ENDOTHELIAL FUNCTION & GENETIC POLYMORPHISMS IN CEREBRAL SMALL VESSEL DISEASE

A study investigating the relationships between endothelial function, genetic polymorphisms and cerebral small vessel disease

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Thesis Abstract

Background

The pathogenesis of cerebral small vessel disease (SVD), encompassing lacunar infarction (LI) and leukoaraiosis (LA), is heterogeneous, with impaired endothelial function (EF) and altered fibrinolysis proposed as important contributors. Genetic factors are involved and may exert their influence via the above mechanisms.

The aim of this study was to explore the relationship between EF and SVD, and to examine the role of candidate polymorphisms in both EF and SVD.

Methods

The study cohort consisted of patients who had undergone a brain magnetic resonance image (MRI) scan for non-vascular indications. Vascular risk factors were collected by interviewing participants. SVD was classified using a modified Fazekas rating scale, where SVD burden was divided into three categories: absent/mild, moderate and severe. LI was graded separately.

EF was assessed using applanation tonometry (ApT) and the radial pulsewave. A global EF score that accounts for both endothelium-dependant and –independent vasodilation was used as the index for comparison. A higher global EF score indicated better EF.

Participants were genotyped using the sequence-specific polymerase chain reaction (PCR-SS) for eight candidate polymorphisms chosen based on biological plausibility and/or previous study evidence: interleukin-6 (IL-6) -174 G/C, NADPH oxidase p22 phox 242 C/T, tissue plasminogen activator (tPA) 20324 C/T, tPA -4360 G/C, tPA -7351 C/T, endothelial nitric oxide synthase (eNOS) -786 T/C, endothelin-1 (ET-1) 138 D/I and paraoxonase-1 (PON1) -107 C/T.

Statistical analyses were performed using Intercooled Stata 9.2, GraphPad Prism and the SNPstats. Regression models were adjusted for the appropriate variables.

Results

A total of 132 participants were assessed. All participants were genotyped and 84 of these 132 participants also had their EF assessed using ApT, but only 72 participants were successful.

Participants were graded separately for LI and LA. LA controls (n=119) were defined as participants with absent/mild LA, and LA cases (n=13) were participants with moderate or severe LA. LI controls (n=126) were participants without a radiologically defined LI and LI cases (n=6) were participants with radiologically defined LI.

The results of the study can be summarised as follows:

1. there was no significant difference between the EF of cases and controls. Subgroup analyses showed that the risk of LA decreased as the global EF values increased after adjusting for confounding influences, but the relationship was not significant (p=0.23);
2. there were no significant differences in EF between the genotypes of the eight candidate polymorphisms, except for the tPA 20324 C/T, where the TT genotype was associated with higher EF compared to the CC/CT genotypes (p=0.02);
3. the tPA 20324 TT genotype was significantly associated with an increased risk of LI compared to the CC/CT genotypes (p=0.03), although the association is under powered. No other significant associations were found.

Although the intent was to achieve a pre-determined sample size, the methodology, and in particular the exclusion criteria, restricted recruitment and consequently the study was under powered to achieve its goals. The study could therefore be considered a pilot study and any conclusions forthwith require validation in a larger sample.

Conclusion

The tPA 20324 TT genotype was significantly associated with LI, while also being significantly associated with better EF. This result may be a Type I error reflective of the small sample size. However, the result does support the hypothesis that impaired fibrinolysis has an important pathogenic role in LI. This study does not support impaired EF as a significant pathogenic contributor to SVD.

Declaration

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McLennan SN, **Lam AK**, Mathias JL, Koblar SA, Hamilton-Bruce MA, Jannes J; Vasodilation reponse and cognition; *Cerebrovascular Diseases* 2010; in submission.

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ACE	angiotensin converting enzyme
ACEI	angiotensin converting enzyme inhibitor
ADMA	asymmetric dimethylarginine
AGE	advanced glycation end products
AIx	augmentation index
AngII	angiotensin II
ApT	applanation tonometry
ARB	angiotensin receptor blocker
ATP	adenosine triphosphate
ATR	angiotensin receptor
BH4	tetrahydrobipterin
Ca^{2+}	calcium ions
CAD	coronary artery disease
CADASIL	cerebral autosomal dominant arteriopathy stroke and ischaemic leukoencephalopathy
CarVD	cardiovascular disease
CF-PWV	carotid-femoral pulsewave velocity
cGMP	cyclic guanosine monophosphate
CRP	C-reactive protein
DAG	1,2-diacylglycerol
DDAH	dimethylarginine dimethylaminohydrolase
DWM	deep white matter
ECE	endothelin converting enzyme
ED	endothelial dysfunction
EDCF	endothelium derived contracting factor
EF	endothelial function
eNOS	endothelial nitric oxide
ET-1	endothelin-1
ET_A	endothelin receptor type A
ET_B	endothelin receptor type B
FLAIR	fluid attenuated inversion recovery
FMC	Flinders Medical Centre, Bedford Park, Adelaide, SA
FMD	flow-mediated dilation

GTN	glyceryl trinitrate
GTP	guanosine triphosphate
HDL	high density lipoprotein
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercelllar adhesion molecule-1
LA	leukoaraiosis
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
LDL	low density lipoproteins
LMH	Lyell McEwin Hospital, Elizabeth Vale, Adelaide, SA
LSM	lymphocyte separation medium
MCP-1	monocyte chemoattractant protein-1
MI	myocardial infarction
MMP	metalloproteinase
MRI	magnetic resonace imaging
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NO	nitric oxide
N-Ox	NADPH oxidase
NSF	N-ethylmaleimide-sensitive factor
nNOS	neuronal nitric oxide synthase
OCSP	Oxfordshire Community Stroke Project
PAI-1	plasminogen activator inhibitor-1
PBS	Dulbecco's Phosphate Buffered Solution (Calcium and Magnesium free)
PCR-SS	polymerase chain reaction (sequence specific)
PGI_2	prostacyclin
PKC	protein kinase C
PLC	phospholipase C
PON-1	paraoxonase-1
PV	periventricular
PWA	pulse-wave analysis
RAH	Royal Adelaide Hospital, Adelaide, SA
RAS	renin-angiotensin system
ROS	reactive oxygen species

SGP	strain gauge plethysmography
SM	smooth muscle
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SVD	small vessel disease (cerebral)
TIA	transient ischaemic attack
TNF-α	tumour necrosis factor-α
TOAST	Trial of Org 10172 in Acute Stroke Treatment
tPA	tissue plasminogen activator (protein)
TQEH	The Queen Elizabeth Hospital, Woodville South, Adelaide, SA
VCAM-1	vascular adhesion molecule-1
vWF	von Willebrand factor
WMH	white matter hyperintensity

Introduction

Cerebral small vessel disease (SVD) encompasses two main phenotypes: lacunar infarction (LI) and leukoaraiosis (LA). LI, a stroke subtype arising from disease of the small vessels in the white matter of the brain, may not often be fatal [1, 2] and thus it does not receive the attention that large-vessel strokes receive. However, LI is associated with dementia [3, 4], as is LA [5, 6]. Essentially, LI and LA arise when there are changes in blood flow due to alterations in blood vessel structure and function. Risk factors, such as hypertension and genetics, have been shown to modify LI and LA risk, but the evidence identifying the connection between risk factors and disease development is lacking.

The concept of endothelial function (EF) is emerging as an interesting one. In essence, the endothelium is responsible for maintaining vascular health. Thus, knowing that cerebral small vessel disease results from diminishing vessel structure and function (i.e. poor vessel health), it would be logical to infer that EF has a role in SVD development and that the endothelium may be the elusive link between some risk factors and disease development. The fact that endothelial dysfunction and SVD have the same risk factors provides strength to this inference. The concept of EF and its relationship to SVD will be further elaborated upon in the background and discussion of results.

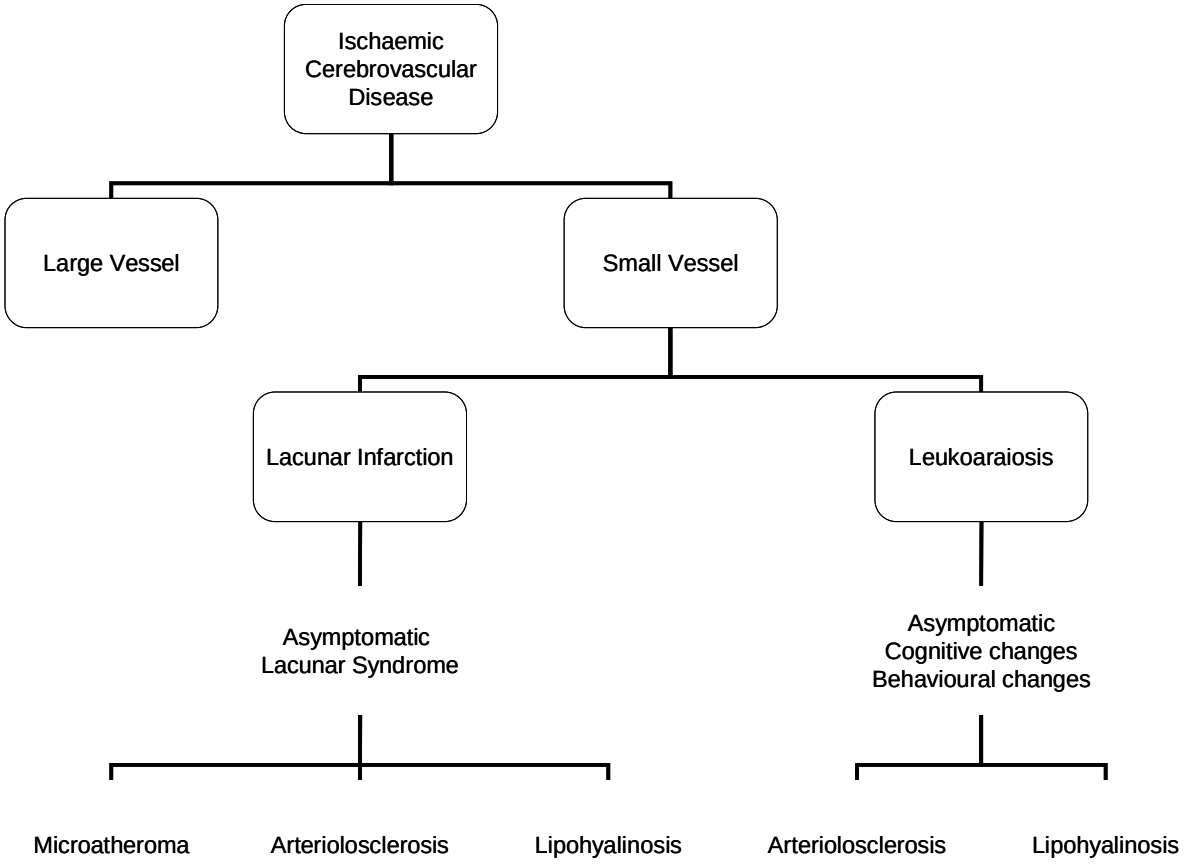
Previous evidence has affirmed the involvement of genetics in modifying EF and SVD risk. Many studies have followed the candidate gene approach to determine the role and effects of specific polymorphisms on both EF and SVD. Of the plethora of genetic polymorphisms in the human body, which genetic polymorphisms are important and which ones are not? Eight candidate genetic polymorphisms have been chosen based on their potential to cause disease.

This study aims to provide further insight into the development of SVD by exploring the relationship between EF and SVD, and to examine the role of candidate polymoprhisms in both EF and SVD.

CHAPTER 1 CEREBRAL SMALL VESSEL DISEASE

Ichaemia accounts for 85% of all cerebral infarctions, while the remainder is accounted for by haemorrhagia. Ischaemic cerebrovascular disease (CVD) can be further classified into large vessel and small vessel disease. Ischaemic CVD most commonly affects the large vessels, which include the middle cerebral artery, common and external carotid arteries, vertebral artery and the Circle of Willis. Small vessel disease (SVD) affects the deep, penetrating arteries of the brain, which arise from the larger arteries. These small arteries are usually non-branching. SVD can present as two main phenotypes: lacunar infarction and leukoaraiosis (**Figure 1.1**).

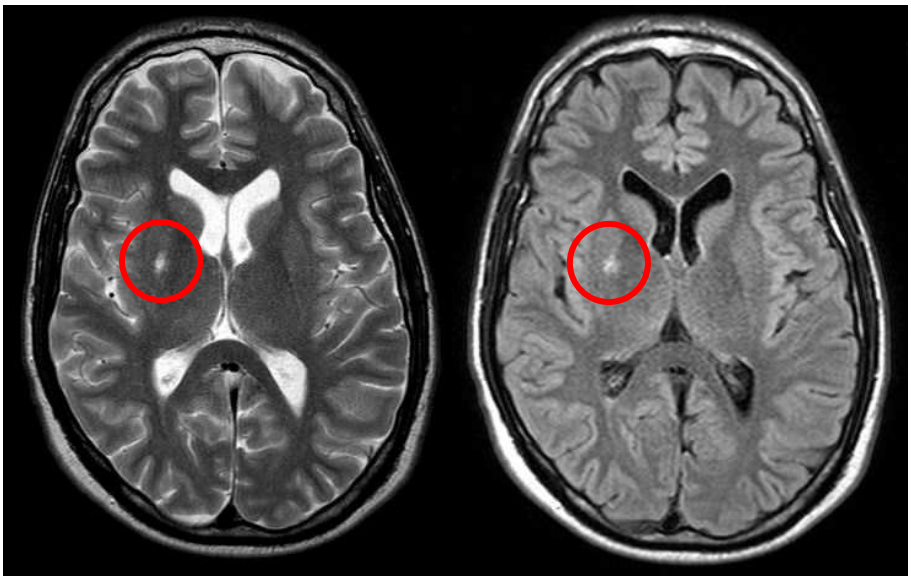
Figure 1.1 – Classification of ischaemic CVD. Small Vessel Disease is expanded, showing phenotype (3rd row), clinical presentation (4th row) and pathophysiological basis (5th row).



1.1 Lacunar Infarction

The term ‘lacunar’ is derived from the Latin word *lacuna* meaning ‘empty space’. This term describes the small cavity (less than 15mm in diameter) remaining after the necrotic tissue of the infarct has been resorbed. Hence infarctions in the white matter with this description are known as ‘lacunar infarcts’.

Figure 1.2 – T_2 MRI of the brain (left) showing a lacunar infarction (circled), confirmed by the FLAIR (right). Courtesy of the Department of Radiology, The Queen Elizabeth Hospital.



Approximately 20% of first-ever strokes are due to small vessel strokes (lacunar infarction (LI)) [7, 8]. LI may be asymptomatic or may present clinically as one of four lacunar syndromes: pure motor stroke, pure sensory stroke, sensorimotor stroke and ataxic hemiparesis. The pure motor stroke is seen most commonly. The prognosis following LI is better than for large-vessel strokes. The Oxfordshire Community Stroke Project percentage survival rate following LI at 1-month is 99% and 90% at 12-months [7]. A more recent study found the survival rate at 1-month to be 95.7% and 87% at 12-months [1] and similar results were seen in a recent French study (96% and 86%, respectively) [2]. Comparatively, the survival rate for a large-vessel ischaemic stroke is 80% at 1-month and 67% at 12-months [9]. Approximately 15-20% of people will develop dementia following LI [3, 4].

Based on the findings of Fisher in the 1960’s during autopsies [10-12], it has been proposed that lacunar infarcts can also be divided into two subtypes based on the pathophysiology of the infarction [13]. The first subtype is caused by microatheromas and is unaccompanied by

leukoaraiosis. These resulted in single, larger lacunes (5mm or greater) and were more symptomatic as they affect slightly larger small arteries (200-800μm) [14, 15].

The second subtype is characterised by multiple, smaller lacunes and the suggested pathophysiology is arteriolosclerosis and lipohyalinosis [14]. Fisher found that it was predominantly hypertensive patients that exhibited this subtype [10-12].

Arteriolosclerosis

Arteriolosclerosis, also known as 'simple SVD' [16], occurs when there is remodelling of the arterial wall caused by concentric hyaline wall thickening. There is hypertrophy of the medial smooth muscle, which is then replaced by extracellular and matrix proteins [17]. The surrounding tissue then suffers changes that include reduced myelination, gliosis and complete axonal destruction [18].

It has been hypothesised that the thickening of the arterial wall may impinge on the lumen and result in reduced blood flow, but later pathological studies have not verified this [19]. It is more likely that these vessel changes are due to an initial leakage of plasma constituents from cerebral spinal fluid leading to increased permeability of the blood-brain barrier (i.e. the cerebral endothelium) – animal studies have shown that the leakage of plasma enzymes leads to the development of arteriosclerosis and lipohyalinosis [20-22].

Lipohyalinosis

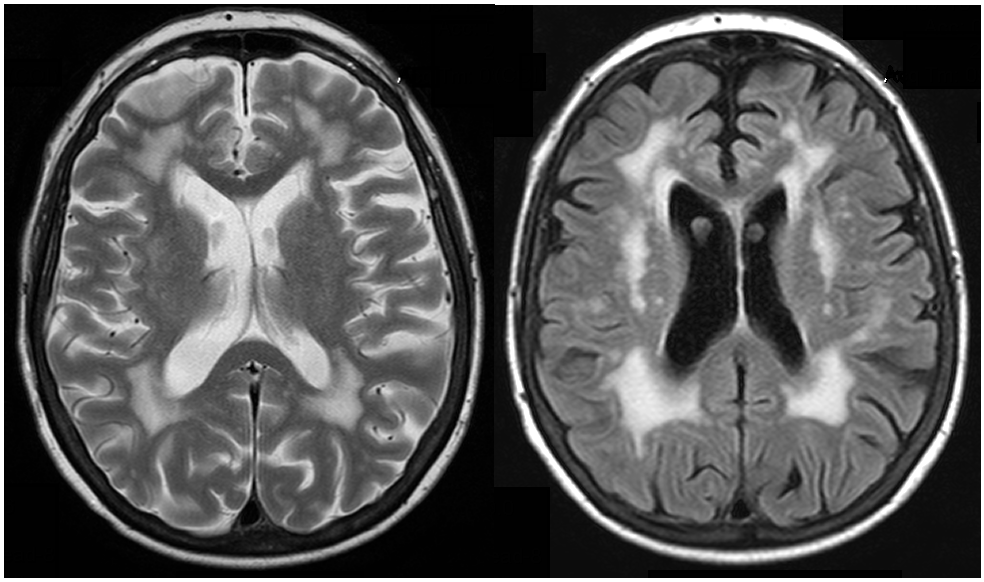
Lipohyalinosis, or 'complex SVD', [16] is described as the loss of the normal arterial wall structure and collection of mural foam cells usually in arteries 40 to 200 μm in diameter, causing lesions 3 to 7 mm in diameter [14, 23]. This weakens the affected artery and predisposes it to thrombosis. Lipohyalinosis can progress further to fibroid necrosis.

1.2 Leukoaraiosis

Leukoaraiosis (LA) is a term used to describe diffuse abnormalities seen in the deep white matter on radiological scans [24]. These abnormalities tend to be seen around the horns of the lateral ventricle and in the centrum semiovale.

It is accepted that LA reflects chronic ischemia, although there may be non-ischemic causes of LA. On MRI, LI tend to be well demarcated and follows cerebrovascular territories, whilst LA is less well-defined (**Figure 1.3**) and frequently involves the temporal and occipital border zone [25].

Figure 1.3 – T_2 MRI of the brain (left) showing severe LA confirmed by the FLAIR (right). Courtesy of the Department of Radiology, The Queen Elizabeth Hopsital.



LA can present as cognitive decline, behavioural changes or it may be asymptomatic. Although LA can be found as part of the normal aging process, it is more strongly associated with dementia, with a reported 35% of demented patients showing LA on their CT scan [26]. One study reported the incidence of LA to be 50% in people with vascular dementia [27]. LA is also seen in patients with dementia of Alzheimer's type, with 25 to 65% of these patients exhibiting changes in the subcortical white matter [5, 6]. LA is also a prognostic indicator for LI, with a significantly higher recurrent LI rate ($p=0.004$) and a significantly lower survival rate ($p=0.012$) in patients with LA compared to those without LA [28].

The pathogenesis of LA remains unclear, although chronic ischaemia is suggested [25, 29]. The argument for ischaemia is that the histological changes seen in the white matter in known cases of hypoxia or ischaemia are also seen in the white matter of patients with LA [30]. Szolnoki proposes that chronic hypoperfusion (leading to chronic ischaemia) leads to changes in the synthesis of the myelin sheath, thus propagating demyelination [31].

A possible explanation of why LA appears to be more common in the periventricular region is that the periventricular region of white matter is very susceptible to injury when there are decreases in cerebral blood flow, for example, due to arteriolosclerosis [32]. Alterations to the permeability of the blood brain barrier during hypertensive episodes, changes in cerebrospinal fluid and cerebral oedema have also been proposed [22, 25]. The combined observations of increased leakage of MRI contrast agents [33] and the post-mortem detection of extravasated proteins [34] in the brains of patients exhibiting white matter lesions suggest that the initiating step for SVD is the breakdown of the blood-brain barrier (BBB), i.e. the cerebral arteriolar endothelium.

One study has recently identified a relationship between specific inflammatory markers and LA [35], highlighting the role of inflammation in LA. Higher concentrations of Lipoprotein-associated phospholipase A_2 (Lp-PLA2) and myeloperoxidase (MPO) were associated with a greater white matter hyperintensity (WMH) volume in a stroke-free cohort. Inflammation is also implicated in increasing BBB permeability [36].

LA can also be caused by cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). This is a genetic disorder linked to a mutation in the Notch3 gene on chromosome 19, which predisposes an individual to stroke and ischaemic leukoencephalopathy. The Notch3 gene encodes the Notch3 protein, which is responsible for building vascular smooth muscle. Abnormal Notch3 proteins encoded from the Notch3 mutation lead to the degeneration of the smooth muscle, resulting in functional loss of blood vessels of the brain and heart. The white matter hyperintensities seen in these CADASIL patients are very similar to the abnormalities seen in severe LA [37].

1.3 Methods to categorise SVD

1.3.1 Clinical subtyping

There are two main classification systems that subtype ischaemic stroke: Trial of Org 10172 in Acute Stroke Treatment (TOAST) and the Oxfordshire Community Stroke Project (OCSP). Both of these subtype ischaemic stroke by assessing the clinical presentation and using imaging such as MRI or CT.

The TOAST definition for "small-artery occlusion (lacune)" is: one of the traditional clinical lacunar syndromes with no evidence of cerebral cortical dysfunction, and a normal CT/MRI or a brain stem or subcortical lesion with a diameter less than 1.5 cm [38]. The OCSP definition for LI is: a case of stroke with one of the recognised lacunar syndromes (pure motor, pure sensory, sensorimotor and ataxic hemiparesis) with CT data compatible with cerebral infarction due to primary disease of a single perforating artery of the brain [7].

It is evident from the definitions that TOAST and OCSP classify LI only, as LA is not included in either definition. These methods have been shown to be able to differentiate well between ischaemic stroke subtypes, and would be useful in studies investigating associations between stroke subtypes. However, these defintions are not useful when investigating a certain subtype that has different phenotypes, which is the situation with SVD. Also, these definitions have a bias towards identifying the larger infarcts that are symptomatic and tend to have an occlusive cause, as opposed to the smaller infarcts, which are comparatively less symptomatic and have an arteriolosclerotic cause [39].

1.3.2 WMH rating scales

Traditionally, SVD burden has been assessed using a visual rating scale by blinded radiologists or neurologists. The classification of LA severity is highly dependant on the interpreter and the rating scale chosen. Thirteen different rating scales for LA on MRI were published between 1986 and 1994 [40], but since 1994, even more scales have been published and used.

The range of existing rating scales differs in the grading range, morphological description and the description of the distribution of hyperintensities. The rating scales, as summarised in **Table 1.1**, can be grouped into four main categories:

1. periventricular (PV) hyperintensities only [41, 42]
2. deep white matter (DWM) hyperintensities only [43, 44]
3. PV and DWM with distinct scores [45-50]
4. PV and DWM with combined scores [51-56]

Table 1.1 – A summary of different visual rating scales used in previous studies, showing the wide variation in grading between the scales

Periventricular ONLY

Year published	Scale Name	Number of grades	MRI type
1986	Gerard & Weisberg [41]	5 (1-5)	PD
1990	Shimada [42]	4 (1-4)	T2/PD

Deep White Matter ONLY

Year published	Scale Name	Number of grades	MRI type
1989	Hunt [43]	3 (for size) + 4 (for number) + 4 (for overall severity)	T2
1990	Herholz [44]	5 (0-4)	T2

Periventricular & Deep White Matter DISTINCT

Year published	Scale Name	Number of grades	MRI type
1987	Fazekas [45]	PV: 4 (0-3) DWM: 4 (0-3)	T2/PD
1991	Mirsen [46]	PV: Present/absent DWM: 5 (0-4)	T2/PD
1992	Scheltens [47, 48]	PV: 3 (0-2) DWM: 7 (0-6)	T2/PD
1993	Ylikoski [49]	PV: 4 on both sides in 4 areas (score 0-24) DWM: 4 on both sides in 4 areas (score 0-24) Combine for total overall score = 0-48	T2
1994	Erkinjuntti [50]	PV: 5 (0-4) DWM: 5 (0-4)	T2/PD

Periventricular & Deep White Matter COMBINED

Year published	Scale Name	Number of grades	MRI type
1990	Van Swieten [53]	3 (0-2, anterior and posterior coded separately)	T2
1992	Schmidt [55]	4 (0-3)	T2/PD
1994	Breteler [51]	3 (0-2)	T2
1994	Manolio [56]	10 (0-9)	T1/T2
1996	Longstreth [52]	10 (0-9)	T2
2001	Wahlund [54]	6 (1-3)	T2

There are also scales that have independent scores for different regions of the brain [53, 57], and 'new' scales that are modifications of older, established scales (e.g. the modified Fazekas scale [58]). Thus, an assessment of a single cohort of patients may elicit different results depending on the rating scale used and the complexity of the scale. It may also vary depending on the assessor and their judgement and experience. The use of many different rating scales has been suggested as a reason for the variation in results seen in previous studies regarding LA [59].

Because of these variabilities, there have been some studies that have compared these scales against each other for inter-rater reliability. One such study compared the Manolio [56], Fazekas [45], and Scheltens [47] scales [60]. These were compared against each other for inter-rater variability and correlated with a quantitative measure. It was found that although the inter-rater variability was high between all scales, but there was better inter-rater agreement with the Fazekas and Scheltens scale than with the Manolio. Another study by Wardlaw et al. compared seven different scales and found that the reliability of these scales could be improved, although performance varied with the degree of WMH and subject cohorts [61].

Although those with a larger number of grades showed greater inter-rater variability, these would be preferable for longitudinal studies due to the ability to detect smaller degrees of change as long as the inter-rater variability is acceptable. More grades imply that the degree of WMH can be more specific, so progression in disease is easier to identify [61]. Scales with fewer grades have less variability, but the potential for monitoring disease progression is diminished as progression can only be defined in broad terms (e.g. from 'mild' to 'moderate').

1.3.3 Quantitative Measures

Although commonly used, WMH visual rating scales have a high degree of subjectivity when categorising SVD burden, depending on the assessor's opinion and experience. Recently, the method of volumetric imaging has been developed and it involves the use of specialised software to calculate the volume of WMH directly from MRI sequences. The software, such as SPM2 (Statistical Parametric Mapping), has the capability of identifying the white matter on FLAIR, T_1 and T_2 sequences. When SPM2 is used in combination with other software, such as Matlab (TheMathWorks Inc, Natick, USA), WMH can be identified by voxel signal

intensity and thus the volume of WMH can be calculated by summing the number of voxels with signal intensity higher than a pre-determined level.

Studies using quantitative measures are starting to appear in the literature. Volumetric imaging was used to quantify SVD burden in a non-demented population with brain injury [62], while volumetric imaging was also used to investigate WMH and cortical perfusion [63].

