software tracked down each vessel to a point two parent vessel widths from the bifurcation (where it is suggested from theoretical modelling that turbulent flow after the bifurcation becomes laminar (Tadjfar 2004), and drew a line reflecting the course of the vessel. The branching angle was calculated using the Cosine rule (figure 7.7). Due to the large variation in angles within each eye, we only included patients in the analysis in whom we were able to perform 5 angle measurements to provide a more reliable average than 3 angle measurements. As we could not assume normal distribution of angles within each eye we took the median of the five angles from each eye.

Figure 7.7. Illustration of measurement of branching angle – the angle represented by the dotted white line. Please note for illustrative purposes the vessel in the figure is a venule but we measured branching angles of arterioles.



The correlation between angles in the left and right eye was poor (Pearson correlation co-efficient 0.23 for 27 randomly chosen patients) and we therefore measured angles in both eyes where possible to give a median value for each eye. We then took the mean of these two values to give an angle measurement for each patient.

An alternative approach to assessing vascular branching efficiency is to measure deviation from the optimum branching angle (theoretically calculated as 75 degrees) (Murray 1926; Patton *et al.* 2007) for each bifurcation and we assessed the median deviation from the optimum for each eye. In a random sample of 10 photographs graded 2 weeks apart the intra-rater class correlation coefficient for median angle was excellent at 0.961.

7.3.5 Statistical analysis

All analyses were performed within Minitab (version 14, Minitab Inc, PA, USA). We compared baseline characteristics between the two stroke groups with t-tests, Mann-Whitney U tests and differences in proportions. The branching coefficients and branching angles were normally distributed, as were the deviations from the optimum branching coefficient and angles (after square root transformation) and we therefore performed multivariable linear regression with branching co-efficient and branching angles as the continuous outcomes and vascular risk factors, stroke subtype and WMH as the independent explanatory variables. We set an alpha level for significance of 0.05.

7.4 Results

We recruited 205 patients with mean age 68.0 years (SD11.6). There were 104 lacunar strokes (51%) and 101 cortical strokes (49%) and 135 patients were male (66%). We could not measure at least 3 branching co-efficients in 24 patients (due to poor quality of the photograph, a paucity of bifurcations in the field of view, or local anatomical variations precluding computer measurements of vessel widths). Therefore 181 patients were included in the analysis of branching co-efficients. We were not able to measure 5 branching angles in at least one eye in 61 patients and therefore included 144 patients in the analysis of branching angles. The 24 patients excluded from the branching co-efficient analysis were older (74.5 SD 8.52 years v 67.1 SD 11.7 years) and the 61 patients excluded from the branching angle analysis more often had hypertension (75% v 56%) but did not differ in other respects from the patients in whom these measurements were possible. The baseline characteristics of the 181 patients with at least 3 branching co-efficients are shown in table 7.1.

Table 7.1. *Baseline characteristics of 181 patients included with at least 3 measurable branching co-efficients.*

Characteristic	**Lacunar stroke**	**Cortical stroke**	**P value for difference**
n	94	87	
Mean age (SD) years	65.2 (11.5)	69.2 (11.5)	0.02
Male n (%)	53 (56%)	62 (71%)	0.04
AF n (%)	4 (4%)	11 (13%)	0.05
Carotid stenosis>50% n (%)	4 (5%)	10 (12%)	0.08
Median Deep WMH Fazekas score (IQR)	1 (1-2)	1 (1-2)	0.97
Median periventricular WMH Fazekas score (IQR)	1 (1-2)	1 (1-1)	0.50
Past medical history of			
Hypertension n (%)	57 (66%)	53 (56%)	0.21
Diabetes n (%)	18 (19%)	10 (11%)	0.15
Ischaemic Heart Disease n (%)	13 (14%)	23 (26%)	0.03
Peripheral Vasc. Disease n (%)	3 (3%)	1 (1%)	0.62
Previous Stroke/TIA n (%)	20 (21%)	17 (20%)	0.77

AF = atrial fibrillation, WMH = white matter hyperintensity, TIA = Transient Ischaemic Attack, IQR = inter quartile range SD = standard deviation.

7.4.1 Arteriolar branching co-efficients

Of the 181 patients in the branching co-efficient analysis, the mean branching co-efficient was 1.44 with a standard deviation of 0.19. There was no difference in mean branching co-efficients between lacunar (1.43, SD 0.17) and cortical stroke (1.44, SD 0.20). On multivariable linear regression (Table 7.2), the presence of IHD and increased periventricular white matter hyperintensities were both significantly and independently associated with an increased branching co-efficients (representing

wider daughter vessels in relation to the parent vessel) and increased deep white matter hyperintensities was significantly and independently associated with a decreased branching co-efficients (representing narrower daughter vessel diameters in relation to the parent vessel). In our pre-specified analysis of patients with 5 branching co-efficients measured in an eye (n=119) the relationships between branching co-efficient and IHD and deep white matter hyperintensities remained but the association between periventricular white matter hyperintensities and branching co-efficient was attenuated and became non-significant (data not shown). When we looked at deviation from the optimum branching co-efficient we found that the results did not change from those in Table 7.2.

Table 7.2. Multivariable adjusted associations with absolute retinal arteriolar branching co-efficient. All values are corrected for the presence of all of the other variables in the table. TIA = transient ischaemic attack, WMH = white matter hyperintensity,

Variable	**Beta co-efficient**	**P value**
Lacunar stroke subtype	-0.001	0.96
Age	-0.001	0.70
Deep WMH	-0.076	0.003
Periventricular WMH	0.072	0.006
Past history of:		
Hypertension	-0.020	0.50
Diabetes	-0.032	0.38
Ischaemic heart disease	0.155	<0.001
Stroke/TIA	0.040	0.25
Peripheral vascular disease	0.032	0.73

7.4.2 Arteriolar branching angles

In the 144 patients with 5 angles measured per eye, we found that the mean branching angle was 84.1º with a standard deviation of 7.1º. Median arteriolar

branching angles did not significantly differ between lacunar (mean 85.2º SD 7.3º) and cortical stroke (mean 83.0º SD 7.3º, difference = 2.3 95% CI 0.0 to 4.6 p=0.054). On univariable and also on multivariable analysis, only a history of PVD was associated with increased branching angle (Table 7.3), but note there were very few patients with PVD on which to base this analysis (n=4). Retinal branching angles were not associated with either deep or periventricular white matter hyperintensities. We assessed deviation from an optimum branching angle of 75º but the associations shown in Table 7.3 did not change.

Table 7.3. Multivariable adjusted associations with absolute retinal arteriolar branching angles. *All values are corrected for the presence of all of the other variables in the table. TIA = transient ischaemic attack, WMH = white matter hyperintensity,*

Variable	Beta co-efficient	P value
Lacunar stroke subtype	2.22	0.07
Age	-0.03	0.61
Deep WMH score	1.12	0.34
Periventricular WMH score	-0.52	0.65
Past history of:		
Hypertension	1.27	0.33
Diabetes	0.95	0.57
Ischaemic heart disease	0.51	0.75
Stroke/TIA	-0.33	0.83
Peripheral vascular disease	9.05	0.006

7.5 Discussion

We have shown that increased retinal arteriolar branching co-efficients are associated with increased periventricular white matter hyperintensities and a history of ischaemic heart disease in patients presenting with mild stroke. Decreased retinal arteriolar branching co-efficient are associated with increased deep white matter hyperintensities but branching co-efficients are not associated with ischaemic stroke subtype. We have not demonstrated significant associations between retinal arteriolar branching angles and ischaemic stroke subtype, white matter hyperintensities or most other vascular risk factors. No previous studies have assessed retinal vascular geometry within ischaemic stroke subtypes or their relationship with white matter hyperintensities.

It is intriguing that deep and periventricular white matter hyperintensities appear to be associated with opposing directions of altered branching co-efficient. A decreased branching co-efficient indicates that the daughter vessels are narrower with respect to the parent vessel and an increased branching co-efficient that the daughters are wider. Pathological studies have indicated that the mechanism of fluid accumulation and subsequent tissue damage in deep and periventricular white matter hyperintensities may differ (Fernando *et al.* 2006) as deep lesions may have more "ischaemic" causes whilst periventricular changes may be secondary to disruption of the ependymal lining of the ventricles (Fazekas *et al.* 1998). Deep and periventricular white matter hyperintensities may have slightly different associations with vascular risk factors (de Leeuw *et al.* 2000). Decreased total cerebral blood flow is associated with the development and worsening of periventricular but not deep white matter hyperintensities (ten Dam *et al.* 2007). Lower B12 levels in lacunar stroke patients are associated with increased periventricular white matter hyperintensities but not deep white matter hyperintensities (Pieters *et al.* 2009), a finding replicated independently in a separate cohort (de Lau *et al.* 2009). Although not definitive, these results suggest that the two types of white matter hyperintensities may have differing causes and we would suggest that deep and

periventricular hyperintensities should, at least for the present, be considered separately in the assessment of white matter disease (Sachdev and Wen 2005).

Nitric oxide levels are associated with altered branching co-efficients (Chapman *et al.* 2000). Endothelial dysfunction may have an aetiological role in the development of white matter hyperintensities (Markus 2008) so it is possible that disordered nitric oxide production may cause both branching co-efficient changes and white matter disease. Clearly more work is needed to determine whether these observations are true and if so to clarify their association.

We did not find an association between lacunar stroke subtype and retinal arterial branching co-efficient or branching angles. There were limited data on which to base sample size calculations and this study may be underpowered to identify differences between stroke subtypes, particularly when features such as white matter hyperintensities were common in both cortical and lacunar stroke subtypes and may have interacted in the analysis of lacunar versus cortical stroke.

The association between IHD and increased daughter vessel width with respect to the parent vessel is interesting and validates a previous study showing that increased branching co-efficients in a cohort of middle aged adults without vascular disease at baseline predicted death with ischaemic heart disease but not stroke, although the number of stroke deaths was small, n=28 (Witt *et al.* 2006). The exact mechanisms behind this observation are unclear. It is probably not simply attributable to medication as both our patient groups were taking similar medications and medication is not known to affect certainly retinal vessel widths (Wong *et al.* 2005b) although the associations with branching co-efficient have not been studied. The true pathophysiological significance of branching coefficients is not known, nor whether these are fixed from birth, alter with age, predispose to or change in the presence of disease. It is therefore difficult to speculate on whether increased branching coefficients might predispose to or be a response to large artery disease. Further studies are needed to examine this interesting finding.

We found no significant associations with branching angles (the apparent association with PVD is based on too few patients so is unlikely to be clinically significant), consistent with some previous studies finding no link between angles and hypertension (Chapman *et al.* 1997), peripheral vascular disease (Chapman *et al.* 2002) and death with IHD and stroke (Witt *et al.* 2006).

The concept of measuring deviation from optimality deserves further comment. In our cohort, the observed mean of 1.43 (SD 0.19) was higher than the optimum theoretically derived branching co-efficient of 1.26. As the majority of our patients had positive deviations from the theoretical optimum, our results did not alter when we assessed deviation from the optimum branching co-efficient rather than absolute values, consistent with other studies (Witt *et al.* 2006). In study populations where the mean branching co-efficient (or branching angle) is nearer to the theoretical optimum, assessing deviation rather than absolute angles leads to more diverse results (Patton *et al.* 2007). It is appealing to use the deviation away from the optimum but not all theories attempting to explain biological systems hold true in vivo. Data are too sparse to know whether optimality of branching coefficients and angles differ between arteriolar beds or patient populations and until more is known further studies should assess both absolute and deviation from optimum values.

The strengths of this study include prospective recruitment and careful assessment of all patients at the time of the stroke by an experienced physician with diagnostic MRI scans graded by an experienced neuroradiologist. Assessment of retinal images was blind to clinical and imaging details. We used a specifically written semi-automated software program to assess retinal vessels which minimized human operator variability resulting in excellent intra-rater repeatability scores. We found that angles did not correlate well between left and right eyes so measured both eyes where possible. This poor correlation between left and right eye further questions whether angles have anything to do with systemic disease. Previous studies either did not specify which eye was measured (Patton *et al.* 2007), looked at one eye only or combined eyes to achieve 5 measurements (Witt *et al.* 2006). This latter approach assumes that angles do not differ between the eyes. There is no published data on the

strength of the association between left and right eyes. That we did not find a strong association between left and right eyes suggests that retinal arteriolar branching angles may not predispose to or change in response to systemic disease. We would suggest that future studies investigating retinal branching angles stipulate which eye they used and presented data regarding left-right correlation.

Weaknesses should also be acknowledged. Although use of the semi-automated software improved measurement accuracy and repeatability, it limited the number of patients that we were able to include because, unlike a human operator, the semi-automated software is not able to make allowances for anatomically difficult vessels ie those with indistinct edges or where a venule is in close proximity to an arteriole. The sample size may not have been large enough to account for interactions between stroke subtype, WML and other key variables like age. We designed our study using all available data to date on retinal changes and stroke (Doubal *et al.* 2009). However despite including over 60,000 patients, this literature is limited by lack of precision in stroke diagnosis and subtyping and included few strokes. The theoretical optimum angles and coefficients are just that – theories based on modelling of the circulation as a series of tubes. It is a further limitation that due to a lack of resources we were unable to perform inter-rater reliability testing for retinal arteriolar branching co-efficients and angles which may limit the usefulness of this metric.

Although the concept of using retinal vascular geometrical parameters to reflect cerebral disease is appealing, these techniques are in their infancy and more research in different vascular beds, in response to pharmacological challenges, at different ages, and in the presence of different diseases is required before the real implications of vascular geometry become clear.

Chapter Eight. Fractal analysis of retinal vessels in ischaemic stroke subtypes

8.1 Aims of chapter

In this chapter we describe the how fractal analysis can be applied to the retinal vascular tree and how the fractal properties of the retinal vasculature differ in ischaemic stroke subtypes.

8.2 Introduction

Cerebral small vessel disease causes lacunar stroke, white matter hyperintensities (WMH) on magnetic resonance scanning, cognitive decline, dementia and depression. The exact small vessel abnormalities are uncertain as they are difficult to study. Retinal microvascular abnormalities are associated with white matter disease (Wong *et al.* 2002a). I have demonstrated earlier in this thesis that retinal microvascular abnormalities are associated with future risk of stroke and having had a stroke and differ in ischaemic stroke subtypes, findings confirmed elsewhere (Lindley *et al.* 2009; McGeechan *et al.* 2009a). Therefore, retinal microvascular abnormalities may act as a surrogate marker for cerebral small vessel disease.

Prior research investigating retinal abnormalities in cerebral small vessel disease has focussed on focal retinal abnormalities (eg retinopathy), vessel widths (Lindley *et al.* 2009) or simple geometrical abnormalities (eg branching angles and branching co-efficients) (Witt *et al.* 2006). The retinal tree is a complex structure and these techniques may not adequately characterise the retinal vascular tree as most of these techniques focus on sampling small sections of the vascular tree. Width measurements, for example are derived from 6 short arteriolar and 6 short venular segments and the geometrical abnormalities presented in chapter 7 of this thesis rely on data from 3 or 5 bifurcations. These techniques therefore do not assess a

considerable proportion of the retinal vascular tree and as a result may have a low sensitivity for the identification of subtle global changes. An approach which uses a global measure of the entire visible retinal vascular tree might be more sensitive at identifying subtle early retinal vascular changes perhaps before macroscopic abnormalities are present.

"Fractal analysis" is a complex geometrical measure that can be used to characterise whole structures which branch in a repetitive manner, are self-similar at different scales, and can be applied to assessment of the retinal vascular tree (Masters 2004). The fractal dimension measures how a structure fills space or the degree of branching complexity of a structure. An increased fractal dimension represents increased branching complexity and a decreased fractal dimension represents decreased branching complexity. Initially, assessment of retinal vessel fractals involved laborious and time consuming (up to one hour per eye to process) hand tracing of the vascular tree to obtain segmented representations of the vascular tree. This effectively precluded the assessment of large numbers of patients and therefore hindered the detection of small differences requiring large samples. We have previously published details of a computer assisted automated technique for the assessment of fractal dimensions using skeletonised images (MacGillivray *et al.* 2007). This technique enables a large number of images to be processed much quicker than previously – 5 minutes rather then 60 minutes per image. Recently, increased retinal vessel fractal dimensions have been associated with retinopathy in type 1 diabetic patients aged 12-20 (Cheung *et al.* 2009) and decreased fractal dimensions with older age and increasing blood pressure (Liew *et al.* 2008b).

In this chapter we investigate the associations between fractal properties of the retinal vessels in patients with clinical and MRI features of lacunar stroke and white matter disease to test the hypothesis that patients with lacunar stroke (as a distinct marker of cerebral small vessel disease) will have altered fractal properties of their retinal vessels compared with a cortical stroke control group (as a distinct marker of large artery atherothromboembolic disease).

8.3 Methods

These are detailed in chapter 2 of this thesis but in brief we recruited patients presenting with clinical lacunar or minor cortical stroke.

Patients had cerebral Magnetic Resonance Imaging (MRI) at presentation, on a 1.5-T MR scanner (Signa LX; General Electric) with 22 mT m^{-1} maximum strength gradients. All patients had six field retinal photography (centred on the disc, macula, lateral macula, nasal to the disc, upper arcade and lower arcade) of the left and right eyes, with 1% tropicamide eye drops where mydriasis was necessary, using a Canon CR-DGi digital retinal camera (Canon USA Inc.).

8.3.1 MRI analysis

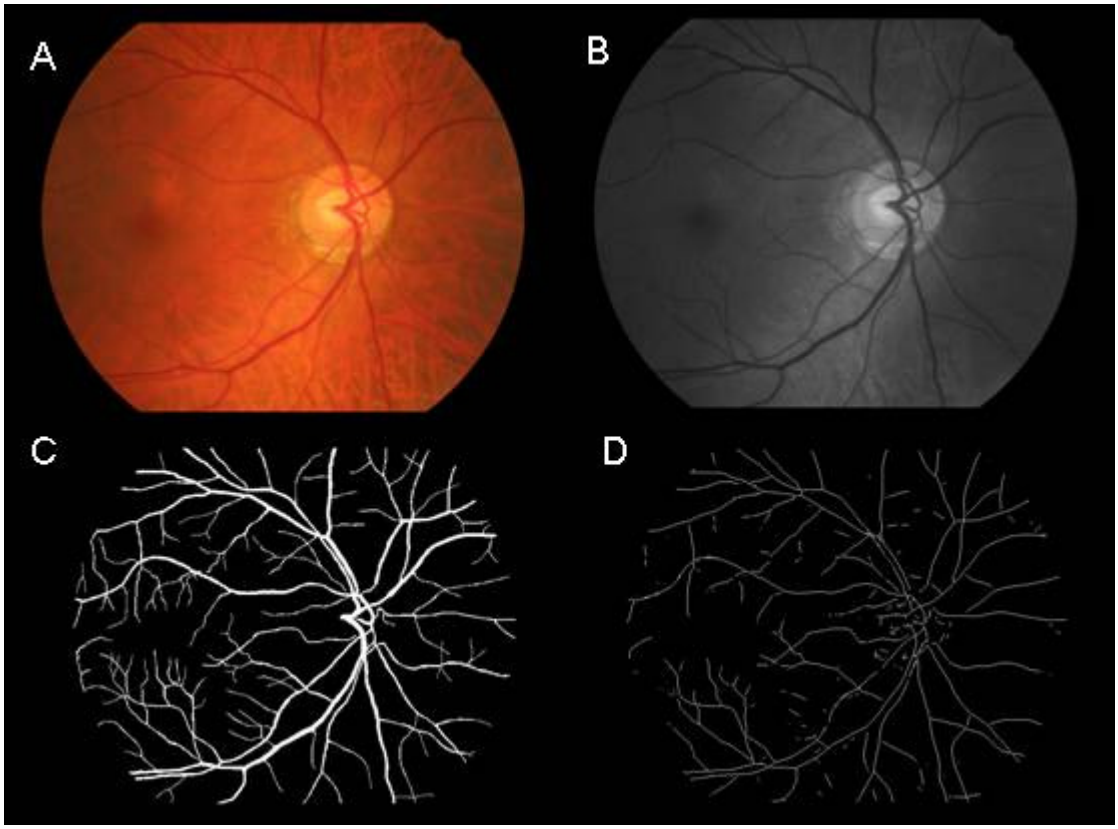
All MRI scans were coded for the presence, location and size of the recent infarct and any old infarcts or haemorrhages and white matter hyperintensities by an experienced neuroradiologist. Scans were coded for deep and periventricular WMH according to the Fazekas scale (Fazekas *et al.* 1987) from 0-3.

8.3.2 Retinal image analysis

For each eye we chose the image centred on the optic disc. Retinal images were analyzed in Matlab (The Mathworks Inc., USA). Image processing was performed on the green channel of the colour fundus images as this typically exhibits the greatest contrast between vessel and background. All algorithms were performed with custom written software (by Dr Tom MacGillivray) within Matlab (The Mathworks Inc., USA). We combined fractal analysis with an automatic vessel segmentation procedure to speed up analysis. We found that the left and right eye fractal dimensions were correlated (Pearsons co-efficient 0.53), replicating previous results (Masters 1994) and we therefore chose the right eye, where available, for analysis. If the right eye retinal photograph was not suitable we used the left eye.