1.4 Risk Factors of SVD

The traditional risk factors of SVD are the same as those for large-vessel CVD: age, hypertension, hypercholesterolaemia, Type II diabetes mellitus and cigarette smoking. Specifically for LA, the major risk factor is age [64-66], while the major risk factors for LI are hypertension and diabetes [67]. LA and LI are also risk factors for each other [68, 69].

These risk factors are the same as those for endothelial dysfunction, which will be discussed in detail in Chapter 2.3.

1.4.1 Age

Age is an important risk factor for SVD. The majority of studies involving LA have shown a positive association between LA and age [28, 70-72]. One study found that age was the only factor that increased the risk of LA significantly (odds ratio (OR) per 10 year increase: 2.4, 95% confidence interval (CI) 1.8 to 3.1) [71].

Another study has shown that the incidence of LI increases with age [2]. There was a significant increase in the incidence rate of LI between 1989 and 2006 in Dijon, France (relative risk (RR) 1.02, 95% CI 1.02 – 1.08, p=0.007). Incidence of LI increased with each 10-year age group for both men and women.

1.4.2 Hypertension

Hypertension is especially important in SVD as it is one of the strongest risk factors for this disease [25] and the main risk factor for lacunar infarction [73]. Hypertension is an independent risk factor for multiple LI, with diastolic blood pressure appearing to determine the association between hypertension and multiple lacunar infarctions [74]. The LADIS study

showed that the frequency of hypertension increased with the degree of age-related WMH in patients without a previous stroke history, further confirming that hypertension is one of the major determinants of age-related WMH [68]. Another Hungarian study showed similar results – 30% of hypertensive patients had a lacunar stroke, while only 19% of non-hypertensive patients had a lacunar stroke (RR 1.14, 05% CI 1.04 to 1.27) [75]. Of the entire lacunar stroke subgroup, 82% of patients had hypertension, while 70% had elevated cholesterol levels. In this same study, 80% of patients with LA also had hypertension compared to hypertension in 75% of patients without LA. Patients with LA had a significantly ($p<0.04$) higher average systolic blood pressure (BP) at admission compared to patients without LA, although there was no difference in diastolic BP.

Animal studies have shown that fibroid necrosis can be induced after hypertension-induced vasospasm [76], although the degree of hypertension required to cause these vasospasms is rarely seen in humans [77]. Small vessels in humans with hypertension show "eutrophic remodelling", which is described as a rearrangement of the smooth muscle cells to reduce the lumen diameter without changes in the media volume [73, 78]. There is no change in vascular elasticity at this stage. Vessels in patients with severe hypertension show vascular remodelling with increased vascular stiffness [78]. There may be changes in the media volume and increased collagen deposition in the extracellular matrix. EF of the small arteries may also be decreased in hypertensive patients [78, 79]. The description of the remodelling of the small arteries in hypertensive patients is very similar to arteriolosclerosis and lipohyalinosis, the basis to SVD.

*1.4.3 **Hypercholesterolaemia***

Hypercholesterolaemia is a known risk factor for cardiovascular and cerebral large-vessel disease. To a lesser extent, it is also associated with SVD. For total cholesterol levels, an association was found for LI when comparing quintiles (OR 2.2, 95% CI 1.4 – 3.4) [80].

A meta-analysis consisting of 28 studies (a total of 21,980 patients of whom 5379 had LI) could not identify high cholesterol levels to be a significant risk factor [66]. In this study, Jackson et al. calculated a pooled RR of 1.22, indicating that higher cholesterol levels predispose more to LI than non-lacunar stroke. However, when including only studies with risk factor-free ischaemic stroke subtype definitions, there was no association between high cholesterol levels with lacunar versus non-lacunar stroke.

1.4.4 Diabetes Mellitus

Elevated insulin levels have been correlated with the prevalence of SVD [81]. Higher serum insulin and C-peptide concentrations were found in the microangiopathy group (which included LI and subcortical arteriosclerotic encephalopathy) when compared with normal controls and patients with large-vessel disease ($p<0.05$).

Diabetes has been associated with multiple, but not single lacunes (OR 2.3, 95% CI 1.1 – 4.5) [74]. Another study showed similar results, with the association between diabetes and LI onserved only if there was more than one silent infarct (OR 2.95, 95% CI 1.56 – 5.57) [82]. A large study consisting of more than 3500 people over 65 years old showed that 23% of their population had LI, with diabetes being independently associated with a radiologically evident LI (OR 1.33, $p<0.05$) [83].

1.4.5 Homocysteine

Homocysteine is not a risk factor for ischaemic stroke, but it has been identified as a specific risk factor for SVD [29, 84]. The proposed mechanism for this is via ED [29]. Homocysteine levels were found to be higher in patients with LA compared with patients with isolated LI and controls. These results showed a concentration-dependant risk, with the risk of SVD increasing as concentrations of homocysteine increased. Another study also showed that the mean homocysteine concentrations were higher in SVD cases compared to controls [84]. Mean asymmetric dimethylarginine (ADMA) levels were also found to be higher in cases than controls when adjusting for age, gender, vascular risk factors and creatinine clearance. The grade of LA was positively correlated with homocysteine ($p=0.003$) as well as ADMA ($p=0.026$), while the grade of LI was only correlated with homocysteine ($p=0.017$).

1.5 Genetics in SVD

The evidence for a genetic influence in SVD is strong. CADASIL is arguably the most well documented monogenic cause of SVD. Involving the Notch3 gene on chromosome 19, the mutation leads to the degeneration of smooth muscle of the blood vessels in the brain. Clinically, the phenotype exhibits multiple subcortical strokes in the absence of vascular risk factors, and a subcortical dementia [85]. The similarity between the vascular physiologies of

CADASIL and non-CADASIL SVD suggest that genes affecting the integrity of blood vessels may be important in SVD.

Monogenic causes are rare in the population, accounting for less than 1% of ischaemic strokes. Thus, these are usually only considered in the differential diagnosis for young patients or middle-aged adults with none of the traditional risk factors for stroke. For the majority of those who present with SVD, a monogenic cause will be disregarded. It is more likely that the aetiology in these people will be 'complex', which is defined as a phenotype that does not follow the classic Mendelian inheritance [86], and the same genotype may result in different phenotypes due to interactions with the environment or other genes.

Evidence suggests that SVD is a polygenic disease, with multiple genetic influences acting together to effect disease. Genetic factors may exert the risk of SVD via the following pathways: (i) the genes are causative, with a direct effect independent of other risk factors and environmental influences; and (ii) indirectly, with the gene exerting an effect by influencing susceptibility to established risk factors and disease expression is dependant on a gene/risk factor interaction.

Discrete polygenic traits, which SVD can be classified as, tend to represent a 'threshold model', where the genetic factor itself may not cause an expression of disease, but when combined with other genetic factors or environmental influences, the likelihood of disease exceeds the threshold to result in the expression of a particular phenotype [86]. Genetic factors remain an important contributor to overall risk, but individually may not be sufficient to cause the overall likelihood of SVD to exceed the threshold. This may explain why some people who possess a genetic polymorphim specific to SVD but do not exhibit an SVD phenotype.

1.5.1 Evidence of a Genetic Predisposition in SVD

1.5.1.1 Twin Studies

A twin study involving monozygotic (identical) and dizygotic (non-identical) twins has shown that genetics has a large role in the development of LA. Using 74 monozygotic and 71 dizygotic white male twins, concordance rates for large amounts of white matter hyperintensities were found to be 61% in monozygotic twins, compared to 38% in dizygotic

twins [87]. In addition to this, the study results indicated that 71% of the variation in WMH volume could be accounted for by genetic factors.

1.5.1.2 Family Studies

The heritability of LA has been estimated at 80±10% in non-Hispanic white subjects. This estimation decreases to 67±11% when adjusting for sex, age, systolic blood pressure and WMH volume [88]. This study involed 483 hypertensive subjects from 210 sibships providing 434 sibling pairs, with a mean±SD age of 65.2±7.3 years.

Atwood et al. had similar findings in the Framingham study in 1330 individuals and their offspring [89]. The overall WMH heritability was 0.55. In subset analyses, the heritability rates for individual sexes were 0.78 and 0.52 for women and men, respectively.

Jerrard-Dunne et al. showed family history was a significant risk factor for developing SVD, with an OR of 3.15 ($p<0.001$) when only considering cases less than 65 years old [90]. This is higher than the reported OR in large-vessel disease with the same parameters (OR 2.93, $p<0.001$). **Table 1.2** shows that the odds ratio of small vessel disease is up to 1.93 (univariate analysis) if there is a family history of stroke at age less than 65 years. Results from the same study show that the younger a person was when they suffered a stroke, the more likely that genetics were a significant risk factor (**Table 1.3**), with an odds ratio of 4 for sufferers less than 55 years old.

*Table 1.2 – Multivariate odds ratio and 95% CIs for a family history of stroke ≤65 years for SVD (Data from [90]) *p<0.05*

	n	OR (95% CI) Univariate	OR (95% CI) Multivariate
Small Vessel Disease	232	1.93 (1.25-2.97) *	1.49 (0.94-2.37)

*Table 1.3 – Relationship between age of stroke and positive family history of stroke ≤ 65 years for SVD (Data from [90]). The multivariate analyses have been adjusted for: age, sex, arterial hypertension, diabetes mellitus, serum cholesterol, and smoking status. *p<0.05*

	No. of Cases	OR (95% CI) Multivariate
Stroke ≤ 55 y	47	3.99 (1.25-12.7) *
Stroke ≤ 60 y	71	2.70 (1.18-6.18) *
Stroke ≤ 65 y	117	2.69 (1.46-4.96) *
Stroke ≤ 70 y	149	1.91 (1.11-3.28) *
Stroke ≤ 75 y	186	1.55 (0.94-2.53)
Stroke ≤ 80 y	216	1.55 (0.96-2.48)
All strokes	232	1.49 (0.94-2.37)

Other studies have shown similar results where a positive family history of stroke ($p<0.05$) increases LI risk signficantly, with OR ranging from 1.79 to 2.76 [91-93]. One study did not find an association [94], while another only observed a trend [95].

1.5.2 Genetic polymorphisms in SVD

Various genetic factors have been proposed as risk factors for LA and LI. A plethora of individual polymorphisms have been investigated in relation to SVD. A review by Lam et al. (Appendix 1) has discussed a genetic approach to SVD, identifying several polymorphisms that are thought to be important in the development of SVD [96]. The single nucleotide polymorphisms (SNPs) discussed in the review included the following genes: endothelial nitric oxide synthase (eNOS), endothelin-1 (ET-1), endothelin-receptor A and B, angiotensin-converting enzyme (ACE), angiotensinogen, methylenetetrahydrofolate (MTHFR), fibrinogen, tPA, IL-8, IL-6 and tumour necrosis factor (TNF)-α.

In the recent literature, SNPs of particular interest have been in the MTHFR, ACE and apolipoprotein E (APOE) 2 or 4 genes [97-99].

1.5.3 Lacunar Infarction

The role of the MTHFR 667 C/T in SVD has been well investigated. One Korean study has shown that the TT genotype is associated with multiple small-artery occlusions (adjusted OR

2.92, 95% CI 1.01 – 8.48) when compared to controls [100]. The same study also showed that the TT genotype was significantly associated specifically with multiple small-artery occlusions when compared to the single small-artery occlusion (adjusted OR 6.90, 95% CI 1.70 – 27.99), as well as being associated with higher plasma homocysteine levels. Other studies in both Caucasian and non-Caucasian populations have shown similar results [101-103]. However, a meta-analysis involving 14,870 participants found no association between the polymorphism and LI [104].

As mentioned in the 'threshold theory', a single mutation may not have sufficient power to elicit disease, but when combined with other mutations, the threshold may be exceeded. This appears to be the situation with the MTHFR 677 T allele. The genotype distribution of MTHFR 667 C/T does not significantly differ between cases and controls [98]. However, when the MTHFR 667 T allele is present with the ACE DD polymorphism, the combination increases the risk of LI (OR 6.2, $p<0.0001$) [99]. This same combination was not found to alter OR significantly in large-vessel stroke. Adding the APOE4 allele to the combination increases the LI risk further (OR 11.9, $p<0.0001$) [99]. All three polymorphisms affect vasoregulation and this relationship is specific to SVD.

There is the suggestion that the ACE I/D (DD genotype) itself may be associated with LI [99, 105]. The same allele has also been reported to be an independent predictor of LA in patients presenting with lacunar syndrome [98, 106]. The investigation between ACE D/D and LI resulted in a significant OR of 2.8 ($p<0.003$) [99]. The ACE D/D + APOE 4 allele combination resulted in the OR for LI increasing to 10.9 ($p<0.0001$).

A specific polymorphism has been identified as a risk factor for lacunar infarction. This polymorphism was found to be tPA -7351 C/T, with a significant association between the TT genotype and lacunar stroke (OR 2.7, 95% CI 1.1 – 6.7) [107]. However, this result has not been replicated in other studies [108, 109]. This polymorphism is dicussed further in detail in Chapter 3.4.1.

1.5.4 Leukoaraiosis

The same polymorphisms in the MTHFR, ACE and APOE genes have been implicated in LA. In a study involving patients with LA presenting with a lacunar syndrome, the ACE DD genotype was found to be more frequent in patients with LA compared to those without LA

[106]. The study also showed that the ACE DD genotype was an independent predictor of LA in patients presenting with lacunar syndromes. Similar results were seen in another study, where the OR for ACE DD genotype was 1.79 (95%CI 1.08 to 2.94) in patients with well-defined LA with multiple LI [98]. This OR increased to 3.92 (95%CI 1.3 to 11.83) for the combined ACE D/D + MTHFR 677 TT in the same patient group. In patients with LA only (i.e. no LI), OR was 8.16 (95%CI 2.83 to 23.36) for ACE D/D + MTHFR 677 TT [98]. Results were adjusted for age, sex, hypertension and diabetes.

Other polyrmorphisms showing a positive effect on LA include: Angiotensinogen M235T [110] and Paraoxanase 1 (PON1) Met54Leu [111]. The role of ApoE polymorphisms has been conflicting, with some studies reporting that the ApoE ε2 or ε4 alleles were associated with a higher prevelance of LA [112], or the ε2/ ε3 genotype associated with LA and LI [113] , while others have found no relationship between LA on MRI and ApoE variants [114].

1.6 Summary

SVD is a complex disease with different phenotypes and multiple pathophysiologies. LI and LA are caused by processes that ultimately result in changes to the vascular structures from a molecular level. Factors such as age and hypertension affect the risk of SVD, as do genetic factors. Although it is understood that the risk factors can cause changes in the blood vessels, the risk factors are exerting their effect via an intermediary that is facilitating these changes. Gaining momentum is the concept of endothelial function (EF), and increasing evidence is suggesting that endothelial dysfunction is a facilitator of disease. This study will aim to explore the relationship between EF and SVD, as well as the role of candidate polymorphisms on SVD risk.

CHAPTER 2 ENDOTHELIAL FUNCTION

The mechanisms behind LI and LA involve ischaemia and changes in arterial structure, suggesting the involvement of unhealthy arteries. Risk factors are exerting their effect via a facilitator and this may be the endothelium. Smooth muscle cells in the cerebral vasculature are completely dependant on a normally functioning endothelium [85], as local vasoregulation of blood flow within the brain relies on the small vessels [31]. Because of this and the role it has in maintaining healthy arteries, it is highly possible that the endothelium and its many functions may be involved in the development of SVD.

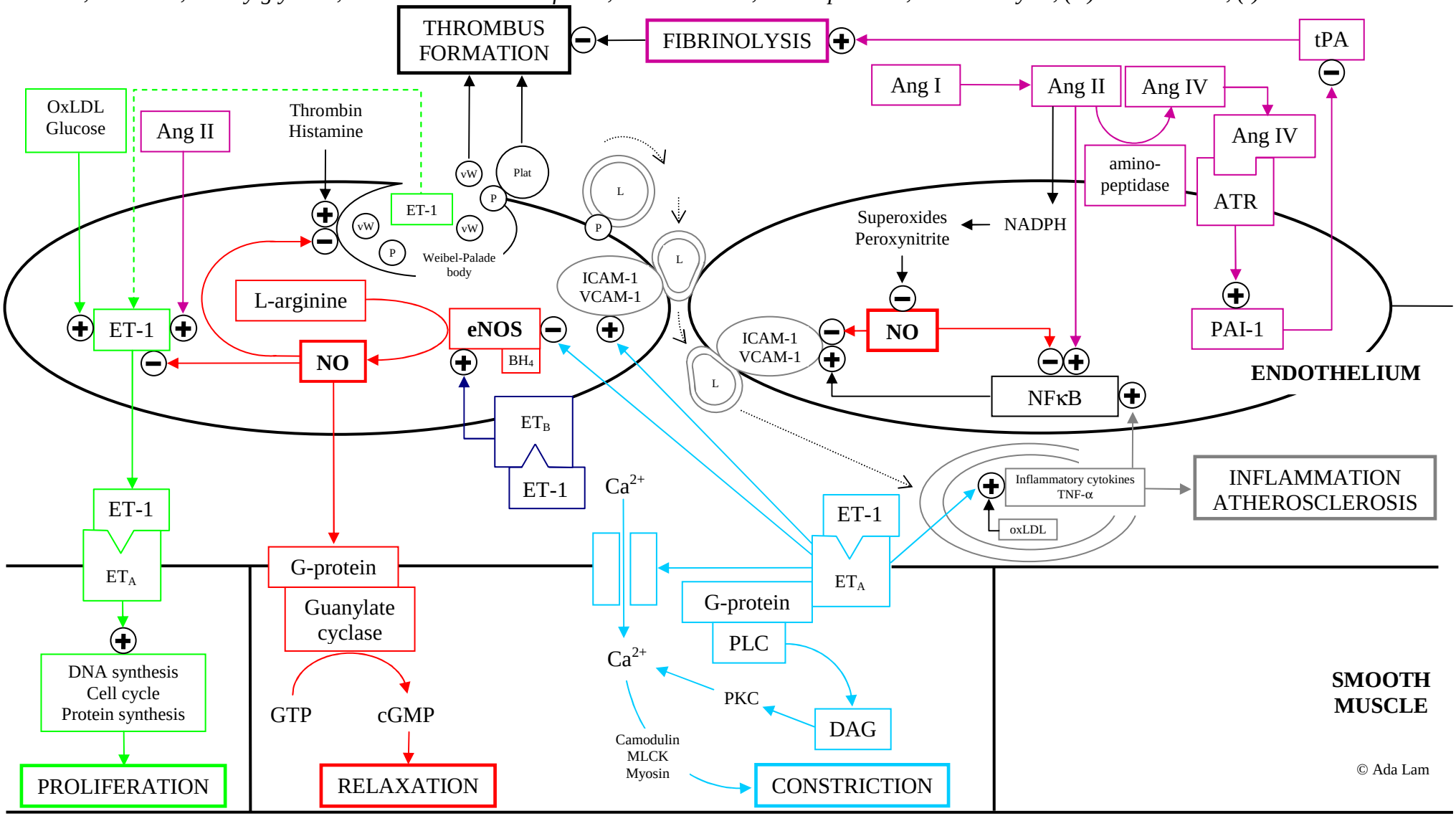
2.1 The endothelium

The endothelium is a monolayer of flat cells lining the inner surface of all blood and lymphatic vessels [115]. Initially thought to be inert, the endothelium is now known to have a vital role in maintaining vascular homeostasis, via vasomodulators and inflammatory mediators released in response to hormonal and mechanical stimuli [116-118]. These substances include vasodilators (e.g. nitric oxide (NO)), vasoconstrictors (e.g. endothelin-1 (ET-1) and angiotensin II (AngII)), inflammatory modulators (e.g. intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1)) and modulators of vascular homeostasis (e.g. plasminogen activator inhibitor-1 (PAI-1) and von Willebrand factor (vWF)) [119, 120]. The endothelium has five main roles:

1. endothelium-dependant vasodilation;
2. anti-inflammatory – to inhibit the migration of inflammatory mediators and inhibit the adhesion of leukocytes to the surface of the endothelium;
3. anti-thrombotic;
4. anti-coagulant and pro-fibrinolytic;
5. anti-hypertrophic.

The pathways showing how the endothelium maintains these effects and how these effects are altered are illustrated in **Figure 2.1** [121-126] and discussed further in Chapter 2.2. EF is an extremely complex process and thus the figure provided only displays some of these pathways. Many of these pathways also interact with each other.

Figure 2.1 – The major modulators and pathways involved in EF & dysfunction. GTP = Guanosine triphosphate, PLC = Phospholipase C, PKC = Protein kinase C, DAG = 1,2-diacylglycerol, vW = von Willebrand factor, P = P-selectin, Plat = platelets, L = leukocytes, (+) = stimulation, (-) = inhibition



2.1.1 Endothelium-Dependant Vasodilation

Under normal circumstances, vasodilation will occur in response to the release of NO from the endothelium. The NO pathway causing vasodilation from smooth muscle relaxation is described in **Figure 2.1** (red pathway). A stimulus, mechanical, chemical or hormonal, causes an influx of calcium ions (Ca^{2+}) into the endothelial cell. The intracellular Ca^{2+} stimulates the endothelial isoform of nitric oxide synthase (eNOS) to convert L-arginine to NO, with L-citrulline as a by-product. L-arginine is produced endogenously from the urea cycle and circulates in the blood. L-arginine is actively uptaken into the endothelial cell by y+ transporters, which are regulated by cytokines [127]. In the absence of extracellular L-arginine, the endothelial cell is capable of resynthesising L-arginine from L-citrulline by argininosuccinate synthetase and argininosuccinate lyase within the cell [128, 129]. Active eNOS exists as a dimer, coupled with its essential cofactor, tetrahydrobiopterin (BH_4). BH_4 helps bind L-arginine to the enzyme and helps the active eNOS dimer maintain its oxygenase activity for the production of healthy NO [130]. The NO produced from L-arginine increases the intracellular concentration of cyclic guanosine monophosphate (cGMP) in the smooth muscle cell via G-proteins and hence causes vascular smooth muscle relaxation [121].

The vasodilating properties of NO are in competition with the vasoconstricting effects of ET-1. ET-1 is a 21 amino-acid peptide with various biological effects, the most important being a vasoconstrictor [131, 132]. ET-1 is a potent vasoconstrictor. The effects of the peptide are mediated via its two major G-protein coupled receptors, endothelin-A (ET_A) and endothelin-B (ET_B). ET_A is found only on smooth muscle cells, whereas the ET_B receptors are found on both endothelial cells and smooth muscle cells. The binding of ET-1 to ET_A activates the accumulation of intracellular calcium (Ca^{+}) [133] via phospholipase C, resulting in a long-lasting vasoconstriction (**Figure 2.1** (light blue pathway)). Binding to ET_B results in NO and prostacyclin (PGI_2), another vasodilator, release and thus results in vaosodilation (**Figure 2.1** (dark blue pathways))[134]. ET-1 binding to the ET_A receptor also leads to the decreased activity and expression of eNOS via the production of hydrogen peroxide [135]. NO, in turn, inhibits the synthesis of ET-1 in the endothelial cell, and thus NO can counter the effects of ET-1 [122].

Like ET-1, AngII opposes the vasodilatory effect of NO. AngII is a vasoconstrictor produced in the renin-angiotensin system (RAS), which is involved in the modulation of blood pressure. RAS becomes activated by the release of renin from the kidneys in response to sympathetic stimulation, hypotension or decreased sodium [136]. Angiotensinogen is released from the liver and renin catalyses the conversion of this to angiotensin I. The angiotensin converting enzyme

(ACE) is responsible for converting angiotensin I to AngII. From here, AngII has various effects including systemic vasoconstriction. AngII can also affect cerebral circulation by increasing oxidative stress via angiotensin type 1 receptors stimulating Nox-2-containing NAPDH oxidase, thereby producing more ROS to further reduce the bioavailability of NO [137, 138]. However, AngII vasoconstriction does not appear to be as evident in the cerebral circuation as compared to the peripheral circulation, possibily due to the large vasodilatory effect of hydrogen peroxide (H_2O_2) on the cerebral vasculature [139].

2.1.1.1 Nitric Oxide and Nitric Oxide Synthase Isoforms

NO can be produced from three distinct isoforms of NO synthase: eNOS, neuronal NO synthase (nNOS) and inducible NO synthase (iNOS) [140, 141]. Each is encoded by a separate gene. eNOS and nNOS produce NO that contributes positively to the five major functions of a healthy endothelium, but each has a distinct role in vascular modulation [142]. However, NO produced from iNOS when stimulated by inflammatory factors (e.g. endotoxin, interleukin-1β, tumour necrosis factor (TNF)-α) can lead to apoptosis [143] and some types of inflammation [144]. Overproduction of NO by nNOS has also been shown to be neurotoxic [145].

Cerebral vasomodulation is regulated by NO from the endothelium [146] or brain neurons [147]. Regardless of the isoform, NO is synthesised from L-arginine in the presence of oxygen by NOS with several cofactors, including BH_4 and NADPH [148]. eNOS is normally bound to caveolin-1 in the endothelial plasma membrane and migrates intracellularly when intraceullar Ca^{2+} concentrations increase in the presence of calmodulin [149]. nNOS is also activated by increases in Ca^{2+} concentrations, but it does not need to migrate as it is normally soluble in the cytoplasm of the nerve terminal. eNOS can also be activated via the phosphatidylinositol-3 (PI_3) kinase-serine/theronine protein kinase (Akt) pathway [150].

The hypothesis for the role of nNOS in regulating cerebral blood flow is based on four main observations [143]:

1. electrical pulse stimulation of efferent nerves leads to increased cerebral blood flow and vasodilation. The response is attenuated by NOS inhibitors and nNOS nerve fibres are located close to the vascular smooth muscle cells;
2. nNOS inhibitors can inhibit the vasodilation and increased blood flow caused by somatosensory stimulation in cerebral arterioles. Intracerebral arterioles and nNOS-positive nerve fibres are located next to each other;

3. N-methyl-D-aspartate (NMDA) is an amino acid that stimulates NO formation by increasing intracellular Ca^{2+} concentrations. NMDA receptor agonists induce cerebral arteriolar dilation and increase blood flow, and this effect is blocked by 7-nitroindazol (a nNOS-specific inhibitor). Increased NO levels were also observed in the brain tissue;
4. nNOS deficient mice showed a decreased cerebral vasodilator response to nerve stimulation, which was not seen in eNOS- and iNOS deficient mice.

The main sites of NO release from nNOS are the parasympathetic post-ganglionic (nitrergic) nerves and glutamatergic nNOS-containing nerves. It is unclear if astrocytes are NO-generating, or an intermediary of neurovascular signalling. NO derived from post-synaptic cells also controls the relase of glutamate, an important neurotransmitter [151]. In the cerebral vasculature, specifically the cerebral artery, the nitrergic nerve has been seen to have a greater influence on vasomodulation than adrenergic and cholinergic nerves [152]. Similar effects of nNOS in the peripheral circulation with regards to increased blood flow and vasodilation were seen, although the nitriergic nerve effects were not as dominant as in the cerebral vasculature.

2.1.2 Anti-inflammatory

The anti-adhesive properties of NO appear to be elicited independent of the G-proteins [153, 154], while the anti-inflammatory effects do not appear to involve cGMP [155, 156]. Vascular inflammation may be regulated by NO via the inhibition of exocytosis of Weibel-Palade bodies at the surface of the endothelial cell [157, 158]. Weibel-Palade bodies in the endothelial cell contain v-WF, P-selectin, CD63 and endothelin-1 (ET-1) [158-161].

ET-1 also has a role in up-regulating ICAM-1 and VCAM-1 via nuclear factor-κB (NFκB), thus promoting leukocyte adhesion and atherosclerotic developments. The endogenous synthesis of ET-1 is stimulated by traditional cardiovascular risk factors, such as high levels of oxidised low-density lipoproteins (LDL) [162], obesity [163] and glucose [164]. Other vasoconstrictors, thrombin and adhesion molecules can also stimulate ET-1 synthesis. NO and prostacyclin are inhibitors of ET-1 synthesis. ET-1 is also stored in Weibel-Palade bodies, and upon exocytosis, ET-1 is released along with v-WF and P-selectins [161].