We converted colour images (figure 8.1, top left image A) to greyscale (figure 8.1, top right image B). We then segmented (traced) the retinal vascular tree (arterioles and venules) using an algorithm previously described (Soares *et al.* 2006) which denotes each pixel in the retinal image as being vessel or non-vessel to produce segmented images (figure 8.1, bottom left image C). We trained and tested our implementation of this algorithm using a set of 20 retinal images (that were sourced from a separate study of patients aged between 70-72) that had been manually segmented by two human observers (Dr Tom MacGillivray and Adria Rovira). Before fractal analysis, we manually inspected and corrected each computational segmented image removing obvious artefacts such as noise introduced by areas of low contrast in the image and "ring" object caused by dust on the camera face. Less than 5% of images in this cohort required manual correction. We then skeletonised each image (figure 8.1, bottom right image D) using Matlab's *bwmorph* algorithm, which is based on iterative deletion of pixels (Lam *et al.* 1992). When the image is skeletonised information regarding vessel width is lost

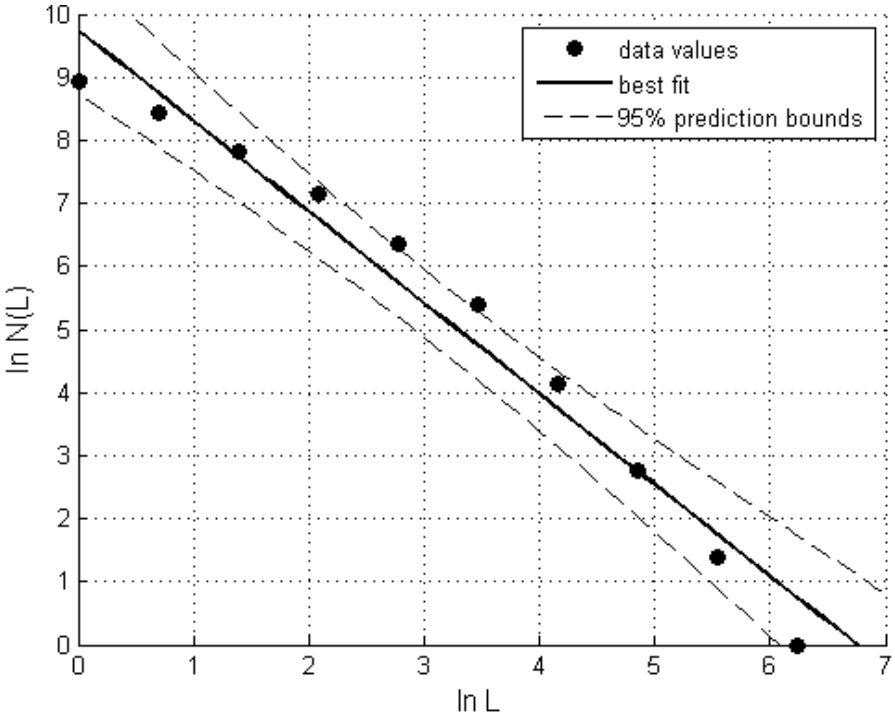
Fig 8.1. Retinal images showing the processing, segmentation and skeletonisation steps. Top left image (A) is the original colour image which is processed to greyscale (top right B) and subsequently segmented to produce the bottom left image (C). Finally, this image is skeletonised to produce the bottom right image (D) upon which fractal analyses are based.



Fractal dimensions can be assessed using a monofractal or a multifractal approach (Stosic and Stosic 2006). We first measured the mono-fractal dimension (Dbox) using the box-counting technique. We covered the skeletonised image with a number of equally sized square boxes and then counted how many of these boxes contained part of the skeletonised retinal vascular tree. We repeated this process with multiple different sized boxes, each time counting how many boxes contained part of the retinal vascular tree and recording the size of the box. We then plotted the logarithm of the number of boxes containing part of the retinal vascular tree against

the logarithm of the size of the box (figure 8.2). The fractal dimension (Dbox) was the slope of the best fit line of these points.

Figure 8.2. Monofractal analysis. Plot showing Logarithm (ln) N(L) where N is the number of boxes containing part of the skeletonised retinal vascular tree and L is the size of the box against the logarithm of L. Dbox (monofractal dimension) is calculated from the slope of the best fit line



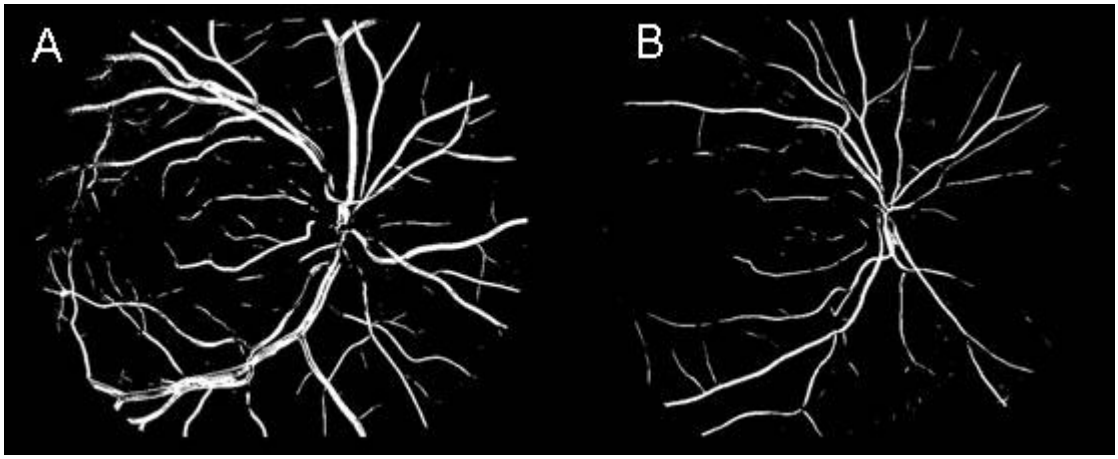
Previous studies have used monofractal analysis of retinal vessels (Cheung *et al.* 2009; Liew *et al.* 2008b) and it is straightforward to calculate monofractal dimensions. It has however been suggested that multi-fractal techniques are better suited to characterizing complex spatial arrangements such as the retinal arteriolar tree which may represent a composite of many monofractal dimensions (Stosic and Stosic 2006). The retinal tree may possess different fractal dimensions depending on the level of scale (or magnification) used. A multi-fractal approach calculates multiple fractal dimensions from randomly chosen points (in this case 1000 points)

within the skeletonised vascular tree (rather than just one dimension from one run with the mono-fractal approach) and is able to assess the effect of scale on the fractal dimension, ie does magnifying the image change the fractal dimension (or degree of branching complexity).

To calculate the multi-fractal dimensions of the retinal vascular tree we used the generalized sand-box method (Stosic and Stosic 2006). We investigated the effect of scale on the fractal dimension and found that the retinal tree clearly exhibited multi-fractal properties as the fractal dimension changed when different scales were used, ie when the image was magnified the fractal dimension changed. If the retinal vasculature was a simple mono-fractal, the fractal dimension would be constant. We used D0 to denote the multi-fractal dimension.

When a mono-fractal dimension is calculated from a retinal vascular tree there is a confidence interval or uncertainty associated with it that is calculated during the fitting of the straight line to the logarithm graph. We found these uncertainties with multifractal analysis to be much lower (<1%) than the uncertainties from mono-fractal analysis which were in the order of 14%. We assessed both mono-fractal and multi-fractal properties of the retinal vessels to compare methods and to permit comparisons with previous studies which used the mono-fractal approach. Figure 8.3 shows the retinal vascular trees with the highest (left) and lowest (right) multi-fractal dimensions in this study.

Fig 8.3. Segmented vessel maps of two retinal vascular trees. The image on the left (A) was the highest fractal dimension in this study (Multifractal D0 1.736) and the image on the right (B) the lowest fractal dimension (Multifractal D0 1.622). Note these images are segmented vessel maps for illustration purposes. We used the skeletonised images to actually perform the fractal analysis.



The intra-grader reliability estimates for this technique were excellent (in 20 randomly chosen images) with intra-class correlation co-efficients of 0.94 for monofractal Dbox and 0.96 for multifractal D0.

8.3.3 Statistical analysis

All analyses were performed within Minitab (version 15). We assessed differences between the lacunar and cortical groups with t-test, differences in proportions and Mann Whitney U tests. Dbox and D0 were both normally distributed and we therefore used multiple linear regression with Dbox and D0 as the dependent variable with age, stroke subtype, white matter hyperintensity scores as independent variables. We modelled deep and periventricular white matter lesion scores as covariates as they are correlated. There were no immediately applicable data upon which to base sample size calculations for the assessment of fractals in cerebral small vessel disease. The power calculation - based upon our pilot study (MacGillivray *et al.* 2007) – found that with 80% power at the 5% level of significance to find a difference between groups of a Dbox of 0.01 with a two sample two tailed t test

which predicted each group should have a minimum of 88 subjects. There were no missing data.

8.4 Results

8.4.1 Patient characteristics

We recruited 183 patients of whom 17 were excluded with poor photographic quality leaving 166 patients for analysis. The mean age was 67.3 years (SD 11.5 years) with a median NIHSS of 2 (IQR 2-3). There were 86 participants with lacunar stroke and 80 with cortical stroke. The baseline characteristics by stroke subtype are detailed in Table 8.1. The lacunar stroke patients were younger with a higher stroke severity than the cortical stroke patients. The prevalence of diabetes, hypertension and white matter hyperintensities did not differ between the groups.

Table 8.1. *Baseline characteristics of participants by ischaemic stroke subtype.*

Feature	**Lacunar stroke**	**Cortical Stroke**	**Difference (lac-cortical) (95% CI)**	**P value for difference**
N	86	80	-	-
Age (mean/SD yrs)	65 (11)	69 (11)	-4 yrs (-8 to -1)	0.02
Gender (n female)	32 (37%)	24 (30%)	7% (-7 to 21)	0.30
Hypertension n	49 (57%)	50 (62%)	-5% (-20 to 9)	0.46
Diabetes n	17 (19%)	8 (10%)	10% (-1 to 20)	0.07
NIHSS (median/IQR)	2 (2-3)	2 (1-3)	-	0.04
DWMH (median/IQR)	1 (1-2)	1 (1-2)	-	0.9
PWMH (median/IQR)	2 (1-2)	1 (1-2)	-	0.4

SD – standard deviation, CI – confidence interval, NIHSS – National Institutes for Health Stroke Scale, DWMH – deep white matter hyperintensities, PWMH – periventricular white matter hyperintensities.

8.4.2 Fractal dimensions

In this cohort the mean Dbox (monofractal dimension) was 1.42 with SD 0.02, the mean D0 (multifractal dimension) was 1.67 with SD 0.03. With multivariable analysis, decreased Dbox (Table 8.2) and D0 (Table 8.3) (both representing decreased branching complexity) were associated with increasing age and lacunar stroke subtype after correcting for hypertension, diabetes, stroke severity and white matter hyperintensity scores. We did not find associations between Dbox (mono fractal) or D0 (multi fractal) dimensions and the presence of hypertension, diabetes or white matter hypertintensities on either univariable or multivariable analysis.

Table 8.2. Multivariable predictors of increasing Dbox (mono-fractal dimension) corrected for all listed variables in the table.

Variable	Beta co-efficient	P value
Age (per year increase)	-0.001	<0.001
Lacunar stroke subtype	-0.01	<0.001
Hypertension	-0.002	0.63
Diabetes	-0.002	0.71
NIHSS (per point increase)	0.0001	0.52
DWMH (per point Fazekas increase)	-0.002	0.45
PVWM (per point Fazekas increase)	0.002	0.46

NIHSS – National Institutes for Health Stroke Scale, DWMH – deep white matter hyperintensities, PWMH – periventricular white matter hyperintensities.

Table 8.3. Multivariable predictors of increasing D0 (multi–fractal dimension) corrected for all listed variables in the table.

Variable	Beta co-efficient	P value
Age (years)	-0.001	<0.001
Lacunar stroke subtype	-0.011	0.002
Hypertension	-0.004	0.26
Diabetes	0.002	0.73
NIHSS	0.0005	0.71
DWMH (Fazekas score)	-0.002	0.61
PVWM (Fazekas score)	0.002	0.64

NIHSS – National Institutes for Health Stroke Scale, DWMH – deep white matter hyperintensities, PWMH – periventricular white matter hyperintensities

8.5 Discussion

We have demonstrated in this chapter that decreased fractal dimension (decreased branching complexity) whether measured with either mono or multi-fractal methods is associated with lacunar stroke and increasing age after correcting for other vascular risk factors and WMH. We are able to demonstrate this despite the cortical stroke patients being 4 years older than the lacunar stroke patients which would have tended to mask any associations with lacunar stroke. Furthermore we have demonstrated that it is feasible to perform automated fractal analysis on larger numbers of patients thereby making this tool a feasible tool for assessment of fractal dimensions in large population-based and clinical cohort studies.

This is the first study to investigate retinal fractal properties in the context of cerebral small vessel disease. Therefore there are no previous studies with which direct comparisons can be drawn. Until recently, fractal analysis had only been performed on smaller sample sizes reflecting the laborious nature of hand tracing the vessels. We have previously presented details of our semi-automated approach to fractal

analysis (MacGillivray *et al.* 2007) and other similar yet independently developed techniques have recently been published. One study used 300 patients from a population based study of eye disease (Blue Mountains Eye Study - BMES) and found using a mono-fractal semi-automated approach that decreased fractal dimension was associated with both the presence of hypertension and increasing age (Liew *et al.* 2008b). Interestingly, the mean Dbox from this sample of 300 community dwelling Australian patients, mean age 66 years, was 1.44 with a SD 0.02 closely matching our mean of 1.43 with SD 0.02.

Fractal analysis of the retinal vessels had previously been used with an aim of eventually predicting diabetic retinopathy before macroscopic changes of diabetic retinopathy developed and there have been a number of studies investigating retinal fractals in diabetes but the results from these studies have been inconsistent. An initial small study (n=10) using hand drawn segmentations found that decreased fractal dimension was associated with retinopathy (Avakian *et al.* 2002) but a subsequent slightly larger and more recent study (n=50) did not replicate this finding, finding no association between fractal dimension and retinopathy (Kunicki *et al.* 2009). Recent larger studies which use semi-automated techniques of retinal vessel segmentation still produce incongruous results. Two large studies from the same group found that in young (12-18 year old type one diabetic patients) increased fractal dimension was associated with prevalent retinopathy (which was not caused by new vessel formation) (Cheung *et al.* 2009) but also paradoxically that fractal dimension did not predict incident retinopathy in this same cohort of young diabetic patients (Lim *et al.* 2009). Others have found that in Type 1 diabetic patients (mean age 57 years) decreased fractal dimension was associated with multivariable analysis with prevalent retinopathy (Grauslund *et al.* 2010). All of these studies used the same software to assess mono-fractal dimension and the differences between them perhaps either reflect the different ages of participants or that mono-fractal assessment might not be a reliable measure of retinal vascular abnormalities (Anjos *et al.* 2010; Stosic and Stosic 2006). Further studies are required to assess the associations and determine the clinical implications of retinal fractal analysis.

The strengths of this study are the prospective, accurate and detailed clinical characterisation including MRI assessment of all patients to be certain of stroke subtypes to reduce observer dependency, the use of automated fractal analysis software and the correction with multivariable analysis for vascular risk factors. We used risk factor free definitions of lacunar and cortical stroke to reduce confounding. We were able to assess both monofractal and multifractal measures of the fractal dimension and the automated nature of the work was not labour intensive. We were also able to measure left and right eyes in most of our patients.

We were unable to assess fractal dimension in 10% of patients due to photographic issues but this was similar across both stroke subtypes. Ideally this proportion would have been smaller as we barely made the sample size calculation but we note that this is similar to previous exclusion rates (Cheung *et al.* 2009) albeit in young patients and our results reflect the difficulties in taking retinal photographs in older patients who have had a stroke. Problems exist with the computerized segmentation: the edge of the optic disk is sometimes wrongly detected as a vessel, incorrect detections result from the underlying choroidal vessels, and some vessel paths are broken. More importantly, very small vessels are frequently missed off the segmentation. All of these issues lead to a reduction in segmentation accuracy and mask the true fractal dimension of the retinal vasculature. Improving segmentation performance by increasing the number of true vessel detections and reducing artefacts would generate a binary object that better reflects the true retinal vascular pattern in the image and thus increase the sensitivity of fractal analysis detecting small changes associated with disease.

In this study we assessed the retinal vascular tree, that is arterioles and venules combined. It is a limitation of the segmentation process that the software cannot differentiate between arteriole and venule and therefore we cannot attribute these changes in branching complexity to either subset of the vascular tree. It is also possible that arterioles and venules behave differently and that assessing both may underestimate changes in one or the other. There has been little work in this area. One study of only 23 normal humans subjects using fluorescein angiography to

delineate the arteries and venules (a technique that is perhaps less reliable than using red free high quality digital photographs as the boundaries of the vessels are less distinct) found no difference between arteriolar and venular tree mono fractal dimensions (Landini *et al.* 1993) although this study may have been underpowered for this analysis as they did not find an association between fractal dimension and age (over a range of 14-73 yrs) and reflects the time consuming nature of manual vessel segmentation leading to small sample sizes.

It is intriguing that decreased branching complexity is associated with both increasing age and lacunar stroke. It has been suggested previously that many physiological systems become less complex with increasing age (Lipsitz and Goldberger 1992). The results from this study strengthen this theory and suggests a role for fractal analysis in the study of senescence. The exact cause of lacunar stroke is unclear and it has been postulated that lacunar stroke is the focal manifestation of a widespread small vessel vasculopathy perhaps resulting from increased blood brain barrier permeability and possibly associated with endothelial dysfunction. We have demonstrated earlier in this thesis that although the presence of retinopathy (haemorrhages and exudates) does not differ, retinal venules are wider in lacunar compared to cortical stroke which others have confirmed and in addition found narrower arterioles. The presence of these small yet important differences in the retinal vessels point towards a distinct vasculopathy causing lacunar stroke. We did not find an association with white matter hyperintensities and fractal dimension. Although it has been suggested that lacunar stroke and white matter hyperintensities may share a common aetiology (Wardlaw *et al.* 2003), WMH are common in older people exposed to risk factors like hypertension and diabetes and therefore occur in cortical stroke and lacunar stroke. This study may not have been powered to detect WMH differences between cortical and lacunar stroke patients.

Automated fractal analysis of retinal vessels is an emerging technique that has multiple possible applications. We have used fractals to elucidate underlying pathological mechanisms but future uses might include using the subtle changes visible in the retina as a marker for future development of retinal and cerebral

disease. There is also scope for improving the vessel segmentation which will improve reliability of automated processing of retinal images for fractal analysis and it would be fascinating (although technically difficult) to delineate isolated arteriolar and venular fractal dimensions. We would be delighted to see this work replicated in other populations. Subsequent work should concentrate on clarifying associations between fractal and cognitive function and assessing the predictive power of fractal analysis for future eye or systemic disease.

Chapter 9: Enlarged perivascular spaces on MRI in ischaemic stroke subtypes.

9.1 Aims

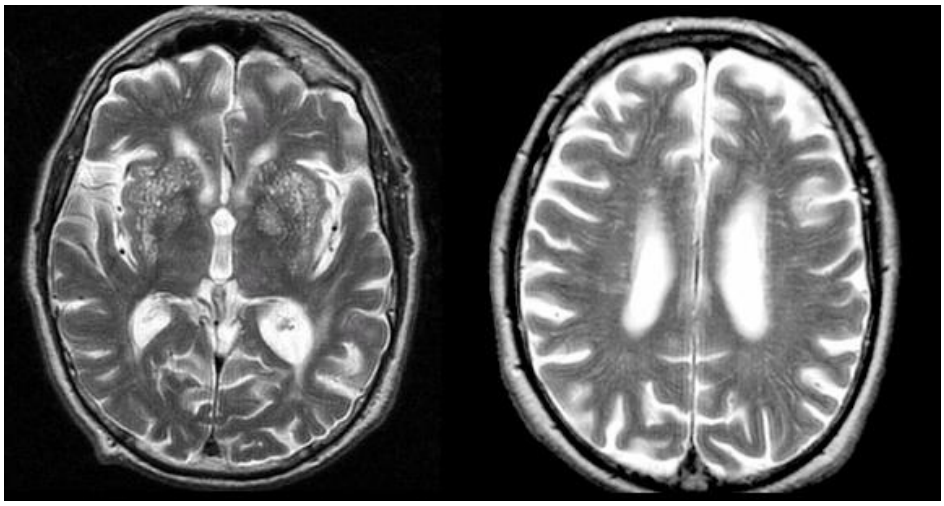
Previous chapters have highlighted a possible role for venular disease in lacunar stroke. In chapter 9 we investigate MR markers of cerebral small vessel disease concentrating on enlarged perivascular spaces in ischaemic stroke subtypes to assess the hypothesis that patients with lacunar stroke have higher rates of enlarged perivascular spaces.

9.2 Introduction

Enlarged perivascular spaces (EPVS) or Virchow-Robin spaces are cerebrospinal fluid filled cavities that surround small penetrating cerebral arterioles and venules and correspond with extensions of the subarachnoid space (Braffman *et al.* 1988). The exact anatomy differs depending on the site and nature of the penetrating vessels but EPVS are contiguous with the perivascular spaces of larger blood vessels in the subarachnoid space (Kwee and Kwee 2007). The exact role of perivascular spaces is not yet clear but tracer studies indicate that there is drainage of interstitial brain fluid down these spaces (Weller and Nicoll 2003) although again the mechanism behind this is unclear (Schley *et al.* 2006). These may communicate with the lymphatic drainage of the neck and this is reflected by the immunological importance of perivascular spaces – they maintain a stable population of migratory macrophages (Bechmann *et al.* 2001). EPVS are visible on axial T2-weighted cerebral magnetic resonance imaging as characteristic small high signal areas in the basal ganglia and centrum semiovale that follow the orientation of penetrating arterioles: they appear linear when parallel and dot like when perpendicular to the imaging plane. Although

common and seen at all ages in adulthood, their aetiology and significance are uncertain.

Figure 9.1. Axial T2 weighted MRI scans showing multiple punctuate hyperintensities characteristic of EPVS in the basal ganglia on the left and linear hyperintensities characteristic of EPVS in the centrum semiovale on the right.



Although a few EPVS are commonly observed in people at all adult ages, EPVS are associated with ageing (Heier *et al.* 1989), impaired cognitive function (MacLullich *et al.* 2004), retinopathy in type I diabetes (Ferguson *et al.* 2003), hypertension (Hiroki and Miyashita 2001), and are found in and around active lesions in multiple sclerosis (Wuerfel *et al.* 2008). A possible association with cerebral small vessel disease is suggested by their greater frequency in patients diagnosed as having vascular dementia as opposed to Alzheimer's disease (Patankar *et al.* 2005), and their association with white matter hyperintensities (WMH) in patients with lacunar stroke (Rouhl *et al.* 2008). However, vascular dementia is not *per se* a marker of small vessel disease and a control group with stroke of a different aetiology (e.g. large artery territorial atherothromboembolic ischaemic stroke) would be required to demonstrate a specific association with lacunar stroke. It is unclear therefore whether EPVS are a marker of small vessel disease or simply represent a generalised brain response to exposure to a range of vascular or other risk factors, or increasing

age. For example, EPVS are present in active MS lesions in association with inflammation, and subside as the inflammation subsides (Wuerfel *et al.* 2008).

Pathologic studies suggest that EPVS are present at all degrees of WMH severity (Udaka *et al.* 2002). EPVS defined pathologically may be related to a widespread microangiopathy resulting in WMH which are commonly associated with age related cognitive impairment, (Gunning-Dixon and Raz 2000) vascular dementia (Sitoh *et al.* 2004) and lacunar stroke (Wiszniewska *et al.* 2000). The aetiology of EPVS in various brain locations may differ, as distinct associations for basal ganglia and centrum semiovale EPVS have been suggested (Rouhl *et al.* 2008).

Many lacunar strokes are caused by an intrinsic disease of cerebral small vessels, and cortical strokes are generally caused by large artery atherosclerosis and cardio-embolism (Wardlaw 2005). If EPVS are a specific marker of small vessel disease, then they should be more closely associated with lacunar stroke and WMH than with cortical stroke. We investigated firstly whether MRI-defined EPVS (subdivided into basal ganglia and centrum semiovale EPVS) are associated with ischaemic stroke subtype, secondly whether EPVS are associated with WMH, thirdly whether EPVS are associated with any ischaemic stroke, and fourthly whether EPVS are associated with demographic and vascular risk factors.

9.3 Methods

Details of patient recruitment have been given in Chapter 2 but in brief we recruited patients with clinical lacunar or mild cortical stroke at presentation to a teaching university hospital. All patients were examined by an experienced stroke physician with MRI at presentation and classified into lacunar or cortical stroke clinical syndromes according to the Oxfordshire Community Stroke Project classification (Bamford *et al.* 1991).

Whilst much of the work in this thesis concentrates on phenotypical differences between ischaemic stroke subtypes and no previous studies have investigated this for enlarged perivascular spaces we were also keen to look at associations between the presence of a stroke and non stroke controls addressing the hypothesis that patients with stroke would have more EPVS than subjects without stroke. In order to achieve this we used a control group of patients who had been recruited for a separate study within the same department which had been established to investigate cognition in healthy males. These patients had been scanned in 2002 and were healthy 65-70 year old males who were recruited through primary care. All non-stroke controls were reviewed by a previous research fellow (Alasdair MacLullich) and were free from significant diseases (including diabetes, cancer, heart disease, neurological disease) and were not taking regular medication at the time of the scan. Full details of this cohort have been presented previously (MacLullich *et al.* 2004).

These non-stroke controls were scanned with a GE prestige scanner (GE Medical, Haifa, Israel) which was a different scanner from that used for the stroke patients. Whilst this was a potential weakness in this study, we felt that this was mitigated as the two groups of patients were imaged in the same centre with the same procedure and techniques. Although the stroke patients were scanned on a 1.5T GE scanner and the controls on a 2T GE scanner the T2 axial sequences were optimised for both scanners, we ran extensive phantom testing to be sure that sequences were optimised and that we could translate for complex computational image analysis between both scanners, and several patient studies were conducted on both scanners (including some of the same subjects) which allowed us to make sure that the change in scanner did not affect structural images. Furthermore, assessing EPVS on optimised T2 images is a robust clinical radiological technique, similar to any other radiological diagnostic visual assessment exercise, and, in contrast to some computational image analysis methods, is not affected by minor between-scanner differences.

9.3.1 Brain image analysis

Images were coded by an experienced neuroradiologist (Joanna Wardlaw) using a standardized classification system for the presence, site and size of the recent infarct (hyperintense on DWI, hypointense on ADC, and possibly also hyperintense on FLAIR and/or T2, hypointense on T1), and also for the presence, site and size of any old infarcts and /or haemorrhages. Scans were coded for deep and periventricular WMH according to the Fazekas scale (Fazekas *et al.* 1987) from 0-3. Cerebral atrophy was defined as deep (enlargement of the ventricles) or peripheral (enlargement of the gyri) and rated on a subjective scale of 0-3 (absent (0), mild (1), moderate (2), severe (3)) against a reference MR brain template of normal subjects (Farrell *et al.* 2008).

EPVS were defined as small, sharply delineated structures of cerebrospinal fluid intensity on imaging that followed the orientation of the perforating vessels and ran perpendicular to the brain surface. Therefore they appeared round in axial section (in the basal ganglia) and linear if in longitudinal sections (in the centrum semiovale) and were less than 3 mm wide. They were of high signal on T2 and low signal on T1 and FLAIR sequences. We assessed EPVS in the basal ganglia and centrum semiovale separately as it is possible that due to their different locations and features they may have separate pathophysiologies. EPVS in both the basal ganglia and centrum semiovale were coded with the following scale: (MacLullich *et al.* 2004) 0 = no EPVS, 1 = <10 EPVS, 2 = 10-20 EPVS, 3 = 30-40 EPVS and 4 = >40 EPVS. The numbers refer to EPVS on one side of the brain; the higher score was used if there was asymmetry between the sides. We summed basal ganglia and centrum semiovale EPVS to form a total EPVS score (0-8).

9.3.2 Statistical Analysis

Total EPVS was normally distributed and analyzed with multiple linear regression. Basal ganglia EPVS and centrum semiovale EPVS were not normally distributed and, to permit binary logistic regression, we dichotomized basal ganglia and centrum

semiovale EPVS into 0 (EPVS scores 0,1) and 1 (EPVS scores 2,3,4). We used t-tests, Mann Whitney U tests and differences in proportions for association to test for differences between the lacunar and cortical groups. We used multiple regression to assess effects of potential explanatory variables in predicting numbers of EPVS, both total EPVS in the acute stroke group and healthy controls combined, and basal ganglia and centrum semiovale EPVS separately within the acute stroke group. We entered both stroke subtype (lacunar versus cortical) and WMH separately into the model as the association between lacunar stroke and WMH is not strong and WMH are found commonly in patients with cortical stroke and normal older people. The alpha value for statistical significance was set at 0.05. All analyses were performed with Minitab software (version 14, Minitab Inc, PA, USA). There were no suitable data upon which to base meaningful study size calculations. There was one patient with missing data regarding MRI analysis and this patient was excluded from all totals given in the paper.

9.4 Results

We recruited 350 patients in total, 253 patients with acute stroke and 97 normal healthy age matched controls (Table 9.1). Of the 253 patients with stroke, the mean age was 68.1 years (SD 11.6 years, range 34-95 years), median NIHSS 2 (interquartile range 2-3), 65% were male and there were 129 patients with lacunar stroke and 124 patients with cortical stroke. Of the 129 lacunar stroke patients, 91 had a recent infarct identified on MRI and of the 124 cortical patients, 95 had a recent infarct identified. No patients had concurrent acute lacunar and cortical infarcts. The mean age of the healthy controls was 66.9 years (SD 1.3 years) and they were all male. Amongst the acute stroke patients, those with cortical stroke were older (70 v 66 years p= 0.01), had less severe strokes ($p<0.001$) and, in keeping with atherothromboembolic causes for their stroke, had a higher prevalence of atrial fibrillation (p=0.02), carotid stenosis >50% (p=0.02) and ischaemic heart disease (p=0.004) than the lacunar stroke patients. There were no significant differences in sex, race or the prevalence of diabetes, PVD, previous TIA/stroke or hypertension between the stroke subtypes.

Table 9.1 Baseline characteristics of subjects

Characteristic	**Lacunar Stroke n=129**	**Cortical Stroke n=124**	**Difference, 95% CI and p value between lacunar and cortical groups**	**Healthy controls n=97**
Mean age (SD) years	66.3 (11.6)	70.0 (11.5)	3.6 (0.8, 6.5) p=0.01	66.9 (1.4)
Median NIHSS (IQR)	3	2	p<0.001	-
Male (n) (%)	77 (60%)	88 (70%)	10% (-1%, 22%) P=0.06	97 (100%)
AF	6 (4.6%)	16 (12.90%)	8% (1, 15) p=0.02	0
Carotid Stenosis >50% (NASCET)	5 (3.88%)	14 (11.29%)	7% (1, 13) p=0.02	0
History previous TIA	19 (14.96%)	13 (10.48%)	4% (-4, 12) p=0.29	0
History previous Stroke	9 (6.98%)	14 (11.38%)	4% (-3, 12) p=0.23	0
History IHD	17 (13.18%)	34 (27.42%)	14% (4, 24) p=0.004	0
History Hypertension	84 (65.32%)	70 (56.59%)	9% (-3, 20) p=0.15	0
History Diabetes	22 (17.05%)	14 (11.29%)	6% (-3, 14) p=0.19	0
History Peripheral Vascular Disease	5 (3.88%)	6 (4.84%)	1% (-4, 6) p=0.71	0
Median Deep White Matter Fazekas score (IQR)	1 (1-2)	1 (1-2)	P=0.92	1 (0-1)
Median Periventricular White Matter Fazekas Score (IQR)	1 (1-2)	1 (1-2)	P=0.50	1 (0-1)
Mean Total EPVS Score (SD)	3.81 (1.76)	3.46 (1.72)	0.3 (-0.1, 0.8) p=0.11	1.02 (0.89)

9.4.1 EPVS in ischaemic stroke subtypes

In the 253 patients with acute stroke, total EPVS (basal ganglia and centrum semiovale combined) were independently and significantly associated with lacunar stroke subtype after correction for important confounders including vascular risk factors, age, WMH and deep atrophy, in keeping with our primary hypothesis (Table 9.2). Basal ganglia EPVS were modelled simultaneously with centrum semiovale EPVS to look at separate associations despite being correlated at Spearman's rank rho 0.47 ($p<0.001$). Basal ganglia EPVS were associated with lacunar stroke subtype after correcting for other confounders including WMH and deep atrophy (Table 9.3). Centrum semiovale EPVS did not differ between ischaemic stroke subtypes.

Table 9.2. Multivariable associations with Total EPVS as the dependent variable correcting for all the other explanatory variables in the table in the subgroup of 253 patients with acute stroke

Variable	**Beta Co-efficient**	**P value**
Age	0.02	0.08
Lacunar stroke subtype (compared with cortical stroke)	0.38	0.04
Hypertension	-0.06	0.76
Diabetes	0.067	0.79
Deep WMH	0.71	<0.001
Periventricular WMH	0.46	0.01
Male gender	0.01	0.96
Deep atrophy	-1.4	0.29

Abbreviations

WMH: white matter hyperintensities

Table 9.3 – Univariable and Multivariable associations with basal ganglia EPVS.

Variable	Univariable P value	Univariable OR (95% CI)	Multivariable p value	Multivariable OR (95% CI)
Age	<0.001	1.08 (1.05-1.11)	0.14	1.03 (0.99-1.08)
Hypertension	0.11	1.53 (0.91-2.58)	0.46	1.33 (0.63-2.81)
Diabetes	0.53	0.79 (0.38-1.65)	0.39	0.65 (0.24-1.74)
Deep WMH	<0.001	4.47 (2.94-6.80)	0.02	2.03 (1.10-3.74)
Periventricular WMH	<0.001	4.97 (3.29-7.50)	0.01	2.28 (1.19-4.38)
Male gender	0.53	1.18 (0.69-2.02)	0.13	1.83 (0.84-3.98)
Centrum semiovale EPVS	<0.001	7.58 (4.01-14.32)	<0.001	6.34 (2.87-14.0)
Deep atrophy	<0.001	2.20 (1.60-3.01)	0.58	1.15 (0.70-1.87)
Lacunar stroke subtype	0.03	1.78 (1.07-2.96)	0.003	3.16 (1.49-6.70)

* = significant p<0.05

Abbreviations

WMH: white matter hyperintensities

9.4.2 EPVS and white matter hyperintensities

In all 350 patients, total EPVS were associated with deep and periventricular WMH after adjusting for age, presence of stroke, hypertension, diabetes and sex (Table 9.4). Furthermore, in the acute stroke group of 253 patients we found that basal ganglia EPVS were associated with deep and periventricular WMH adjusting for lacunar stroke subtype, age and deep atrophy. Centrum semiovale EPVS were not associated with any explanatory variables (after correcting for basal ganglia EPVS - data not shown).

Table 9.4 - Multivariable associations with Total EPVS as the dependent variable correcting for all the other explanatory variables in the table in the total study population of 350 patients

Variable	Beta Co-efficient	P value
Age	0.01	0.35
Presence of stroke	0.31	0.21
Hypertension	-0.08	0.69
Diabetes	0.06	0.81
Deep WMH	0.60	<0.001
Periventricular WMH	0.64	<0.001
Male gender	-0.16	0.44

Abbreviations
WMH: white matter hyperintensities

9.4.3 EPVS and the presence of stroke

In all 350 patients, EPVS were associated with the presence of any stroke on univariable analysis, but this association was reduced to below statistical significance after adjustment for WMH.

9.4.4 EPVS, vascular risk factors and demographics

In all 350 patients, EPVS were associated with increasing age on univariable analysis but this association was attenuated to below statistical significance after adjustment for WMH. After full adjustment for other variables, only deep and periventricular WMH remaining significantly associated with EPVS.

9.5 Discussion

Our novel findings in this chapter are that total and basal ganglia EPVS are independently and significantly associated with lacunar compared to cortical ischaemic stroke, in keeping with the hypothesis that EPVS and lacunar stroke share a common aetiology. EPVS were also independently and significantly associated with deep and periventricular WMH after correcting for increasing age and vascular risk factors, further emphasizing the specific association of EPVS with small vessel disease.

No previous study has included a control group without stroke to distinguish the relationship between EPVS and any stroke. No previous study has included a control group with a different stroke mechanism to distinguish the relationship between EPVS and ischaemic stroke subtypes. Lacunar stroke may be the focal manifestation of a widespread small vessel angiopathy consequent upon increased blood brain barrier permeability (Wardlaw *et al.* 2009; Wardlaw *et al.* 2008) which also leads to the development of WMH (Young *et al.* 2008). The apparently different associations for basal ganglia and centrum semiovale EPVS might be explained by anatomical differences between the arterioles in the basal ganglia and elsewhere: the former have two layers of surrounding leptomeninges whereas the latter only have one (Pollock *et al.* 1997).

The exact causes of EPVS are uncertain but the perivascular space is an important conduit for drainage of interstitial fluid to the ventricles (Abbott 2004) and could be affected by various factors including abnormalities at the blood brain interface (Wardlaw *et al.* 2009). Increased blood brain barrier permeability is associated with EPVS in stroke patients (Wardlaw *et al.* 2009), in patients with active multiple sclerosis lesions (Wuerfel *et al.* 2008), and with WMH (Starr *et al.* 2003). WMH are more common in lacunar than cortical stroke (Wardlaw *et al.* 2006), again supporting a common small vessel disease pathophysiology. In the case of multiple sclerosis, the appearance of the EPVS followed active inflammation and were associated with a blood brain barrier breakdown; the EPVS resolved as the active inflammation subsided. Endothelial inflammation may be associated with small vessel disease also (Hassan *et al.* 2003b). It is therefore plausible that EPVS are a manifestation of a cerebral small vessel pathology associated with, and a possible marker for, altered blood brain barrier function (Wuerfel *et al.* 2008). A possible link with venular pathology in small vessel disease and inflammation has been suggested by previous findings in chapter 6 of this thesis and elsewhere (Lindley *et al.* 2009) and cerebral venous insufficiency secondary to collagenosis has been implicated in the aetiology of white matter disease (Black *et al.* 2009; Moody *et al.* 1995) and it has recently been suggested that research should concentrate on the venular pathophysiology of white matter disease (Andersson 2010).

The strengths of this study are that we directly compared EPVS in ischaemic stroke subtypes and between stroke patients and healthy age matched controls. The sample size was relatively large and thus we were able to investigate several explanatory variables with multivariable regression to correct for confounders. The patients had carefully standardised MRI brain imaging on a dedicated research scanner as part of the study assessment, rather than using either retrospectively recruited patients (Heier *et al.* 1989) or those who had had clinically indicated MRI (Rouhl *et al.* 2008). All MRI scans were assessed by one experienced neuroradiologist using a standard EPVS rating scale. Importantly, because we used multi-sequence MRI, we were able to fully characterise WMH and EPVS and simultaneously correct for important

confounding variables, especially WMH and brain atrophy, to ensure that EPVS are not simply caused by parenchymal brain loss.

This study has limitations. The healthy control group did not have basal ganglia and centrum semiovale EPVS characterised separately (though we were able to use a combined total EPVS score to compare groups) and the healthy control group was all male (although sex was not shown to have an independent association with EPVS in the acute stroke group). This is a cross sectional study and therefore can only determine associations not causation, nor the sequence of development of small vessel disease features.

A previous study showed that basal ganglia EPVS were associated on multivariable analysis with periventricular (but not deep) WMH, asymptomatic lacunar infarcts and increasing age in 165 patients with lacunar stroke (Rouhl *et al.* 2008). Centrum semiovale EPVS were associated on multivariable analysis with diabetes and asymptomatic lacunes. Although this study included patients with large artery stroke (n=41) as well as lacunar stroke, they did not report the EPVS associations for cortical stroke. A retrospective study of 816 patients using data from clinically-indicated brain MRI showed that EPVS were strongly associated with age but not WMH, hypertension or dementia on multiple regression (Heier *et al.* 1989). The clinically indicated MRI scan, retrospective design, wide age range and a low rate of WMH, may explain the differences with our results. EPVS and WMH were more frequent in patients with vascular dementia than other types of dementia (35 patients with Alzheimer's, 25 with vascular or fronto-temporal dementia and 35 healthy controls) but this may have been confounded by higher rates of white matter disease in vascular dementia and the small study size (Patankar *et al.* 2005).

Beyond confirming that EPVS seen on MRI are indeed enlarged spaces around the perforating arterioles, pathologic studies of white matter disease produce conflicting information, and shed little light on their aetiology (Pantoni and Garcia 1997). In 122 patients whose MRI findings were compared with pathology, EPVS were present at all Fazekas grades of deep and periventricular WMH (Udaka *et al.* 2002). Other

features present in all grades of WMH were myelin pallor and loss, with gliosis and axonal loss in the more severe cases. In the brains of 19 patients >60 years who had died of non-brain disease causes, comparison of MRI and pathologic appearance suggested that arteriosclerosis leads to demyelination, then axonal loss and then subsequent dilatation of perivascular spaces and WMH (van Swieten *et al.* 1991). However it is difficult to infer a sequence of events from a static picture obtained at post mortem. It is not clear therefore if EPVS precede, follow or appear concurrently with WMH, or if they arise from the same pathologic process or not. The variation in methods for assessing EPVS may account for the discrepancies between studies and points to the need for a robust and reliable classification scheme for EPVS.