AngII is also involved in stimulating to the production of VCAM-1 via NFκB [165], contributing to inflammation and further recruitment of leukocytes.

2.1.3 Anti-thrombotic

The release of factors from Weibel-Palade bodies can encourage platelet adhesion and aggregation (by vWF and tPA) and leukocyte adhesion (by P-selectins). Weibel-Palade body exocytosis can be triggered by histamine, thrombin, fibrin, leukotrienes and adenosine triphosphate (ATP). The exocytosis of these Weibel-Palade bodies is mediated via N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment protein receptors (SNAREs) [166]. NO is capable of inhibiting NSF and thus prevent NSF-activated exocytosis [167]. These effects of NO are described on **Figure 2.1** (black pathway).

Under high shear stress conditions, platelet activation appears to be mediated also by membrane bound P-selectins released from Weibel-Palade body exocytosis [168, 169].

2.1.4 Anti-coagulant & Pro-fibrinolytic

eNOS is also found in platelets and the platelet-derived NO has a role in hemostasis. Bleeding times of mice with eNOS-deficient platelets showed a decrease in bleeding time compared to mice with normal platelets [170]. Endothelium-derived NO also inhibits the expression of P-glycoproteins on the platelet surface, thus preventing platelet aggregation [171].

The NO pathway is an important mediator for the acute release of tPA from the endothelium [172]. The mechanism appears to involve substance P [173] and β-adrenoreceptors [174]. NO also inhibits PAI release from platelets [175].

It has also been found that the RAS is involved in fibrinolytic balance [176, 177], with the endothelium speculated to be the link between the two. The reason for this is that Ang II binds to the endothelium and stimulates the production of PAI-1 [178-180] in a time and dose-dependant manner. The endothelial effect of AngII on PAI-1 expression appears to act exclusively via Angiotensin IV (converted from AngII by the aminopeptidases A & M to an active fragment also known as 'AngII 3-8 fragment') binding specifically to an endothelial angiotensin receptor (ATR) [181]. Although the exact pharmacological subtype of the receptor is unknown, the receptor can be blocked specifically by an angiotensin receptor type 4 antagonist. Tissue plasminogen activator (tPA), released from the endothelium, activates plasminogen to encourage fibrinolysis, thus the inhibition of tPA by PAI-1 decreases fibrinolysis. This pathway is shown on **Figure 2.1** (pink pathway).

2.1.5 Anti-hypertophic

ET-1 may have smooth muscle proliferative activity, since the binding of ET-1 to ET_A also results in the stimulation of DNA synthesis, cell cycles and protein synthesis (**Figure 2.1** (green pathway)). ET-1 can induce eNOS activity and NO production by binding to the ET_B receptor (**Figure 2.1** (dark blue pathway))[134, 182]. The NO produced then inhibits the synthesis of ET-1 in the endothelial cell and thus can inhibit ET-1-associated smooth muscle proliferation. This mechanism, shown in cardiomyocytes, was via NO suppression of an increased *c-fos* mRNA expression caused by ET-1 [182]. *c-fos* is one of the complexes responsible for modulating ET-1 gene transcription [183]. In addition to this, NO was found to directly inhibit extracellular-regulated signal kinase (ERSK) phosphorylation and activation via cGMP [182]. ERSK is involved in ET-1-induced *c-fos* gene expression. The same study also found that the ET-1-stimulated promoter activity of β-myosin heavy chain and the resulting protein synthesis were inhibited by NO.

Past animal studies using NOS inhibitors suggest that the basal release of eNOS-produced NO increases cerebral blood flow and also prevents hypertrophy and remodelling in the cerebral vasculature [143]. One study observed that there was hypertrophy and remodelling in the cerebral arterioles in rats with a NOS-inhibitor-induced hypertension [184]. This same study also observed that the same rats had increased cerebral arteriolar pressure and pulse pressure when compared to controls, but these were still lower than stroke-prone spontaneously hypertensive rats.

AngII has been shown to have hypertrophic properties [185, 186]. AngII, through the angiotensin type 1 receptor (coupled to tyrosine kinases) [187], can induce the expression of growth factors in the smooth muscle, such as platelet-derived growth factor and basic fibroblast growth factor, encouraging smooth muscle proliferation [188].

2.2 Endothelial Dysfunction

Endothelial dysfunction (ED) arises when the endothelium becomes damaged and the equilibrium of vasomodulators and inflammatory mediators become imbalanced. This is characterised by three main processes: (1) endothelial activation; (2) inflammation; and (3) impaired endothelium-mediated vasodilation [119]. Each process of the three processes stimulates the other two, eventually leading to ED in a detrimental cycle.

Endothelial activation occurs when there is an increased endothelium-leukocyte interaction and endothelial permeability. There is the release of mediators that facilitate platelet aggregation and thrombus formation, and impair fibrinolysis. These mediators include tissue factor, which is not normally expressed in endothelial cells, PAI-1 and vWF. The increased 'stickiness' of the endothelium as a result of endothelial activation encourages leukocytes to migrate into the arterial intima, while also upregulating inflammatory mediators such as ICAM-1.

2.2.1 Pathophysiology of ED: Pathways of Dysfunction

Nitric oxide has a key role in EF – it is not only involved in endothelium-mediated vasodilation, but also in preventing thrombus formation, inflammation and the mobilisation of inflammatory mediators and vascular hypertrophy, as shown in **Figure 2.1**. The pathways of dysfunction are centrered around the NO pathway and NO bioavailability [119], thus endothelium-dependant vasodilation is used as an indicator for the other functions of the endothelium [189].

2.2.1.1 Decreased availability of L-arginine

One proposed mechanism is via asymmetric dimethylarginine (ADMA). ADMA is a competitive endogenous antagonist of eNOS formed as a by-product of protein metabolism [119], and as such, it would compete with L-arginine for binding sites on eNOS. ADMA is either excreted via the kidneys or is metabolised to citrulline by dimethylarginine dimethylaminohydrolase (DDAH). Accumulation of ADMA occurs when DDAH is inhibited [190] or when the kidneys are unable to excrete it (e.g. in people with poor renal function) [191]. Antagonism of eNOS by ADMA reduces the enzyme's capacity to convert L-arginine to NO, and hence the production of NO is reduced, resulting in impaired vasodilation.

2.2.1.2 Changes in eNOS expression and function

Since eNOS is fundamental to the production of NO, any change to the functionality or expression of the enzyme could have a major impact on EF. The expression of the enzyme can be altered by various physiological factors, such as exercise. Exercise increases the expression of eNOS and augments the ability for the blood vessels to dilate in response to NO [192]. Other factors, such a TNFα, high concentrations of LDL and hypoxia can have negative effects on eNOS expression The uptake of oxidised LDL by LOX-1 also reduces eNOS

expression [193]. eNOS expression in endothelial cells covering advanced atherosclerotic plaques is lower when compared to the endothelium in normal vessels [194]. This will have an effect on the vasomotor capacity of the endothelium and may be the reason for endothelial dysfunction in atherosclerotic patients.

The binding of ET-1 to its ET_A receptor can also cause decreased expression of eNOS, resulting in a lack of NO-dependant vasodilation to counter the ET-1 induced vasoconstriction.

Another way in which NO production can be affected is via altered eNOS function. Enzyme function can be affected via genetics. The polymorphisms which affect the eNOS enzyme function are discussed later in this chapter.

2.2.1.3 Decreased NO availability

Superoxide has been identified as a source of NO inactivation in diseased blood vessels exhibiting a lesser vasodilatory response per unit of NO when compared to normal vessels [195]. Under normal circumstances, the majority of ROS and superoxide are inactivated by antioxidant enzymes, such as superoxide dismutase (SOD), present in the endothelial cell. Superoxide (O_2^-**)** is so highly reactive with NO, such that its reaction with NO is three times faster than that with SOD [196]. This suggests that the bioavailability of NO is highly dependant upon the local concentration of superoxide. Inactivation of NO leads to a decreased vasodilatory influence on the vessel and results in vasoconstriction [197]. If left activated, superoxide may inactivate NO by producing peroxynitrite:

$$O_2^- + {}^{\cdot}NO \rightarrow O{-}O{-}N{=}O^-$$

Peroxynitrite itself is a potent oxidant. It is capable of initiating lipid peroxidation [198]. Lipid peroxyl radicals produced during this process can again affect NO bioavailability. These lipid peroxyl radicals can react readily with NO to form 'lipid peroxynitrite derivatives' [199] again further reducing the amount of NO available. Lipid peroxidation also has a role in oxidising LDL, which can affect eNOS expression and function [200].

Peroxynitrite and oxidised products from other pathways (e.g. metal ion-induced oxidation, lipoxygenase, glycoxidation and myeloperoxidase) oxidise the LDL to modified lipoproteins. These modified lipoproteins are involved in the formation and progression of atherosclerosis

[126, 201]. Subjects with acute LI have been shown to have higher levels of peroxynitrite production in their plasma compared to healthy controls [202].

One study suggested that the main source of superoxides is from nicotinamide adenine dinucleotide (NADH) oxidoreductase [203]. NADPH oxidase is also involved in the production of superoxide [204-206]. Xanthine oxidase bound to the surface of endothelial cells has also been implicated in the production of superoxide [195, 207, 208].

The role of ROS specifically in the cerebral circulation has been under investigation, as it appears to have slightly different properties when compared with the systemic vasculature. Superoxide produced from xanthine oxidase results in the dilation of cerebral arterioles [209]. Likewise, NADH and NADPH increases in the production of superoxide that causes cerebral vasodilation [210]. However, cerebral arteriolar vasoconstriction is observed when there are higher concentrations of superoxide [210].

2.2.1.4 Altered NO signalling

A proposed mechanism of altered NO signalling is via altered G-protein signalling. Changes in membrane fluidity may prevent G-proteins from interacting with the receptors responsible in activating eNOS. Tissue cultures have shown that the Gαi2 subunit of the G-protein is inhibited by oxidised LDL [211], while research in human coronary arteries has shown G_{ia} may be impaired by physiological states, such as hypertension, age and hypercholesterolaemia [212].

2.2.1.5 Changes to tetrahydrobiopterin

BH_4 is essential for the production of healthy NO. BH_4 itself has properties which allow it to be a producer and a scavenger of oxygen radicals [127]. When attached to eNOS, BH_4 assists with eNOS dimer coupling and activates the eNOS dimer oxygenase so that it can convert L-arginine to NO. In the absence of BH_4, the eNOS dimer uncouples and the reductase activity of eNOS is activated (and oxygenase inactivated), thus producing more ROS and superoxide [130, 213, 214]. Peroxynitrite produced from ROS can degrade BH_4 and create further ROS, hence forming a cycle detrimental to EF. Past studies have shown that eNOS uncoupling has given rise to vascular disorders via ED [215, 216]. Uncoupled eNOS has also been reported to have a role in cerebral hypoxic-ichaemic injury [217].

2.2.1.6 Other proposed mechanisms

ROS have also been suggested to have a role in 'anoikis', which is a form of endothelial cell apoptosis caused by the detachment of the endothelial cells from the cellular matrix [218]. A component of fish oil, eicosapentaenoic acid, was shown to prevent anoikis in endothelial cells [219].

2.3 Methods to measure EF

Arterial stiffness has been implicated in isolated systolic hypertension and is possibly an indicator of the risk of developing atherosclerosis and its associated complications [220]. Arterial stiffness occurs when the vessel is beginning to lose its ability to undergo positive remodelling. ED and arterial stiffness are coupled because the endothelium must have undergone some degree of damage for the arteries to lose the ability to remodel positively, thus arterial stiffness can be an indicator of ED. The importance of ED and arterial stiffness is that any changes in these measures can be detected before any of the clinical signs are present. The general principle of all methods to measure ED is to compare how well the endothelium dilates an artery against how well an artery dilates without the involvement of the endothelium.

The inability of an artery to react to a stimulus (i.e. arterial stiffness) is a major component of ED. However, ED is a complicated condition and arterial stiffness does not reflect the total degree of ED – arterial stiffness can only give a good estimate of the degree of ED. The majority of studies investigating ED in various vascular conditions only measure arterial stiffness. Other factors to consider in determining ED include platelet activation, measures of inflammation and changes to vessel wall thickness. However, the evaluation of vascular responsiveness is accepted as the standard test for EF [221, 222].

2.3.1 Invasive Methods

2.3.2 Intra-arterial Infusions

The methods requiring a direct infusion of vasoactive substances are regarded as invasive techniques. This was the method originally used to assess EF [223].

Many of the initial studies testing EF used methods that required surgery or the infusion of vasoactive substances directly into the vessels of interest. Al Suwaidi et al. inserted an infusion catheter into a coronary artery and intracoronary infusions of adenosine and acetylcholine were given [224]. A Doppler wire was used to measure Doppler flow velocity. EF was calculated from the dose-response curves calculated from volumetric coronary blood flow. Halcox et al. [225] and Targonski et al. [226] had similar methods.

Although accurate and reproducible, there is a considerable amount of risk associated with these invasive techniques. If intra-arterial infusions were to be used to measure cerebral EF directly, the potential adverse effects associated with this would be dangerous. Intra-arterial infusions into the brachial artery to measure peripheral EF would also carry the risk of damaging the artery or the median nerve [227]. It is also an expensive procedure.

Thus, as a method for testing EF clinically, intra-arterial infusions may not be the most efficient, easiest or the safest.

2.3.2.1 Skin Biopsies

Some studies have used skin biopsies from the abdominal or gluteal regions to examine EF. Rizzoni et al. showed that the small resistance arteries in the abdomen have the same function and morphology as those in the gluteal regions [228]. This group used subcutaneous skin biopsies (3 cm long x 0.5 cm wide x 1.5 cm deep) to show that EF was impaired in all groups of hypertensive patients regardless of the aetiology of the hypertension. However, the method they used to measure vessel reactivity was to isolate arteries in the biopsy and infuse specific vasoactive substances into the artery in the laboratory under biological conditions. This method is very similar to the acetylcholine infusion and requires some degree of speciality (e.g. taking the biopsy, isolating the artery, keeping the artery under biological conditions) and may not be suitable as a routine clinical test.

2.3.2.2 Markers of ED

A normal endothelium is anti-thrombotic. Thrombomodulin, tissue factor pathway inhibitor and protein S are examples of endothelium-derived anti-coagulants [221]. ED is partly characterised by endothelial activation, which occurs when there is an increased endothelium-leukocyte interaction and endothelial permeability, encouraging inflammation. The endothelium then also releases mediators that facilitate platelet aggregation and thrombus

formation, and impair fibrinolysis. These mediators include tissue factor, which is not normally expressed in endothelial cells, PAI-1 and vWF.

Hassan et al. [58] investigated the ratio of inflammatory markers related to endothelial dysfunction in patients with LI and LA. These markers were: ICAM-1, thrombomodulin, tissue factor, and tissue factor pathway inhibitor. It was found that patients with isolated LI had a different inflammatory marker profile to those with ischaemic LA (defined as LA with LI). The results showed that the levels of some markers were significant in both isolated LI and ischaemic LA when compared to the controls. However, significance was not found when comparing the two groups with each other.

Although ICAM-1 and VCAM-1 are elevated in ED and coronary artery disease, these biomarkers lack specificity and thus may be raised in other inflammatory conditions [229]. E-selectins, as a marker, are more specific for the endothelium, and likewise thrombomodulin. Thrombomodulin is also associated with severe coronary artery disease and stroke. tPA release and PAI-1 have also been of interest in studies using endothelial markers as indicators of EF. vWF is considered the gold standard in the measurement of endothelial damage, and thus is the best endothelial biomarker [230].

Another marker used is C-reactive protein (CRP). CRP is produced from the liver in response to systemic inflammation [231]. CRP is often found to be elevated in patients following acute ischaemia and myocardial infarction. CRP can also be used as a predictor of future ischaemic events [232]. It is known that CRP has a role in the atherosclerotic process and thus may be an indirect indicator of ED [233, 234]. Fichltscherer et al. found that elevated levels of CRP were associated with blunted endothelial vasodilator responses, thus further emphasizing that ED is associated with systemic inflammation and that CRP may be an indicator of ED [235]. However CRP is not specific to SVD or even neurological inflammation and thus may also be elevated in other inflammatory conditions.

Inflammatory markers provide an easy alternative to determining ED, as tests can be done from blood samples taken as part of routine therapy. They are accurate, reproducible and simple. However, because many of the markers may be influenced by other physiological conditions, and since there is currently no ‘cut-off point’ at which inflammatory markers can indicate ED separately from other inflammatory conditions, further research is required to refine the use of inflammatory markers for this to be clinically applicable.

2.3.3 Non-invasive Methods

Although there has been some debate as to whether all vascular territories exhibit the same degree of ED, two studies have shown a close relationship between EF in the coronary and brachial arteries [236, 237]. Due to the exclusive nature of cerebral blood vessels, there has been very little work done aimed at correlating assessments of EF between the brain and the periphery.

As a clinical tool, the non-invasive methods are preferred, as they cause less harm to the patient and are simpler to perform.

2.3.3.1 Transcranial Doppler Sonography (TDS)

This has traditionally been the method to measure cerebrovascular reactivity to L-arginine, and it is believed to indicate cerebral EF [238-240]. Patients with LI have been found to have a decreased cerebrovascular reaction to L-arginine [241].

TDS is used clinically to measure the velocity of blood flow in the cerebrovascular system [242] by analysing the Doppler frequency shifts from red blood cells moving through a prespecificed arterial volume [242]. Although TDS can monitor cerebral blood flow, measurements must be performed using the carotid arteries.

TDS is a specialised technique and requires a high degree of operator training.

2.3.3.2 Carotid-Femoral Pulsewave Velocity (CF-PWV)

CF-PWV is considered one of the best methods of measuring arterial stiffness [243-245]. Arterial stiffness is determined using the time required for an arterial pulse to propagate from the carotid to the femoral artery [246]. There are currently two commonly used systems for CF-PWV: SphygmoCor® (AtCor Medical) and Complior® (Artech Medical). SphygmoCor® uses an arterial tonometer to measure the wave forms, and the propogation time is determined from the foot of the carotid waveform to the foot of the femoral waveform. Complior® uses mechanotransducers to simultaneously measure both carotid and femoral waveforms and time is determined by the points of maximum systolic upstroke.

2.3.3.3 Flow-mediated dilation (FMD)

The techniques of FMD are based on vasodilation in response to shear stress, which is likely to represent the main pshyiological stimulus [221]. There are two main techniques that utilise FMD: ultrasound and plethysmography.

Using ultrasound to measure EF is easy and reproducible, but to some extent, user-dependant [223]. The user measures the diameter of the artery at baseline. The baseline result is compared to the diameter of the same artery after 5 minutes of ischaemia. It is important that the same image of the artery is maintained. A sufficient time interval is given before baseline is measured again and compared to the arterial diameter after glyceryl trinitrate (GTN) administration to measure the endothelium-independent vasodilation. This technique is relatively simple and is therefore suited for use in large populations. However, ultrasound FMD is highly user-dependant and often requires months of hands-on training to ensure the skills of the users are adequate and consistent. There may also be some intraobserver variability when measuring the vessel diameter, but specialised computer software has aided in reducing this variability [247]. It also relies on good patient cooperation and is limited to the larger and more accessible arteries, such as the aorta and carotid arteries [220]. Small changes in vessel diameter are also hard to detect using ultrasound. As there is no standard protocol for ultrasound FMD, there may be differences in the position of the cuff, duration of occlusion and dose of GTN between studies, making comparisons between studies difficult. Regardless, FMD ultrasound was found to be the method of choice when investigating EF due to its high reproducibility and low variability [248].

Strain gauge plethysmography (SGP) is a relatively simple test measuring the percentage change in forearm blood flow (brachial artery) between baseline and maximum flow [223]. The change in blood flow can be caused by reactive hyperaemia or forearm perfusion of pharmacological agents (which is invasive). A strain gauge, usually a stretchable tube filled with a liquid metal, is tied around the forearm and changes in blood flow alter the forearm circumference, thus affecting the cross-sectional area of the tube. The circumference of the tube and the electrical resistance of the metal are measured using a plethysmograph. EF is measured indirectly as the overall dilatory capacity of resistance arteries, which is calculated by the area under the curve of the flow response during reactive hyperaemia [249, 250]. Although results from SGP are less specific than FMD in ultrasound, SGP is less user-dependant and less reliant on highly trained personnel. Also, if forearm perfusion is the method of choice, it is not suited for large populations or studies that require multiple repeated measurements.

2.3.3.4 Digital Pulse Amplitude Augmentation (DPAA)

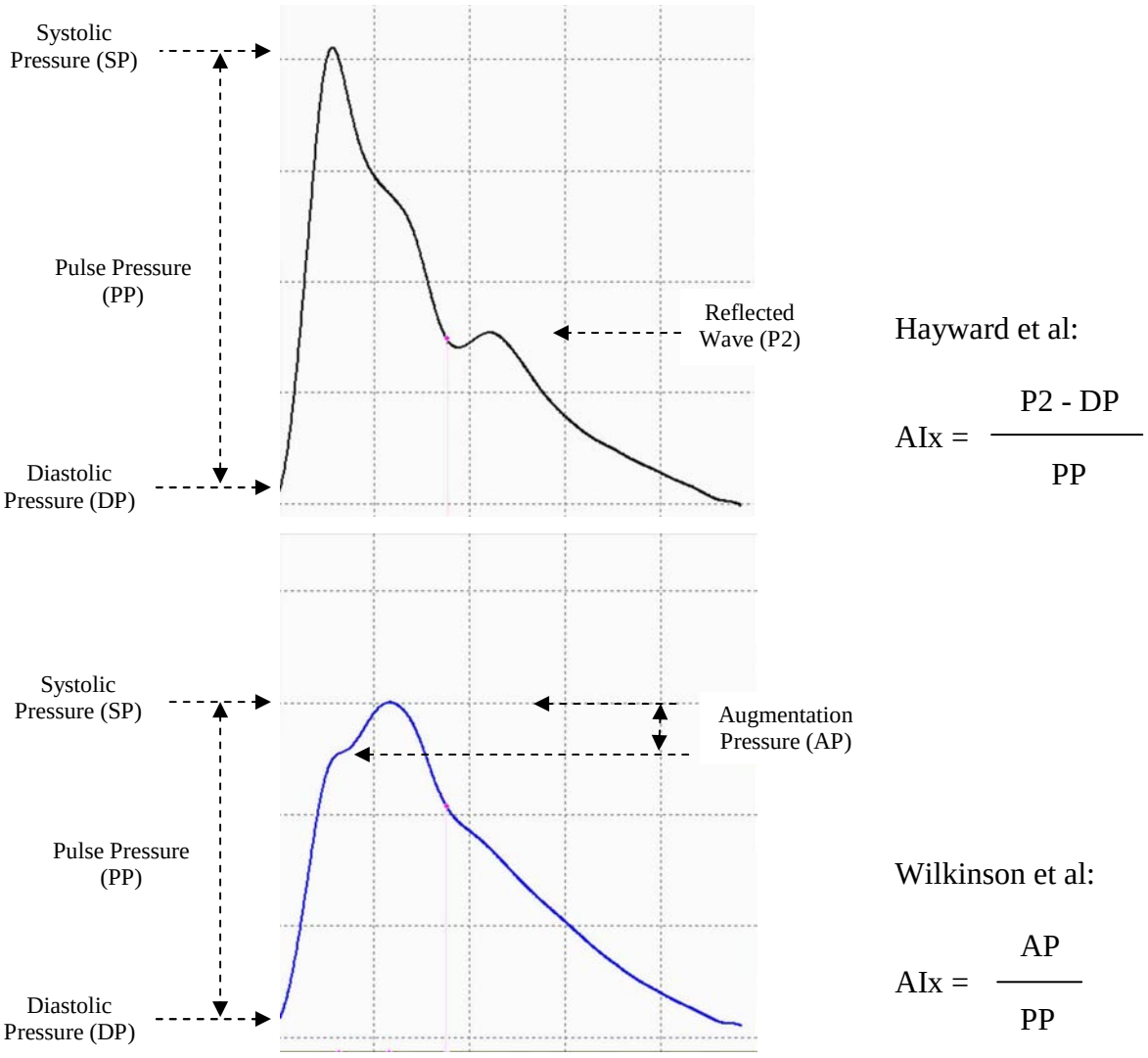
DPAA is a relatively new technique currently being trialled in the Framingham Heart Study [251]. This technique measures a hyperaemic vasodilator response using a fingertip pulse amplitude tonometry (PAT) device. An impaired pulse amplitude hyperaemic response was associated with cardiovascular ED [252, 253].

2.3.3.5 Applanation Tonometry (ApT)

ApT uses pulsewave analysis (PWA) and determines EF by testing how well the forearm radial artery responds to pharmacological stimuli. ApT for EF was modified from the technique used in measuring intraocular pressure. The waveform from the radial arterial is transformed using a transfer function to derive an aortic waveform. Studies using this technique have reported both peripheral [254] and aortic [255] results.

A probe is placed over the point of maximum arterial pulsation on a superficial artery (usually the radial artery in the wrist) to depress the arterial wall gently. The electrical resistance of a crystal on the tip of the probe varies with arterial pressure, thus resulting in an arterial waveform (**Figure 2.2**). This technique is called PWA. The measurement is called an 'augmentation index' (AIx).

Figure 2.2 – Top: peripheral waveform; Bottom: arterial waveform derived from the peripheral waveform using a validated transfer function. The waveforms shown are from a participant (control) in our study.



AIx can be calculated using either the peripheral or aortic waveform. Hayward et al. used the AIx derived from the radial (peripheral) artery using the first reflected wave [254]: (P2 – Diastolic Pressure) / (Pulse Pressure). Wilkinson et al. calculated AIx from the central pulse wave, which is derived from the peripheral pulse wave using a validated transfer function [256]. This is the difference between the two peaks expressed as a percentage of the pulse pressure. This is calculated as: (Augmentation pressure) / (Pulse pressure) [243]. A higher AIx indicates increased arterial stiffness.

Two pharmacological agents are given to determine EF. The first is GTN, a NO donor to elicit endothelium-independent vasodilation. The GTN is given to demonstrate that the change seen after salbutamol administration is due to deficiencies in NO and the NO pathway

(a key pathway of EF) and not due to signalling problems within the smooth muscle. The second agent is salbutamol, a β_2-adrenoceptor agonist and an endothelium-dependant vasodilator. Previous studies have shown that β_2-adrenoceptor stimulation induces NO to be released from the endothelium in humans [257] and animals [258]. Hayward et al. showed that the peripheral arterial waveform is sensitive to β_2-receptor stimulation by low-dose inhaled salbutamol [254]. These results are supported by the findings of Wilkinson et al. [256]. Both these studies show that the salbutamol waveform, when compared to the waveform of GTN, can be used in the non-invasive measurement of EF.

According to McEniery et al., global EF can be calculated as a ratio of the change after salbutamol administration to the change after GTN administration [259]. Thus, a higher ratio indicates better EF.

ApT versus other techniques

One study compared the use of the invasive acetylcholine infusion (into the peripheral circulation), ultrasound-based FMD and applanation tonometry in elderly subjects [260]. All three techniques have been used to relate EF with the Framingham risk score (associated with coronary heart disease). The authors indicated that their results strongly suggest that endothelium-dependant vasodilation in resistance arteries and in conduit arteries differ significantly and may have different mechanisms. They also raise the point that FMD relies on shear stress as the stimulus for vasodilation, whereas acetylcholine infusion and applanation tonometry induce vasodilation pharmacologically, implying greater similarity between the gold standard (acetylcholine infusion) and applanation tonometry than FMD and infusion. The conclusion from this study was that all three techniques to measure endothelium-dependant vasodilation would be feasible to use in the general elderly population, but if the acetylcholine infusion can not be used, then applanation tonometry would be a suitable alternative.