EPVS are relatively under-researched but easy to recognise. Amongst older people, they are part of the spectrum of small vessel disease associated with widespread white matter lesions, lacunar stroke and cognitive impairment. We propose that EPVS should be incorporated into white matter rating scales because the associations and significance of EPVS will only be elucidated once EPVS have been studied in more depth. Future research should investigate longitudinal associations between EPVS, WMH, stroke and cognitive function to fully ascertain temporal associations between these common and important abnormalities. More information is needed to provide accurate prognostic indicators and guide development of future therapies for small vessel disease, especially with small vessel disease related to cognitive impairment.

Chapter Ten: Conclusions, implications and suggestions for future research.

10.1 Aims of chapter

In this chapter I summarise the findings of this thesis and compare these results to recently published data from other groups. I will discuss generic strengths and weaknesses of this study before discussing the pathological and clinical implications of these results. Finally I detail ongoing work that has arisen from these results and provide suggestions for further research.

10.2 Summary of findings

We performed a systematic review of retinal microvascular abnormalities in lacunar stroke to clarify associations and identify where further research was required. We then established a cohort of patients presenting with lacunar stroke with cortical stroke controls to investigate retinal microvascular abnormalities in ischaemic stroke subtypes. All patients had MRI brain at presentation and retinal photography of both eyes with a digital retinal camera. We investigated the prevalence of retinopathy (hard and soft exudates or haemorrhages or microaneurysms), retinal arteriolar and venular widths, focal arteriolar narrowing, arteriovenous nicking, retinal arteriolar geometry (branching co-efficients (change in arteriolar cross sectional area across a bifurcation) and branching angles) and fractal dimensions (reflecting branching complexity) of the vasculature. We also assessed MRI parameters in lacunar stroke. We used multivariable analysis to correct for baseline imbalances in vascular risk factors.

From the systematic review we demonstrated that retinal microvascular abnormalities are associated with incident and prevalent stroke but in general stroke was inadequately characterised and there were no data regarding retinal

microvascular abnormalities in ischaemic stroke subtypes. We recruited 253 patients, 129 lacunar stroke and 124 cortical strokes, mean age 68 years. In this cohort of patients presenting with a clinical diagnosis of mild stoke we found that diffusion weighted MRI at presentation was negative for ischaemia in 33% of patients and negative when all sequences were included in 24%. We found no difference in prevalence of retinopathy, arteriovenous nicking, focal arteriolar narrowing or arteriolar widths between lacunar and cortical stroke subtypes. We found that venules were wider in lacunar stroke. We found no differences in arteriolar branching co-efficients or arteriolar branching angles between lacunar and cortical strokes but found that deep white matter hyperintensities on MRI were associated with increased branching co-efficients and periventricular white matter hyperintensities associated with decreased branching co-efficients. We found that fractal dimensions were decreased in lacunar stroke. Furthermore we found that enlarged perivascular spaces on MRI are associated with lacunar stroke and white matter disease.

10.3 Validation by other work

Much of the work presented and previously published from this thesis has been subsequently validated by results from other centres. A recent individual patient based meta-analysis investigated whether retinal arteriolar or venular widths predicted incident stroke (McGeechan *et al.* 2009a). This systematic review identified 6 population based studies - ARIC, Ausdiab, CHS, BMES, BDES and the Rotterdam study, contacted the principal investigators of each study and merged the data. Their main findings confirmed the results of our systematic review as increased venular width predicted incident stroke but arteriolar width did not predict future stroke. This analysis was limited to studies which assessed arteriolar and venular widths (and did not investigate the predictive nature of other retinal microvascular abnormalities such as retinopathy or retinal arteriolar occlusion) and incident stroke or indeed any associations with prevalent stroke. There will inevitably have been heterogeneity between studies in the definition of stroke and in general stroke was diagnosed by review of medical records (reflecting limitations of

the published literature detailed in chapter 3 of this thesis) but this study included unpublished data from the ARIC and Ausdiab studies.

As detailed previously, the Mild Stroke Study in Edinburgh was designed to complement the similar Multicentre Retinal Stroke (MCRS) study co-ordinated in Sydney, Australia (Lindley and Multi-Centre Retinal Stroke Study Collaborative Group 2008). The MCRS study recruited in three centres – Sydney and Melbourne, Australia and Singapore. The inclusion criteria were slightly different from the Mild Stroke Study – the MRCS recruited patients who were less than 7 days post stroke onset who were admitted to hospital with stroke (including posterior circulation and haemorrhagic stroke). All patients had standard investigations for stroke (plus retinal photographs) which meant that a significant proportion of patients had CT (60%) rather than MRI (40%) which were clinically indicated, to diagnose and subtype ischaemic stroke. To correct for this they performed a series of sensitivity analyses using different methods of subtyping stroke with a hierarchy of classifications. The authors subtyped stroke using lacunar stroke diagnosed with the OCSP classification first, followed by the TOAST method, followed by those with lacunar stroke who had a MRI scan and finally those who had a DWI positive lacunar lesion. There were decreasing numbers of patients in each of these categories. They subsequently repeated all of these analyses in patients without diabetes.

The MCRS study recruited 1321 patients, mean age 66 years and reported that in general, associations between retinal microvascular abnormalities and lacunar stroke subtype strengthened with increasing precision of lacunar stroke diagnosis. Using the OCSP classification in all patients they found no difference in focal arteriolar narrowing, arteriovenous nicking, arteriolar width or venular width between lacunar and non-lacunar stroke. When this analysis was restricted to those patients who had had a MRI , a group perhaps most comparable to those presented in this thesis, (146 patients with lacunar stroke) they found that focal arteriolar narrowing and arteriovenous nicking were more prevalent in lacunar stroke, there was a borderline significant narrowing of arteriolar diameter (OR for the smallest quintile versus the

largest quintile 2.4 (95% CI 1.0 to 5.8) and that retinal venules were not wider in lacunar stroke (OR 1.4 (95% CI 0.9 to 2.3).

When patients with diabetes were excluded from the analysis in the cohort who had had brain MRI (which included 94 patients with lacunar stroke) focal arteriolar narrowing (OR 2.4, 1.1 to 4.5) and arteriovenous nicking (OR 2.4, 1.3 to 4.5) were more prevalent in lacunar stroke, arterioles were narrower (OR 2.1, 1.1 to 4.2) and venules were wider (OR 2.1, 1.1 to 3.9). It is interesting to note however when this analysis was restricted to those with DWI lesions of lacunar stroke (89 patients with lacunar stroke) the association with narrowed arterioles became non-significant. The reported association between narrowed arterioles and lacunar stroke seems inconsistent and perhaps reflect imperfect stroke subtyping as most patients only had CT as their brain imaging which may introduce bias but it would appear that venules are reproducibly wider in lacunar stroke.

Each study independently developed its own protocol for measuring arteriolar widths (although they were based on the same iterative process for combining widths to produce CRAE and CRVE). It is interesting to note that in the MCRS study the absolute mean arteriolar width in lacunar patients was 132 microns and the mean venular width was 214 microns which are similar to our measurements of 161 microns and 208 microns respectively.

Although our study and that of our collaborators were designed to complement each other there were several key methodological and analytical differences between the two studies. All of the patients in the Edinburgh based Mild Stroke Study had diagnostic MRI at presentation and were carefully characterised clinically by a single research fellow using risk factor free definitions. There were slightly more restricted inclusion criteria – we did not include patients with stroke equivalent to total anterior circulation stroke or those with haemorrhage although it is interesting to note that there were only small numbers of patients with haemorrhage in a subset of the MCRS (Baker *et al.* 2010) – 51 of 630 patients had deep cerebral haemorrhage.

10.4 Variability of retinal features

We have used a cross sectional study design to investigate associations between retinal features and stroke subtypes. Retinal features may vary over short time periods. For certain retinal features such as retinal vessel geometry or fractal analysis it is not clear whether the abnormalities are present at birth, appear at a later stage and precede cerebrovascular disease or are a response to lifetime accumulation of risk factors.

It has been suggested that retinal vessel widths differ at different stage in the cardiac cycle (Chen *et al.* 1994) but subsequent studies of retinal photographs performed with a digital retinal camera found that the variability in widths from the point in the cardiac cycle was less than the between image variability due to compromised image quality and therefore efforts should be made to ensure maximal image quality rather than co-ordinating photos with the cardiac cycle (Knudtson *et al.* 2004).

Certain retinal microvascular abnormalities might be present from a young age. A study from Singapore investigated retinal vessel widths in children aged 7-9 years found no relationships between birth markers (weight, length, gestational period, and head circumference) and retinal vessel widths although there were differences in width parameters between children of Chinese, Malay and Indian origin (Cheung *et al.* 2007b). These finding suggest that retinal vessel width associations develop in response to disease later in life.

If retinal markers can be used as a surrogate marker of disease it is possible that they could be used to assess response to treatment of the associated systemic disease such as hypertension or diabetes. In a small study (n=25) investigating retinal changes before and after treatment for hypertension, both ACE inhibitor and calcium channel treatment were associated with comparable blood pressure falls and a reduction in arteriolar narrowing, widening of arteriolar branching angles and an increase in arteriolar density (Hughes *et al.* 2008). A study from the same group compared retinal length/diameter ratios (a marker of arteriolar narrowing) in a randomized trial

of antihypertensive regimes finding that treatment with amlodipine was associated with lower ratios than treatment with beta-blockers (Thom *et al.* 2009). Both of these studies suggest that arteriolar parameters are not fixed and may respond to external factors.

The mechanisms behind these responses are yet to be fully elucidated. In chapter 7 we suggested that endothelial dysfunction (possibly acting via nitric oxide dependent processes) may affect retinal arteriolar geometry. Advanced laser scanning doppler cameras can measure dynamic changes in retinal arterial and capillary blood flow in response to flicker stimuli which under normal circumstances promotes NO dependent vasodilatation. Using laser scanning dopplers, young hypertensive patients had impaired arterial vasodilatation and blood flow increases in response to the flicker light stimulus compared to non-hypertensive controls. Blockage of NO (with N(G)-monomethyl-L-arginine – L-NMA) abolished the dilatation in non-hypertensive controls. Subsequent administration of candesartan (an angiotensin-1 receptor blocker (AT-1)) restored dilatation in response to the flicker light stimulus (Delles *et al.* 2004). This suggests a role for NO in the control of retinal blood flow and that blockage of AT-1 receptors may reverse the impaired endothelial function present in hypertensive subjects. No studies have reported associations between the retinal features described in this thesis and response to stimuli.

10.5 Why did we not find a difference in arteriolar widths?

Initial pathological reports suggested that a selection of the key changes apparent in the arterioles are thickened walls with lipohyalinosis and fibrinoid necrosis with associated destruction of the arteriolar wall and finally narrowing and eventual blockage of the arteriolar lumen.(Lammie 2000) We found that arteriolar widths were 2 microns narrower in patients with lacunar stroke, a difference which did not reach statistical significance. There was a paucity of data upon which to base sample size calculations and this study may have been underpowered to detect this small difference. Subsequent publication of results from the Multi-centre retinal

stroke study with a total sample size of 1321 patients of whom 410 had lacunar stroke from our collaborators in Sydney found that there arterioles were 2 microns narrower in lacunar stroke – a similar difference to ours - suggesting that with more power our difference may have reached statistical significance (Lindley *et al.* 2009). There are inconsistencies however as the MCRS study did not consistently find decreased arteriolar widths in lacunar stroke after multivariable correction for vascular risk factors. Other than our sample being underpowered to look for arteriolar widths differences in lacunar stroke might there be other explanations for the lack of an association between lacunar stroke and decreased retinal arteriolar widths?

Other studies have investigated associations between venular widths and arteriolar widths for the following systemic variables: cognitive function (Liew *et al.* 2009), incident stroke (Wong *et al.* 2006), decreased arteriolar saturations and cerebral blood flow (De Jong *et al.* 2008), MRI evidence of small vessel disease (Ikram *et al.* 2006b) and serum markers of systemic inflammation (C-reactive protein) (Yim-Lui *et al.* 2010). All of these studies found associations between increased venular width but not arteriolar width and their variable of choice. It is possible that the retinal venules are more reactive than the retinal arterioles to systemic influences (or their larger size makes any changes easier to identify). The exact role and influences behind alterations in arteriolar and venular widths are imperfectly understood. In general narrower retinal arterioles are associated with hypertension and increased age whilst it is felt that wider venules are associated with younger age (in population based studies of middle aged individuals), hypertension, increased body mass index and increased inflammation (measured with serum markers) (Liew *et al.* 2008a). It is likely that a genetic influence determines retinal vessel widths (Sun *et al.* 2009).

Arteriolar narrowing has been demonstrated in cerebral pathological studies yet important information regarding the sequencing of events (which is absent in human pathological studies) is beginning to emerge from animal studies of lacunar stroke (Bailey *et al.* 2009; Hainsworth and Markus 2008). In the spontaneously hypertensive stroke prone rat the narrowing and subsequent blocking of the lumen of

the arteriole is a late event which follows increased blood brain barrier permeability and parenchymal damage from leaked plasma proteins. It is possible that in this cohort of patients with lacunar stroke the disease process is not far enough advanced for us to be able to identify arteriolar narrowing. In retinal photographs the vessel wall is transparent and what can actually be seen is the red blood cell column in the lumen thereby we are measuring the luminal width rather than the widths of the arterioles. It is possible that even though the arteriolar wall has thickened this has not necessarily resulted in a narrowing of the lumen.

10.6 Why might the retinal venules be wider in lacunar stroke?

The exact reason for this is not clear. There is a paucity of information regarding the cerebral venules in lacunar stroke both from pathological studies (Lammie 2000) or animal models of lacunar stroke (Bailey *et al.* 2009). Increased retinal venular widths have been associated with decreased oxygen saturations and decreased cerebral blood flow (De Jong *et al.* 2008), increased serum markers of inflammation (De Jong *et al.* 2007) and decreased small artery compliance but not large artery compliance (Cheung *et al.* 2007a). Recent results have found that increased venular width is associated with decreased flow mediated dilatation of the brachial artery (which represent NO mediated endothelial related dilatation) (Nguyen *et al.* 2010). There is increasing evidence of venular abnormalities in lacunar stroke and altered venular function may underlie lacunar stroke but the venular signs may simply be a sign of the presence of the process that leads to lacunar stroke. At present we would recommend that future research include venular pathology.

10.7 The role of fractals in assessment of the retinal vasculature

When initially presented, the data presented in chapter 8 of this thesis regarding the use of fractals were novel and preliminary. They suggest that patients with lacunar stroke have less complex branching patterns of the retinal vasculature. It is difficult to draw exact pathological correlations from these findings but they reinforce the concept that lacunar stroke is caused by a distinct vasculopathy. Ideally these results

would be replicated elsewhere by another centre. Subsequently our MCRS collaborators have presented data in a subgroup of their cohort study showing that patients with lacunar stroke had increased monofractal dimensions compared to patients with non-lacunar stroke (Cheung *et al.* 2010), the opposite to our finding of decreased fractal dimensions with lacunar stroke although they also showed that increasing age was associated with decreased fractal dimension. This apparently contradictory result reinforces the nascent nature of retinal fractal associations but it is worthwhile investigating possible causes for this discrepancy.

There are important differences between the two cohorts of patients. Cheung report on a subgroup of 392 patients from a total of 1321 patients recruited for the Multicentre Retina and Stroke Study (Lindley *et al.* 2009). They used the TOAST criteria to subtype ischaemic stroke. There were 79 patients with lacunar stroke who were compared to patients with non-lacunar stroke. The authors do not detail how many of the non-lacunar stroke were cardio-embolic, large artery, mixed, rare or unknown aetiology. Patients either had a MRI or CT as the brain investigation– but the proportions or reasons for choosing either were not given (the rate of DWI was approximately 40% in the entire MCRS from previous publications ie approximately 30/79 lacunar patients in this sub study). Reliance on CT for the rest will have resulted in noise due to incorrect classification of stroke subtype. All patients presented in this thesis had brain MRI at presentation to subtype stroke and all patients either had a lacunar or cortical stroke using non-risk factor based definitions. Cheung et al report supplementary analysis for patients with MRI but is not clear if these analyses are corrected for age and other vascular risk factors and, given the small number of patients and outcomes it is possible that these regression models are overfitted. These features may explain the differing inter study results.

Is there any evidence from other sources which may indicate which direction fractal dimensions may differ in the presence of cerebral small vessel disease? No other studies have reported on retinal fractal dimensions in stroke but there is evidence of inconsistent associations between retinopathy and fractal dimensions even when the same semi-automated fractal technique is used: Cheung reported that increased

fractal dimension was associated with prevalent retinopathy in young type I diabetic patients (Cheung *et al.* 2009) but Lim in the same cohort found that fractal dimension did not predict incident retinopathy (Lim *et al.* 2009). Using the same technique for assessing fractal dimensions, Grauslund found that decreased fractal dimension was associated with prevalent diabetic retinopathy in middle aged adults with type I diabetes (Grauslund *et al.* 2010). Studies that were performed prior to the recent advent of semi-automated fractal analysis – that were much smaller – again found conflicting results: Avakian found decreased fractal dimension with retinopathy in 5 patients (Avakian *et al.* 2002) but Kunicki in 35 patients found no relationship between fractal dimension and diabetic retinopathy(Kunicki *et al.* 2009).

Sng (Sng *et al.* 2010) investigated retinal fractal dimension and chronic kidney impairment and found that although retinal fractals were lower in those with kidney disease there was evidence for a U shaped relationship between fractal dimension and renal disease. It was suggested that there may be an optimal fractal dimension and that deviation away from this optimum may be associated with the presence of kidney disease. There have been no other fully published papers that support this notion although preliminary data presented in abstract form hints that the presence of retinopathy in non-diabetic patients may be associated with extremes of fractal dimensions (Mitchell *et al.* 2010). Clearly more work is needed to assess associations and the eventual clinical utility of fractal analysis of the retinal vessels.

10.8 Pathophysiological implications of the work in this thesis

The results in this thesis demonstrate that there are retinal microvascular differences between lacunar and cortical stroke, and perhaps between deep and periventricular white matter hyperintensities on cerebral MRI that are likely to mirror differences in small vessels in the brain. Evidently more work is needed to fully characterise these abnormalities and to ascertain whether they confer clinical risk.

Traditional research into the causes of lacunar stroke has concentrated on the role of the arterioles in an attempt to identify the nature of the underlying arteriopathy. We have demonstrated that venular widths differ between lacunar and cortical stroke which, coupled with the association we demonstrated between enlarged perivascular spaces and lacunar stroke suggests that venular disease may play a prominent role in, or be a major marker for the aetiology of cerebral small vessel disease.

10.9 Clinical implications arising from this thesis

The results in this thesis will not directly alter clinical behaviour although these results reinforce the hypothesis that conventional atherothromboembolic mechanisms do not cause lacunar stroke. If a novel mechanism is responsible for causing lacunar stroke that this subtype of stroke may be treated differently from other subtypes but it would be speculative and is beyond the scope of this thesis to predict how these treatments may differ. There are ongoing clinical trials to assess whether lacunar strokes specifically benefit from conventional secondary stroke prevention strategies (Benavente and Hart 2003). Alternatively many secondary preventative drugs eg statins, ACE inhibitors have other effects such as on the endothelium and this may be effective even if via a different mechanism.

Other than using the retinal vessels as markers of the cerebral vessels to elucidate the underlying pathophysiology are there other applications for assessing the retinal microvasculature? There is current interest in using retinal abnormalities as independent predictors of future and current retinal and systemic disease (McGeechan *et al.* 2009a). Although retinal micro vessels are perhaps most closely linked to the cerebral small vessels they have been assessed as risk predictors or linked to for a variety of different non-stroke and cognitive clinical features including ischaemic heart disease (McGeechan *et al.* 2009b), psychological wellbeing (Jensen *et al.* 2009) and hearing loss (Liew *et al.* 2007b) to fish consumption (Kaushik *et al.* 2008). Although the results presented in this thesis are derived from a cross sectional study the techniques developed and tested in this

thesis, notably concerning vessel geometry and fractal analysis will contribute to the future use of novel retinal markers as risk predictors for incident disease.

10.10 Research implications arising from this thesis

These results contribute to the mounting evidence from other disciplines that lacunar stroke is caused by a distinct vasculopathy to other types of stroke. Lacunar stroke is difficult to study and fully elucidating the underlying aetiology will require close collaboration between researchers currently employing different methodologies in such diverse fields as epidemiology, animal models of disease, genetics, advanced brain imaging, retinal imaging, rheology and pathology. Although there has been a recent decline in the rate of autopsies performed in the UK (Bell 2004), post mortem studies in populations where hypertension has been controlled and with careful pre-morbid characterisation of stroke symptoms would be fascinating.

10.11 Mild Stroke Study Cohort follow up

The results presented in this thesis suggest several potential directions for further research. The results presented are cross sectional analyses and we are therefore only able to report associations without implying causation. In the first instance we have designed follow up for these patients clinically collecting data regarding functional outcome and repeat vascular events (stroke and myocardial infarction) and follow up MRI scans and relating these to original presenting clinical and retinal features. Separately we have established a second study where we are investigating cross sectional and longitudinal associations with blood brain barrier permeability. These results will enable us to investigate the predictive power of both retinal vascular abnormalities and blood brain barrier permeability with regard to both lacunar stroke and white matter disease.

10.12 Individual patient data meta-analysis

One of the limitations of our analysis of retinopathy in ischaemic stroke subtypes presented in chapter 4 of this thesis is that the sample size was powered to detect the presence of retinopathy as a combined outcome measure (comprising hard or soft exudates, microaneurysms or haemorrhages). As previously discussed this may have been too blunt a tool as the components of retinopathy may be caused by different factors. The design of this study and the database in particular was such that it would be easy to merge databases with our collaborators in Australia thus permitting individual patient data meta-analyses. We are planning this analysis which will, with its increased power, be able to study the prevalence of the components of retinopathy in ischaemic stroke subtypes.

10.13 Other retinal markers of disease

We have shown associations between certain retinal vascular markers but other investigators have studied other markers such as vessel tortuosity. From the early pathological studies of lacunar stroke it was suggested that arterioles were more tortuous (Fisher 1969) but it is difficult to quantify tortuosity. Previous studies have used the subjective view of an ophthalmologist to ascertain degree of tortuosity against standardised scoring techniques (Kwa *et al.* 2002) or used semi-automated software to quantify tortuosity (Witt *et al.* 2006). We are currently developing software to assess retinal arteriolar tortuosity and plan to analyse this parameter in the cohort of patients with mild stroke.

As reported in chapter 9 of this thesis, fractal analysis of the retinal vessels included both the arteriolar and venular trees because segmentation techniques cannot yet reliably distinguish arteriole from venule. We are developing techniques to overcome this difficulty and plan to analyse fractal dimensions of the separate vascular trees.

In our assessment of retinal vascular geometry we had originally concentrated on arteriolar disease as in keeping with other groups we felt the likely pathology was probably arteriolar. The results from our other investigations challenge this assertion and suggest that venular pathology may also be important. Other groups have recently presented abnormalities in venular geometry in preadolescent children (Bayu *et al.* 2010) and there would be scope to investigate venular geometrical abnormalities in our cohort of stroke patients.

10.14 Associations with serum markers of inflammation, fibrinolysis and coagulation.

In a subset of 125 patients in the Mild Stroke Study (59 patients with cortical stroke and 66 patients with lacunar stroke) we drew serum samples at a median time of 54 days post stroke. These have been analysed in a collaboration with Prof Gordon Lowe in the University of Glasgow for the following serum markers: von-Willebrand factor (vWF), D-dimer, fibrinogen, tissue plasminogen activator antigen(t-PA), tumour necrosis factor alpha (TNF alpha), C-reactive protein (CRP), intracellular adhesion molecule (ICAM) and interleukin-6 (IL-6). We plan to investigate associations between these serum markers with both MRI markers of small vessel disease and retinal vascular abnormalities.

10.15 Summary

We have clearly demonstrated that retinal microvascular abnormalities differ between lacunar and cortical stroke suggesting that a distinct small vessel vasculopathy may cause lacunar stroke. We have also identified MR markers of lacunar stroke. These results suggest that venular disease (a hitherto under-researched area) may play a role in the pathophysiology of lacunar stroke. Retinal microvascular abnormalities can act as markers for cerebral small vessel disease. We plan collaborative analyses with colleagues who have performed similar studies to further assess retinal abnormalities in lacunar stroke and have followed up this cohort to assess longitudinal associations with retinal abnormalities.

Appendix 1. Medline search strategy for systematic review of serum markers of endothelial dysfunction in lacunar stroke.

1. brain ischemia/ or brain infarction/ or brain stem infarctions/ or cerebral infarction/ or hypoxia-ischemia, brain/ or stroke/
2. (isch?emi$ adj6 (stroke$ or apoplex$ or cerebral vasc$ or cerebrovasc$ or cva or attack$)).tw.
3. ((brain or cerebr$ or cerebell$ or vertebrobasil$ or hemispher$ or intracran$ or intracerebral or infratentorial or supratentorial or middle cerebr$ or mca$ or anterior circulation) adj5 (isch?emi$ or infarct$ or thrombo$ or emboli$ or occlus$ or hypoxi$)).tw.
4. 1 or 2 or 3
5. (lacun$ or small vessel$ or small infarct$ or microinfarct$ or subcortical lesion$ or subcortical infarct$ or microvascular$ or microcirculation$).tw.
6. 4 and 5
7. blood-brain barrier/ or endothel$, vascular/ or tunica intima/ or microcirculation/
8. (endotheli$ adj5 (function$ or dysfunction$ or impairment$)).tw.
9. (endogenous tissue plasminogen activator or endogenous tPA).tw
10. thrombosis.tw
11. fibrinogen.tw
12. fibrinolysis.tw
13. homocysteine.tw
14. (ICAM or Intra cellular adhesion molecule).tw
15. (VCAM or Vascular Cell Adhesion Molecule).tw
16. (IL6 or Inter leukin 6).tw
17. (CRP or C reactive protein).tw
18. von Willebrand factor.tw
19. plasminogen activator inhibitor.tw
20. selectin$.tw
21. D-dimer.tw
22. or/7-21
23. 6 and 22
24. limit 23 to humans

Appendix 2. Medline search strategy for systematic review of dynamic markers of endothelial dysfunction in lacunar stroke.

1. brain ischemia/ or brain infarction/ or brain stem infarctions/ or cerebral infarction/ or hypoxia-ischemia, brain/ or stroke/
2. (isch?emi$ adj6 (stroke$ or apoplex$ or cerebral vasc$ or cerebrovasc$ or cva or attack$)).tw.
3. ((brain or cerebr$ or cerebell$ or vertebrobasil$ or hemispher$ or intracran$ or intracerebral or infratentorial or supratentorial or middle cerebr$ or mca$ or anterior circulation) adj5 (isch?emi$ or infarct$ or thrombo$ or emboli$ or occlus$ or hypoxi $)).tw.
4. 1 or 2 or 3
5. (lacun$ or small vessel$ or small infarct$ or microinfarct$ or subcortical lesion$ or subcortical infarct$ or microvascular$ or microcirculation$).tw.
6. 4 and 5
7. blood-brain barrier/ or endothel$, vascular/ or tunica intima/ or microcirculation/
8. (endotheli$ adj5 (function$ or dysfunction$ or impairment$)).tw.
9. ((vascular or capillary) adj5 endotheli$).tw.
10. (endotheli$ adj5 (contraction or relaxation)).tw.
11. vascular tone/ or arterial stiffness.mp. [mp=title, original title, abstract, name of substance word, subject heading word]
12. (vascul$ tone or neurovasc$ coupl$ or arterial stiff$ or vascul$ remodel$ or cerebrovascular reactiv$ or cerebral autoregulation).tw.
13. (Flow mediated adj3 (dilat$ or vasodilat$)).tw.
14. exp Ultrasonography, Doppler, Transcranial/
15. pulse wave analysis.tw.
16. strain gauge plethysmography.tw.
17. (brachial artery or radial artery or popiteal artery or posterior tibial artery).tw.
18. or/7-17
19. 6 and 18
20. limit 19 to yr="1995 - 2008"
21. limit 19 to humans
22. limit 21 to humans
23. from 22 keep 1-376
24. (strain gauge plethysmography or venous occlusion plethysmography).tw.
25. forearm blood flow.tw.
26. (dorsal hand vein technique or aellig technique).tw.
27. stimulated tPA release.tw.
28. or/24-27
29. 18 or 28

Appendix 3. Patient information leaflet for Mild Stroke Study

Patient Information:

The Mild Stroke Study

Dec 2006

What is the study about?

We are investigating patients who have had a mild stroke. We are trying to find out why the different types of mild stroke happen. We aim to find links between brain scan and retinal (back of the eye) appearances. This will hopefully mean that in the future we will be able to better understand, prevent and treat patients who have had mild strokes.

Why have I been asked to take part?

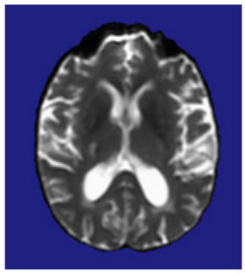
You have been asked because you have recently had a mild stroke.

What will the study involve?

If you agree to be in the study we will change the type of brain scan we do from a CT scan to MRI scan. MRI scans give more detailed pictures. We will also take photographs of your retina (the back of the eye) and a blood sample.

For the brain scans you will need to lie flat and still on your back on a table. The table will move into the scanner so that only your head is in the scanner. The rest of your body is outside the scanner.

For the retinal photographs you will need to sit opposite a camera. We may put eye drops into your eye to make the pupil bigger. You will not be able to drive for four hours after the eye drops are put in.



MRI IMAGE OF THE BRAIN

How long will it take?

For the brain scan you will need to lie down in the scanner for twenty to thirty minutes. The retinal photographs will take about ten minutes.

What is the benefit to me?
You will have a more detailed scan than you would normally have. This will help you and the doctors looking after you. You will also be helping future patients who may have a stroke.

Are there any bad points?
All of the tests performed are relatively safe. Occasionally patients feel claustrophobic in the brain scan and we stop the scan if this is the case. Certain patients cannot go into the scanner and we ask all patients about this – for example if the patient has a pacemaker. I will takea small blood sample from you.

How will I travel to the hospital?
If you can have a lift from a relative or friend I can reserve you a parking space in the hospital. If this is not possible then I will arrange a taxi which we will pay for.

What happens if you find something abnormal on the scans?
We write to your General Practitioner anyway to inform them that you are in the study. If we find any other abnormal results we will write another letter to your GP.

What do you do with the scans?
We store the scans and may share anonymous scans with our other researchers.

Can I withdraw at any time?
Yes

Will being in the trial affect my medical care?
You may get better care as the quality of the scan used is better than normal but otherwise your quality of care will be unchanged.

How can I find out the results of the trial?
If you would like to find out we will send you a letter detailing what the results are when the study is finished.

Can I discuss the trial with another doctor?
You can, if you wish, discuss whether or not to have the scans with one of our doctors who is not involved in the research and can give independent advice. His name is Professor Charles Warlow, Professor of Neurology, Department of Clinical Neurosciences, Western General Hospital. Please ask if you wish to speak to him, or phone 537 1000 to contact him by phone.

If you have any further questions about the study, even after you have left the hospital, you may contact:

Dr. Fergus Doubal- tel number –

07789 792200/ 0131 537 2909

or

Professor Joanna Wardlaw -0131-537-3110

Thank you for taking the time to read this leaflet and considering the study.

Appendix 4. Patient consent form

NHS Lothian

The Lothian University Hospital NHS Trust
Western General Hospital

MAGNETIC RESONANCE IMAGING AND ULTRASOUND STUDIES IN LACUNAR STROKE

PATIENT'S CONSENT FORM

I have read the information leaflet "Magnetic Resonance and Ultrasound Imaging Studies in Lacunar Stroke". I have had time to consider the study and have had all my questions about it answered. ☐

I understand that I am free to withdraw at any time from any part of the study, without giving a reason, and without it adversely affecting my future medical care. ☐

I agree to take part in the above study. ☐

I agree that my anonymous retinal photographs may be shared with other researchers. ☐

Signed:……(patient's signature)

Name:……(patient's name)

Date:……

Signed: ……(signature of the investigator)

Name: ……(name of the investigator)

Date: ……

Please Attach Patient Label

When signed, Please attach this form to patient's MR request card.

Lacunar Stroke Dec 2006

Appendix 5. Patient data collection sheet.

Mild Stroke Study Data Collection Form

(Amended for data punching – if there is a line then answer is numerical and if a box it is a tick – if unticked assume no)

Patient Name - _____ DOB -___/___/____ Sex- M ☐ F ☐
CN number- _____
Date of assessment for study - ___/___/____

PMH of –

TIA ☐
Stroke ☐ (left ☐/right ☐ /both ☐/infarct ☐/haem ☐/cortical ☐/lacunar ☐) –
Ischaemic Heart Disease ☐ (angina ☐ /MI ☐/angioplasty ☐/CABG ☐)
Peripheral Vascular Disease (or symptoms of) ☐
Diabetes Mellitus ☐ (diagnosed at presentation ?)
Hypertension ☐
Atrial Fib ☐
Hyperlipidaemia ☐
LVSD ☐
Structural Heart Disease ☐

Social and family history

Family history of stroke ☐
Cigarette smoker ☐ (ex-<12 months ☐, ex >12 months ☐, never ☐)
Alcohol use ☐ - units per week _____ Previous alcohol excess ☐
Independent prior to stroke ☐

Details of presenting stroke

Date and time of symptom onset ____/_____/_____ - ___.____
Duration of symptoms - _____ days or persisting
Date patient seen by stroke service ____/____/_____
Side of body/vision affected – Left ☐ Right ☐

Weakness ☐ (face ☐, arm ☐, leg ☐)
Sensory abnormality ☐ (face ☐, arm ☐, leg ☐)
Posterior circulation symptoms ☐
Dysphasia ☐ Neglect ☐
Visual field loss ☐

Right ☐ Left ☐ Handed
NIHSS score at assessment _____ and estimated worst _____

Investigations at time of presenting stroke

Total cholesterol _____ Blood glucose _____
ECG - sinus ☐ Atrial Fibrillation ☐ LVH ☐
Carotid doppler/ MRA – Left ICA stenosis _____% Right ICA stenosis _____%
Echocardiology abnormality ☐ If so complete free text
Blood Pressure ____/____ mmHg

Medications at time of stroke	**Medications at time of MRI permeability scan**
Aspirin ☐	Aspirin ☐
Dipyridamole ☐	Dipyridamole ☐
Clopidogrel ☐	Clopidogrel ☐
Warfarin ☐	Warfarin ☐
Diuretic ☐	Diuretic ☐
ACE inhibitor ☐	ACE inhibitor ☐
Angiotensin II R antagonist ☐	Angiotensin II R antagonist ☐
Beta blocker ☐	Beta blocker ☐
Other antihypertensive ☐	Other antihypertensive ☐
Oral Hypoglycaemic ☐	Oral Hypoglycaemic ☐
Insulin ☐	Insulin ☐
Statin ☐	Statin ☐
Other chol low medication ☐	Other chol low medication ☐

Clinical Classification – Lacunar ☐ Cortical ☐
Imaging Classification – Lacunar ☐ Cortical ☐

Final Clinical and Imaging Classification – Lacunar ☐ Cortical ☐ POCS ☐

Free text comments:

Appendix 6. MRI coding form.

MRI NVSI grading for Mild Stroke Study

Patient CN: _____ Date/time of MR: ____/____/____:____.

Sequences done (underline)– DWI/T2/FLAIR/GRE/T1/OTHER

Relevant lesion present (age and location) – **Y**es/ **N**o

Sequence on which lesion present: DWI – **Y**es/ **N**o FLAIR/T2 - **Y**es/ **N**o
Side of brain – **L**eft/ **R**ight

Lacunar (size mm ☐)	**MCA Cortical**	**Other Cortical**
internal capsule ☐	small temporal ☐	anterior ACA ☐
internal border zone ☐	small parietal ☐	posterior ACA ☐
centrum semiovale ☐	basal ganglia ☐	anterior PCA ☐
extends to cortical margin ☐	sub-cortical ☐	posterior PCA ☐
thalamus ☐	ant half periph MCA ☐	ant border zone ☐
lentiform ☐	post half periph MCA ☐	post border zone ☐
brainstem ☐		

Old lesion(s) present Yes/No

	Lesion 1		Lesion 2		Lesion 3
Left/**R**ight	Side ☐	Side	☐	Side	☐
Lac/**C**ortical/**P**ost	Location ☐		Location ☐		Location ☐
Infarct/**H**aemorrhage	Type ☐		Type ☐		Type ☐

Number of microhaemorrhages seen ☐

White matter rating –	Overall	Left	Right
Periventricular lesions-Fazekas **1/2/3** ☐	☐	☐	
Deep white matter lesions - Fazekas **1/2/3**	☐	☐	☐
De Leeuw n<3mm	☐	☐	☐
De Leeuw n3-10mm	☐	☐	☐
De Leeuw n>10mm	☐	☐	☐
Enlarged perivascular spaces			
Basal ganglia 0-4	☐	☐	☐
Centrum semiovale 0-4 ☐	☐	☐	

Deep atrophy **N**one/**M**ild/**M**oderate/**S**evere
Superficial atrophy **N**one/**M**ild/**M**oderate/**S**evere

Appendix 7. Retinal vascular grading form.

Mild Stroke Study Retinal Vascular Grading Form

August 2006
Patient CN
Grading date
Grader
Eye Left =1 Right=2

Field	Macula	Disc	Lat Mac	Upper Arcade	Lower Arcade	Nasal
Ungradable	1 CL PQ	1 CL PQ	1 CL PQ	1 CL PQ	1 CL PQ	1 CL PQ
Missing	2	2	2	2	2	2
Focal Art Narrow						
None	1	1	1	1	1	1
Questionable	2	2	2	2	2	2
Yes Mild	3	3	3	3	3	3
Severe	4	4	4	4	4	4
AVN						
None	1	1	1	1	1	1
Ques	2	2	2	2	2	2
Yes Mild	3	3	3	3	3	3
Moderate	4	4	4	4	4	4
Severe	5	5	5	5	5	5
No of crossings						
Total no of crossings						
No of AV crossings						
No. of AVN 1	1	1	1	1	1	1
2	2	2	2	2	2	2
3+	3	3	3	3	3	3
Hard Exudates (overall)		**Haemorrhage/micro aneurysms**				
None	1	None	1			
Questionable	2	Questionable	2			
Yes	3	MAs only	3			
CG?	4	Haem only	4			
Soft Exudates		HMA<2A	5			
None	1	HMA>2A	6			
Questionable	2	CG	7			
Yes	3					
CG	4					

Appendix 8. Medline search strategy for retinal microvascular abnormalities in stroke systematic review.

On stroke/TIA/asymptomatic stroke:

1. exp Ischemic Attack, Transient
2. cerebrovascular disorders/
3. exp brain ischemia/
4. cerebrovascular accident/
5. exp brain infarction/
6. exp hypoxia-ischemia, brain/
7. exp "Intracranial Embolism and Thrombosis"/
8. exp intracranial hemorrhages/
9. (stroke or cva$ or isch?emi$ attack$ or tia$1 or neurologic$ deficit$).tw.
10. ((brain$ or cerebr$ or cerebell$ or cortical or vertebrobasilar or hemispher$ or intracran$ or intracerebral or infratentorial or supratentorial or MCA or anterior circulation or posterior circulation or basal ganglia) adj10 (isch?emi$ or infarct$ or thrombo$ or emboli$ or occlus$ or hypox$ or vasospasm or obstruction or vasculopathy)).tw.
11. ((lacunar or cortical) adj5 infarct$).tw.
12. ((brain$ or cerebr$ or cerebell$ or intracerebral or intracran$ or parenchymal or intraventricular or infratentorial or supratentorial or basal gangli$ or putaminal or putamen or posterior fossa) adj10 (haemorrhage$ or hemorrhage$ or bleed$)).tw.

On retinal abnormalities:

13. exp *Retina/
14. exp *Retinal Vessels/
15. exp *Retinal Artery/
16. exp *Retinal Vein/
17. exp *Retinal Diseases/
18. exp *Retinal Hemorrhage/
19. exp *Retinal Artery Occlusion/
20. exp *Papilledema
21. (retina$ or retinopath$ or retinal abnormal$).tw

Overall search:

22. or/1-12
23. or/13-21
24. 22 and 23

Reference List

Abbott NJ. Inflammatory mediators and modulation of blood-brain barrier permeability. Cell Mol Neurobiol 2000; 20: 131-147.

Abbott NJ. Evidence for bulk flow of brain interstitial fluid: significance for physiology and pathology. Neurochem Int 2004; 45: 545-552.

Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 2006; 7: 41-53.

Abu El-Asrar AM, Al Rubeaan KA, Al Amro SA, Moharram OA, Kangave D. Retinopathy as a predictor of other diabetic complications. International Ophthalmology 2001; 24: 1-11.

Adams HP, Jr., Bendixen BH, Kappelle LJ *et al*. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke 1993; 24: 35-41.

Adams HP, Jr., Davis PH, Leira EC *et al*. Baseline NIH Stroke Scale score strongly predicts outcome after stroke: A report of the Trial of Org 10172 in Acute Stroke Treatment (TOAST). Neurology 1999; 53: 126.

Ageno W, Finazzi S, Steidl L *et al*. Plasma measurement of D-dimer levels for the early diagnosis of ischemic stroke subtypes. Arch Intern Med 2002; 162: 2589-2593.