Another study found that ApT was not as reproducible as FMD and thus FMD was concluded to be the preferred choice of measuring EF [248]. However, ApT is still a promising technique as long as reproducibility is improved. ApT has been shown to be highly reproducible, with many studies showing good reproducibility of results [261-264].

The advantage in using ApT rather than FMD for studies of the small vessels is that ApT measures the responses in the radial artery, whereas FMD uses the brachial arteries. The

radial artery is a smaller vessel than the brachial artery, and is therefore more likely to have the characteristics of the smaller vessels than the larger brachial artery.

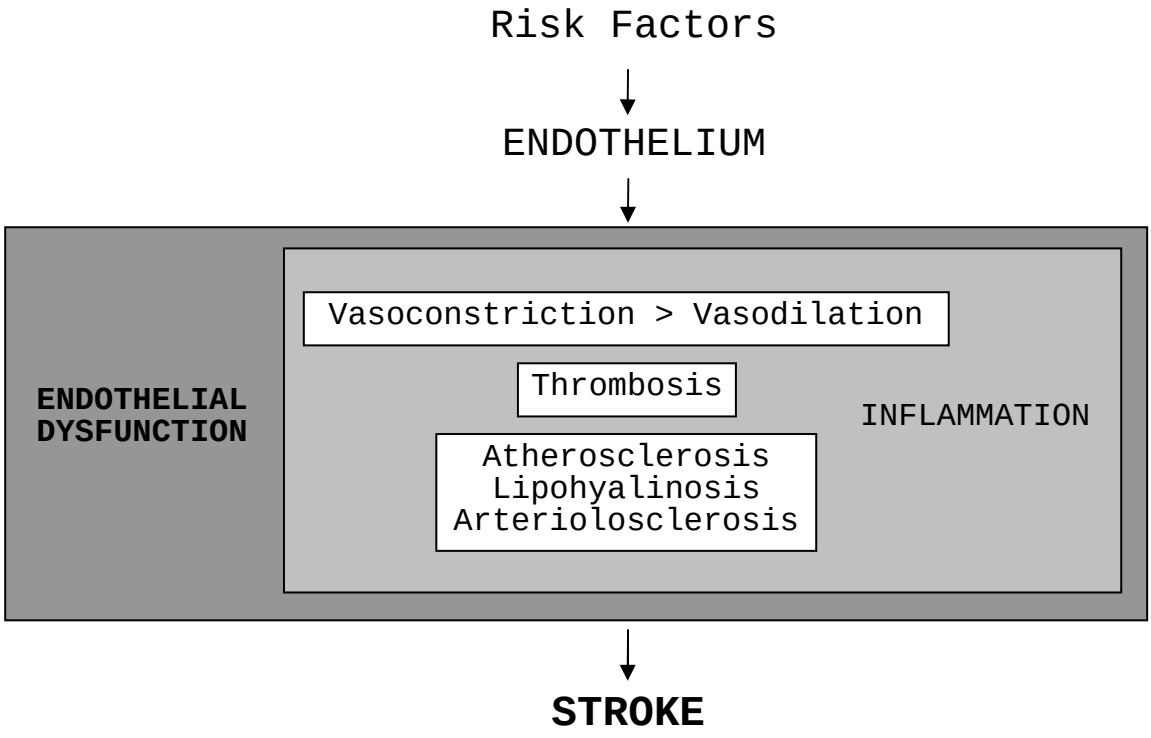
The main disadvantage of ApT is that it requires approximately 1½ hours for the test to be undertaken (including the 30 minute resting time required before testing). ApT also requires the oral administration of drugs to measure the endothelium's vasoreactivity, and therefore introduces the risk of medication-related adverse effects. However, the administration of the medication is easy for both the investigators and patients and adverse effects are minimal and dose-dependant. Thus, when compared with invasive measures, there is less harm and trauma to the participant. When compared with other non-invasive measures, the principles of producing endothelium-dependant vasodilation using ApT are more akin to the gold standard than those of FMD are [260], therefore ApT is considered to be a better alternative to infusions than FMD.

2.4 Influential factors of EF

It has been well established that ED can be influenced by other factors such as age, genetics, smoking, insulin dependant and non-insulin dependant diabetes, hypercholesterolaemia and hypertension, as illustrated in **Figure 2.3**. These factors have been associated with excessive oxidant stress on the endothelium [265]. Oxidative stress is described as an excess of oxidants compared to antioxidants [126]. The main contributors to oxidative stress are superoxide and peroxynitrite. Other contributors include the tyrosyl radical [266] and hypochlorous acid [267] from myeloperoxidases and lipoxygenase [268]. The risk factors identified generate an environment within the vascular wall promoting the production of oxidants such as superoxide and peroxynitrite, which, as described above, exert negative effects on EF. It is important to note that the risk factors for ED are the same risk factors for LA and LI. It may be that the endothelium is the intermediary between the risk factors and the development of disease. EF may be considered the 'sum of all factors', exhibiting the net impact of all risk factors. If so, EF would be a better indicator of the true risk of disease than the individual risk factors themselves.

Pretnar-Oblak et al. published a study showing the effect of EF in the development of SVD using FMD [269]. Healthy controls were shown to have the best EF, followed by people with traditional cardio- and cerebrovascular risk factors, while people with SVD showed the poorest EF.

Figure 2.3 – The various risk factors for endothelial dysfunction are proposed to modulate the vascular environment to result in neurovascular events, such as stroke.



2.4.1 Age

EF is progressively impaired by age [270-275]. Animal models have shown that elderly rats show impaired vasodilation in response to acetylcholine and that this impaired vasodilation is unrelated to cyclooxygenase [276]. Other human studies have also found that age is an independent risk factor for impaired EF in the coronary microvasculature [277, 278]. Gerhard et al. suggested that the reason for this decline in endothelium-dependant vascular reactivity in the elderly human population is due to the decreased bioavailability of endothelial NO [279]. Other proposed factors include the loss of endothelial cells and the increased vasoconstriction in smooth muscle that develops with increasing age [280].

A study by Yavuz et al. showed a significant association between age and EF using a cohort of healthy elderly subjects (mean age 71.3 years) and a cohort of younger subjects (mean age 26.5 years) [281]. The correlation between age and EF was found to have a $r=-0.528$, $p<0.001$

One study has identified a difference in age-related EF decline between males and females, where older females have a faster rate of decline compared to age-matched males [271]. A similar observation was noted in another study [282].

*2.4.2 **Genetics***

As in most diseases, there is some degree of inter-individual variability. Since the pathophysiology, cause and progression of ED is multifactorial, it is highly likely there is a contributing genetic component. With the large number of proteins and factors involved in ED, it is highly likely that genetic polymorphisms that alter protein and factor levels will have a role in ED.

eNOS

Any differences in the genes that encode enzymes is the most likely to affect the clinical outcome. The major enzyme directly involved in ED is eNOS. As described above, eNOS is responsible for converting L-arginine to NO and changes to eNOS expression and function are one of the identified contributors to ED. Three clinically relevant polymorphisms have been identified involving eNOS. The first, which regulates the transcription rate of the eNOS gene, is -786 T/C in the promoter region of the gene [283, 284]. This mutation has been associated with coronary spasms in the Japanese population [285]. However, the association of this mutation with coronary artery disease (CAD) in Caucasians is less consistent [286, 287]. The -786 T/C mutation is discussed in further detail in Chapter 3.

The second involves 27-base pair repeats in intron 4. This polymorphism has been associated with smoking-dependant CAD in some studies [287, 288], while other studies disagree [289, 290].

The third polymorphism, a glutamic acid to aspartic acid swap in position 298 in exon 7 (894 G/T) [283, 291, 292] has been studied extensively and has been associated with a decrease in NO production [293]. With this polymorphism, the T allele is the mutant, with a frequency of 32.5% in a white Australian population (562 men, 201 women) [292]. The overall TT frequency was found to be 11.7%. The association of this polymorphism with CAD has been controversial [287, 294-296].

In terms of this polymorphism altering enzyme activity eNOS, there was no obvious difference in the Michaelis constant, K_m, or V_{max} between recombinant eNOS Asp298 and Glu298 [297-299]. The polymorphism is located in a loop on the external surface of the enzyme and is not in contact with either the active site of the enzyme or the dimerization interface [297]. This suggests that the functional significance of this polymorphism is independent of enzymatic catalysis. The Asp variant has been previously shown to have a shorter half-life in

endothelial cells culture [300]. An Asp298 eNOS was shown to be cleaved into N-terminal 135 kDa and C-terminal 100 kDa fragments, and the blood vessels of individuals with this variant also have an increased reactivity to phenylephrine. This is consistent with decreased eNOS activity [301]. However, there are two reports that disagree with the observations made by Tesauro et al. [299, 302].

The majority of studies investigate associations between these eNOS polymorphisms and CAD. Very few studies have involved CVD. Vascular lesions similar to those in SVD have been observed developing in knockout mice lacking the eNOS gene [303]. The Glu298Asp in exon 7 (894 G/T) has also been associated with LI in a sub-population of 95 patients with LI compared to 95 matched controls [304].

Endothelin-1

As shown in **Figure 2.1**, ET-1 appears to have a large role in the regulation of EF. Many of the effects of ET-1 binding to ET_A are opposing that of NO. Thus, as for eNOS, any change in the function or expression of ET-1 would be important to the functional status of the endothelium. Likewise, polymorphisms involving the ET-1 receptor may be important. ET-1 is derived from a larger peptide, preproendothelin. Any structural or functional changes to any of the proteins and enzymes involved in ET-1 formation could have an effect of ET-1 function and thus affect EF.

A polymorphism in the preproendothelin-1 gene has been identified to affect vascular reactivity in human mammary arteries [305, 306]. The T allele of the 5665 G/T polymorphism on exon 5 has been associated with higher blood pressure levels in overweight individuals. The results from Iglarz et al. suggested that this polymorphism may potentiate the effect of ET-1 and AngII by increasing Ca^{2+} sensitivity [306].

Angiotensin and Angiotensin Converting Enzyme

The ACE insertion/deletion polymorphism has been thought to be involved in ED, LA and LI. There appears to be conflicting evidence for the involvement of the ACE I/D in ED [307-311]. Subjects with the homozygous deletion (DD) allele were reported to be at higher risk of developing LI [105, 312]. Amar et al. also reported that the DD allele was a risk factor for white matter lesions (LA) associated with cerebral infarction [312].

Some studies have shown that the ACE insertion/deletion DD allele may have a role in potentiating ED [307-309]. In contrast, other studies have found that the allele is not involved [310, 311, 313]. In the studies that showed a positive relationship between the DD allele and EF, the mechanism in which the allele negatively affected ED was unknown, with the possibilities including increased AngII-induced NO breakdown (via ROS) [203] or decreased bradykinin-mediated NO release [314]. The frequency of the ACE DD allele was found to be 30% in the general population in one study [315].

2.4.3 Smoking

Both active and passive smoking may damage the endothelium. Long-term smoking has also been associated with impaired endothelium-dependant vasodilation in both the coronary and peripheral vascular systems [117].

The results of one study showed that EF was impaired in middle-aged smokers, but not in younger smokers [316]. From these results, the authors suggested that the duration of smoking is important in determining the degree of damage that smoking has on EF. Decreased EF has been found in chronic smokers [317] and an oxidative stress-related decrease in vasodilator response to acetylcholine was also seen in chronic smokers [318].

Another study showed that concentrations of ICAM-1 and E-selectin, markers of inflammatory endothelial activation, were increased significantly in current smokers, while this same increase was not seen in diabetic and hypertensive patients [319].

The exact component of cigarette smoke that causes ED is unknown. However, it has been suggested that the mechanism involves increasing oxidative stress via a superoxide-anion [320, 321].

2.4.4 Hypercholesterolaemia

Hypercholesterolaemia is a well known risk factor for cardio- and cerebrovascular disease. LDL is intimately linked with the formation and progression of atherosclerotic lesions. EF in the coronary arteries has been shown to be decreased when there are elevated cholesterol levels [277, 322, 323]. This effect is also dependant on the ratio of HDL and LDL – ED is closely associated with LDL levels, but the effect can be reversed with high HDL levels [324]. One study using a relatively young male sample (n=60) has shown that higher HDL levels can

improve EF (r=0.29, p=0.02) [325]. Oxidised LDLs have been shown to stimulate NADPH, a major producer of ROS [326].

It has been shown that the administration of L-arginine can improve EF in hypercholesterolaemic patients. The results imply that there is an L-arginine deficiency or a decreased availability of L-arginine in hypercholesterolaemic patients [327]. Accumulation of ADMA has also been associated with hypercholesterolaemia [328], however, a study by Maas et al. found no difference in plasma ADMA concentrations between hypercholesterolaemic and normocholesterolaemic volunteers [329]. This group also found that hypercholesterolaemic patients showed a 40% lower excretion of nitrate compared to normocholesterolaemic patients 48 hours a L-arginine infusion. FMD was also lower in hypercholesterolaemics, as was normalised eNOS protein expression in platelets.

Circulating ET-1 levels have been found to be elevated in experimental hypercholesterolaemia [330, 331], and it has been implicated in obesity-related hypertension [332].

HMG-CoA inhibitors, or 'statins', are traditionally used as anti-lipid therapy to help patients reduce serum cholesterol levels. Statins help reduce LDL cholesterol. They also increase EF by up-regulating eNOS. Statins also help stabilise atherosclerotic plaque, preventing parts of the plaque from breaking off and becoming emboli. There is also some evidence to show that statins can help increase the number of circulating endothelial progenitor cells in coronary artery disease, which help in neovascularisation [333, 334]. Studies in different population groups have shown that treatment with atorvastatin has resulted in improved EF [335, 336].

2.4.5 Diabetes Mellitus

Animal models of diabetes have shown that ED may be caused by excessive oxidative stress [337-339]. Excessive blood glucose, or hyperglycaemia, can lead to the following [119, 340]:

- Oxidation of glucose leading to oxidative stress;
- Arachadonic acid metabolism arising from Protein Kinase C (PKC) activation leading to the formation of more oxidative stress. Oxidative stress can then lead to further activation of PKC, and thus the cycle self-propagates;
- Decreased production of NO from the production of advanced glycation end products (AGE). AGE, in turn, can also stimulate NADPH oxidase to produce more ROS [341] and thus enhances inflammation by increasing the expression of various inflammatory mediators.

In addition to this, insulin-signalling pathways can become altered. The pathway involving the phosphorylation and activation of eNOS via phosphoinositide 3-kinase, phosphoinositide-dependant kinase-1 and Akt/protein kinase becomes down-regulated, resulting in the decreased production of NO from L-arginine.

Insulin also has both metabolic and mitogenic effects on the small vessel endothelium. Following the primary insult, insulin can also stimulate the HMG-CoA reductase activity in monocytes resulting in increased LDL binding and the formation of foam cells [342, 343]. Insulin can also stimulate the migration and proliferation of smooth muscle cells [344, 345].

Past studies have shown that small vessel endothelium appears to be more susceptible to the effects of insulin compared to the large vessel endothelium [346]. Greater insulin-induced incorporation of glucose into glycogen was observed in retinal endothelial cells compared to aortic endothelial cells [344]. Other studies have also shown that a variety of small vessel endothelia, including the human cerebral microvascular endothelium, have a greater insulin-induced stimulation of DNA synthesis compared to aortic endothelial cells [347-349]. Clinical evidence has also suggested that changes in the cerebral endothelium caused by diabetes leads to an increased incidence of vascular dementia and LI [350].

High levels of insulin and glucose have been shown to stimulate NADPH oxidase, a major producer of ROS [351, 352].

*2.4.6 **Hypertension***

Isolated arteries from experimental models have shown that endothelium-dependant vasodilation is impaired in hypertension [353], although the mechanism of ED varies from one type of hypertension to another. In spontaneously hypertensive rats, aortic endothelium induces smooth muscle contraction via cyclooxygenase in response to various substances, such as acetylcholine, serotonin, arachidonic acid and ET-1 [354]. This leads to the release of "endothelium-derived contracting factors" (EDCF). In contrast, ED in salt-sensitive hypertensive rats is not due to the release of EDCF. These rats show decreased vasodilation in response to NO-donors, indicating that the smooth muscle has become less responsive to NO [355].

Ang II is also a known stimulant of NADPH oxidase [356, 357], which is a prominent producer of ROS and decreases EF [126, 358].

ACE decreases the half-life of bradykinin, an endogenous potent vasodilator, by degrading it to an inactive peptide. Bradykinin couples with transduction proteins to activate phospholipase C in the endothelial cell to stimulate NO synthesis [359]. ACE inhibitors (ACEI) and angiotensin II receptor blockers (ARB) are common medications used in the treatment of hypertension. Both classes of medications inhibit the vasoconstrictive effects of angiotensin II in the renin-angiotensin system. ACEI can also sensitise vascular cells to bradykinin, thus amplifying the vasodilatory effects of bradykinin [360]. Perindoprilat, the active metabolite of perindopril, was shown to induce a 10-fold increase in the potency of bradykinin [360].

50% of people over the age of 60 years have hypertension. 80% of those with hypertension will have isolated systolic hypertension, which is described as an increased systolic blood pressure, but normal diastolic blood pressure [361]. Wallace et al. found that patients with isolated systolic hypertension had significantly decreased EF (as measued using FMD, $p=0.03$) and higher aortic pulse wave velocity ($p<0.01$) when compared with age-matched controls [362]. With other types of hypertension, such as essential hypertension, there are conflicting results regarding the role of EF in hypertension [363-366]. One of the proposed reasons for this was the differences in different vascular beds [367].

Hypertension has also been associated with the accumulation of ADMA [368].

*2.4.7 **Homocysteine***

Although not a traditional risk factor, hyperhomocysteinemia has been implicated in ED because of the prothrombotic properties of homocysteine and its toxicity to endothelial cells. A study using young, healthy males (n=60) with no known CVD risk factors has shown that high homocysteine levels are associated with lower EF as measured by FMD, with $r=-0.231$, $p=0.008$ [325].

Patients without hypertension but with hyperhomocysteinemia show ED [369]. Some research has shown that homocysteine produces oxidative excess that decreases NO bioavailability [369, 370] and that this oxidative stress can be reversed by vitamin C and folic acid [371, 372]. It has been proposed that homocysteine can also cause ED by accumulating ADMA.

2.5 Clinical implications of ED

2.5.1 Atherosclerosis and Inflammation

ED has been implicated in the development of atherosclerosis. Atherosclerosis is a vascular lesion that has been frequently associated with cardiovascular disease (CarVD) and CVD. Atherosclerosis is much more than just the accumulation of lipids – it is an inflammatory disorder [373]. Atherosclerosis is characterised by the progressive accumulation of lipids and fibrous elements in the large arteries [125] and 10% of all ischaemic strokes in Caucasians have an atherosclerotic basis [374]. Although to a lesser extent, SVD is also affected by atherosclerosis.

Bonetti et al. suggest that the endothelium may be the link between these risk factors and vascular effect because of the strategic location of the endothelium between the blood and vessel wall [375]. Blood pressure measurements, cholesterol and glucose levels may only provide a limited insight into the degree of risk an individual has for both cardio- and cerebrovascular events, while the wholistic measurement of EF integrity may provide a better means of assessing overall atherosclerotic risk. Since ED can be affected by traditional risk factors as well as atherosclerosis and hyperhomocysteinemia, and ED is also an independent predictor of adverse events, ED may be able to provide a better understanding of the overall risk of an individual.

It appears that the connection between atherosclerosis and ED involves NO. Animal models of familial hypercholesterolaemia have shown that the progression of atherosclerotic lesions is accelerated when endothelial NO production is inhibited, while treatment with L-arginine reduced lesion development [376]. Another study involving ApoE-knockout mice (with eNOS removed) showed that these mice also had accelerated lesion formation in the coronary vasculature and aorta unrelated to moderate hypertension [377, 378].

The following atherosclerotic & inflammatory pathway is described in **Figure 2.1** (grey pathway). Normally, the endothelium prevents the adhesion of leukocytes to its surface. Experimental studies have shown that pharmacological inhibition of production of NO from the endothelium increases the adhesiveness of the endothelial cell to monocytes [379, 380]. In response to excess oxidative stress, the endothelium begins to attract leukocytes via the activation of adhesion molecules [381]. The process of endothelial activation occurs in two main stages. The first involves the rapid moving of P-selectins to the endothelial cell surface [382].

The second stage involves a slower synthesis and expression of ICAM-1 and other adhesion molecules [166, 383]. At first, the leukocytes are only adhere lightly to the endothelium and roll along the endothelium [375, 384]. This process is mediated by L-selectins on the leukocytes and E- and P-selectins on the endothelium [385]. Oxidative stress initiates the expression of E-selectin and rapidly mobilises the P-selectin towards the endothelial surface, both of which are mediated via nuclear factor (NF)-κB [384].

Following this, leukocytes become more strongly attached to the endothelium. Under oxidative stress, the endothelium upregulates VCAM-1 and ICAM-1, and these are the adhesion molecules that are mainly responsible for the binding of monocytes and T-lymphocytes in this phase [125]. Although both VCAM-1 and ICAM-1 are upregulated in atherosclerosis, some research has shown that VCAM-1 is more influential in early atherosclerosis than ICAM-1 [386]. VCAM-1 induction is activated by inflammation caused by modified lipoproteins particles from oxidised lipoproteins. These lipoproteins and other inflammatory cytokines, such as interleukin 1β and tumour necrosis factor (TNF)-α, increase the transcription of the VCAM-1 gene again via NF-κB [387].

When bound to the endothelium, the monocytes begin to migrate, or 'diapedese', between endothelial cells with the aid of chemoattractant molecules (e.g. monocyte chemoattractant protein (MCP-1)) situated in the arterial intima [125]. Inside the arterial intima, the monocytes develop characteristics of tissue macrophages. A variety of scavenger receptors for oxidised LDL, including LOX-1, become expressed on the surface of the cell and the monocyte internalises the LDL, giving the macrophage the appearance of foam cells [388]. These foam cells, in turn, secrete inflammatory cytokines that produce local inflammation and increase ROS in the area, thus further damaging the endothelium. Foam cells also secrete matrix metalloproteinases (MMP) that degrade the fibrous cap securing the arterial lesion, causing further thrombotic complications when the fibrous cap ruptures and exposes the lesion to various pro-coagulant factors.

As with monocytes, the T-lymphocytes migrate across the endothelial barrier with the aid of VCAM-1 and other chemoattractant molcules. For T-lymphocytes, these chemoattractant molecules include inducible protein (IP)-10, monokines induced by interferon-γ and interferon-inducible T-cell α-chemoattractant [389]. Antigens such as oxidised-LDL presenting in the arterial intima bind to the CXCR3 receptor expressed on the T-lymphocyte surface. This causes the T-lymphocyte to release cytokines into the arterial intima. T-lymphocytes also

express CD154 on the cell surface, which binds to CD40 receptors on macrophages and causes further release of the inflammatory cytokines, TNF-α and MMPs.

Early atherogenesis is characterised by the recruitment of leukocytes, production of inflammatory mediators and the accumulation of lipids in the arterial intima to form a lipid-rich core (also known as the fatty streak) [125]. This core expands into the luminal space, and when other cardiovascular risk factors are present, it can continue to grow. Smooth muscle cells proliferate and enlarge in an attempt to sequester the lipid-rich core to form an atheroma. The atheroma and the lipid-rich core are collectively known as a plaque. This plaque continues to grow into the luminal space, but the vessels have a compensatory mechanism by enlarging themselves such that the lumen is not compromised by the plaque [390]. This is known as 'positive remodelling' [391]. However, the compensatory mechanism is only able to remodel the lumen to a certain extent before the artery becomes stiff.

Thickening plaque also disrupts the exchange of nutrients in the cells of the arterial wall and thus leads to arterial wall degeneration in the local area. Fibroblasts invade the damaged area and form a cap of connective tissue consisting mainly of collagen over the plaque [392]. MMPs are secreted by the foam cells, and MMP2 and 9 degrade the fibrous cap to expose the underlying collagen to platelets [393, 394], hence triggering the coagulation cascade to form a thrombus. NO will normally inhibit coagulation, but when there is ED, NO bioavailability is reduced and the inhibition of coagulation is impaired [119].

2.5.2 Cerebral Small Vessel Disease

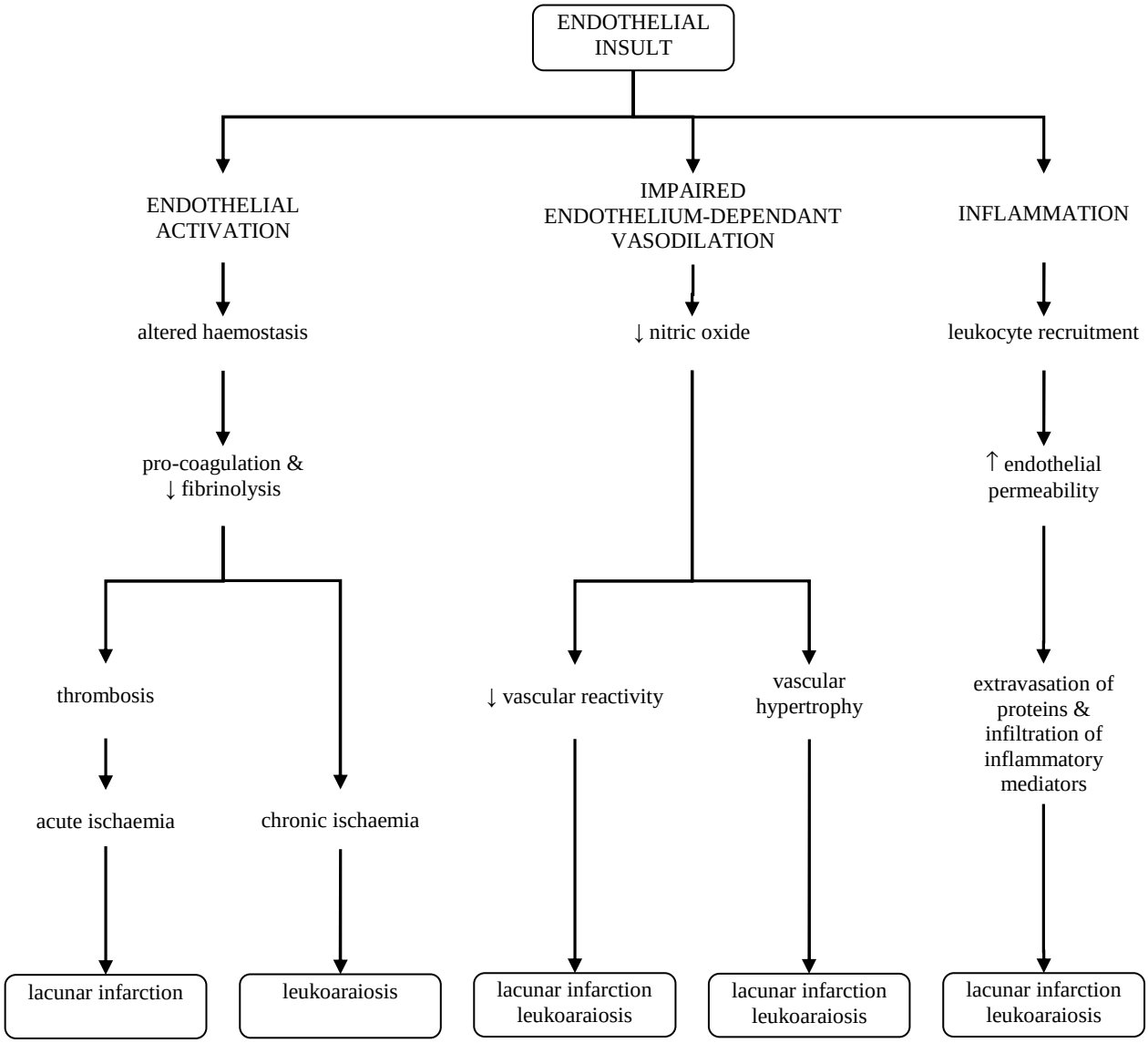
Since the pathophysiologies of LI and LA all involve altered integrity of the cerebral arteries, and a healthy endothelium is important for the proper functioning of the smooth muscle cells of the cerebral small vessels, it is highly likely that the endothelium has a role in genesis and/or progression of SVD. One study has shown a link between ED and cerebral large vessel stroke [226], while ED is a major participant in atherosclerotic lesion formation and progression as described in Chapter 2.5.1. Atherosclerosis is a major contributor to large-vessel stroke. One study has shown that there is an increased release of endothelium-derived adhesion molecules in both patients with cerebrovascular large and small vessel disease, indicating that inflammatory endothelial activation and leukocyte adhesion have similar roles in large and small vessel disease [319].

Although the current literature has much evidence for ED in CarVD and to a lesser extent large-vessel stroke [226, 395], there is growing evidence to suggest an association between ED and SVD.

White et al. showed that NO had a role in cerebral autoregulation, which influences cerebral perfusion, using a nitric oxide synthase inhibitor [396]. Although the inhibitor is a non-specific inhibitor, it was deduced that the main effect was on eNOS rather than nNOS. These results indicate the importance of NO and eNOS activity in maintaining cerebral autoregulation. Since reduced eNOS activity and NO bioavailability is a hallmark of ED, it can be postulated that ED may have a role in the development of cerebral ischaemia and SVD.

Based on the pathophysiology of LA and LI, the proposed pathways of which ED facilitates SVD is shown in **Figure 2.4**. The pathways follow the three hallmarks of ED: endothelial activation (altered haemostasis), impaired endothelium-dependant vasodilation (from decreased nitric oxide) and inflammation (initiated by leukocyte recruitment). Each pathway has a logical role in the pathogenesis of SVD.

Figure 2.4 – Proposed pathways of EF in SVD



Studies involving stroke-prone spontenously hypertensive rats (SP-SHR) have suggested that impaired EF is a factor that predisposes an individual to stroke [397]. Also, knockout mice lacking eNOS, a key enzyme of EF, have been shown to be sensitive to focal ischaemic events [398] while also exhibiting vessel wall abnormalities [303].

Other animal studies have shown that the injection of plasmin, a plasma enzyme, into the brain parenchyma resulted in acute fibroid necrosis (i.e. lipohyalinosis) and increased BBB permeability in the perforating artery [20], while the leakage of plasma in the perforating artery wall initiated the development of lipohyalinosis [21]. A human study has suggested that the blood-brain barrier (or the cerebral endothelium) may be dysfunctional in LI [77]. This was shown using MRI scans before and after contrast enhancement in patients with LI. There was greater post-contrast enhancement in the cerebrospinal fluid of LI patients compared to cortical stroke patients, indicating that the BBB in LI patients was ‘leakier’.

In another human study, cerebral EF in LI patients and healthy controls was determined using L-arginine reactivity and systemic EF was measured using FMD – both L-arginine reactivity and FMD were significantly lower in LI patients compared to healthy controls, as shown in **Table 2.1** [336]. An intermediate group of controls with risk factors was also included in the study. Similar results were observed in other studies [269, 399].

Table 2.1 – Cerebral EF (as indicated by L-arginine reactivity) and systemic EF (as measured using FMD) in LI patients, controls with similar risk factors and healthy controls. A higher percentage value indicates better EF (Data from [336]).

	LI patients	Controls with similar risk factors	Healthy Controls
L-arginine reactivity	13.1 ± 8.4%	13.5 ± 8.3%	21.3 ± 10.9%
FMD	0.06 ± 4.9%	3.1 ± 4.8%	8.1 ± 6.0%

Also using FMD, Stenborg et al. demonstrated poorer endothelium-dependant vasodilation in CADASIL patients [255], while Hoth et al. showed a similar relationship between EF and WMH volume in older adults with CVD [400].

Using another method of assessing EF, Hassan et al. measured markers of endothelial activation and damage to show evidence of an association between cerebrovascular SVD and EF [58]. The markers measured were: soluble ICAM-1, thrombomodulin, tissue factor and tissue factor pathway inhibitor. These markers have previously been found to be elevated in large vessel coronary and CVD [401, 402]. The study by Hassan et al. also showed that patients with LI and LA had a different profile of endothelial markers compared to the patients who also had LI but no LA. Homocysteine levels were also shown to be higher in LA patients when compared to isolated LI patients [29]. However, when adjusting for markers of ED, homocysteine levels between the two groups were not found to be significantly different. This suggests that the effect of the elevated homocysteine levels seen in the patients with LA were mediated via endothelial dysfunction.

Similarly, Wright et al. demonstrated a relationship between specific inflammatory markers and LA [35]. The inflammatory markers lipoprotein-associated phospholipase A2 and myeloperoxidase were associated with greater WMH volume in a stroke-free multiethinic population. Associations between WMH and high-sensitivity C-reactive protein, another inflammatory marker, were not as strong. All three inflammatory markers are known

predictors of vascular risk. Although this study did not investigate EF in SVD directly, an inflammatory response may arise from ED and thus elevated inflammatory markers may provide some indirect insight into the EF of the subject.

2.6 Summary

The endothelium is no longer the inert structure that it was originally believed to be. It has an active role in maintaining vascular health and it has now been shown that the endothelium is vital to the regulation of cerebral vascular tone. The risk factors for SVD are the same as those for ED, which suggest that they may be related. The pathophysiology of SVD can be simply explained as reduced vasoreactivity, inflammation, altered fibrinolysis and thrombus formation, and each of these processes can be explained using the pathways of ED. The evidence between SVD and impaired EF is accumulating, but the relationship is still worthy of further investigation. Many past studies have used FMD to assess EF, but this study will opt to use ApT because it is non-invasive, cheaper, reproducible and will require less operator training. ApT will also use the radial artery in preference to the larger brachial arterty. This study will aim to explore the relationship between EF and SVD using ApT, as well as the effect of candidate polymorphisms on EF.

CHAPTER 3 GENETIC POLYMORPHISMS

3.1 Selection of Candidate Genetic Polymorphisms

The investigations involving polymorphisms can be performed either using the candidate gene approach or a genome-wide association study. The candidate gene approach involves identifying specific polymorphisms within genes of interest and then analysing the genotypes with respect to the study endpoint. Polymorphisms are usually chosen based on biological plausibility, meaning that the polymorphism should have a plausible pathway in which the clinical effect is elicited. The advantage of the candidate gene approach compared to genome-wide association studies is that the sample sizes required are usually smaller. Since it was unlikely that the population of this study would reach the sample sizes required for genome-wide association studies, the candidate gene approach was preferred for this study. Also due to logistical issues and availability of equipment, it was decided that the candidate gene approach would also be the most cost effective.

As there is a plethora of genetic polymorphisms associated with EF and SVD, the decision to select specific polymorphisms was determined using the following criteria:

1. The SNPs selected must have biological plausibility. Ideally, there should be reasonable grounds on which a hypothesis can be generated involving the SNP and a biological outcome. For example, a SNP that alters the binding properties of transcription factors may result in the decreased expression of a gene that encodes an enzyme involved in maintaining a healthy state. This SNP can then be hypothesised to promote illness, and is biologically plausible.
2. The equipment available for genotyping was a 96-well polymerase chain reaction (PCR) thermal cycling machine. After considering both the logistical and economical factors, it was decided that assessing eight polymorphisms would be feasible, thus allowing six patients to be analysed per plate.
3. The genomic sequence of the SNP is required to design oligonucleotide primers. Thus, only the SNPs with known genomic sequences (accessed via Entrez SNP Database, http://www.ncbi.nlm.nih.gov/SNP) were considered.
4. The background frequency of the polymorphism in a Caucasian population is high enough to detect a significant association in the population size expected in this study.

Therefore, based on the above criteria, the following polymorphisms were selected: Interleukin (IL)-6 -174 G/C, NADPH oxidase p22 phox 242 C/T, tissue plasminogen activator

(tPA) -7351 C/T, -4360 G/C, 20324 C/T, endothelial nitric oxide synthase (eNOS) -786 T/C, endothelin (ET)-1 138A deletion (D)/insertion (I) and paraoxonase 1 (PON1) -107 C/T.

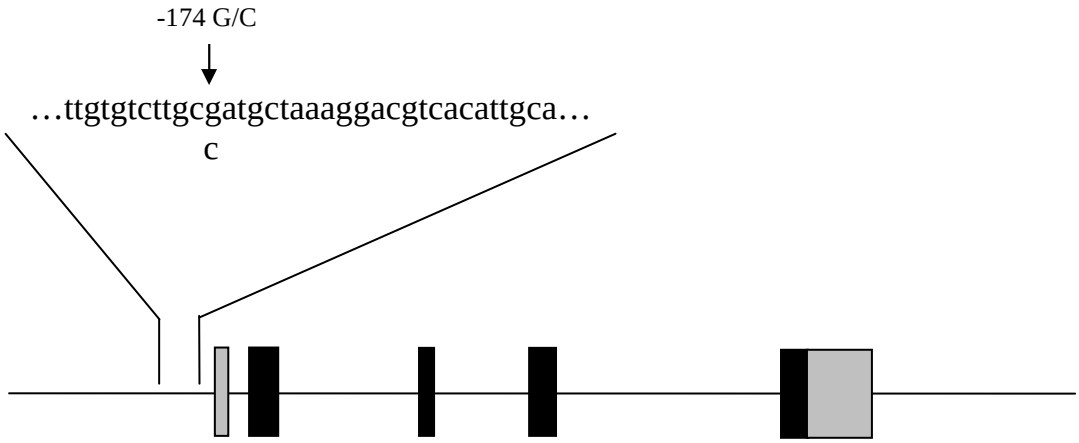
3.2 Interleukin-6 (IL-6) -174G/C

Interleukins are a group of multifunctional cytokines often involved in inflammatory processes. IL-6 is derived from fibroblasts, macrophages and other cells that increase the synthesis and secretion of immunoglobulins by B-lymphocytes. Plasma and cerebrospinal fluid levels of IL-6 have been correlated with infarct size and prognosis following stroke [403-405].

Research by Saura et al. has shown that IL-6 has a direct effect upon endothelial cells by inhibiting eNOS expression through signal transducer and transactivator-3 (Stat3) [406]. Stat3 is phosphorylated, and thus activated upon IL-6 binding to the IL-6 receptor. The data shows that there are decreased steady-state levels of human eNOS mRNA and protein in human aortic endothelial cells, partly caused by IL-6 inhibition of transactivation of the human eNOS promoter. Binding sites for Stat3 were found in the eNOS promoter region at positions -1520, -1024, -840 and -540 and it was found that Stat3 binding at -1024 mediated inhibition of eNOS promoter activity.

The IL-6 gene is located on chromosome 7 (7p21) with an approximate length of 5kb. The gene contains 5 exons and 4 introns. The -174 G/C polymorphism was found to be in the negative regulatory domain (**Figure 3.1**) [407] in the IL-6 gene [408, 409]. There is a glucocorticoid receptor binding region at approximately -201 and because of the close proximity between this binding region and the polymorphism of interest, there is potential that the -174 G/C polymorphism can interfere with glucocorticoid receptor binding.

Figure 3.1 – Gene structure of IL-6. Exons are indicated by the boxes and the coding regions are indicated in black. The sequence neighbouring the -174 G/C polymorphism is also indicated (adapted from [408] and [410]).



The G to C change in the -174 position creates a potential binding site for nuclear factor-1 (NF-1), a transcription factor [408]. NF-1 has been shown to be a repressor of gene expression in HeLa cells [411]. Experiments by Fishman et al. showed that upon stimulation by lipopolysaccheride and interleukin-1, expression from the G construct showed a 2.35±0.10-fold and 3.60±0.26-fold increase ($p<0.001$ for both), respectively, compared to an unstimulated construct [408]. Stimulated expression of the C construct did not vary much from the unstimulated construct. These results indicate that the reporter gene expression from the C allele is repressed in HeLa cells.

The G allele is also associated with higher IL-6 plasma levels in normal individuals, as is expected with in vitro data [408, 412, 413]. However, other studies have reported either no difference in IL-6 plasma levels between GG and CC carriers [414], or that CC carriers have higher IL-6 plasma levels than GG carriers [415].

Brull et al. have shown previously that there is a trend between greater FMD in healthy volunteers with the CC genotype compared with those with the GG or GC gentoypes, suggesting that CC carriers had better EF compared to GG or GC carriers [416]. However, this trend was not found to be significant ($p=0.14$).

Ridker et al. had previously shown that IL-6 plasma concentrations in apparently healthy men were independently associated with increased coronary risk [417]. For CVD, Revilla et al. found that the CC genotype was significantly higher in patients with LI compared to healthy controls [418], and these results were replicated in a later study by Chamorro et al. [419]. The latter

study showed an OR of 3.22 (95% CI 1.12 – 9.09, p=0.03) for LI in patients with the CC genotype.

IL-6 is strongly involved in the regulation of C-reactive protein (CRP) production [420]. CRP is an acute phase reactant and a marker for systemic inflammation. IL-6 has been reported to increase CRP gene expression in adult hepatocytes and adult hepatoma cell lines [421-423]. Specifically, the -174 G/C has been associated with increased plasma IL-6 and CRP levels [424, 425]. CRP itself has also been shown to decrease eNOS expression and bioactivity by destabilising eNOS mRNA [426, 427]. Thus, the SNP that results in less IL-6 production would also affect CRP levels, thereby improving EF by reducing the inhibition of eNOS (via Stat3) and down-regulating CRP.

3.3 NADH/NADPH-oxidase (N-Ox) p22 phox 242 C/T

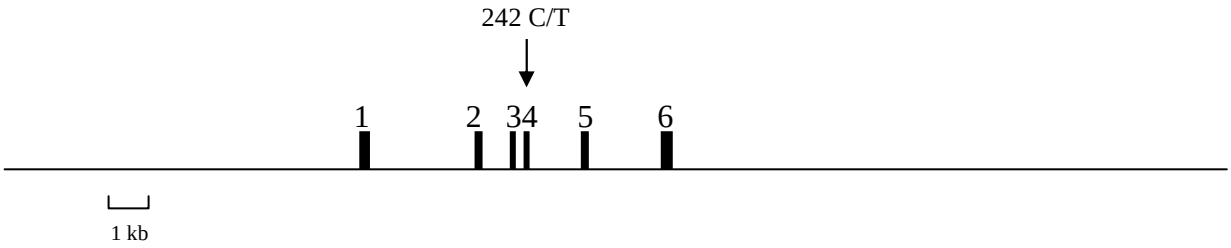
The NADH/NAPDH-oxidase (N-Ox) system is a source of superoxide, a contributor to ED by reducing NO bioavailability [126]. Stimulants of endothelial N-Ox activity include AngII [356, 357], ET-1 [428], TNF-α [429, 430], increased glucose [351], insulin [352], AGEs [341] and oxidised lipids [326]. Some studies hypothesise that N-Ox may be the main source of superoxide [203]. The inhibition of N-Ox has been shown to improve EF in both animals and humans by increasing NO bioavailability [358].

N-Ox has different isoforms, each differing in the catalytic subunit required [431]. The Nox1 and Nox2 isoforms of N-Ox consist of a flavocytochrome (cytochrome b_{558}) consisting of two subunits, p22 phox and gp91 phox, and four cystolic proteins (p47 phox, p67 phox, p40 phox and GTPase Rac2) [432-434]. Cytochrome b_{558} is an essential component of N-Ox with regards to enzymatic activity and stability, and it is postulated that this cytochrome is the last redox carrier in the transfer of electrons from NADPH to oxygen. However, study results have suggested that the Nox4 isoform only requires p22 phox [435].

Although p22 phox and gp91 phox have a close physical interaction, each subunit has mRNA that is independently regulated. p22 phox, also known as the α-light chain, is crucial for superoxide production in vascular cells. The p22 phox gene is located on chromosome 16 (16q24). It consists of 6 exons and 5 introns, and the entire gene spans 8.5 kb (**Figure 3.2**) [434]. Exon 6 encodes for approximately 35% of the 195 amino acid-protein. The 242 C/T SNP, found in exon 4, results in a histidine to tyrosine change in position 72 of the protein and this change is located in a potential heme-binding site. Cytochrome b_{558} contains two non-

indentical hemes that transfer electrons to oxygen, each with a different redox potential. Heme groups are predicted to exist bound to histidine residuals within the membrane. gp91 phox has several histidine residuals, whereas p22 phox only has the single histidine residual at codon 94 [436]. The 242 C/T SNP alters the histidine residual in p22 phox, thus potentially affecting heme binding and electron transfer.

Figure 3.2 – Gene structure of N-Ox p22 phox (adapted from [434]). The 242 C/T polymorphism in exon 4 is also indicated.



According to the hypothetical structure of cytochrome b_{558} proposed by Quinn in 1992, at least one heme group would reside in the gp91 phox subunit and another one would be shared between gp91 phox and p22 phox [437]. More recent research has proposed that both the hemes are located within gp91 phox, and thus the histidine in p22 phox would not be required for electron transfer or heme binding, but would affect the stability of the entire subunit [438].

In endothelial cells, it is thought that the Nox2 and Nox4 isoforms of N-Ox are most important in generating ROS [439, 440]. In the rat basilar endothelial cells, it was found that mRNA was expressed for Nox1, Nox2 and Nox4 [441]. Although there is limited evidence regarding the cerebral N-Ox isoform expression profile, Nox1, Nox2 and Nox4 are believed to be involved in ROS generation in the cerebrovascular cells [431]. Endothelial cells appear to have a greater expression of Nox4 compared to the other isoforms [440]. The difference between neutrophilic N-Ox and endothelial N-Ox is that endothelial N-Ox has a continuous production of relatively small amounts of superoxide. Production can be increased but to a lesser degree compared to neutrophilic N-Ox [204]. Another difference is that endothelial N-Ox predominantly generates intracellular superoxide, whereas neutrophilic N-Ox superoxide generation is extracellular.

The activity and expression of N-Ox is 10-100 times higher in cerebral arteries compared to systemic arteries [139]. Superoxide is generated by Nox1, 2 and 4, and is metabolised by SOD to form H_2O_2. H_2O_2 can also be generated directly by Nox4 and other vascular oxidases such as xanthine oxidase [435, 442, 443]. H_2O_2 is a potent vasodilator in the cerebral vasculature and this

has been shown in both in vitro and in vivo situations [139, 209, 444, 445]. Low concentrations of H_2O_2 have been shown to elicit cerebrovasodilation, but higher concentrations (>1mM) have been shown to elicit vasoconstriction first before following with vasodilation [431]. AngII-induced vasoconstriction also appears to be selectively less in cerebral arteries because of the endogenous H_2O_2 [139]. It will be interesting to see whether there will be any differences in EF or SVD risk resulting from the polymorphism, especially with the opposing effects of SOD and H_2O_2 in the cerebral vasculature.

Initial experiments on human saphenous veins and mammary arteries showed that the T allele was associated with lower levels of basal NADH-stimulated superoxide production compared to the C allele [446]. Another study showed that TT carriers had approximately 30% of the superoxide production of the carriers of the wild-type [447]. Similarly, another study showed that the T allele was associated with reduced oxidative stress [448]. Although it was not measured directly, the result suggested that carriers of the T allele have better EF than carriers of the C allele.

From these results, it could be hypothesised that if the TT carriers have less superoxide production, then they would also have better EF, due to the detrimental effect of superoxide on EF. The results of a study by Schachinger et al. agreed with this hypothesis, showing that the 242 C/T polymorphism of the N-Ox p22 phox gene was an independent determinant of coronary EF [449]. In a group of 93 patients where 12% had the TT genotype (CC 47%, CT 41%), it was found that carriers of the CC genotype had blunted endothelial vasodilatory responses compared to TT carriers.

Ito et al. found an association between the T allele and CVD in a Japanese population [450]. Patients with ischemic CVD had a higher frequency of the T allele compared to controls. The odds ratio for the TC + TT genotype versus the CC genotype was found to be highest for atherothrombotic stroke at 2.22 (95% CI 1.15 – 2.86), while the same odds ratio for LI was 1.71 (95% CI 1.01 – 2.88). Shimo-Nakanishi et al. and Kuroda et al. did not find any association between LI and the T allele or the TT genotype in a Japanese population [451, 452], although Kuroda et al. had results that suggested that the 242 C/T polymorphism was protective against cardioemobolic stroke.

In a study specifically focusing on SVD in a Caucasian population, Khan et al. found there was no significant association between SVD and the 242 C/T genotype when selecting SVD patients based on the TOAST criteria [453]. There was also no association between isolated LI

(defined as LI with absent/mild LA using MRI) or ischaemic LA (defined as LI with moderate/severe LA) and the SNP. The results of this study are shown in **Table 3.1**. None of their controls were imaged.

Table 3.1 – Effect of the N-Ox p22 phox 242 C/T polymorphism on SVD (Data from [453])

NOTE:
This table is included on page 57
of the print copy of the thesis held in
the University of Adelaide Library.

The issue with the TOAST classification is that the definition of SVD is based on the clinical presention of a lacunar syndrome without cortical or cerebellar dysfunction with a subcortical or brain stem infarct less than 1.5cm [38]. Thus this definition tends to only include the larger LI caused by occlusion (which tend to be more symptomatic), while overlooking the smaller LI caused by arteriolosclerosis and lipohyalinosis (which can be asymptomatic) [14]. It also does not include LA.

This deficiency in the TOAST classification may be the reason why the isolated LI and ischaemic LA definitions were also included in the study and separate analyses were performed in this group. Perhaps the only subgroup lacking is the LA only group, where there may be LA of ischaemic nature without LI. Our study intends to include the LA only subgroup and to investigate its relationship with this SNP.

3.4 Tissue Plasminogen Activator (tPA)

Tissue plasminogen activator (tPA) belongs to the family of serine proteases and is a promoter of fibrinolysis. It cleaves plasminogen into plasmin, which then acts to dissolve the fibrin network in the blood clot. Occlusive thrombi arising as a complication from atherosclerotic plaque, contribute to both large vessel and small vessel strokes. When the atherosclerotic plaque is ruptured, collagen is exposed, thus triggering the coagulation cascade. The degree of thrombosis is determined by the balance of the pro-thrombotic coagulation cascade and the endogenous fibrinolytic system. The importance of the role of tPA in thrombolysis has been highlighted in studies involving tPA-deficient mice [454], while other studies have found associations between plasma tPA levels and MI or stroke [455-457]. Animal brain studies show that tPA expression is limited to the endothelial cells of the brain

capillaries with no expression of tPA in the larger arteries under normal circumstances [458-460], highlighting the importance of tPA in the cerebral small vessels.

tPA is synthesised as a single-chain glycoprotein consisting of 530 amino acids and is activated by plasmin, trypsin or Factor Xa by cleaving arginine and isoleucine (at positions 278 and 279, respectively) into two chains (heavy and light) linked by a disulfide bond [461, 462]. The active site of tPA is located in the carboxyl terminus light chain. As circulating tPA is derived from the endothelium, it can be hypothesised that ED may alter the secretion of tPA into the circulation, therefore encouraging the balance to favour thrombosis. A previous study has shown that the release of endogenous tPA by the endothelium is the most important mechanism in dissolving an arterial thrombus [463].

The tPA gene can be found on chromosome 8, localised to 8p12. The gene spans 32 kilobase pairs, consisting of 14 exons and 13 introns [461]. Exons range from 43 to 914 base pairs, while introns are 111 to 14,257 base pairs in size. Regulation of this gene is mainly at the transcription level, with two transcription sites in the promoter region dependant on an initiation element and a TATA box [461, 464]. Two elements within the proximal promoter have been identified as being responsible for constitutive expression and induction by the protein kinase C pathway [465].

3.4.1 tPA -7351 C/T

The -7351 C/T SNP can be found in the enhancer region of the gene and influences the regulation of gene expression. The T allele has been shown to influence Sp1 binding and also has been associated with a reduced tPA release compared to those with the homozygous C allele. It was found that CC subjects had a two-fold higher tPA release rate compared to CT or TT subjects [466], however another paper by the same authors concluded that genetic variation in the tPA gene was not a strong predictor of plasma tPA levels [467].

A study in 2006 incorporating four tPA polymorphisms (*Alu*-repeat insertion/deletion, -7351 C/T, 20099 T/C and 27445 T/A) hypothesised that the polymorphisms would alter vasomotor and fibrinolytic function in patients with coronary heart disease to cause athero-thrombotic events [468]. The results showed that there were no differences in basal or net tPA antigen concentrations between genotypes, indicating that these four polymorphisms were unlikely affect acute tPA release in patients with existing coronary heart disease. The TT genotype has been previously shown to influence risk of MI in another study [469].

An Adelaide-based study was the first to identify an association between this polymorphism and LI (OR 2.7, 95% CI 1.1 – 6.7) [107]. However, these results were not replicated in two other independent studies by Jood et al. [108] and Armstrong et al. [109], who both found non-significant OR of 1.05 and 1.34, respectively. The original significant association was identified in an ischaemic stroke population after stratifying for stroke subtypes, and the LI subtype was identified using non-radiological methods (OCSP criteria). Jood et al. also used a non-radiological method (TOAST criteria) in determining stroke subtype, while Armstrong et al. defined LI primarily based on presentation with a lacunar syndrome with an accompanying radiologically defined lesion. The common factor between the three studies is that the classification of LI is based on the presentation of lacunar syndromes. Jannes et al. and Jood et al. also do not include LA in their studies due to the limitations of the classification criteria. The proposed study intends to use MRI only to identify participants with SVD, which will therefore include participants with non-symptomatic LI as well as paticipants with LA with and without LI.

Since endogenous tPA release from the endothelium is the most important mechanism in dissolving an aterial thrombus and the important role of tPA in brain capillaries, further investigations of this polymorphism in a dedicated SVD population is warranted.

3.4.2 tPA -4360 G/C, 20324 C/T

Another study has shown that a particular combination of SNPs contributed to the risk of ischaemic stroke in a Japanese population [470]. However, results were not stratified into stroke subtypes. It was found that G-T haplotype at -4360 G/C (rs7007329) and 20324 C/T (rs8178750) had a significant odds ratio of 11.4. The 95% confidence interval ranged from 1.32 to 97.9. The other three SNPs investigated were: +15827 C/A (rs732612), +27271 T/A (rs2020922) and +624 G/A (rs4471024). All five SNPs were intronic SNPs and as such, the authors did not believe that these would be functional. It was inferred that the G-T haplotype of -4360 G/C and 20324 C/T was not functional, but was linked to another functional mutation with a physiological role in the development of ischaemic stroke.

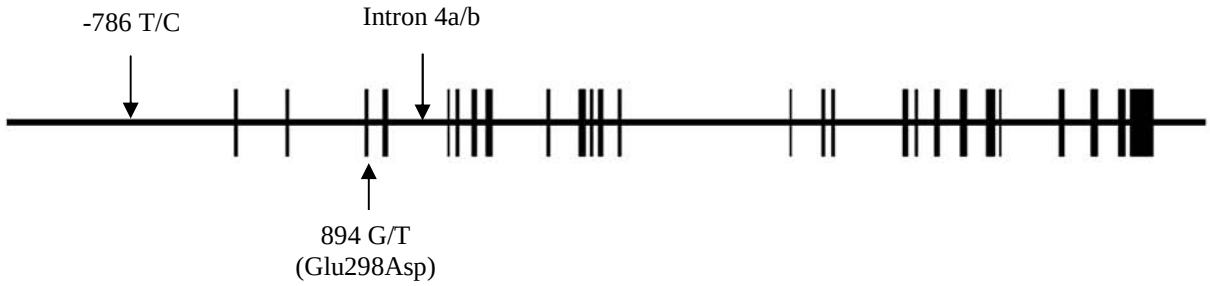
The functional significance of these two SNPs is unknown and the biological plausibility is undetermined. Therefore according to our selection criteria, both these SNPs should be excluded from investigation in this study. However, for the following reasons, these two tPA SNPs have been included in the study:

1. Due to the previous finding of the involvement of tPA -7351 C/T in SVD (although later studies have conflicting results), other tPA SNPs may also have a role in SVD;
2. To determine whether the result published by Saito et al. could be replicated in a SVD population;
3. There is very little information regarding either of these two SNPs in the literature, thus any finding made in this study could contribute to the overall knowledge of these SNPs;
4. To determine the SNP freqencies in an Australian population.