Aho K, Harmsen P, Hatano S, Marquardsen J, Smirnov VE, Strasser T. Cerebrovascular disease in the community: results of a WHO collaborative study. Bull World Health Organ 1980; 58: 113-130.

Albers GW. Advances in intravenous thrombolytic therapy for treatment of acute stroke. Neurology 2001; 57: 77S-781.

Allder SJ, Moody AR, Martel AL, Morgan PS, Delay GS, Gladman JR. Limitations of clinical diagnosis in acute stroke. Lancet 1999; 354: 1523.

Andersson T. What do white matter hyperintensities really represent? Stroke 2010; 41: 574.

Anjos EG, Mendonca M, Orefice F. Retinal Vessels, " To Be or Not to Be" Fractal? Log-log plots are Invalid Paradigms of "The Fractal Geometry of Nature". Invest Ophthalmol Vis Sci 2010; 51: E-abstract 6441.

Arboix A, Ferrer I, Marti-Vilalta JL. Analisis clinico-anatomopatologico de 25 pacientes con infartos lacunares. Rev Clin Esp 1996; 196: 370-374.

Arboix A, Lopez-Grau M, Casasnovas C, Garcia-Eroles L, Massons J, Balcells M. Clinical study of 39 patients with atypical lacunar syndrome. J Neurol Neurosurg Psychiatry 2006; 77: 381-384.

Avakian A, Kalina RE, Sage EH *et al*. Fractal analysis of region-based vascular change in the normal and non-proliferative diabetic retina. Curr Eye Res 2002; 24: 274-280.

Ay H, Buonanno FS, Rordorf G *et al*. Normal diffusion-weighted MRI during stroke-like deficits. Neurology 1999a; 52: 1784-1792.

Ay H, Oliveira-Filho J, Buonanno FS *et al*. Diffusion-weighted imaging identifies a subset of lacunar infarction associated with embolic source. Stroke 1999b; 30: 2644-2650.

Bailey EL, McCulloch J, Sudlow C, Wardlaw JM. Potential animal models of lacunar stroke: a systematic review. Stroke 2009; 40: e451-e458.

Baker ML, Hand PJ, Liew G *et al*. Retinal microvascular signs may provide clues to the underlying vasculopathy in patients with deep intracerebral hemorrhage. Stroke 2010; 41: 618-623.

Baker ML, Hand PJ, Wang JJ, Wong TY. Retinal signs and stroke: revisiting the link between the eye and brain. Stroke 2008; 39: 1371-1379.

Ball CJ, Henkind P. Retinal arteriolar collaterals in man. Br J Ophthalmol 1967; 51: 688-691.

Bamford J, Sandercock P, Dennis M, Burn J, Warlow C. Classification and natural history of clinically identifiable subtypes of cerebral infarction. Lancet 1991; 337: 1521-1526.

Bamford JM, Warlow CP. Evolution and testing of the lacunar hypothesis. Stroke 1988; 19: 1074-1082.

Bang OY, Joo SY, Lee PH *et al*. The course of patients with lacunar infarcts and a parent arterial lesion: similarities to large artery vs small artery disease. Arch Neurol 2004; 61: 514-519.

Bang OY, Yeo SH, Yoon JH *et al*. Clinical MRI cutoff points for predicting lacunar stroke may not exist: need for a grading rather than a dichotomizing system. Cerebrovasc Dis 2007; 24: 520-529.

Baumgartner RW, Sidler C, Mosso M, Georgiadis D. Ischemic lacunar stroke in patients with and without potential mechanism other than small-artery disease. Stroke 2003; 34: 653-659.

Bayu SM, Wang JJ, Donaghue KC *et al*. Alterations in Retinal Microvascular Geometry in Young Type 1 Diabetes. Diabetes Care 2010; 33: 1336.

Bechmann I, Priller J, Kovac A *et al*. Immune surveillance of mouse brain perivascular spaces by blood-borne macrophages. Eur J Neurosci 2001; 14: 1651-1658.

Bell JE. Is Post Mortem Practice in Terminal Decline and Should We Care? Practical Neurology 2004; 4: 257-259.

Benavente O, Hart RG. Secondary prevention of small subcortical strokes (SPS3). Seminars in Cerebrovascular Diseases and Stroke 2003; 3: 8-17.

Benisty S, Gouw AA, Porcher R *et al*. Location of lacunar infarcts correlates with cognition in a sample of non-disabled subjects with age-related white-matter changes: the LADIS study. J Neurol Neurosurg Psychiatry 2009; 80: 478-483.

Bernacki J, Dobrowolska A, Nierwinska K, Malecki A. Physiology and pharmacological role of the blood-brain barrier. Pharmacol Rep 2008; 60: 600-622.

Black S, Gao F, Bilbao J. Understanding white matter disease: imaging-pathological correlations in vascular cognitive impairment. Stroke 2009; 40: S48-S52.

Boiten J, Lodder J. Large striatocapsular infarcts: clinical presentation and pathogenesis in comparison with lacunar and cortical infarcts. Acta Neurol Scand 1992; 86: 298-303.

Boiten J, Lodder J, Kessels F. Two clinically distinct lacunar infarct entities? A hypothesis. Stroke 1993; 24: 652-656.

Boiten J, Rothwell PM, Slattery J, Warlow CP. Ischaemic lacunar stroke in the European Carotid Surgery Trial. Cerebrovasc Dis 1996; 6: 281-287.

Braffman BH, Zimmerman RA, Trojanowski JQ, Gonatas NK, Hickey WF, Schlaepfer WW. Brain MR: pathological correlation with gross and histopathology. 1. Lacunar infarction and Virchow-Robin spaces. AJR Am J Roentgenol 1988; 151: 551-558.

Bruno A, Jones WL, Austin JK, Carter S, Qualls C. Vascular outcome in men with asymptomatic retinal cholesterol emboli. A cohort study. Annals of Internal Medicine 1995; 122: 249-253.

Burton JL, Underwood J. Clinical, educational, and epidemiological value of autopsy. Lancet 2007; 369: 1471-1480.

Castellanos M, Castillo J, Garcia MM *et al*. Inflammation-mediated damage in progressing lacunar infarctions: a potential therapeutic target. Stroke 2002; 33: 982-987.

Chalela JA, Kidwell CS, Nentwich LM *et al*. Magnetic resonance imaging and computed tomography in emergency assessment of patients with suspected acute stroke: a prospective comparison. Lancet 2007; 369: 293-298.

Challa VR, Bell MA, Moody DM. A combined hematoxylin-eosin, alkaline phosphatase and high-resolution microradiographic study of lacunes. Clin Neuropath 1990; 9: 196-204.

Chamorro A, Saiz A, Vila N *et al*. Contribution of arterial blood pressure to the clinical expression of lacunar infarction. Stroke 1996; 27: 388-392.

Chan-Ling T, Hughes S, Baxter L *et al*. Inflammation and breakdown of the blood-retinal barrier during "physiological aging" in the rat retina: a model for CNS aging. Microcirculation 2007; 14: 63-76.

Chapman N, Baharudin S, King L, Stanton A, Thom S, Hughes A. Acute effects of L-NMMA on the retinal circulation in man. Journal of Human Hypertension 2000; 14: 841-842(abstract).

Chapman N, Dell'Omo G, Sartini MS *et al*. Peripheral vascular disease is associated with abnormal arteriolar diameter relationships at bifurcations in the human retina. Clin Sci (Lond) 2002; 103: 111-116.

Chapman N, Mohamudally A, Cerutti A *et al*. Retinal vascular network architecture in low-birth-weight men. J Hypertens 1997; 15: 1449-1453.

Chen HC, Patel V, Wiek J, Rassam SM, Kohner EM. Vessel diameter changes during the cardiac cycle. Eye (Lond) 1994; 8 (Pt 1): 97-103.

Chen PL, Wang PY, Sheu WH *et al*. Changes of brachial flow-mediated vasodilation in different ischemic stroke subtypes. Neurology 2006; 67: 1056-1058.

Cheung N, Donaghue KC, Liew G *et al*. Quantitative assessment of early diabetic retinopathy using fractal analysis. Diabetes Care 2009; 32: 106-110.

Cheung N, Islam FM, Jacobs DR, Jr. *et al*. Arterial compliance and retinal vascular caliber in cerebrovascular disease. Ann Neurol 2007a; 62: 618-624.

Cheung N, Islam FM, Saw SM *et al*. Distribution and associations of retinal vascular caliber with ethnicity, gender, and birth parameters in young children. Invest Ophthalmol Vis Sci 2007b; 48: 1018-1024.

Cheung N, Liew G, Lindlay RI *et al*. Retinal fractals and acute lacunar stroke. Ann Neurol 2010; 68: 107-111.

Cheung N, Rogers S, Couper DJ, Klein R, Sharrett AR, Wong TY. Is diabetic retinopathy an independent risk factor for ischemic stroke? Stroke 2007c; 38: 398-401.

Cho AH, Kang DW, Kwon SU, Kim JS. Is 15 mm size criterion for lacunar infarction still valid? A study on strictly subcortical middle cerebral artery territory infarction using diffusion-weighted MRI. Cerebrovasc Dis 2007; 23: 14-19.

Chowdhury D, Wardlaw JM, Dennis MS. Are multiple lacunar infarctions caused by embolic mechanisms? J Neurol Neurosurg Psychiatry 2004; 75: 1416-1420.

Cohen JA, Estacio RO, Lundgren RA, Esler AL, Schrier RW. Diabetic autonomic neuropathy is associated with an increased incidence of strokes. Autonomic Neuroscience-Basic & Clinical 2003; 108: 73-78.

Cooper LS, Wong TY, Klein R *et al*. Retinal microvascular abnormalities and MRI-defined subclinical cerebral infarction: the Atherosclerosis Risk in Communities Study. Stroke 2006; 37: 82-86.

Cordonnier C, Al-Shahi SR, Wardlaw J. Spontaneous brain microbleeds: systematic review, subgroup analyses and standards for study design and reporting. Brain 2007; 130: 1988-2003.

Cugati S, Wang JJ, Knudtson MD *et al*. Retinal Vein Occlusion and Vascular Mortality. Pooled Data Analysis of 2 Population-Based Cohorts. Ophthalmology 2007; 114: 520-524.

Cupini LM, Diomedi M, Placidi F, Silvestrini M, Giacomini P. Cerebrovascular reactivity and subcortical infarctions. Arch Neurol 2001; 58: 577-581.

Cupini LM, Pasqualetti P, Diomedi M *et al*. Carotid artery intima-media thickness and lacunar versus nonlacunar infarcts. Stroke 2002; 33: 689-694.

Davis SM, Donnan GA. Why lacunar syndromes are different and important. Stroke 2004; 35: 1779-1780.

De Jong FJ, Ikram MK, Witteman JC, Hofman A, de Jong PT, Breteler MM. Retinal vessel diameters and the role of inflammation in cerebrovascular disease. Ann Neurol 2007; 61: 491-495.

De Jong FJ, Vernooij MW, Ikram MK *et al*. Arteriolar oxygen saturation, cerebral blood flow, and retinal vessel diameters. The Rotterdam Study. Ophthalmology 2008; 115: 887-892.

de Jong G, van RL, Kessels F, Lodder J. Stroke subtype and mortality. a follow-up study in 998 patients with a first cerebral infarct. J Clin Epidemiol 2003; 56: 262-268.

de Lau LM, Smith AD, Refsum H, Johnston C, Breteler MM. Plasma vitamin B12 status and cerebral white-matter lesions. J Neurol Neurosurg Psychiatry 2009; 80: 149-157.

de Leeuw FE, de Groot JC, Achten E *et al*. Cerebral white matter changes and atherosclerosis - the Rotterdam Scan Study. J Neurol 1998; 245: 345.

de Leeuw FE, de Groot JC, Bots ML *et al*. Carotid atherosclerosis and cerebral white matter lesions in a population based magnetic resonance imaging study. J Neurol 2000; 247: 291-296.

de Leeuw FE, van Huffelen A, Kappelle J. Cerebrovascular reactivity in patients with a recent lacunar infarction. J Neurol 2003; 250: 232-233.

De Reuck J, Nieuwenhuis L, Hemelsoet D. Do corticosubcortical lacunes exist? A clinicopathological case report. Cerebrovasc Dis 2004; 18: 342-344.

Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation 2007; 115: 1285-1295.

Dechambre A. Memoire sur la curabilite du ramoulissement cerebral. Gaz Med Paris 1838; 6: 305-314.

DellaCroce JT, Vitale AT. Hypertension and the eye. Curr Opin Ophthalmol 2008; 19: 493-498.

Delles C, Michelson G, Harazny J, Oehmer S, Hilgers KF, Schmieder RE. Impaired endothelial function of the retinal vasculature in hypertensive patients. Stroke 2004; 35: 1289-1293.

Department of Health, Vascular Programme, Stroke. National Stroke Strategy. 2007.

Dewey HM, Thrift AG, Mihalopoulos C *et al*. Cost of stroke in Australia from a societal perspective. Results from the North East Melbourne Stroke Incidence Study (NEMESIS). Stroke 2001; 32: 2409-2416.

Dimmitt SB, West JN, Eames SM, Gibson JM, Gosling P, Littler WA. Usefulness of ophthalmoscopy in mild to moderate hypertension. Lancet 1989; 1: 1103-1106.

Ding J, Patton N, Deary IJ *et al*. Retinal microvascular abnormalities and cognitive dysfunction: a systematic review. Br J Ophthalmol 2008; 92: 1017-1025.

Doubal FN, Hokke PE, Wardlaw JM. Retinal microvascular abnormalities and stroke: a systematic review. J Neurol Neurosurg Psychiatry 2009; 80: 158-165.

Eastwood JD, Engelter ST, MacFall JF, DeLong DM, Provenzale JM. Quantitative assessment of the time course of infarct signal intensity on diffusion-weighted images. AJNR Am J Neuroradiol 2003; 24: 680-687.

Farrall AJ, Wardlaw JM. Blood brain barrier: ageing and microvascular disease - systemic review and meta-analysis. Neurobiol Aging 2007; 30: 337-352.

Farrell C, Chappell F, Armitage PA *et al*. Development and initial testing of normal reference MR images for the brain at ages 65-70 and 75-80 years. Eur Radiol 2008; 19: 177-183.

Fazekas F, Chawluk JB, Alavi A, Hurtig HI, Zimmerman RA. MR signal abnormalities at 1.5T in Alzheimer's dementia and normal aging. AJR Am J Roentgenol 1987; 149: 351-356.

Fazekas F, Schmidt R, Scheltens P. Pathophysiologic mechanisms in the development of age-related white matter changes of the brain. Dement Geriatr Cogn Disord 1998; 9: 2-5.

Ferguson SC, Blane A, Perros P *et al*. Cognitive ability and brain structure in type 1 diabetes: relation to microangiopathy and preceding severe hypoglycaemia. Diabetes 2003; 52: 149-156.

Fernando MS, Simpson JE, Matthews F *et al*. White matter lesions in an unselected cohort of the elderly: molecular pathology suggests origin from chronic hypoperfusion injury. Stroke 2006; 37: 1391-1398.

Fiebach JB, Schellinger PD, Jansen O *et al*. CT and diffusion-weighted MR imaging in randomized order: diffusion- weighted imaging results in higher accuracy and lower interrater variability in the diagnosis of hyperacute ischemic stroke. Stroke 2002; 33: 2206-2210.

Fisher CM. Lacunes: small, deep cerebral infarcts. Neurology 1965; 774-784.

Fisher CM. The arterial lesions underlying lacunes. Acta Neuropathol 1969; 12: 1-15.

Fisher CM. Lacunar strokes and infarcts: a review. Neurology 1982; 32: 871.

Frank RN, Turczyn TJ, Das A. Pericyte coverage of retinal and cerebral capillaries. Invest Ophthalmol Vis Sci 1990; 31: 999-1007.

Fuller JH, Stevens LK, Wang S-L *et al*. Risk factors for cardiovascular mortality and morbidity: The WHO multinational study of vascular disease in diabetes. Diabetologia 2001; 44: S54-S64.

Fulton A, Stanton A, Moroney J. Direct comparison of microvascular endothelial function and carotid intima-media thickness in patients with large vessel and small vessel ischaemic stroke. Cerebrovasc Dis 2006; 21: 22.

Futrell N, Millikan C, Watson BD, Dietrich WD, Ginsberg MD. Embolic stroke from a carotid arterial source in the rat: pathology and clinical implications. Neurology 1989; 39: 1050-1056.

Gass A, Ay H, Szabo K, Koroshetz WJ. Diffusion-weighted MRI for the "small stuff": the details of acute cerebral ischaemia. Lancet Neurol 2004; 3: 39-45.

Geijer B, Lindgren A, Brockstedt S, Stahlberg F, Holtas S. Persistent high signal on diffusional-weighted MRI in the late stages of small cortical and lacunar ischaemic lesions. Neuroradiology 2001; 43: 115-122.

Giles MF, Rothwell PM. Risk of stroke early after transient ischaemic attack: a systematic review and meta-analysis. Lancet Neurol 2007; 6: 1063-1072.

Gold G, Kovari E, Herrmann FR *et al*. Cognitive Consequences of Thalamic, Basal Ganglia, and Deep White Matter Lacunes in Brain Aging and Dementia. Stroke 2005; 36: 1184-1188.

Goto I, Katsuki S, Ikui H, Kimoto K, Mimatsu T. Pathological studies on the intracerebral and retinal arteries in cerebrovascular and noncerebrovascular diseases. Stroke 1975; 6: 263-269.

Gouw AA, van der Flier WM, Fazekas F *et al*. Progression of white matter hyperintensities and incidence of new lacunes over a 3-year period. The leukoaraiosis and disability study. Stroke 2008; 39: 1414-1420.

Grau AJ, Weimar C, Buggle F *et al*. Risk Factors, Outcome, and Treatment in Subtypes of Ischemic Stroke: The German Stroke Data Bank. Stroke 2001; 32: 2559-2566.

Grauslund J, Green A, Kawasaki R, Hodgson L, Sjolie AK, Wong TY. Retinal Vascular Fractals and Microvascular and Macrovascular Complications in Type 1 Diabetes. Ophthalmology 2010; 117: 1400-1405.

Gunning-Dixon FM, Raz N. The cognitive correlates of white matter abnormalities in normal aging: a quantitative review. Neuropsychology 2000; 14: 224-232.

Gur AY, Gucuyener D, Uzuner N *et al*. Cerebral vasomotor reactivity of patients with acute ischemic stroke: cortical versus subcortical infarcts: an Israeli-Turkish collaborative study. J Neurol Sci 2007; 257: 121-125.

Hainsworth AH, Markus HS. Do in vivo experimental models reflect human cerebral small vessel disease? A systematic review. J Cereb Blood Flow Metab 2008; 28: 1877-1891.

Hand PJ, Wardlaw JM, Rowat AM, Haisma JA, Lindley RI, Dennis MS. Magnetic resonance brain imaging in patients with acute stroke: feasibility and patient-related difficulties. J Neurol Neurosurg Psychiatry 2005; 76: 1525-1527.

Hassan A, Hunt BJ, O'Sullivan M *et al*. Homocysteine is a risk factor for cerebral small vessel disease, acting via endothelial dysfunction. Brain 2003a; 127: 212-219.

Hassan A, Hunt BJ, O'Sullivan M *et al*. Markers of endothelial dysfunction in lacunar infarction and ischaemic leukoaraiosis. Brain 2003b; 126: 424-432.

Hatano S. Experience from a multicentre stroke register: a preliminary report. Bull World Health Organ 1976; 54: 541-553.

Hayreh SS, Zimmerman B, McCarthy MJ, Podhajsky P. Systemic diseases associated with various types of retinal vein occlusion. American Journal of Ophthalmology 2001; 131: 61-77.

Heier LA, Bauer CJ, Schwartz L, Zimmerman RD, Morgello S, Deck MDF. Large Virchow-Robin spaces: MR-clinical correlation. AJNR Am J Neuroradiol 1989; 10: 929-936.

Heuschmann PU, Grieve AP, Toschke AM, Rudd AG, Wolfe CDA. Ethnic Group Disparities in 10-Year Trends in Stroke Incidence and Vascular Risk Factors. The South London Stroke Register (SLSR). Stroke 2008; 39: 2204-2210.

Hirai FE, Moss SE, Knudtson MD, Klein BEK, Klein R. Retinopathy and Survival in a Population without Diabetes: The Beaver Dam Eye Study. Am J Epidemiol 2007; 166: 724-730.

Hiroki M, Miyashita K. Linear hyperintensity objects on magnetic resonance imaging related to hypertension. Cerebrovasc Dis 2001; 11: 164-168.

Howard RS, Russell RWR. Prognosis of patients with retinal embolism. Journal of Neurology, Neurosurgery & Psychiatry 1987; 50: 1142-1147.

Hubbard LD, Brothers RJ, King WN *et al*. Methods for evaluation of retinal microvascular abnormalities associated with hypertension/sclerosis in the Atherosclerosis Risk in Communities Study. Ophthalmology 1999; 106: 2269-2280.

Hughes AD, Stanton AV, Jabbar AS, Chapman N, Martinez-Perez ME, McG Thom SA. Effect of antihypertensive treatment on retinal microvascular changes in hypertension. J Hypertens 2008; 26: 1703-1707.

Hund-Georgiadis M, Zysset S, Naganawa S, Norris DG, Yves von Cramon D. Determination of cerebrovascular reactivity by means of fMRI signal changes in cerebral microangiopathy: a correlation with morphological abnormalities. Cerebrovasc Dis 2003; 16: 158-165.

Ikeda T, Gomi T, Kobayashi S, Tsuchiya H. Role of hypertension in asymptomatic cerebral lacunae in the elderly. Hypertension 1994; 23: I259-I262.

Ikram MA, Vernooij MW, Hofman A, Niessen WJ, van der Lugt A, Breteler MMB. Kidney function is related to cerebral small vessel disease. Stroke 2008; 39: 55-61.

Ikram MK, De Jong FJ, Bos MJ *et al*. Retinal vessel diameters and risk of stroke: the Rotterdam Study. Neurology 2006a; 66: 1339-1343.

Ikram MK, De Jong FJ, Van Dijk EJ *et al*. Retinal vessel diameters and cerebral small vessel disease: the Rotterdam Scan Study. Brain 2006b; 129: 182-188.

Ikram MK, De Jong FJ, Vingerling JR *et al*. Are retinal arteriolar or venular diameters associated with markers for cardiovascular disorders? The Rotterdam Study. Invest Ophthalmol Vis Sci 2004; 45: 2129-2134.

Immink RV, van Montfrans GA, Stam J, Karemaker JM, Diamant M, van Lieshout JJ. Dynamic cerebral autoregulation in acute lacunar and middle cerebral artery territory ischemic stroke. Stroke 2005; 36: 2595-2600.

Inoue T, Fushimi H, Yamada Y, Udaka F, Kameyama M. Asymptomatic multiple lacunae in diabetics and non-diabetics detected by brain magnetic resonance imaging. Diabetes Research & Clinical Practice 1996; 31: 81-86.

Inzitari D, Eliasziw M, Sharpe BL, Fox AJ, Barnett HJ. Risk factors and outcome of patients with carotid artery stenosis presenting with lacunar stroke. North American Symptomatic Carotid Endarterectomy Trial Group. Neurology 2000; 54: 660-666.

Iso H, Moriyama Y, Sato S *et al*. Serum total homocysteine concentrations and risk of stroke and its subtypes in Japanese. Circulation 2004; 109: 2766-2772.

Jackson CA, Sudlow CLM. Are lacunar strokes really different? A systematic review of differences in risk factor profiles between lacunar and non-lacunar infarcts. Stroke 2005; 36: 891-904.

Jensen RA, Shea S, Ranjit N *et al*. Psychosocial Risk Factors and Retinal Microvascular Signs: The Multi-Ethnic Study of Atherosclerosis. Am J Epidemiol 2009.

Jung DK, Devuyst G, Maeder P, Bogousslavsky J. Atrial fibrillation with small subcortical infarcts. J Neurol Neurosurg Psychiatry 2001; 70: 344-349.

Kang CK, Park CW, Han JY *et al*. Imaging and analysis of lenticulostriate arteries using 7.0-Tesla magnetic resonance angiography. Magn Reson Med 2009; 61: 136-144.

Kang DW, Chalela JA, Ezzeddine MA, Warach S. Association of ischemic lesion patterns on early diffusion-weighted imaging with TOAST stroke subtypes. Arch Neurol 2003a; 60: 1730-1734.

Kang DW, Latour LL, Chalela JA, Dambrosia J, Warach S. Early ischemic lesion recurrence within a week after acute ischemic stroke. Ann Neurol 2003b; 54: 66-74.

Kaposzta Z, Young E, Bath PM, Markus HS. Clinical application of asymptomatic embolic signal detection in acute stroke: a prospective study. Stroke 1999; 30: 1814-1818.

Kataoka S, Hirose G, Hori A, Shirakawa T, Saigan T. Activation of thrombosis and fibrinolysis following brain infarction. J Neurol Sci 2000; 181: 82-88.

Kaur C, Foulds WS, Ling EA. Blood-retinal barrier in hypoxic ischaemic conditions: basic concepts, clinical features and management. Prog Retin Eye Res 2008; 27: 622-647.

Kaushik S, Wang JJ, Flood V, Liew G, Smith W, Mitchell P. Frequency of fish consumption, retinal microvascular signs and vascular mortality. Microcirculation 2008; 15: 27-36.

Kazui S, Levi CR, Jones EF, Quang L, Calafiore P, Donnan GA. Risk factors for lacunar stroke: a case-control transesophageal echocardiographic study. Neurology 2000; 54: 1385-1387.

Khan U, Crossley C, Kalra L *et al*. Homocysteine and its relationship to stroke subtypes in a UK black population: the south London ethnicity and stroke study. Stroke 2008; 39: 2943-2949.

Khan U, Porteous L, Hassan A, Markus HS. Risk factor profile of cerebral small vessel disease and its subtypes. J Neurol Neurosurg Psychiatry 2007; 78: 702-706.

Kilpatrick TJ, Matkovic Z, Davis SM, McGrath CM, Dauer RJ. Hematologic abnormalities occur in both cortical and lacunar infarction. Stroke 1993; 24: 1945-1950.

Kim Y-H, Hong M-K, Lee CW *et al*. Diabetic retinopathy as a predictor of late clinical events following percutaneous coronary intervention. Journal of Invasive Cardiology 2002; 14: 599-602.

Klein BEK, Klein R, McBride PE *et al*. Cardiovascular disease, mortality, and retinal microvascular characteristics in type 1 diabetes: Wisconsin epidemiologic study of diabetic retinopathy. Archives of Internal Medicine 2004; 164: 1917-1924.

Klein R, Klein BE, Knudtson MD, Wong TY, Tsai MY. Are inflammatory factors related to retinal vessel caliber? The Beaver Dam Eye Study. Arch Ophthalmol 2006; 124: 87-94.

Klein R, Klein BEK, Jensen SC, Moss SE, Meuer SM. Retinal emboli and stroke: The beaver dam eye study. Archives of Ophthalmology 1999a; 117: 1063-1068.

Klein R, Klein BEK, Moss SE, Cruickshanks KJ. Association of ocular disease and mortality in a diabetic population. Archives of Ophthalmology 1999b; 117: 1487-1495.

Klein R, Klein BEK, Moss SE *et al*. Retinal emboli and cardiovascular disease: The Beaver Dam Eye Study. Transactions of the American Ophthalmological Society 2003; 101: 173-182.

Klein R, Sharrett AR, Klein BEK *et al*. Are retinal arteriolar abnormalities related to atherosclerosis? The atherosclerosis risk in communities study. Arteriosclerosis, Thrombosis & Vascular Biology 2000; 20: 1644-1650.

Knottnerus IL, Govers-Riemslag JW, Hamulyak K *et al*. Endothelial Activation in Lacunar Stroke Subtypes. Stroke 2010; E-pub ahead of print July 1st 2010.

Knottnerus IL, Ten CH, Lodder J, Kessels F, Van Oostenbrugge RJ. Endothelial dysfunction in lacunar stroke: a systematic review. Cerebrovasc Dis 2009; 27: 519-526.

Knudtson MD, Klein BE, Klein R *et al*. Variation associated with measurement of retinal vessel diameters at different points in the pulse cycle. Br J Ophthalmol 2004; 88: 57-61.

Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BEK. Revised formulas for summarising retinal vessel diameters. Current Eye Research 2003; 27: 143-149.

Kobayashi S, Okada K, Koide H, Bokura H, Yamaguchi S. Subcortical silent brain infarction as a risk factor for clinical stroke. Stroke 1997; 28: 1932-1939.

Kozuka K, Kohriyama T, Nomura E, Ikeda J, Kajikawa H, Nakamura S. Endothelial markers and adhesion molecules in acute ischemic stroke--sequential change and differences in stroke subtype. Atherosclerosis 2002; 161: 161-168.

Krogsaa B, Lund-Andersen H, Mehlsen J, Sestoft L, Larsen J. The blood-retinal barrier permeability in diabetic patients. Acta Ophthalmol (Copenh) 1981; 59: 689-694.

Kunicki AC, Oliveira AJ, Mendonca MB, Barbosa CT, Nogueira RA. Can the fractal dimension be applied for the early diagnosis of non-proliferative diabetic retinopathy? Braz J Med Biol Res 2009; 42: 930-934.

Kwa VIH, Van der Sande JJ, Stam J, Tijmes N, Vrooland JL. Retinal arterial changes correlate with cerebral small-vessel disease. Neurology 2002; 59: 1536-1540.

Kwee RM, Kwee TC. Virchow-Robin spaces at MR imaging. Radiographics 2007; 27: 1071-1086.

Kwon H-M, Kim BJ, Oh JY *et al*. Retinopathy as an indicator of silent brain infarction in asymptomatic hypertensive subjects. Journal of the Neurological Sciences 2007; 252: 159-162.

Lam L, Lee SW, Suen CY. Thinning methodologies-a comprehensive survey. IEEE Trans Pattern Analysis Machine Intelligence 1992; 14: 869-885.

Lammie GA. Pathology of small vessel stroke. British Medical Bulletin 2000; 56: 296-306.

Lammie GA, Brannan F, Slattery J, Warlow C. Nonhypertensive cerebral small-vessel disease. An autopsy study. Stroke 1997; 28: 2222-2229.

Lammie GA, Brannan F, Wardlaw JM. Incomplete lacunar infarction (type 1b lacunes). Acta Neuropath 1998; 96: 163-171.

Landau WM, Nassief A. Editorial comment--time to burn the TOAST. Stroke 2005; 36: 902-904.

Landini G, Misson GP, Murray PI. Fractal analysis of the normal human retinal fluorescein angiogram. Curr Eye Res 1993; 12: 23-27.

Landmesser U, Hornig B, Drexler H. Endothelial function: a critical determinant in atherosclerosis? Circulation 2004; 109: II27-II33.

Lansberg MG, Albers GW, Beaulieu C, Marks MP. Comparison of diffusion-weighted MRI and CT in acute stroke. Neurology 2000a; 54: 1557-1561.

Lansberg MG, Norbash AM, Marks MP, Tong DC, Moseley ME, Albers GW. Advantages of adding diffusion-weighted magnetic resonance imaging to conventional magnetic resonance imaging for evaluating acute stroke. Arch Neurol 2000b; 57: 1311-1316.

Leibson CL, Naessens JM, Brown RD, Whisnant JP. Accuracy of hospital discharge abstracts for identifying stroke. Stroke 1994; 25: 2348-2355.

Leppala JM, Virtamo J, Heinonen OP. Validation of Stroke Diagnosis in the National Hospital Discharge Register and the Register of Causes of Death in Finland. European Journal of Epidemiology 1999; 15: 155-160.

Lesage SR, Mosley TH, Wong TY *et al*. Retinal microvascular abnormalities and cognitive decline: the ARIC 14-year follow-up study. Neurology 2009; 73: 862-868.

Licata G, Tuttolomondo A, Di RD, Corrao S, Di SR, Pinto A. Immuno-inflammatory activation in acute cardio-embolic strokes in comparison with other subtypes of ischaemic stroke. Thromb Haemost 2009; 101: 929-937.

Liew G, Mitchell P, Wong TY *et al*. Retinal microvascular signs and cognitive impairment. J Am Geriatr Soc 2009; 57: 1892-1896.

Liew G, Sharrett AR, Kronmal R *et al*. Measurement of retinal vascular caliber: issues and alternatives to using the arteriole to venule ratio. Invest Ophthalmol Vis Sci 2007a; 48: 52-57.

Liew G, Sharrett AR, Wang JJ *et al*. Relative importance of systemic determinants of retinal arteriolar and venular caliber: the atherosclerosis risk in communities study. Arch Ophthalmol 2008a; 126: 1404-1410.

Liew G, Wang JJ. Retinal vascular signs in diabetes and hypertension--review. Arq Bras Endocrinol Metabol 2007; 51: 352-362.

Liew G, Wong TY, Mitchell P, Newall P, Smith W, Wang JJ. Retinal microvascular abnormalities and age-related hearing loss: the Blue Mountains hearing study. Ear Hear 2007b; 28: 394-401.

Liew G, Wang JJ, Cheung N *et al*. The Retinal Vasculature as a Fractal: Methodology, Reliability, and Relationship to Blood Pressure. Ophthalmology 2008b; 115: 1951-1956.

Liew G, Wang JJ, Mitchell P, Wong TY. Retinal Vascular Imaging: A New Tool in Microvascular Disease Research. Circ Cardiovasc Imaging 2008c; 1: 156-161.

Lim SW, Cheung N, Wang JJ *et al*. Retinal vascular fractal dimension and risk of early diabetic retinopathy: A prospective study of children and adolescents with type 1 diabetes. Diabetes Care 2009; 32: 2081-2083.

Lindgren A, Lindoff C, Norrving B, Astedt B, Johansson BB. Tissue plasminogen activator and plasminogen activator inhibitor-1 in stroke patients. Stroke 1996; 27: 1066-1071.

Lindgren A, Staaf G, Geijer B *et al*. Clinical lacunar syndromes as predictors of lacunar infarcts. A comparison of acute clinical lacunar syndromes and findings on diffusion-weighted MRI. Acta Neurol Scand 2000; 101: 128-134.

Lindley RI, Multi-Centre Retinal Stroke Study Collaborative Group. Retinal microvascular signs: a key to understanding the underlying pathophysiology of different stroke subtypes? International Journal of Stroke 2008; 3: 297-305.

Lindley RI, Wang JJ, Wong MC *et al*. Retinal microvasculature in acute lacunar stroke: a cross-sectional study. Lancet Neurol 2009; 8: 628-634.

Lipsitz LA, Goldberger AL. Loss of 'complexity' and aging. Potential applications of fractals and chaos theory to senescence. JAMA 1992; 267: 1806-1809.

Lloyd-Jones D, Adams RJ, Brown TM *et al*. Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation 2010; 121: 948-954.

Lodder J, Bamford J, Kappelle J, Boiten J. What causes false clinical prediction of small deep infarcts? Stroke 1994; 25: 86-91.

Longstreth W, Jr., Larsen EK, Klein R *et al*. Associations between findings on cranial magnetic resonance imaging and retinal photography in the elderly: the Cardiovascular Health Study. Am J Epidemiol 2007; 165: 78-84.

Longstreth WT, Jr., Bernick C, Manolio TA, Bryan N, Jungreis CA, Price TR. Lacunar infarcts defined by magnetic resonance imaging of 3660 elderly people. Arch Neurol 1998; 55: 1217-1225.

Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJL. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 2006; 367: 1747-1757.

Luijckx GJ, Boiten J, Kroonenburgh Mv *et al*. Systemic small-vessel disease is not exclusively related to lacunar stroke. A pilot study. J Stroke Cerebrovasc Dis 1998; 7: 52-57.

Lutsep HL, Albers GW, DeCrespigny A, Kamat GN, Marks MP, Moseley ME. Clinical utility of diffusion-weighted magnetic resonance imaging in the assessment of ischemic stroke. Ann Neurol 1997; 41: 574-580.

Ma K-C, Olsson Y. The role of chronic brain oedema in the formation of lacunes in Binswanger's encephalopathy. Cerebrovasc Dis 1997; 7: 324-331.

Macdonald RL, Kowalczuk A, Johns L. Emboli enter penetrating arteries of monkey brain in relation to their size. Stroke 1995; 26: 1247-1251.

MacGillivray TJ, Patton N, Doubal FN, Graham C, Wardlaw JM. Fractal analysis of the retinal vascular network in fundus images. Conf Proc IEEE Eng Med Biol Soc 2007; 2007: 6456-6459.

MacLullich AM, Wardlaw JM, Ferguson KJ, Starr JM, Seckl JR, Deary IJ. Enlarged perivascular spaces are associated with cognitive function in healthy elderly men. J Neurol Neurosurg Psychiatry 2004; 75: 1519-1523.

Maeda H, Matsumoto M, Handa N *et al*. Reactivity of cerebral blood flow to carbon dioxide in various types of ischemic cerebrovascular disease: evaluation by the transcranial Doppler method. Stroke 1993; 24: 670-675.

Markus HS. Genes, endothelial function and cerebral small vessel disease in man. Exp Physiol 2008; 93: 121-127.

Masters BR. Fractal analysis of normal human retinal blood vessels. Fractals 1994; 2: 103-110.

Masters BR. Fractal analysis of the vascular tree in the human retina. Annu Rev Biomed Eng 2004; 6: 427-452.

McGeechan K, Liew G, Macaskill P *et al*. Prediction of incident stroke events based on retinal vessel caliber: a systematic review and individual-participant meta-analysis. Am J Epidemiol 2009a; 170: 1323-1332.

McGeechan K, Liew G, Macaskill P *et al*. Meta-analysis: retinal vessel caliber and risk for coronary heart disease. Ann Intern Med 2009b; 151: 404-413.

McLeod D. Why cotton wool spots should not be regarded as retinal nerve fibre layer infarcts. Br J Ophthalmol 2005; 89: 229-237.

Mead GE, Lewis SC, Wardlaw JM, Dennis MS, Warlow CP. How well does the Oxfordshire community stroke project classification predict the site and size of the infarct on brain imaging? J Neurol Neurosurg Psychiatry 2000; 68: 558-562.

Mead GE, Lewis SC, Wardlaw JM, Dennis MS, Warlow CP. Severe ipsilateral carotid stenosis and middle cerebral artery disease in lacunar ischaemic stroke: innocent bystanders? J Neurol 2002; 249: 266-271.

Micheli S, Agnelli G, Palmerini F *et al*. Need for extensive diagnostic work-up for patients with lacunar stroke. Journal of Neurology 2008; 255: 637-642.

Mitchell P, Smith W, Chang A. Prevalence and associations of retinal vein occlusion in Australia: The blue mountains eye study. Archives of Ophthalmology 1996; 114: 1243-1247.

Mitchell P, Wang JJ, Wong TY, Smith W, Klein R, Leeder SR. Retinal microvascular signs and risk of stroke and stroke mortality. Neurology 2005; 65: 1005-1009.

Mitchell PJ, Rochtchina E, Liew G, Li H, Wong TY, Wang J. Association of retinal vascular fractal dimension and retinopathy lesions in older people without diabetes. Invest Ophthalmol Vis Sci 2010; 51: E-abstract 1242.

Mochizuki Y, Oishi M, Takasu T. Cerebral blood flow in single and multiple lacunar infarctions. Stroke 1997; 28: 1458-1460.

Mok VC, Wong A, Lam WW *et al*. Cognitive impairment and functional outcome after stroke associated with small vessel disease. J Neurol Neurosurg Psychiatry 2004; 75: 560-566.

Molina C, Sabin JA, Montaner J, Rovira A, Abilleira S, Codina A. Impaired cerebrovascular reactivity as a risk marker for first-ever lacunar infarction: a case-control study. Stroke 1999; 30: 2296-2301.

Moody DM, Brown WR, Challa VR, Anderson RL. Periventricular venous collagenosis: Association with leukoaraiosis. Radiology 1995; 194: 469-476.

Moseley ME, Kucharczyk J, Mintorovich J, Cohen Y, Kurhanewicz J, Derugin N. Diffusion-weighted MR imaging of acute stroke: correlation with T2-weighted and magnetic susceptibility-enhanced MR imaging in cats. Am J Neuroradiol 1990; 11: 423-429.

Moulin T, Tatu L, Vuillier F, Berger E, Chavot D, Rumbach L. Role of a stroke data bank in evaluating cerebral infarction subtypes: patterns and outcome of 1,776 consecutive patients from the Besancon stroke registry. Cerebrovasc Dis 2000; 10: 261-271.

Moustafa RR, Izquierdo-Garcia D, Jones PS *et al*. Watershed Infarcts in Transient Ischemic Attack/Minor Stroke With >=50% Carotid Stenosis. Hemodynamic or Embolic? Stroke 2010; 41: 1410-1416.

Mullins ME, Schaefer PW, Sorensen AG *et al*. CT and conventional and diffusion-weighted MR imaging in acute stroke: Study in 691 patients at presentation to the emergency department. Neuroradiology 2002; 224: 353-360.

Murray CD. The Physiological Principle of Minimum Work: I. The Vascular System and the Cost of Blood Volume. Proc Natl Acad Sci U S A 1926; 12: 207-214.

Murray CJL, Lopez AD. Global mortality, disability, and the contribution of risk factors: Global burden of disease study. The Lancet 1997a; 349: 1436.

Murray CJL, Lopez AD. Mortality by cause for eight regions of the world: global burden of disease study. Lancet 1997b; 349: 1269-1276.

Nakase T, Yamazaki T, Ogura N, Suzuki A, Nagata K. The impact of inflammation on the pathogenesis and prognosis of ischemic stroke. J Neurol Sci 2008; 271: 104-109.

Newsom RS, Sullivan PM, Rassam SM, Jagoe R, Kohner EM. Retinal vessel measurement: comparison between observer and computer driven methods. Graefes Arch Clin Exp Ophthalmol 1992; 230: 221-225.