3.5 Endothelial Nitric Oxide Synthase (eNOS)

The eNOS gene is found on human chromosome 7 and has been localised to 7q36 [471]. The gene contains 26 exons (**Figure 3.3**), spanning 4.4 kb, which encodes mRNA to produce a 135-kD protein of 1,203 amino acids [472]. The eNOS gene appears to lack the typical TATA box, but there are several *cis*-regulatory DNA sequences such as a CCAT box, Sp1, GATA motifs, CACCC boxes, AP-1 and AP-2 sites, a p53 binding region, NF-1 elements and acute phase reactant regulatory elements [472]. Three clinically relevant polymorphisms in eNOS have been identified previously: -786 T/C, 894 G/T and intron 4 a/b [283, 284, 291].

Figure 3.3 – Structure of the eNOS gene with the clinically relevant polymorphisms indicated. Exons are indicated by the vertical black boxes (adapted from [472]).



3.5.1 eNOS -786 T/C

Apart from the 894 G/T, the other two clinically relevant eNOS polymorphisms are -786 T/C in the promoter region of the gene [283, 284] and 27-base pair repeats in intron 4 (intron 4 a/b).

The -786 T/C SNP has been associated with coronary spasms in the Japanese population (OR 5.2, $p<0.001$) [285], although associations between this SNP and coronary artery disease (CAD) in Caucasians have been less consistent [286, 287, 473]. The CC genotype has been found to be

more common in Caucasians than in Japanese (17.7% and 2%, respectively) [285, 474]. Likewise, associations between the 27-base pair repeats in intron 4 and smoking-dependant CAD are inconsistent [287-290].

As the -786 T/C mutation is in the promoter region, it was reported that the C allele reduced eNOS gene transcription by 50±11% in HUVEC [285]. Another study has demonstrated differences in a nuclear protein binding to the T and C alleles [475]. Miyamoto et al. purified a protein that was identical to replication protein A1 (RPA1), and this purified protein bound specifically to the mutant allele in HeLA cells [476]. RPA1 is a DNA binding protein essential for DNA repair, replication and recombination. It was found that transcription of the eNOS gene was restored when the RPA1 was inhibited by anti-sense oligonucleotides. Over-expression of RPA1 further inhibited gene transcription. The authors concluded from these results that RPA1 appears to be a repressor protein in the presence of the -786 T/C mutation.

The -786 T/C and intron 4 a/b are have been shown to be in linkage disequilibrium with a coefficient of 0.86 [477, 478]. A review of genetic studies involving the 894 G/T and -786 T/C shows that there is a significant linkage disequilibrium between these two polymorphisms in Caucasian populations [474, 478, 479], although another study also in a Caucasian population has shown that the linkage coefficient is less than 0.5 [477].

The majority of studies investigate the association between the eNOS polymorphisms and CAD. Few studies have investigated these polymorphisms in cerebrovascular disease (CVD) and even fewer have included SVD. One of these studies was by Hassan et al. [477] who showed that the -786 T/C did not independently alter SVD risk when measuring plasma NO levels. The current literature is lacking in studies that investigate SVD directly with this polymorphism.

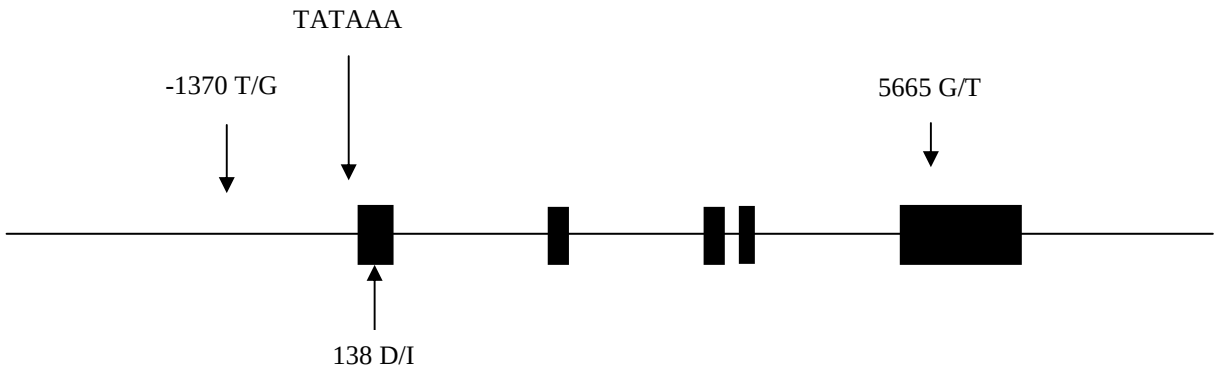
3.6 Endothelin-1 (ET-1)

ET-1 is a potent endogenous vasoconstrictor. The entire ET-1 gene is 6836 base pairs in length. It is located at 6p24 and consists of 5 exons encoding for a 2026-nucleotide mRNA [480]. Exon 2 (amino acid 53 to 73 of preproendothelin) encodes the complete sequence for ET-1 [481, 482]. The preproendothelin peptide must be further cleaved by furin covertase to Big ET_{1-38} [483, 484] and again by endothelin-converting enzyme (ECE)-1 and ECE-2 to form $ET\text{-}1_{1-21}$ [485-487]. A second product, $ET\text{-}1_{1-31}$, is cleaved from Big ET_{1-38} by chymase [488].

Transcription of this gene is regulated via phorbol-ester-sensitive *c-fos* and *c-jun* complexes [183], nuclear factor-1 [489], AP-1, GATA-2 [490] and acute phase reactant regulatory elements [480].

The most commonly investigated SNP in the ET-1 gene is the 5665 G/T, which causes a lysine (K) to asparagine (N) amino acid change in the primary protein structure. Hence it is otherwise known as ET-1 K198N. The mechanism of how the 5665 G/T SNP influences clinical presentation is unknown. Since the SNP is located in exon 5, a non-regulatory region of the gene, it is unlikely to influence gene expression directly. However, data shows that the SNP is in linkage disequilibrium with another SNP, -1370 T/G, located in the promoter region of the gene (**Figure 3.4**). Linkage disequilibrium data for K198N with other identified SNPs can be found at http://genecanvas.idf.inserm.fr./

Figure 3.4 – The gene structure of preproendothelin, showing the relative positions of the -1370 T/G, 138 D/I and 5665 G/T polymorphisms (Adapted from [480])



The 5665 G/T SNP in the ET-1 gene has been found to be associated with higher blood pressure levels in overweight individuals [305]. It is also associated with potentiating the effects of ET-1 and AngII by increasing calcium sensitivity [306]. It could be hypothesised that because the polymorphism potentiates ET-1, the polymorphism encourages vasoconstriction, smooth muscle proliferation and inflammation via ICAM-1 and VCAM-1 up-regulation ultimately resulting in ED. Although there is a hypothetical link between ED and this SNP, due to the unknown functional significance and the lack of evidence for biological plausibility, this SNP was excluded from further investigation in this study.

3.6.1 ETN-1 +138A D/I

This deletion (D)/insertion (I) polymorphism is located in exon 1. The wild-type variant is the DD, with an allele frequency of 0.72 [491]. In vitro assays using the luciferase reporter gene reported that the 4A (insertion) allele showed increased ET-1 expression when compared to

the 3A (deletion) allele. Assays in HUVECs from 4A homozygotes also showed a significant increase in ET-1 protein expression due to increased mRNA stability [491]. Another study showed significantly higher concentrations of ET-1 were found in hypertensive Japanese subjects with the 4A allele (insertion), compared to 3A (deletion) homozygotes [492]. It is possible that this adenine insertion could affect the secondary structure and stability of mRNA, translation initiation efficacy or the binding of sequence specific mRNA binding proteins [491].

The 3A/4A heterozygote in conjunction with another heterozygote allele (8002 G/A) has been shown to be associated with higher Big ET-1 levels in subjects with congestive heart failure [493].

An extensive literature search has revealed that there have been no studies that have investigated this SNP directly in association with EF. There is also very little information regarding the role of this SNP in SVD. Any findings regarding this SNP in this study will be novel. We hypothesise that this polymorphism will lead to poorer EF due to increased vasoconstriction, and likewise for SVD.

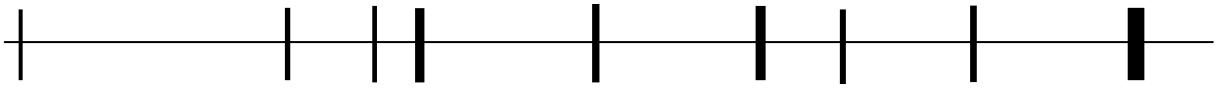
3.7 Paraoxonase (PON1)

Oxidised LDLs are implicated in atherogenesis, as well as the development of ED. Oxidised LDL induces the release of chemoattractant molecules that encourage monoctyes to diapedese between endothelial cells [125]. Monocytes then express scavenger receptors on the cell surface for oxidised LDL, such as LOX-1, so that the monocyte can internalise the LDL [388]. Inflammatory cytokines are secreted from these cells, encouraging local inflammation and the atherosclerotic process.

The oxidation of LDL is limited by high-density lipoproteins (HDL). PON1 is an enzyme produced by the liver and is bound exclusively to HDL. PON1 hydrolyses sub-endothelial lipid peroxides before it can accumulate in LDL particles [494]. Shih et al. showed that PON1 knockout mice were more susceptible to diet-induced atherosclerosis because the HDL had lost its protective effect on the oxidation of LDL [495].

The PON1 gene is located at 7q21-q22 [496]. The entire gene spans approximately 27kb, with a coding sequence consisting of nine exons [497] (**Figure 3.5**).

Figure 3.5 – Gene structure of PON1 (adapted from [497]). Only the exons and introns are shown. The promoter region is not shown here. The size of exons 1 and 9 are unknown.



A commonly investigated PON1 polymorphism is the Q192R. This polymorphism causes a change from glutamine to arginine in position 192 in the primary protein structure, and it has a significant effect on the activity of PON1 [498]. The Q allele has previously been found to be associated with greater ED than the R allele [499], and similar results were seen in another study in people with peripheral artery disease [500]. The latter study demonstrated a direct relationship between PON1 activity and brachial FMD ($r=0.46$, $p=0.004$), and they also showed higher PON1 activity in carriers of the R allele when compared to the Q allele ($p<0.001$).

3.7.1 PON1 -107 C/T

Levels of PON1 appear to be constitutive, rather than inducible, indicating that the different levels of PON1 activity seen in various individuals may be pre-determined by genetic factors. Many polymorphisms have been identified in both the regulatory and coding regions. Leviev et al. in 2000 found that the -107C variant had approximately twice the transcriptional activity than the -107T variant ($p<0.05$) [501]. Conclusions from this study were that the -107 C/T was the main contributor to PON1 concentrations, as this SNP accounted for 24.7% of the variance seen in serum PON1 levels. Similar results were seen in another study by Brophy et al. [502], where it was shown that the -107 C/T and M54L SNPs accounted for 22.8 and 5% of the variance in PON1 activity, respectively.

The -107 C/T SNP occurs in the Sp1 transcription factor binding site, thus potentially altering transcription of the gene [501]. Sp1 binding affinity changes depending on the allele present, with the C allele showing better binding affinity [503]. Thus it is hypothesised that those with the C allele would have more PON1, leading to increased hydrolysis of lipid peroxides and improved EF.

3.8 Haplotype Studies

SNPs can be useful genetic markers, but single SNPs may not provide an accurate indication of the full potential of the SNP on biological outcomes as the development of disease may not

be totally reliant upon a single SNP. However, SNPs in combinations may provide a truer picture as it shows both the effect of the single SNPs as well as the effect of the interaction of the SNPs. For example, in the study by Saito et al., there were no significant differences between the 5 SNPs in cases and controls [470]. However, when the authors performed diplotype analyses, it was found that the tPA -4360 G / 20324 T haplotype was highly significant (p=0.013).

Studies are now tending towards the use of Genome Wide Association Studies (GWAS), where the decrease in cost had made investigations using this technique more cost-effective. These GWAS have the capability of analysing many thousands of SNPs in a single gene simultaneously and also have the advantage of being able to use the results in multiple SNP analyses, or haplotype analyses.

3.9 Sample size calculations

For genotyping, the frequencies and estimated sample size for the candidate polymorphisms are listed in **Table 3.2**. All calculations were performed using the Sample Size and Power Determination calculator included in the STATA Statistical Data Analysis 9.2 software package (StataCorp, USA). Estimated sample sizes were determined using the frequency of the polymorphism in healthy populations found in previous studies. All estimates had a confidence level of 95%, power of 80% and a minimum detectable relative risk of 2. Since it was estimated that our study population would have greater number of controls compared to cases, a second calculation was performed assuming a 2:1 ratio of controls to cases with the same confidence level, power and minimum relative risk.

Table 3.2 – Sample size estimates for candidate polymorphisms based on the genotype frequency in healthy Caucasian populations (unless indicated otherwise). All estimates have been verified by a statistician.

Candidate polymorphism	Frequency of polymorphism	Estimated sample size			Reference
		Cases	Controls	Total	
IL-6 -174 G/C					
CC	18%	106	106	212	[408]
		77	154	231	
N-Ox p22 phox 242 C/T					
TT	9.4%	235	235	470	[504]
		170	340	510	
tPA -7351 C/T					
TT	9%	247	247	494	[107]
		179	357	536	
TT	12%	176	176	352	[466]
		128	255	383	
tPA -4360 G/C					
CC	1%	2515	2515	5030	[505]
		1805	3610	5415	
tPA 20324 C/T					
CT	4% (Japanese)	602	602	1204	[470]
		433	865	1298	
TT	1.7%	1464	1464	1928	[506]
		1052	2103	3155	
eNOS -786 T/C					
CC	16%	123	123	246	[477]
		90	179	269	
CC	14.5%	140	140	280	[478]
		102	203	305	
ET-1 +138 D/I					
Ins/Ins	7%	328	328	656	[491]
		237	473	710	
Ins/Ins	8.8%	254	254	508	[507]
		183	366	549	
PON1 -107 C/T					
TT	32%	44	44	88	[501]
		32	64	96	
TT	21.2%	83	83	166	[508]
		61	121	182	

3.10 Summary

The candidate polymorphisms chosen have plausible involvement in either EF or SVD based on the possible effects they may have biologically. The aim of this study is to determine whether the candidate polymorphisms will affect EF or SVD as hypothesised.

CHAPTER 4 STUDY AIMS AND RATIONALE

4.1 Study Aims & Hypotheses

This study aims to explore the relationship between EF and SVD, and to examine the role of candidate polymorphisms.

The hypotheses for the study were:

1. SVD is associated with altered EF. Those with SVD will exhibit lower values of global EF compared to those without SVD;
2. EF is genetically determined. Different genotypes will have varying values of global EF;
3. SVD is genetically determined. Cases and controls will exhibit different genotype distributions.

4.2 Study Design

A retrospective case-control study was used to address the study aims.

4.3 Justification of Study

SVD is not often fatal, but it does have a large impact on quality of life. Twin studies have shown high hereditability rates for SVD, and previous studies have shown the importance of certain genotypes. The pathophysiology is well defined for LI. It is known that arteriolosclerosis and lipohyalinosis are the main causes of LI, but the trigger for these causes is not yet clear. What is causing the cerebral small vessels to lose their normal structure and function?

Based on the pathophysiology of SVD, it can be hypothesised that ED may have a role in triggering the onset of disease. The proper functionality of blood vessels is important in preventing SVD and because an endothelium that is functioning normally is vital for the smooth muscle cells of the cerebral small vessels [85], we can infer that a damaged endothelium is involved in the development of SVD. Animal studies have supported this hypothesis [20, 21] and likewise in a recent human study [77]. EF has already been shown to be impaired in LI patients [336], as well in older patients with LA [400]. Since EF is affected by the traditional cardio- & cerebrovascular risk factors, EF may be an indicator of the risk of SVD i.e. the

indicator that assumes the effects of all the individual risk factors to determine an overall risk of disease in certain CVD phenotypes.

Previous studies have already identified EF to be an indicator of cardiovascular health, and limited studies have shown the same effect for cerebrovascular health [58, 226, 269]. Studies have focused on patients with LI and LI with LA, but very few studies have investigated LA without LI. The method described in Hassan et al. uses markers of endothelial activation, but the disadvantage is that some of these markers can be elevated in other inflammatory conditions. Pretnar-Oblak et al. [269] and Chen et al. [399] use FMD, which is used commonly to measure EF and is less likely to be affected by other inflammatory conditions. Both found associations between EF and LI, but both have similar flaws that potentially bias their results. There is the argument that FMD investigates a large artery (the brachial artery) which may not be relevant to a small vessel disease. ApT uses the radial artery, which is a smaller artery compared to the brachial artery and Lind et al. have made the suggestion that ApT may be a better alternative to FMD when an acetylcholine infusion is unsuitable [260]. ApT has been reported to be a valid, repeatable method of measuring EF in a clinical situation [254, 256]. This study aims to use ApT to measure EF. There are currently no studies that have investigated the role of EF in SVD, especially LA, using ApT in an Australian population. There are also no studies that have used the modified Fazekas visual rating scale [58] in conjunction with ApT and EF. We believe that the expected sample of approximately 300 participants will be sufficient to achieve the minimum sample sizes required for statistical significance at 0.05 and a power of 80%.

SVD is a disease that progresses very slowly, with the effects only becoming clinically evident after many years. SVD is irreversible and once effects are clinically evident, treatment is extremely limited. Thus, for SVD, prevention is paramount. The advantage of using EF as a risk indicator is that detrimental changes in EF are detectable before factors are clinically evident. By combining a relatively inexpensive and easy method of assessing EF with genetic screening for associated SNPs, people at elevated risk of SVD can be identified and preventative measures, such as pharmacological interventions, can be implemented earlier in the disease process, thus reducing the burden of disease on the individual, their family and the Australian community.

CHAPTER 5 RESEARCH METHODS

5.1 Clinical Methods

5.1.1 Study Design

The research aims were addressed using a case-control study.

5.1.2 Participating Hospitals

All participants were recruited from patients who had undergone an MRI brain within the recruitment period at any of the four major public hospitals within metropolitan Adelaide, South Australia: The Queen Elizabeth Hospital (TQEH), Lyell McEwin Health Service (LMHS), the Royal Adelaide Hospital (RAH) and Flinders Medical Centre (FMC). This study has been approved by the following clinical research ethics committees:

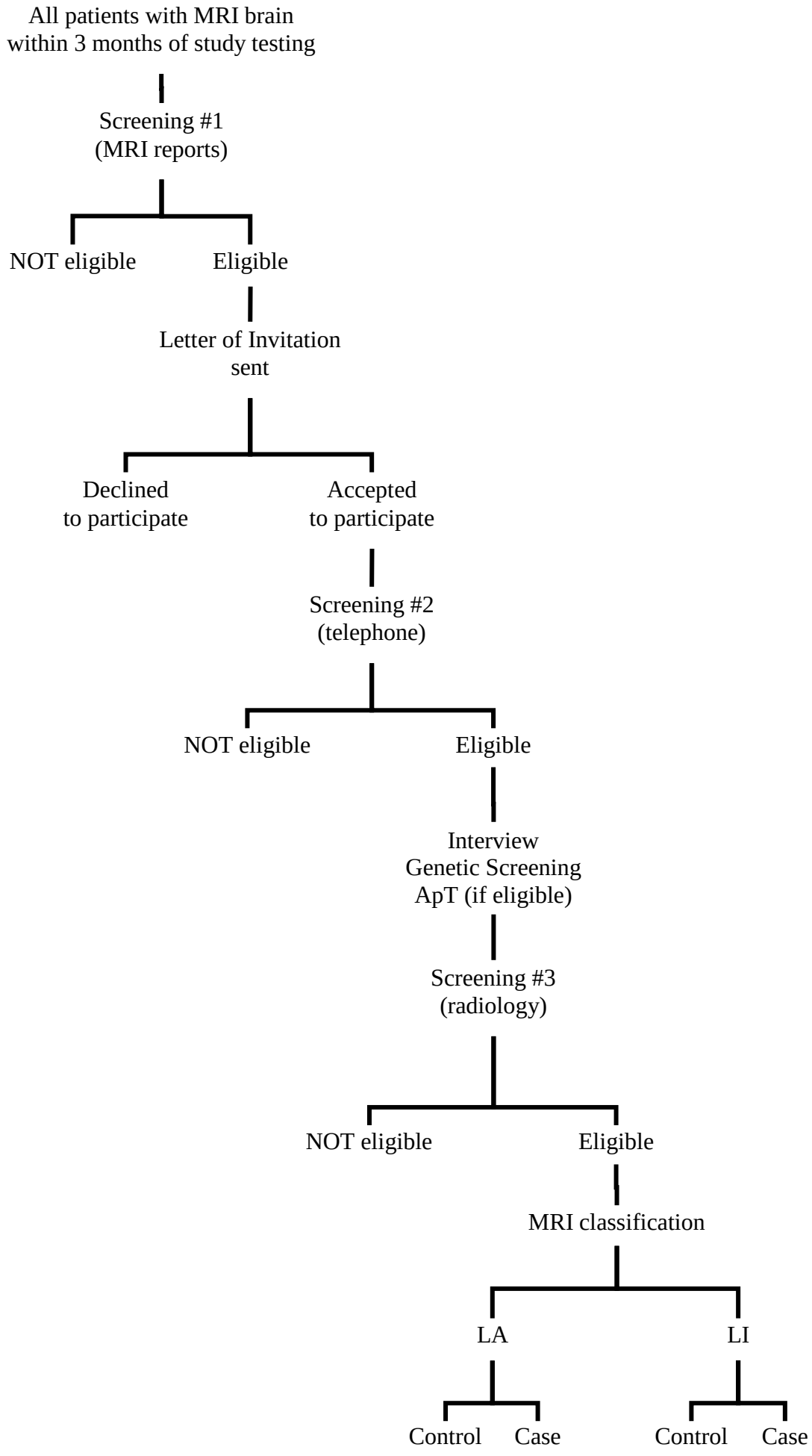
- The Central Northern Adelaide Health Service, Ethics of Human Research Committee for TQEH, LMHS and RAH;
- The Flinders Clinical Research Ethics Committee for FMC.

5.1.3 Recruitment of Participants

Recruitment from TQEH and LMHS began in March 2007, and patients from the RAH and FMC were included from January 2008 onwards. Recruitment was completed in October 2008.

The pathway for recruitment is shown in **Figure 5.1**. All participants were selected from consecutive lists of patients who had had recent MRI of the brain at a participating hospital. Patient lists for the previous month were forwarded from each participating Radiology Department at the beginning of every month.

Figure 5.1 – Recruitment pathway



Screening #1 consisted of exluding participants according to the following criteria using the radiology reports accessed via the public hospital network:

- younger than 18 or older than 75 years old;
- radiological evidence of previous large-vessel ischaemic stroke, tumour, demyelination or other CNS conditions affecting SVD classification;
- significant change to the normal brain structure;
- unable to provide informed consent;
- no axial FLAIR or T_2, or poor image quality.

After the initial screening, which consisted of including and excluding participants based on the criteria above, the potential participant was contacted via a letter with the information sheet attached. A form for the potential participant to indicate interest and their preferred contact methods (e.g. home/work telephone, mobile telephone or email) was attached to the letter with a pre-paid self-addressed envelope. The investigator then contacted the interested participants for Screening #2 using their preferred contact method. Participants were further excluded based on the following exclusion criteria:

- history of brain diseases, dementia or Alzheimer's Disease;
- have had another TIA since the MRI;
- currently participating in another clinical trial, or have been in one within the last 3 months.

A positive response to any of these three questions excluded the participant from all sections of the study.

To determine eligibility for ApT, the following criteria were used:

- concomitant use of vasoactive medication (eg. ACE inhibitors, Angiotensin II receptor blockers, calcium channel blockers, long and short acting nitrates or beta-blockers);
- known cardiac arrhythmias;
- currently using Viagra®, Cialis® or Levitra®;
- currently pregnant or breastfeeding.

A positive response to any of these questions excluded the participant from ApT, but not from genetic testing.

A record of all patients screened and contacted was kept on a secure electronic database (Microsoft Access 2003). Participants were allocated a 3-hour time frame for assessment and

a sequentially-assigned study specific number on the day of assessment. All assessments were performed and completed in the morning.

Every participant undergoing testing also underwent Screening #3, which was based on the MRI. The exact MRI sequences undertaken or poor image quality was not always obvious when assessing the radiology reports. Thus, although these were exclusion criteria at Screening #1, not all participants with these criteria could have been excluded then. The participants that did not have the correct sequences or had images of poor quality were excluded at this stage. The remaining eligible participants were classified into cases and controls based on the presence/absence of LI and the severity of LA.

5.1.4 Radiological Assessment of Participants

MRIs were taken using the details provided in **Table 5.1**.

Table 5.1 – MRI details; T=Tesla

Hospital	Machine Model	Strength	Repetition/echo time (TR/TE)	Slice thickness
TQEH	Philips Intera	1.5T	T2 3700/100ms FLAIR 6000/100ms	5mm
LMH	Philips Achieva	1.5T	T2 4424/100ms FLAIR 11000/140ms	5mm
RAH	Siemans Avanto	1.5T	T2 3890/106ms FLAIR 9000/90ms	4.5mm
	Siemans Triotim	3T	T2 4130/105ms FLAIR 9000/93ms	4.5mm
FMC	Philips Intera	1.5T	T2 4690/90ms FLAIR 6000/100ms	5mm

Wardlaw et al. suggested that if visual rating scales are to be used, then a rating scale that was first validated in a patient population similar to the population of interest should be used [61]. Therefore we chose to use a modified Fazekas scale that had been previously used by Hassan et al. in their studies investigating EF in SVD patients [58].

The final grading of MRIs was performed by two neuroradiologists who interpreted the scans on eFilm (eFilm Workstation version 2.1.2, Merge Healthcare) and conferred on a LA grade according to a modified Fazekas scale used by Hassan, et al. [58]:

1. absent or mild (includes periventricular capping, halo, punctate foci, but ≤20% of white matted affected);
2. >20% but ≤50% of white matter affected;
3. >50% of white matter affected.

Both neuroradiologists were blinded to the original MRI report. All scans were graded in a single session, except for six participants where the MRIs were unavailable at that time. All remaining six participants were graded in a final single session.

The presence/absence of LI was also recorded.

5.1.5 Questionnaire Assessment of Participants

After informed consent was obtained from each participant, the participant was interviewed and all information collected was hand-written on to an assessment template. All data was transferred into a secure electronic database (Microsoft Access 2003) within 24 hours of data collection. The following information was recorded:

1. Demographics: Gender, date of birth, ethnic origin, contact details;
2. Clinical data: Weight, height, pulse, blood pressure;
3. History of diagnosed cerebrovascular risk factors as a yes/no response for: stroke/TIA, hypertension, hypercholesterolaemia, diabetes mellitus (Type I or II), peripheral vascular disease, ischaemic heart disease, known family history of stroke;
4. Medications currently being taken and those taken on a regular basis for at least 3 months prior to testing. All dosages were also recorded. Medications of interest were: antiplatelets & anticoagulants, antihypertensives, lipid lowering, antidiabetics and fish oil. All other medications, including herbal and complementary medicines, were also documented;
5. Lifestyle information: tobacco smoking, alcohol consumption and physical activity.

Since a cohort of this sample was used for a separate cognitive study [509] and a retinal nerve study [510], other data were also collected but not used in the analyses for this study.

5.1.6 Clinical Assessment of Participants

5.1.6.1 Blood pressure and ECG

Blood pressure (BP) was determined using the World Health Organisation recommendations for the assessment of BP. All measurements were taken using an automated Hartmann Tensoval or Omron T8 blood pressure machine in the seated position after a minimum of five minutes of rest. Three measurements were taken with a five minute interval between measurements. The three measurements were averaged.

A 12-lead resting ECG was performed on each participant in the supine position. ECGs were performed by qualifed ECG technicians and read by a single investigator (AL).

5.1.6.2 Applanation Tonometry

The technique and the advantages and disadvantages of ApT have been discussed previously in Chapter 2.3.3.5. ApT was selected as the technique to measure EF due to it using the radial artery rather than the larger brachial artery, good evidence for reproducibility, ease of use for both the operator and participant and availability of equipment.

All volunteers and participants were assessed using the SphygmoCor® applanation tonometer and software (SphygmoCor PX, AtCor Medical Pty Ltd, Australia). The Cardiology Department, TQEH, kindly allowed the use of their equipment and facilities for this study.

Six people independent of the study volunteered prior to recruitment so that the ApT technique and method could be practised. None of these volunteers were used in any statistical analyses.

All study participants were asked to fast for at least six hours prior to testing and were asked to rest supine for 30 minutes before a baseline measurement were taken. 50μL of a 1μg/μL GTN solution (equivalent to 50μg) prepared from a 5mg/mL stock solution and water on the morning of testing was administered when the baseline measurement had been successfully recorded. Measurements were recorded at 3, 5 10, 15 and 20 minutes following administration. After this, the participant was given five minutes to have a brief stretch or a drink of water (while remaining supine) before another baseline measurement was taken. A total of 400 μg of Ventolin® was administered in two divided doses via a spacer.

Measurements were again recorded at 3, 5, 10, 15 and 20 minutes following administration. Only measurements with a quality index (as calculated by the SphygmaCor® software based on the uniformity of the pulse-waves over an 11-second period) over 95% were accepted.

Since AIx is known to vary with heart rate, all measurements for AIx were corrected for a pulse rate of 75 beats per minute (as calculated by the Sphygmacor® software). BP measurements were required to be taken prior to each time point. This was done using an automated Omron T8 blood pressure machine.

The response for GTN and salbutamol was defined as the maximum change in AIx following drug administration. Using the definition set by McEniery et al. [259], global EF was defined as the ratio of the maximum change in AIx after salbutamol administration relative to the maximum change in AIx after GTN . Thus, a smaller ratio is indicative of poorer EF.

Both PAIx and AAIx were recorded and used in statistical analyses.

All participants, including the six volunteers, were assessed over a 19-month period by a single operator (AL) to eliminate inter-operator variability.

5.1.6.2.1 Repeatability

Five people independent of the study (and separate from the initial six volunteers) volunteered to be tested under normal study conditions. All five people were retested after one week. Readings were compared and checked for variability using correlation and a paired t-test. For results to be reproducible the correlation analysis should be significant and the paired t-test should be non-significant (indicating the means are not significantly different). The results should show: r >0.9, slope=1 and y-intercept=0.

The operator (AL) who assessed the participants also assessed these five people.

5.2 Laboratory Methods

5.2.1 Blood Analysis

All participants were asked to fast for at least 6 hours prior to testing. Venous blood was collected and sent to the Institute of Veterinary and Medical Science (IMVS) clinical

biochemistry laboratory at TQEH for the determination of fasting plasma glucose, lipid studies (HDL, LDL, triglycerides, total cholesterol) and serum creatinine concentrations.

5.2.2 DNA extraction

DNA was extracted from 10mL of EDTA venous whole blood using a validated protocol with LSM Lymphocyte Separation Medium (MP Biomedicals) and DNAzol (Molecular Research Centre Inc). Details of the method and the reagents used are given in **Appendix 2.**

A Nano-drop spectrophotometer was used to confirm the presence of DNA in the sample and to quantify the amount of DNA present. The method above produced samples with sufficient quantities of DNA with acceptable 260:280 absorbance ratios.

5.2.3 Genotyping Methods

The polymerase chain reaction sequence-specific primer (PCR-SSP) method was used to determine whether a SNP was present or absent [511]. For this method to be successful, the terminal 3'-nucleotide of the primer must base pair with the polymorphic site of interest. A consensus primer is designed to target an area approximately 300 base pairs away from the SNP. Amplification of the wild-type is facilitated since the Taq polymerase lacks exonuclease activity and is therefore unable to repair the deliberately mismatched terminal 3'-nucleotide primer.

5.2.3.1 Primer design

All primers were designed such that a SNP-dependant nucleotide was located on the 3'-terminus, except for the ET-1 +138A D/I, where it was necessary to create a mismatch within the primer due to the type of polymorphism.

Primers were designed using known sequences in Entrez Nucleotide. The primers used are listed below in ***Table 5.2***. The online primer design tool, Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) [512], was used to assist with primer design and selection. All the default settings were used, except for "product size", which was changed from an optimum of 200 to 300 base pairs. A BLAST search was performed on all primer sequences (www.ncbi.nlm.nih.gov/BLAST) to check for cross-reactivity with other sites within other human DNA regions.

Table 5.2 – Oligonucleotide primer sequences

Polymorphism	Primer Sequence (5'-3')	Genbank Accession No.	Position (5'-3')	Primer Size	Product Size
IL-6 -174 G/C		AF372214			
G	CAA TgT gAC gTC CTT TAg CAT C		1531-1510	22	
C	CAA TgT gAC gTC CTT TAg CAT g		1531-1510	22	340
Consensus	gCg ATg gAg TCA gAg gAA AC		1191-1211	20	
N-Ox p22 phox 242 C/T		M61107			
C	CAC CAC ggC ggT CAT gTg		701-684	18	
T	CAC CAC ggC ggT CAT gTA		701-684	18	292
Consensus	Tgg TgA gTC TCC TCC TgC TC		410-429	20	
tPA -7351 C/T		Z48484			
C	ATg gCT gTg TCT ggg gCg		2245-2228	18	
T	ATg gCT gTg TCT ggg gCA		2245-2228	18	405
Consensus	ATT ggC gCA AAC TCC TCA CA		1841-1860	20	
tPA -4360 G/C		NT_007995			
G	ggA gTA CTg TCA TTA TTg gTg		12389924-12389944	21	
C	ggA gTA CTg TCA TTA TTg gTC		12389924-12389944	21	304
Consensus	ggA gAA TCA gCA AAC CAA CC		12390219-12390200	20	
tPA 20324 C/T		AY291060			
C	gAA TCT TTC CCC AgC TgT Ag		22342-22324	20	
T	gAA TCT TTC CCC AgC TgT AA		22342-22324	20	300
Consensus	TCC TgA ggg CTg AAT gAA Ag		22043-22062	20	
eNOS -786 T/C		AF519768			
T	CAT CAA gCT CTT CCC Tgg CT		1113-1132	20	
C	CAT CAA gCT CTT CCC Tgg CC		1113-1132	20	335
Consensus	TgC Agg TTC TCT CCT TCA CC		1447-1428	20	
ET-1 +138A D/I		AY434104			
D	TTC AgC CCA AgT gCC CTT TA		2036-2016	20	
I	TCA gCC CAA gTg CCC TTT TA		2035-2016	20	277
Consensus	ACg TTg CCT gTT ggT GAC TA		1160-1179	20	
PON1 -107 C/T		AF539592			
C	CCg ATT ggC CCg CCC Cg		1712-1696	17	
T	CCg ATT ggC CCg CCC CA		1712-1696	17	274
Consensus	CAA ggA CCg ggA Tgg CAC		1439-1456	18	

All oligonucleotide primers were synthesised by Geneworks Pty Ltd (Adelaide, South Australia).

The following two primers encoding for a 600 base pair fragment of the HLA-DRB3 gene were used as positive controls:

Forward primer	TGC CAA GTG GAG CAC CCA A
Reverse primer	GCA TCT TGC TCT GTG CAG AT

5.2.3.2 Primer Preparation & Optimization

The control primer stock containing the HLA-DRB3 positive controls primers was made using the following formula:

Cresol Red	62.5 μg/mL
HLA-DRB3 forward primer	5 μg/mL
HLA-DRB3 consensus primer	5 μg/mL

All SNP-specific primers were optimised by comparing the bands produced by different concentrations of primers in control stock. The concentrations of primers were adjusted where necessary and the concentrations that produced bands of approximatly equal intensity for the primer and control were used as the final concentration.

The final primer concentrations are listed in **Table 5.3**.

Table 5.3 – Final concentrations of primers used in the reaction mix for each polymorphism

Polymorphism	Allele-specific primer (μg/mL)	Consensus primer (μg/mL)
IL-6 -174 G/C	5	5
N-Ox p22 phox 242 C/T	40	40
tPA 20324 C/T	20	20
tPA -4360 G/C	60	60
tPA -7351 C/T	10	10
eNOS -786 T/C	20	20
ET-1 +138A D/I	10	10
PON1 -107 C/T	40	40

5.2.3.3 DNA Preparation

The following reagent formulae were prepared as stock solutions and used to prepare the DNA for PCR:

10x PCR buffer

Tris Base 670 mM	(Sigma Chemicals)
Ammonium sulphate 170 mM	(BDH Laboratory Supplies)
pH 8.9 with concentrated hydrocholoric acid	(BDH Laboratory Supplies)
5 mL Tween 20 per 500 mL	(BDH Laboratory Supplies)

TMDH Mixture

6 mL 10x PCR buffer	
6 mL 10 mM dNTP (nucleotides)	(Applied Biosystems)
5.1 mL 25 mM Magnesium chloride	(Applied Biosystems)
6 mL autoclaved, deionised water	

The DNA solution was prepared as follows:

100 μL TMDH mixture	
57 μL autoclaved, deionised water	
4.25 units AmpliTaq DNA polymerase	(Applied Biosystems)
3 μL genomic DNA	

5 μL of the control/SNP-primer solution was placed into the bottom of the appropriate wells of a 96-well plate. To this, 8 μL of the above DNA solution was added. A colour change in the control/SNP-primer solution from yellow to red indicated the presence of DNA. 10 μL of liquid paraffin was also added to each well to prevent evaporation during thermal cycling.

5.2.3.4 PCR Amplification Program

The PTC-200 Peltier Thermal Cycler (MJ Research) was used for PCR using the following program:

90^{o}C for 60 seconds;

5 cycles of 96^{o}C for 25 seconds, 70^{o}C for 45 seconds, 72^{o}C for 45 seconds;

21 cycles of 96^{o}C for 25 seconds, 65^{o}C for 50 seconds, 72^{o}C for 45 seconds;

4 cycles of 96^{o}C for 25 seconds, 55^{o}C for 60 seconds, 72^{o}C for 120 seconds.

Following PCR, 5 μL of orange glycerol buffer was added to each well and centrifuged briefly. The orange glycerol buffer was mixed with the post-PCR mixture in the well using the pipette before being transferred into the gel.

5.2.3.5 Genotype Determination

PCR products were fractionated using 1.33% agarose gels comprising of 4 g of agarose made up to 300 mL with 1x TBE. 1x TBE was made from 5x TBE using the following formula:

53.9 g TRIZMA base

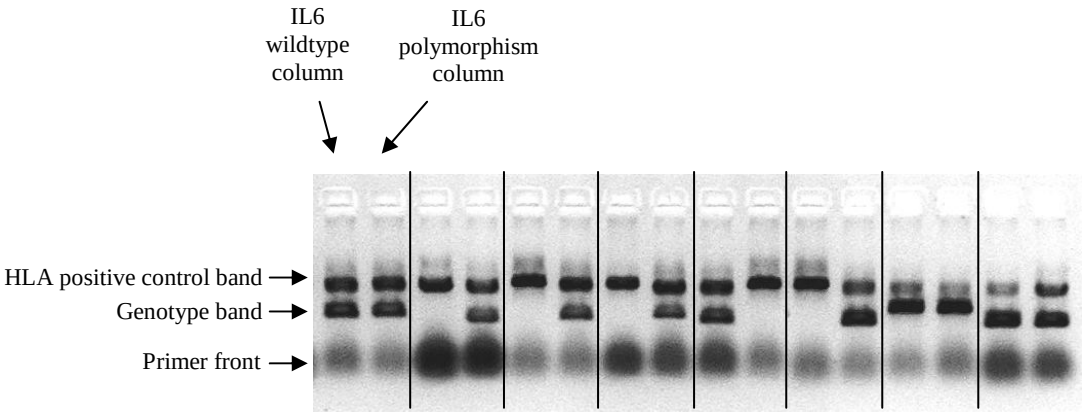
27.1g boric acid

1.865g EDTA

800mL purified water

40μL of ethidium bromide was used as the staining agent. The gel was submerged in 1x TBE when it had set. The post-PCR mixture was carefully transferred to the gel before undergoing electrophoresis at 100 volts for exactly 30 minutes. The bands were illuminated using UV light and the results were photographed using a high resolution digital camera (Olympus ColourPix X990). If necessary, brightness and contrast of the photographs were digitally enhanced uniformly across gels to aid the visual inspection of the bands, as shown in **Figure 5.2**.

Figure 5.2 – Digitally enhanced photograph of the gel under UV light following 30 minutes of gel electrophoresis. Each polymorphism is represented by two columns, with the order of polymorphisms as follows: IL-6, N-Ox p22, tPA 20324, eNOS, tPA -4360, ET-1, tPA -7351, PON-1.



5.2.4 Quality Assurance

5.2.4.1 Sequence Confirmation

The PCR products of one forward primer and the consensus primer of each polymorphism were purified using ExoSAP-IT (USB/Affymetrix) and then a Big Dye Terminator Reaction mix PCR was performed to produce a product that was cleaned and sent to the IMVS for sequencing.

The ExoSAP-IT clean-up consisted of adding 5 μL of PCR product to 2 μL ExoSAP-IT then placing the mixture into the thermocycler for a single pass through the following process:

1. 37^{o}C for 15 minutes to allow the enzyme to remove any single-stranded DNA, primers and unincorporated deoxynucleotide triphosphates;
2. 80^{o}C for 15 minutes to destroy the enzymes used in the clean-up.

The concentration of the 'clean' PCR product was determined using a UV NanoDrop Spectrophotometer (Thermo Scientific).

For the Big Dye Terminator reaction version 3.1, the sample was prepared as follows:

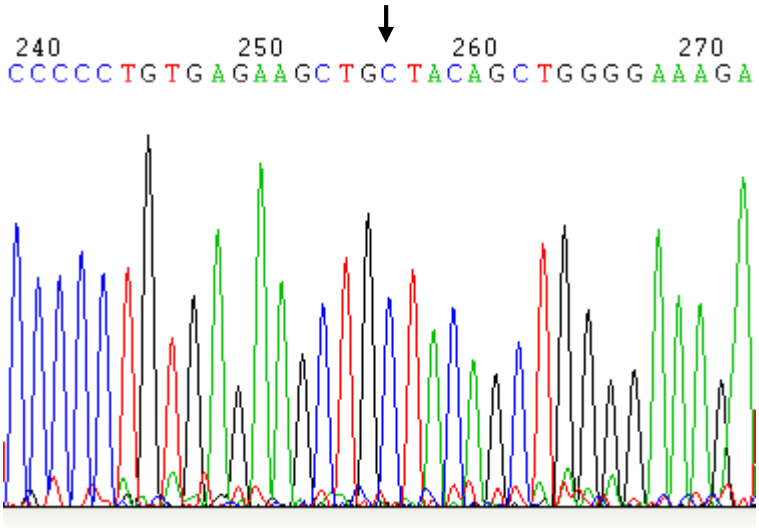
0.1 μg primer
1 μL Big Dye Terminator dye mix
3.5 μL 5x Sequencing buffer
20-100 ng clean PCR product (determined by NanoDrop Spectrophotometer)
to 20 μL with autoclaved, deionised water

The sample was then placed into the thermal cycler:

96^{o}C for 2 minutes
25 cycles of 96^{o}C for 30 seconds, 50^{o}C for 15 seconds, 60^{o}C for 4 minutes
25^{o} for 10 minutes

Isopropanol (75%) was used to remove the low-molecular weight, unused reaction ingredients and the higher molecular weight Big Dye Terminator reaction products were air-dried and sent to the IMVS for sequencing on an ABI 3730 capillary sequencer. An example of a resultant chromatogram is shown in **Figure 5.3**. Product sequences were compared to the original NCBI Entrez Nucleotide sequence and checked for discrepancies.

Figure 5.3 – Chromatogram showing the DNA sequence of the tPA 20324 C/T polymorphism using the consensus primer. The C allele is indicated by the arrow at position 256.



5.2.4.2 Genotype Reproducibility

Thirteen participants (10% of the total sample) for each polymorphism were randomly selected to be re-analysed. There were no discrepancies when samples were repeated.

5.3 Statistical Methods

All analyses were performed using Intercooled Stata 9.2 (StataCorp LP, USA), GraphPad Prism 5.01 (GraphPad Software, Inc, USA) and the online genetic statistical software package, SNPstats (http://bioinfo.iconcologia.net/index.php?module=Snpstats) [513, 514].

All tests were two-sided and statistical significance was defined at a p-value of less than 0.05.

5.3.1 Classification of SVD subtypes

Analyses were performed using two definitions of case and control:

1. Leukoaraiosis (LA) [58]:
    a. Control = no or mild LA on MRI (modified Fazekas Grade 1);
    b. Case = moderate or severe LA on MRI (modified Fazekas Grade 2 or 3).
2. Lacunar infarction (LI):
    a. Control = absence of LI on MRI;
    b. Case = one or more LI on MRI (infarction may be asymptomatic).

5.3.2 Univariate analyses

Comparison of continuous variables between cases and controls were made using the Mann-Whitney test. The relationships investigated using this test included:

- comparison of age, systolic blood pressure and other continuous variables in the descriptive statistics between SVD cases and controls;
- comparison of global EF between SVD cases and control.

Binary logistic regression (for age) and Fisher's exact test was used with 2x2 contingency tables (for other variables) to determine the effect of known cerebrovascular risk factors on LA and LI. Cerebrovascular risk factors were defined as follows:

- Age: age in years divided into categories each spanning 10 years (ie 20-29, 30-39…60-69, 70+);
- Hypertension: systolic > 140mmHg and/or diastolic > 90mmHg and/or a doctor's previous diagnosis of hypertension;
- Hypercholesterolaemia: total cholesterol > 5.5mmol/L on the day of testing and/or a doctor's previous diagnosis of high cholesterol levels and/or currently taking lipid-lowering medication;
- Diabetes: glucose > 5.5mmol/L on the day of testing and/or a doctor's previous diagnosis of diabetes mellitus;
- Smoking: has smoked within the last 5 years (i.e. a current smoker or has quit for less than 5yrs);
- Family history of stroke: stroke in a 1st degree relative (i.e. mother, father, brother or sister).

Binary logistic regression was used to determine the univariate odds ratio of LA and LI for known cerebrovascular risk factors using the definitions described previously.

Binary logistic regresson was used to calculate univariate odds ratios for LA and LI for each polymorphism. As binary logistic regression requires the dependant variable to be dichotomous, the relationship between LA and LI and each polymorphism was analysed using three different models:

1. by individual genotype: wildtype, heterozygous and homozygous, with wildtype as the reference category;
2. Dominant model (aa vs ab+bb): wildtype, and heterozygous and homozygous combined, with the wildtype as the reference category;

3. Recessive model (aa+ab vs bb): wildtype and heterozygous combined and homozygous, with the wildtype/heterozygous as the reference category.

The aim of using logistic regression is to find a model that best predicts the outcome for individual cases that includes all independant variables that are useful in determining that outcome. Logistic regression will also provide information about the strength of the relationship between independent and dependant variables, as well between independent variables. Using SVD and polymorphisms as an example, the equation for a univariate logistic regression can be described as follows:

Logit(SVD) = log(odds of SVD) = constant + coefficient*genotype

Mathematic manipulations will show that the odds ratio will be determined as e^{coeff}.

Linear regression was used to determine the association between EF and each polymorphism. The three different models as described previously were also used. Mathematically, results for a linear univariate analysis can be interpreted as follows:

Global EF = constant + coefficient*genotype

$\therefore$ Global EF = constant + [($coeff_{ab}$*ab) + ($coeff_{bb}$*bb)]

ab and bb = 1 (if present) or 0 (if absent). Thus, for the homozygous wildtype, [($coeff_{ab}$*ab) + ($coeff_{bb}$*bb)] cancels out, leaving Global EF = constant.

5.3.3 Multivariate analyses

Binary logistic regression was used to determine the risk of SVD associated with each SNP and the risk of SVD associated with global EF, adjusting for known cerebrovascular risk factors using the definitions described previously.

To determine which variables would be included in the final multivariate model, a set of confounding variables were identified. Variables were identified as confounding if the incorporation of that variable into a bivariate logistic regression model altered the unadjusted OR by at least 10% [515].

For the analyses involving EF, extra dichotomous variables were used:

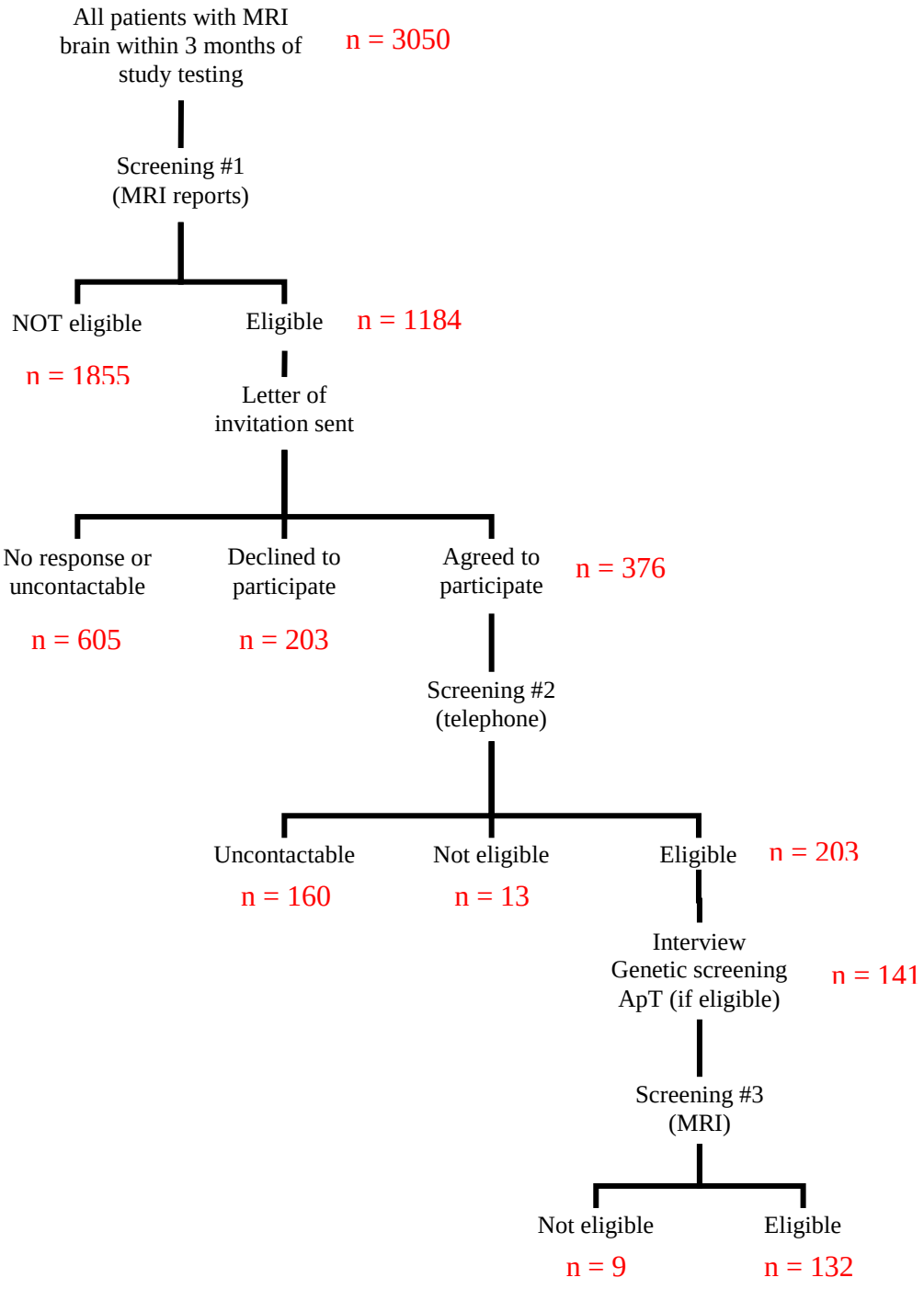
- Fish oil – using fish oil at the time of testing;
- Physical exercise – reported to participate in dedicated physical activity equivalent to twenty minutes, three times a week (total 60 minutes per week).

CHAPTER 6 RESULTS

6.1 Study Sample

A total of 3050 people from LMH, QEH, RAH and FMC were screened during the recruitment period (**Figure 6.1**).

Figure 6.1 – Breakdown of the recruitment process. The numbers of cases and controls are not listed here as it varied depending on the LI/LA definition used.



From these, 1184 people were eligible according to the primary inclusion/exclusion criteria. The reasons for exclusion at this stage are listed below in **Table 6.1**. Of these people, 567 people did not respond to the invitation, 38 were unable to be contacted, 376 agreed to participate, 203 declined due to various reasons (**Table 6.2**) and one person died in the time between having the MRI and being invited into the study.

Table 6.1 – Reasons for exclusion after Screening #1

Reason not eligible based on primary criteria	Number of people
Age (<18 or >75)	727
Evidence of stroke/TIA	282
Tumour	246
Incorrect MRI (no FLAIR, T_2 or has motion artefact)	226
Multiple sclerosis or primary demyelination	170
Already in another clinical trial	62
Radiotherapy	32
Unable to provide informed consent (e.g. severe psychiatric illnesses)	27
Has Alzheimer's Disease or dementia	24
Other (includes deceased, significant changes to brain structure, haemorrhage, encephalitis)	59

Table 6.2 – Reasons why the invitees declined to participate. These reasons were listed on the reply slip and invitees were asked to select a reason why they chose not to participate.

Reason declined	Number of people
Not interested	81
Inconvenient (e.g. travel distance, language barrier)	77
No time	44

Of the 376 people who agreed, 203 were considered eligible during the telephone interview, 13 were not eligible and 160 were unable to be contacted within the set timeframe. Reasons for ineligibility at this stage are shown in **Table 6.3**. Twenty people eligible at Screening #2 withdrew before the assessment day, citing illness or loss of interest as the reasons. Fourteen people failed to attend the scheduled appointment and declined to reschedule. Twenty-eight people (also eligible at Screening #2) requested more time to think about participation but never replied or were uncontactable thereafter.

Table 6.3 – Reasons for exclusion after Screening #2

Reason not eligible at Screening #2	Number of people
Further stroke	5
In another clinical trial	4
Has dementia/Alzheimer's Disease	3
Unidentified brain disease	1

A total of 141 participants were assessed and of these, 132 were eligible. The remaining nine participants who were assessed were not eligible due to incorrect MRI sequences (e.g. sagittal FLAIR instead of axial FLAIR). All eligible participants were graded for LA severity using the modified Fazekas score [58], as described in Chapter 5.1.4. There were 119 controls (grade 1) and 13 cases (grade 2 n=7 and grade 3 n=6).