Nguyen TT, Islam FM, Farouque HM *et al*. Retinal Vascular Caliber and Brachial Flow-Mediated Dilation. The Multi-Ethnic Study of Atherosclerosis. Stroke 2010; 41: 1343-1348.

Norrving B. Lacunar infarcts: no black holes in the brain are benign. Pract Neurol 2008; 8: 222-228.

Ogata J. Symposium Article: The arterial lesions underlying cerebral infarction. Neuropathology 1999; 19: 112-118.

Ohira T, Shahar E, Chambless LE, Rosamond WD, Mosley TH, Jr., Folsom AR. Risk factors for ischemic stroke subtypes: the Atherosclerosis Risk in Communities study. Stroke 2006; 37: 2493-2498.

Oppenheim C, Stanescu R, Dormont D *et al*. False-negative diffusion-weighted MR findings in acute ischemic stroke. Am J Neuroradiol 2000; 21: 1434-1440.

Panczel G, Bonoczk P, Voko Z, Spiegel D, Nagy Z. Impaired vasoreactivity of the basilar artery system in patients with brainstem lacunar infarcts. Cerebrovasc Dis 1999; 9: 218-223.

Pantoni L, Garcia JH. Pathogenesis of leukoaraiosis: a review. Stroke 1997; 28: 652-659.

Parnetti L, Caso V, Santucci A *et al*. Mild hyperhomocysteinemia is a risk-factor in all etiological subtypes of stroke. Neurol Sci 2004; 25: 13-17.

Patankar TF, Mitra D, Varma A, Snowden J, Neary D, Jackson A. Dilatation of the Virchow-Robin space is a sensitive indicator of cerebral microvascular disease: study in elderly patients with dementia. AJNR Am J Neuroradiol 2005; 26: 1512-1520.

Patton N, Aslam T, Macgillivray T, Dhillon B, Constable I. Asymmetry of retinal arteriolar branch widths at junctions affects ability of formulae to predict trunk arteriolar widths. Invest Ophthalmol Vis Sci 2006a; 47: 1329-1333.

Patton N, Aslam T, Macgillivray T, Pattie A, Deary IJ, Dhillon B. Retinal vascular image analysis as a potential screening tool for cerebrovascular disease: a rationale based on homology between cerebral and retinal microvasculatures. J Anat 2005; 206: 319-348.

Patton N, Aslam TM, Macgillivray T *et al*. Retinal image analysis: concepts, applications and potential. Prog Retin Eye Res 2006b; 25: 99-127.

Patton N, Pattie A, Macgillivray T *et al*. The association between retinal vascular network geometry and cognitive ability in an elderly population. Invest Ophthalmol Vis Sci 2007; 48: 1995-2000.

Patwardhan JR, Gadgil RK, Pandit YK, Velankar BB. Comparative histopathological study of arteriosclerosis of the retina, the brain and the circle of Willis. J All India Ophthalmol Soc 1970; 18: 155-161.

Petitti DB, Bhatt H. Retinopathy as a risk factor for nonembolic stroke in diabetic subjects. Stroke 1995; 26: 593-596.

Pieters B, Staals J, Knottnerus I *et al*. Periventricular white matter lucencies relate to low vitamin B12 Levels in patients with small vessel stroke. Stroke 2009; 40: 1623-1626.

Piriyawat P, Smajsova M, Smith MA *et al*. Comparison of Active and Passive Surveillance for Cerebrovascular Disease: The Brain Attack Surveillance in Corpus Christi (BASIC) Project. Am J Epidemiol 2002; 156: 1062-1069.

Poirier J, Derouesne C. [The concept of cerebral lacunae from 1838 to the present]. Rev Neurol (Paris) 1985; 141: 3-17.

Pollock H, Hutchings M, Weller RO, Zhang ET. Perivascular spaces in the basal ganglia of the human brain: their relationship to lacunes. J Anat 1997; 191 (Pt 3): 337-346.

Potter GM, Doubal FN, Jackson CA *et al*. Counting cavitating lacunes underestimates the burden of lacunar infarction. Stroke 2010; 41: 267-272.

Potter GM, Wardlaw JM. Imaging definitions of lacunar lesions amongst researchers with an interest in cerebral small vessel disease - an online, worldwide survey. Cerebrovasc Dis 2010; 29:Supp 2: 240.

Pretnar-Oblak J, Sabovic M, Pogacnik T, Sebestjen M, Zaletel M. Flow-mediated dilatation and intima-media thickness in patients with lacunar infarctions. Acta Neurol Scand 2006a; 113: 273-277.

Pretnar-Oblak J, Sabovic M, Sebestjen M, Pogacnik T, Zaletel M. Influence of atorvastatin treatment on L-arginine cerebrovascular reactivity and flow-mediated dilatation in patients with lacunar infarctions. Stroke 2006b; 37: 2540-2545.

Pretnar-Oblak J, Zaletel M, Zvan B, Sabovic M, Pogacnik T. Cerebrovascular reactivity to L-arginine in patients with lacunar infarctions. Cerebrovasc Dis 2006c; 21: 180-186.

Qaum T, Xu Q, Joussen AM *et al*. VEGF-initiated blood-retinal barrier breakdown in early diabetes. Invest Ophthalmol Vis Sci 2001; 42: 2408-2413.

Qiu C, Cotch MF, Sigurdsson S *et al*. Microvascular lesions in the brain and retina: The age, gene/environment susceptibility-Reykjavik study. Ann Neurol 2009; 65: 569-576.

Rasquin SMC, Van Oostenbrugge RJ, Verhey FRJ, Lodder J. Vascular Mild Cognitive Impairment Is Highly Prevalent after Lacunar Stroke but Does Not Increase over Time: A 2-Year Follow-Up Study. Dement Geriatr Cogn Disord 2007; 24: 396-401.

Rosenberg GA. Inflammation and white matter damage in vascular cognitive impairment. Stroke 2009; 40: S20-S23.

Rothwell PM, Coull AJ, Silver LE *et al*. Population-based study of event-rate, incidence, case fatality, and mortality for all acute vascular events in all areterial territories (Oxford Vascular Study). Lancet 2005; 366: 1773-1783.

Rothwell PM, Giles MF, Chandratheva A *et al*. Effect of urgent treatment of transient ischaemic attack and minor stroke on early recurrent stroke (EXPRESS study): a prospective population-based sequential comparison. Lancet 2007; 370: 1432-1442.

Rouhl RPW, Van Oostenbrugge RJ, Knottnerus ILH, Staals JEA, Lodder J. Virchow-Robin spaces relate to cerebral small vessel disease severity. Journal of Neurology 2008; 255: 692-696.

Sacco S, Marini C, Totaro R, Russo T, Cerone D, Carolei A. A population-based study of the incidence and prognosis of lacunar stroke. Neurology 2006; 66: 1335-1338.

Sachdev P, Wen W. Should we distinguish between periventricular and deep white matter hyperintensities? Stroke 2005; 36: 2342-2343.

Salobir B, Sabovic M. Possible vascular-bed-specific role of interleukin-6 in young women with a history of myocardial infarction, lacunar cerebral infarction and deep vein thrombosis. Cytokine 2004; 25: 265-272.

Salobir B, Sabovic M, Peternel P, Stegnar M, Grad A. Classic risk factors, hypercoagulability and migraine in young women with cerebral lacunar infarctions. Acta Neurol Scand 2002; 105: 189-195.

Sandercock PAG, Warlow CP, Jones LN, Starkey IR. Predisposing factors for cerebral infarction: The Oxfordshire community stroke project. Br Med J 1989; 298: 75-80.

Saposnik G, Young B, Silver B *et al*. Lack of improvement in patients with acute stroke after treatment with thrombolytic therapy: predictors and association with outcome. JAMA 2004; 292: 1839-1844.

Scher AI, Gudmundsson LS, Sigurdsson S *et al*. Migraine Headache in Middle Age and Late-Life Brain Infarcts. JAMA 2009; 301: 2563-2570.

Schley D, Carare-Nnadi R, Please CP, Perry VH, Weller RO. Mechanisms to explain the reverse perivascular transport of solutes out of the brain. J Theor Biol 2006; 238: 962-974.

Schmidt D. The mystery of cotton-wool spots - a review of recent and historical descriptions. Eur J Med Res 2008; 13: 231-266.

Schulz UGR, Briley D, Meagher T, Molyneux A, Rothwell PM. Abnormalities on diffusion weighted magnetic resonance imaging performed several weeks after a minor stroke or transient ischaemic attack. J Neurol Neurosurg Psychiatry 2003; 74: 734-738.

Schulz UGR, Flossmann E, Francis JM, Redgrave JN, Rothwell PM. Evolution of the diffusion-weighted signal and the apparent diffusion coefficient in the late phase after minor stroke. Journal of Neurology 2007; 254: 375-383.

Schulz UG, Briley D, Meagher T, Molyneux A, Rothwell PM. Diffusion-Weighted MRI in 300 Patients Presenting Late With Subacute Transient Ischemic Attack or Minor Stroke. Stroke 2004; 35: 2459-2465.

Scottish Intercollegiate Guidelines Network. Management of patients with stroke or TIA: Assessment, investigation, immediate management and secondary prevention. Guideline No.108. (accessed 4th July 2009) 2008; ISBN 978 1 905813 40 7.

Seifert T, Enzinger C, Storch MK, Pichler G, Niederkorn K, Fazekas F. Acute small subcortical infarctions on diffusion weighted MRI: clinical presentation and aetiology. J Neurol Neurosurg Psychiatry 2005; 76: 1520-1524.

Sharrett AR. A review of population-based retinal studies of the microvascular contribution to cerebrovascular diseases. Ophthalmic Epidemiol 2007; 14: 238-242.

Sherry LM, Wang JJ, Rochtchina E *et al*. Reliability of computer-assisted retinal vessel measurement in a population. Clinical and experimental ophthalmology 2002; 30: 179-182.

Sitoh YY, Sitoh YY, Sahadevan S. Clinical significance of cerebral white matter lesions in older Asians with suspected dementia. Age Ageing 2004; 33: 67-71.

Sng CC, Sabanayagam C, Lamoureux EL *et al*. Fractal analysis of the retinal vasculature and chronic kidney disease. Nephrol Dial Transplant 2010; 25: 2252-2258.

Snowdon DA, Greiner LH, Mortimer JA, Riley KP, Greiner PA, Markesbery WR. Brain infarction and the clinical expression of Alzheimer disease. The Nun Study. JAMA 1997; 277: 813-817.

Soares JV, Leandro JJ, Cesar Junior RM, Jelinek HF, Cree MJ. Retinal vessel segmentation using the 2-D Gabor wavelet and supervised classification. IEEE Trans Med Imaging 2006; 25: 1214-1222.

Srikanth VK, Anderson JFI, Donnan GA *et al*. Progressive dementia after first-ever stroke: A community-based follow-up study. Neurology 2004; 63: 785-792.

Staals J, van RL, Hilton A, Lodder J. Differences in long-term survival in two lacunar stroke types: a 15-year follow-up study in 782 cerebral infarct patients. Cerebrovasc Dis 2008; 25: 26-31.

Stanford MR. The pathogenesis of diabetic retinopathy. Br J Ophthalmol 2004; 88: 444-445.

Stanton AV, Wasan B, Cerutti A *et al*. Vascular network changes in the retina with age and hypertension. J Hypertens 1995; 13: 1724-1728.

Starr JM, Wardlaw J, Ferguson K, MacLullich A, Deary IJ, Marshall I. Increased blood-brain barrier permeability in type II diabetes demonstrated by gadolinium magnetic resonance imaging. J Neurol Neurosurg Psychiatry 2003; 74: 70-76.

Stosic T, Stosic BD. Multifractal analysis of human retinal vessels. IEEE Trans Med Imaging 2006; 25: 1101-1107.

Strong K, Mathers C, Bonita R. Preventing stroke: saving lives around the world. Lancet Neurol 2007; 6: 182-187.

Sudlow CLM, Warlow CP. Comparable studies of the incidence of stroke and its pathological types. Results from an international collaboration. Stroke 1997; 28: 491-499.

Sun C, Wang JJ, Mackey DA, Wong TY. Retinal vascular caliber: systemic, environmental, and genetic associations. Surv Ophthalmol 2009; 54: 74-95.

Sylaja PN, Coutts SB, Krol A, Hill MD, Demchuk AM. When to expect negative diffusion-weighted images in stroke and transient ischemic attack. Stroke 2008; 39: 1898-1900.

Tadjfar M. Branch angle and flow into a symmetric bifurcation. J Biomech Eng 2004; 126: 516-518.

Takano K, Yamaguchi T, Uchida K. Markers of a hypercoagulable state following acute ischemic stroke. Stroke 1992; 23: 194-198.

Tejada J, Diez-Tejedor E, Hernandez-Echebarria L, Balboa O. Does a relationship exist between carotid stenosis and lacunar infarction? Stroke 2003; 34: 1404-1411.

ten Dam VH, van den Heuvel DMJ, deCraen AJM *et al*. Decline in total cerebral blood flow is linked with increase in periventricular but not deep white matter hyperintensities. Radiology 2007; 243: 198-203.

Thom S, Stettler C, Stanton A *et al*. Differential effects of antihypertensive treatment on the retinal microcirculation: an anglo-scandinavian cardiac outcomes trial substudy. Hypertension 2009; 54: 405-408.

Thompson CS, Hakim AM. Living beyond our physiological means: small vessel disease of the brain is an expression of a systemic failure in arteriolar function: a unifying hypothesis. Stroke 2009; 40: e322-e330.

Topakian R, Barrick TR, Charlton RA, Schiavone F, Howe FA, Markus HS. Increased blood-brain permeability in normal-appearing white matter in subjects with small vessel disease. A MRI study. Cerebrovasc Dis 2008; 25: 21.

Tousoulis D, Antoniades C, Stefanadis C. Evaluating endothelial function in humans: a guide to invasive and non-invasive techniques. Heart 2005; 91: 553-558.

Tsaloumas MD, Kirwan J, Vinall H *et al*. Nine year follow-up study of morbidity and mortality in retinal vein occlusion. Eye 2000; 14: 821-827.

Udaka F, Sawada H, Kameyama M. White matter lesions and dementia: MRI-pathological correlation. Ann N Y Acad Sci 2002; 977: 411-415.

Ueda Y, Kanazawa S, Ohira A *et al*. Retinal vascular obstruction and asymptomatic cerebral infarction. Jpn J Ophthalmol 2002; 46: 209-214.

Urbach H, Flacke S, Keller E *et al*. Detectability and detection rate of acute cerebral hemisphere infarcts on CT and diffusion-weighted MRI. Neuroradiology 2000; 42: 722-727.

van den Born BJ, Hulsman CA, Hoekstra JB, Schlingemann RO, van Montfrans GA. Value of routine funduscopy in patients with hypertension: systematic review. BMJ 2005; 331: 73.

van Swieten JC, van den Hout JH, van Ketel BA, Hijdra A, Wokke JH, van Gijn J. Periventricular lesions in the white matter on magnetic resonance imaging in the elderly. A morphometric correlation with arteriolosclerosis and dilated perivascular spaces. Brain 1991; 114: 761-774.

Vernooij MW, Ikram MA, Tanghe HL *et al*. Incidental Findings on Brain MRI in the General Population. The New England Journal of Medicine 2007; 357: 1821-1828.

Wang JJ, Cugati S, Knudtson MD *et al*. Retinal arteriolar emboli and long-term mortality: pooled data analysis from two older populations. Stroke 2006; 37: 1833-1836.

Wardlaw JM. What causes lacunar stroke? J Neurol Neurosurg Psychiatry 2005; 76: 617-619.

Wardlaw JM. What is a lacune? Stroke 2008; 39: 2921-2922.

Wardlaw JM, Doubal F, Armitage P *et al*. Lacunar stroke is associated with diffuse blood-brain barrier dysfunction. Ann Neurol 2009; 65: 194-202.

Wardlaw JM, Farrall A, Armitage PA *et al*. Changes in background blood-brain barrier integrity between lacunar and cortical ischemic stroke subtypes. Stroke 2008; 39: 1327-1332.

Wardlaw JM, Lewis SC, Keir SL, Dennis MS, Shenkin S. Cerebral microbleeds are associated with lacunar stroke defined clinically and radiologically, independently of white matter lesions. Stroke 2006; 37: 2633-2636.

Wardlaw JM, Sandercock PA, Dennis MS, Starr J. Is breakdown of the blood-brain barrier responsible for lacunar stroke, leukoaraiosis, and dementia? Stroke 2003; 34: 806-812.

Wardlaw JM, Sellar RJ. A simple practical classification of cerebral infarcts on CT and its interobserver reliability. Am J Neuroradiol 1994; 15: 1933-1939.

Warlow C, Sudlow C, Dennis M, Wardlaw J, Sandercock P. Stroke. The Lancet 2003; 362: 1211-1224.

Weller RO, Nicoll JA. Cerebral amyloid angiopathy: pathogenesis and effects on the ageing and Alzheimer brain. Neurol Res 2003; 25: 611-616.

Wessels T, Rottger C, Jauss M, Kaps M, Traupe H, Stolz E. Identification of embolic stroke patterns by diffusion-weighted MRI in clinically defined lacunar stroke syndromes. Stroke 2005; 36: 757-761.

WHO Collaborative Group. Atlas of Heart Disease and Stroke. Geneva: World Health Organisation; 2004.

Wiszniewska M, Devuyst G, Bogousslavsky J, Ghika J, Van Melle G. What is the significance of leukoaraiosis in patients with acute iscahemic stroke? Arch Neurol 2000; 57: 967-973.

Witt N, Wong TY, Hughes AD *et al*. Abnormalities of retinal microvascular structure and risk of mortality from ischemic heart disease and stroke. Hypertension 2006; 47: 975-981.

Wong TY. Is retinal photography useful in the measurement of stroke risk? Lancet Neurol 2004; 3: 179-183.

Wong TY, Barr ELM, Tapp RJ *et al*. Retinopathy in persons with impaired glucose metabolism: The Australian diabetes obesity and lifestyle (AusDiab) study. American Journal of Ophthalmology 2005a; 140: 1157-1159.

Wong TY, Kamineni A, Klein R *et al*. Quantitative retinal venular caliber and risk of cardiovascular disease in older persons: the cardiovascular health study. Archives of Internal Medicine 2006; 166: 2388-2394.

Wong TY, Klein R, Couper DJ *et al*. Retinal microvascular abnormalities and incident stroke: the Atherosclerosis Risk in Communities Study. Lancet 2001; 358: 1134-1140.

Wong TY, Klein R, Sharrett AR *et al*. Cerebral white matter lesions, retinopathy, and incident clinical stroke. JAMA 2002a; 288: 67-74.

Wong TY, Klein R, Sharrett AR *et al*. The prevalence and risk factors of retinal microvascular abnormalities in older persons: The cardiovascular health study. Ophthalmology 2003; 110: 658-666.

Wong TY, Klein R, Sharrett AR *et al*. Retinal microvascular abnormalities and cognitive impairment in middle-aged person. The Atherosclerosis Risk in Communities Study. Stroke 2002b; 33: 1487-1492.

Wong TY, Knudtson MD, Klein R, Klein BE, Meuer SM, Hubbard LD. Computer-assisted measurement of retinal vessel diameters in the Beaver Dam Eye Study: methodology, correlation between eyes, and effect of refractive errors. Ophthalmology 2004; 111: 1183-1190.

Wong TY, McIntosh R. Hypertensive retinopathy signs as risk indicators of cardiovascular morbidity and mortality. Br Med Bull 2005b; 73-74: 57-70.

Wong TY, McIntosh R. Systemic associations of retinal microvascular signs: a review of recent population-based studies. Ophthalmic Physiol Opt 2005a; 25: 195-204.

Wong TY, Mitchell P. Hypertensive retinopathy. N Engl J Med 2004; 351: 2310-2317.

Wong TY, Mitchell P. The eye in hypertension. Lancet 2007; 369: 425-435.

Wong TY, Knudtson MD, Klein BEK, Klein R, Hubbard LD. Medication use and retinal vessel diameters. American Journal of Ophthalmology 2005b; 139: 373-375.

Wuerfel J, Haertle M, Waiczies H *et al*. Perivascular spaces--MRI marker of inflammatory activity in the brain? Brain 2008; 131: 2332-2340.

Yim-Lui CC, Wong TY, Lamoureux EL *et al*. C-reactive protein and retinal microvascular caliber in a multiethnic asian population. Am J Epidemiol 2010; 171: 206-213.

Young VG, Halliday GM, Kril JJ. Neuropathologic correlates of white matter hyperintensities. Neurology 2008; 71: 804-811.

Yu T, Mitchell P, Berry G, Li W, Wang JJ. Retinopathy in older persons without diabetes and its relationship to hypertension. Arch Ophthalmol 1998; 116: 83-89.

Zamir M. Nonsymmetrical bifurcations in arterial branching. J Gen Physiol 1978; 72: 837-845.