Table 6.4 shows the descriptive statistics for all eligible participants according to the LA classification. Participants had a mean time of 97 ± 32.2 days (range 33 to 179) between having the MRI and study assessment. Medications used for 'high blood pressure' as indicated by the participant were: ACE-inhibitors (captopril, enalapril, perindopril, and trandolapril), ARBs (candesartan, irbesartan, and telmisartan), combination ACE-inhibitor or ARB with thiazide diuretic (Atacand Plus®, Avapro HCT®, and Karvezide®), beta-blockers (metoprolol, pindolol and propranolol), calcium-channel blockers (lercanidipine) and thiazide diuretics (hydrochlorthiazide and Hydrene®). Medications used for 'high cholesterol' as indicated by the participant were: atorvastatin, rosuvastatin and simvastatin. Use of regular aspirin, warfarin and dipyridamole was also noted. A variety of complementary and herbal medicines were used by the participants, all of which were recorded, but the use of fish oil was of particular interest due to its potential anti-inflammatory and blood thinning properties.

*Table 6.4 – Descriptive statistics of all 132 participants based on the LA classification. All values are mean ± SD for continuous variables. Significance was determined using the Mann-Whitney test and all significant results are indicated by *.*

	Controls	Cases	Significance (p)	
n	119	13		
n female (%)	62 (52.10)	8 (61.54)		
n Caucasian (%)	115 (96.64)	13 (100)		
age (years)	54.23 ± 11.74	67.46 ± 7.32	<0.001	*
height (m)	1.66 ± 0.18	1.64 ± 0.10	0.25	
weight (kg)	80.05 ± 19.87	67.69 ± 23.95	0.13	
BMI (kg/m^2)	28.40 ± 6.27	25.74 ± 9.26	0.54	
Systolic BP (mmHg)	130.27 ± 20.27	155.47 ± 22.16	<0.001	*
Diastolic BP (mmHg)	82.52 ± 10.76	91.24 ± 14.65	0.02	*
Pulse (beats per minute)	68.93 ± 12.49	69.23 ± 10.75	0.61	
Total cholesterol (mmol/L)	5.14 ± 1.14	5.65 ± 2.08	0.36	
LDL (mmol/L)	3.05 ± 1.01	3.03 ± 1.05	0.92	
HDL (mmol/L)	1.41 ± 0.39	1.31 ± 1.40	0.47	
Triglycerides (mmol/L)	1.56 ± 0.86	3.45 ± 5.97	0.28	
Glucose (mmol/L)	5.45 ± 1.83	6.15 ± 1.76	0.15	
n aspirin or warfarin (%)	31 (21.05)	6 (46.15)		
n medication for BP (%)	34 (28.57)	7 (53.85)		
n statin (%)	31 (21.05)	6 (46.15)		
n fish oil (%)	33 (27.73)	3 (23.08)		

Participants were also classified separately based on the presence/absence of LI. The classification is as follows: control = no LI, case = presence of LI. Using this definition, there were 126 controls and six cases. The descriptive statistics of the sample according to the LI definition are shown in **Table 6.5.** Of these LI cases, three were classified as isolated LI (LI with modified Fazekas grade 1), and three were classified as ischaemic LA (LI with modified Fazekas 2 or 3) [58]. Thus, because of the small number of patients with isolated LI, genetic analyses were not able to be further stratified into isolated LI versus ischaemic LA. Of the two LI cases that also underwent ApT, one had isolated LI and the other had ischaemic LA. Again because of the small number of patients in each subgroup cohort, the EF analyses were not stratified further into individual subgroups.

*Table 6.5 – Descriptive statistics of all 132 participants based on the LI classification. All values are mean ± SD for continuous variables. Significance was determined using the Mann-Whitney test and all significant results are indicated by *.*

	Control	Case	Significance (p)	
n	126	6		
n female (%)	66 (55.47)	4 (66.67)		
n Caucasian (%)	122 (96.83)	6 (100)		
age (years)	55.09 ± 11.90	64.83 ± 11.91	0.05	*
height (m)	1.66 ± 0.18	1.60 ± 0.14	0.06	
weight (kg)	79.23 ± 20.73	70.50 ± 14.83	0.19	
BMI (kg/m^2)	28.38 ± 6.16	27.87 ± 7.06	0.85	
Systolic BP (mmHg)	132.11 ± 20.65	156.28 ± 20.93	0.01	*
Diastolic BP (mmHg)	83.22 ± 11.54	87.06 ± 8.77	0.36	
Pulse (beats per minute)	67.69 ± 12.07	73.33 ± 12.01	0.28	
Total cholesterol (mmol/L)	5.14 ± 1.12	6.10 ± 2.99	0.44	
LDL (mmol/L)	3.06 ± 0.99	2.94 ± 1.45	0.99	
HDL (mmol/L)	1.40 ± 0.39	1.44 ± 0.44	0.54	
Triglycerides (mmol/L)	1.58 ± 0.88	5.13 ± 7.79	0.50	
Glucose (mmol/L)	5.33 ± 0.98	9.30 ± 6.78	0.18	
n aspirin or warfarin (%)	36 (28.57)	1 (16.67)		
n medication for BP (%)	38 (30.16)	3 (50.00)		
n statin (%)	35 (27.78)	2 (33.33)		
n fish oil (%)	35 (27.78)	5 (83.33)		

Using Fisher's exact test, the risk of LA in the sample associated with documented cerebrovascular risk factors (as definined in Chapter 5.3.2) is shown in **Table 6.6.** The analyses were repeated to determine the risk of LI in the sample with the same cerebrovascular risk factors (**Table 6.7**).

Table 6.6 – Univariate analyses of known cerebrovascular risk factors with LA.

Risk Factor	Control		Case		OR (95%CI)	p
	No	Yes	No	Yes		
Age	-	-	-	-	3.41 (1.65, 7.03)	<0.01
Hypertension	55	64	2	11	4.73 (1.00, 22.25)	0.05
Hypercholesterolaemia	44	75	1	12	7.04 (0.97, 307.61)	0.03
Diabetes Mellitus	84	35	6	7	2.80 (0.74, 10.78)	0.07
Smoking	89	30	9	4	1.32 (0.28, 5.16)	0.66
Family history	86	33	10	3	0.78 (0.13, 3.30)	0.72

Table 6.7 – Univariate analyses of known cerebrovascular risk factors with LI.

Risk Factor	Control		Case		OR (95%CI)	p
	No	Yes	No	Yes		
Age	-	-	-	-	2.31 (0.98, 5.44)	0.06
Hypertension	57	69	0	6	-	-
Hypercholesterolaemia	43	83	2	4	1.04 (0.14, 11.88)	0.97
Diabetes Mellitus	88	38	2	4	4.63 (0.63, 52.56)	0.06
Smoking	94	32	4	2	1.47 (0.13, 10.78)	0.66
Family history	91	35	5	1	0.52 (0.01, 4.89)	0.55

6.2 Association Between Cerebral Small Vessel Disease and Endothelial Function

Eighty-four of the 132 participants were eligible for ApT. The other 48 participants were not eligible for ApT because they were taking medication for hypertension. Twelve of the 84 participants failed ApT because a sufficient number of readings with a quality index of over 95% were unable to be obtained. Reasons observed for low quality index readings were:

1. irregular pulse-wave indicating an irregular pulse;
2. insufficient pulse-wave signal, especially in overweight females.

6.2.1 Applanation Tonometry Repeatability

Five people (4 female, 1 male) aged between 23 and 58 years old and independent of the study were tested using ApT under standardised study conditions. The second test was performed exactly one week after the first test. The Pearson correlation analysis showed a significant correlation between the original and repeated global EF AAIx results (r=0.98,

p<0.01). Slope was calculated to be 1.00 and the y-intercept was 0.04. The paired t-test was not significant (p=0.79).

Global EF PAIx results were found not to be repeatable. The paired t-test was not significant (p=0.91), and neither was the correlation analysis (r=0.18, p=0.82). Slope of the correlation graph was 0.38 and the y-intercept was calculated to be 0.44.

6.2.2 Associaton Between Leukoaraiosis and Endothelial Function

Figure 6.2 shows a box and whiskers plot of the global EF AAIx. The descriptive statistics of all the participants who underwent ApT are shown below in **Table 6.8**.

Figure 6.2 – Box and whiskers (minimum to maximum) plots showing the distribution of global AAIx.

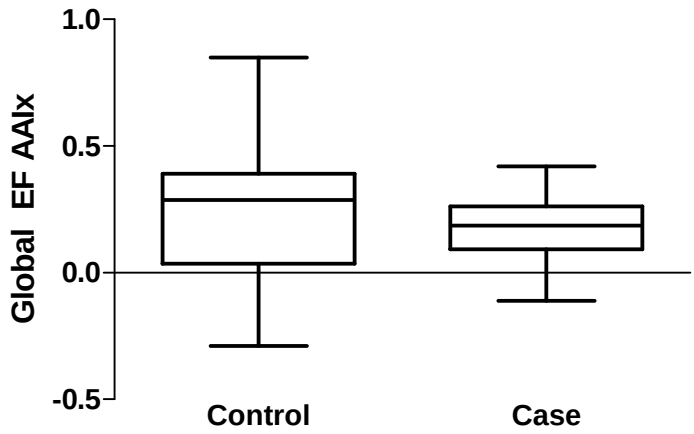


*Table 6.8 – Descriptive statistics of all the participants who underwent ApT based on the LA classification. All values are mean ± SD. Significance was determined using the Mann-Whitney test and all significant results are indicated by *. GTN = glyceryl trinitrate, SALB = salbutamol.*

	Control	Case	Significance (p)	
n	66	6		
% female	53.03	66.67		
age (y)	51.26 ± 11.07	66.00 ± 9.78	<0.01	*
height (m)	1.66 ± 0.23	1.65 ± 0.08	0.39	
weight (kg)	75.29 ± 19.78	65.67 ± 10.76	0.19	
BMI ($kg.m^{-2}$)	26.45 ± 6.29	24.26 ± 3.72	0.39	
Systolic BP (mmHg)	121.84 ± 16.34	142.61 ± 17.35	0.02	*
Diastolic BP (mmHg)	78.82 ± 10.46	82.95 ± 13.86	0.40	
Pulse ($beats.min^{-1}$)	67.30 ± 12.86	65.67 ± 11.09	0.88	
Total cholesterol ($mmol.L^{-1}$)	5.32 ± 1.14	5.40 ± 1.09	0.82	
LDL ($mmol.L^{-1}$)	3.28 ± 0.99	3.37 ± 0.84	0.81	
HDL ($mmol.L^{-1}$)	1.42 ± 0.40	1.50 ± 0.41	0.66	
Triglycerides ($mmol.L^{-1}$)	1.46 ± 0.83	1.23 ± 0.27	0.87	
Glucose ($mmol.L^{-1}$)	5.14 ± 0.60	5.28 ± 0.57	0.46	
% taking fish oil	25.76	50.00		
GTN Baseline AAIx (%)	21.03 ± 11.03	33.00 ± 4.58	<0.01	*
GTN AAIx (%)	7.12 ± 10.83	19.47 ± 6.82	<0.01	*
GTN change	-13.91 ± 5.50	-13.53 ± 3.03	0.89	
SALB Baseline AAIx (%)	17.89 ± 11.44	28.90 ± 4.46	<0.01	*
SALB AAIx (%)	14.82 ± 11.32	26.47 ± 5.09	<0.01	*
SALB change	-3.07 ± 3.50	-2.43 ± 1.81	0.55	
Global EF AAIx	**0.23 ± 0.27**	**0.17 ± 0.17**	**0.61**	
GTN Baseline PAIx (%)	76.05 ± 15.16	94.60 ± 5.84	<0.01	*
GTN PAIx (%)	56.94 ± 13.81	73.12 ± 7.49	<0.01	*
GTN change	-19.11 ± 5.61	-21.48 ± 7.37	0.46	
SALB Baseline PAIx (%)	72.70 ± 15.90	87.63 ± 5.87	<0.01	*
SALB PAIx (%)	66.10 ± 15.49	81.25 ± 5.65	<0.01	*
SALB change	-6.60 ± 6.00	-6.38 ± 4.73	0.96	
Global EF PAIx	**0.37 ± 0.37**	**0.28 ± 0.21**	**0.65**	

The method as described by Rothman et al. [515] was used to determine the variables that the model had to be adjusted for (**Table 6.9**). When adjusting for age, gender, hypertension, diabetes, fish oil and physical activity, the OR for LA status and global EF AAIx was 0.10 (95%CI 0.0007 – 13.40, p=0.36). The OR for global EF PAIx was 0.24 (95% CI 0.005 – 11.71, p=0.47), adjusting for age, hypertension, diabetes, fish oil and physical activity.

Table 6.9 – Determination of which variables should be included in the multiple logistic regression model for LA and ED.

Variable	Global EF AAIx		Global EF PAIx	
	OR	% change in OR	OR	% change in OR
None	0.42	-	0.42	-
Age	0.52	25	0.54	24
Gender	0.37	-11	0.42	-2
Family History	0.44	6	0.43	1
Hypertension	0.47	12	0.30	-29
Hypercholesterolaemia	0.45	9	0.46	9
Diabetes	0.51	24	0.29	-31
Smoker	0.42	1	0.44	4
Fish Oil	0.35	-17	0.37	-12
Physical Activity	0.50	19	0.53	26

6.2.3 Association Between Lacunar Infarction and Endothelial Function

Of the 72 ApT participants, only two had LI. Of these cases, one was classified as isolated LI and the other was ischaemic LA. The descriptive statistics of the participants based on the LI classification is displayed in **Table 6.10**.

The unadjusted OR for LI status and global EF AAIx was 15.66 (95%CI 0.06 – 4404.62, p=0.34) and PAIx was 2.01 (95%CI 0.74 – 54.81, p=0.68). Due to the low number of LI cases, the OR adjusted for confounding variables was not able to be accurately determined.

*Table 6.10 – Descriptive statistics of participants who underwent ApT based on the LI classification. All values are mean ± SD. Significance was determined using the Mann-Whitney test and all significant results are indicated by *. GTN = glyceryl trinitrate, SALB = salbutamol.*

	Control	Case	Significance (p)	
n	70	2		
% female	52.86	100		
age (y)	51.91 ± 11.30	72.50 ± 2.12	0.02	*
height (m)	1.66 ± 0.22	1.59 ± 0.03	0.15	
weight (kg)	74.90 ± 19.36	60.00 ± 15.56	0.20	
BMI ($kg.m^{-2}$)	26.34 ± 6.16	23.64 ± 5.31	0.43	
Systolic BP (mmHg)	123.76 ± 16.19	117.0 ± 55.15	0.05	*
Diastolic BP (mmHg)	79.15 ± 10.85	79.84 ± 5.42	0.69	
Pulse ($beats.min^{-1}$)	66.0 ± 9.14	108.0 ± 46.67	0.08	
Total cholesterol ($mmol.L^{-1}$)	5.32 ± 1.11	5.45 ± 2.33	0.96	
LDL ($mmol.L^{-1}$)	3.29 ± 0.96	3.30 ± 1.84	0.99	
HDL ($mmol.L^{-1}$)	1.42 ± 0.40	1.70 ± 0.28	0.21	
Triglycerides ($mmol.L^{-1}$)	1.46 ± 0.80	1.0 ± 0.57	0.37	
Glucose ($mmol.L^{-1}$)	5.15 ± 0.57	5.15 ± 1.48	0.96	
% taking fish oil	27.14	50.00		
GTN Baseline AAIx (%)	21.76 ± 11.17	31.55 ± 2.76	0.14	
GTN AAIx (%)	7.87 ± 11.09	17.90 ± 2.69	0.12	
GTN change	-13.88 ± 5.41	-13.65 ± 0.71	0.97	
SALB Baseline AAIx (%)	18.57 ± 11.49	27.20 ± 3.11	0.18	
SALB AAIx (%)	15.62 ± 11.50	21.60 ± 0.85	0.45	
SALB change	-2.94 ± 3.38	-5.60 ± 3.96	0.30	
Global EF AAIx	**0.22 ± 0.26**	**0.41 ± 0.29**	**0.32**	
GTN Baseline PAIx (%)	77.28 ± 15.58	88.60 ± 1.70	0.18	
GTN PAIx (%)	58.05 ± 14.23	66.75 ± 2.05	0.86	
GTN change	-19.23 ± 5.81	-21.85 ± 0.35	0.22	
SALB Baseline PAIx (%)	73.80 ± 16.05	79.00 ± 1.41	0.54	
SALB PAIx (%)	67.32 ± 15.69	68.80 ± 4.81	0.86	
SALB change	-6.48 ± 5.88	-10.20 ± 6.22	0.40	
Global EF PAIx	**0.36 ± 0.36**	**0.47 ± 0.29**	**0.52**	

6.3 Association Between Genetic Polymorphisms and Endothelial Function

Table 6.11 shows the genotype distribution for the ApT participants only (n=72).

Sub-analyses were performed in participants that were free of SVD (n=65, defined as LA grade 1 only with no LI) to eliminate the possibility of confounding the results with the influence of SVD. The genotype distribution for this sub-cohort is shown in **Table 6.12**.

Table 6.11 – Genotype distribution for all ApT participants (n=72) with percentages in brackets.

	aa (%)	ab (%)	bb (%)
IL-6 -174 G/C	32 (44.4)	27 (37.5)	13 (18.1)
N-Ox p22 242 C/T	29 (40.3)	33 (45.8)	10 (13.9)
tPA -7351 C/T	31 (43.1)	38 (52.8)	3 (4.3)
tPA -4360 G/C	40 (55.6)	29 (40.3)	3 (4.2)
tPA 20324 C/T	51 (70.8)	19 (26.4)	2 (2.8)
eNOS -786 T/C	26 (36.1)	35 (48.6)	11 (15.3)
ET-1 +138 D/I	32 (44.4)	33 (45.8)	7 (9.7)
PON1 -107 C/T	13 (18.1)	42 (58.3)	17 (23.6)

Table 6.12 – Genotype distribution for SVD-free controls (n=65) with percentages in brackets.

	aa (%)	ab (%)	bb (%)
IL-6 -174 G/C	31 (47.7)	23 (35.4)	11 (16.9)
N-Ox p22 242 C/T	23 (35.4)	32 (49.2)	10 (15.4)
tPA -7351 C/T	27 (41.5)	36 (55.4)	2 (3.1)
tPA -4360 G/C	36 (55.4)	26 (40.0)	3 (4.6)
tPA 20324 C/T	46 (70.8)	18 (27.7)	1 (1.5)
eNOS -786 T/C	25 (38.5)	29 (44.6)	11 (16.9)
ET-1 +138 D/I	31 (47.7)	28 (43.1)	6 (9.2)
PON1 -107 C/T	12 (18.5)	36 (55.4)	17 (26.2)

Linear regression was used to investigate the association between EF (as described by global EF AAIx and PAIx) and genetics. Univariate analyses identified the tPA 20324 C/T and tPA -4360 G/C polymorphisms to be of interest (**Table 6.13**). As these are univariate analyses, the p-values given for the coefficient is the same are the p-value for the overall model.

*Table 6.13 – Unadjusted linear regression of individual genotypes with global EF (n=72) using the equation: Global EF = constant + $coeff_{ab}$*ab + $coeff_{bb}$*bb, where by default the constant = $coeff_{aa}$.*

	Global EF AAIx			Global EF PAIx		
	Coefficient	p	R^2	Coefficient	p	R^2
IL-6 -174 G/C			<0.01			0.03
GG	0.23			0.43		
GC	0.26	0.80		0.32	0.25	
CC	0.25	0.82		0.31	0.29	
N-Ox p22 242 C/T			0.05			0.06
CC	0.19			0.33		
CT	0.22	0.65		0.33	0.99	
TT	0.37	0.07		0.57	0.07	
tPA -7351 C/T			0.01			0.03
CC	0.25			0.42		
CT	0.20	0.44		0.31	0.21	
TT	0.31	0.72		0.50	0.72	
tPA -4360 G/C			0.02			0.08
GG	0.25			0.43		
GC	0.19	0.38		0.25	**0.04**	
CC	0.31	0.70		0.64	0.32	
tPA 20324 C/T			0.11			0.03
CC	0.23			0.36		
CT	0.18	0.50		0.36	0.94	
TT	0.73	**<0.01**		0.70	0.18	
eNOS -786 T/C			<0.01			0.03
TT	0.22			0.29		
TC	0.23	0.91		0.43	0.15	
CC	0.28	0.54		0.36	0.62	
ET-1 +138 D/I			0.03			0.02
DD	0.26			0.41		
DI	0.23	0.69		0.32	0.32	
II	0.09	0.14		0.41	0.99	
PON1 -107 C/T			<0.01			0.01
CC	0.25			0.35		
CT	0.22	0.67		0.34	0.93	
TT	0.25	0.94		0.43	0.56	

However, when applying the analyses to the dominant and recessive models of all polymorphisms, only the tPA 20324 C/T recessive model remained to show a significant difference in EF between the CC/CT and TT genotypes. The N-Ox p22 242 C/T recessive model also showed a significant difference in EF between the CC/CT and TT genotypes (**Table 6.14**).

*Table 6.14 – Unadjusted linear regression of dominant and recessive models with global EF (n=72) using the equation: Global EF = constant + coeff*genotype.*

	Global EF AAIx				Global EF PAIx			
	Const	Coeff	p	R^2	Const	Coeff	p	R^2
IL-6 -174 G/C								
GG vs GC/CC	0.23	-0.01	0.93	<0.01	0.43	-0.11	0.18	0.03
GG/GC vs CC	0.22	0.03	0.73	<0.01	0.38	-0.08	0.50	<0.01
N-Ox p22 242 C/T								
CC vs CT/TT	0.19	0.06	0.32	0.01	0.33	0.06	0.51	<0.01
CC/CT vs TT	0.21	0.16	0.08	0.04	0.33	0.24	**0.05**	0.06
tPA -7351 C/T								
CC vs CT/TT	0.25	-0.04	0.51	<0.01	0.42	-0.10	0.26	0.02
CC/CT vs TT	0.23	0.09	0.58	<0.01	0.36	0.14	0.51	<0.01
tPA -4360 G/C								
GG vs GC/CC	0.25	-0.05	0.46	<0.01	0.43	-0.14	0.10	0.04
GG/GC vs CC	0.23	0.09	0.59	<0.01	0.36	0.28	0.18	0.03
tPA 20324 C/T								
CC vs CT/TT	0.23	0.01	0.93	<0.01	0.36	0.04	0.67	<0.01
CC/CT vs TT	0.22	0.52	**<0.01**	0.10	0.36	0.34	0.18	0.03
eNOS -786 T/C								
TT vs TC/CC	0.22	0.02	0.76	<0.01	0.29	0.12	0.18	0.03
TT/TC vs CC	0.22	0.06	0.53	<0.01	0.37	-0.01	0.92	<0.01
ET-1 +138 D/I								
DD vs DI/II	0.26	-0.05	0.42	<0.01	0.41	-0.07	0.39	0.01
DD/DI vs II	0.25	-0.15	0.15	0.03	0.36	0.04	0.76	<0.01
PON1 -107 C/T								
CC vs CT/TT	0.25	-0.03	0.73	<0.01	0.35	0.02	0.89	<0.01
CC/CT vs TT	0.23	0.02	0.78	<0.01	0.35	0.08	0.40	0.01

When adjusting the tPA 20324 C/T recessive and N-Ox p22 242 C/T recessive models for age, gender, hypertension, hypercholesterolaemia, diabetes, smoking, family history, fish oil use and physical activity, none of these factors were found to significantly affect the result. For both tPA 20324 C/T recessive and the N-Ox p22 242 C/T recessive models, none of the individual variables were significant and neither were the overall models (**Table 6.15**).

Table 6.15 – Adjusted values of the tPA 20324 C/T recessive and N-Ox p22 242 C/T recessive linear regression models (n=72)

	tPA 20324 C/T recessive	N-Ox p22 242 C/T
Constant	0.23	0.21
Coefficient	0.53 (p=0.01)	0.23 (p=0.07)
Overall model p-value	0.30	0.23
R^2	0.17	0.18

The regression results for the SVD-free controls are shown in **Table 6.16**. Only the tPA 20324 C/T recessive model was observed to have significantly different EF between the CC/CT and TT genotype groups.

*Table 6.16 – Unadjusted linear regression of dominant and recessive models with global EF in SVD-free controls (n=65) using the equation: Global EF = constant + coeff*genotype.*

	Global EF AAIx				Global EF PAIx			
	Const	Coeff	p	R^2	Const	Coeff	p	R^2
IL-6 -174 G/C								
GG vs GC/CC	0.24	-0.03	0.67	<0.01	0.45	-0.15	0.11	0.04
GG/GC vs CC	0.23	0.15	0.87	<0.01	0.38	-0.07	0.58	<0.01
N-Ox p22 242 C/T								
CC vs CT/TT	0.18	0.08	0.27	0.02	0.33	0.07	0.50	<0.01
CC/CT vs TT	0.20	0.16	0.08	0.05	0.33	0.24	0.06	0.06
tPA -7351 C/T								
CC vs CT/TT	0.24	-0.02	0.79	<0.01	0.42	-0.09	0.35	0.01
CC/CT vs TT	0.23	0.15	0.47	<0.01	0.36	0.26	0.33	0.02
tPA -4360 G/C								
GG vs GC/CC	0.24	-0.02	0.73	<0.01	0.43	-0.13	0.15	0.03
GG/GC vs CC	0.23	0.09	0.59	<0.01	0.36	0.28	0.20	0.03
tPA 20324 C/T								
CC vs CT/TT	0.23	0.004	0.95	<0.01	0.36	0.05	0.63	<0.01
CC/CT vs TT	0.22	0.63	**0.02**	0.08	0.37	0.36	0.33	0.02
eNOS -786 T/C								
TT vs TC/CC	0.21	0.03	0.64	<0.01	0.29	0.13	0.18	0.03
TT/TC vs CC	0.22	0.06	0.53	<0.01	0.37	-0.02	0.90	<0.01
ET-1 +138 D/I								
DD vs DI/II	0.25	-0.05	0.50	<0.01	0.41	-0.08	0.40	0.01
DD/DI vs II	0.25	-0.17	0.14	0.03	0.36	0.07	0.64	<0.01
PON1 -107 C/T								
CC vs CT/TT	0.24	-0.01	0.89	<0.01	0.36	0.02	0.90	<0.01
CC/CT vs TT	0.22	0.02	0.77	<0.01	0.35	0.08	0.43	0.01

When adjusting the tPA 20324 C/T recessive model for age, gender, hypertension, hypercholesterolaemia, diabetes, smoking, family history, fish oil use and physical activity, none of these factors were found to affect the result significantly – none of the individual variables were significant and neither was the overall model (**Table 6.17**).

Table 6.17 – Adjusted values of the tPA 20324 C/T recessive linear regression model (n=65)

	tPA 20324 C/T recessive
Constant	0.19
Coefficient	0.79 (p=0.01)
Overall model p-value	0.37
R^2	0.17

6.4 Association Between Genetic Polymorphisms and Cerebral Small Vessel Disease

6.4.1 Hardy-Weinberg Equilibrium

Hardy-Weinberg equilibrium was determined using the online genetic statistical software package, SNPstats [513, 514]. The results are shown in **Table 6.18**. All polymorphisms are in Hardy-Weinberg equilibrium, except for IL-6 -174 G/C.

Table 6.18 – Subject numbers (total n=132) and p-values for Hardy-Weinberg calculations for each polymorphism

Polymorphism	Subject count			p-value
	aa	ab	bb	
IL-6 -174 G/C	59	49	24	0.02
N-Ox p22 242 C/T	60	54	18	0.33
tPA -7351 C/T	52	68	12	0.18
tPA -4360 G/C	65	56	11	1.00
tPA 20324 C/T	93	34	5	0.36
eNOS -786 T/C	50	61	21	0.72
ET-1 +138 D/I	57	64	11	0.32
PON1 -107 C/T	30	74	28	0.22

6.4.2 Association Between Genetic Polymorphisms and Leukoaraiosis

6.4.2.1 Univariate Analyses

Table 6.19 shows the allele and genotype distribution of all participants for all eight polymorphisms according to the LA definition of cases and controls. There were no significant unadjusted associations between genotype and SVD in this sample. No significant associations were observed in the univariate analyses between SVD and the dominant and recessive models of each polymorphism (**Table 6.20**).

Table 6.19 – Genotype distribution based on LA, with the percentage in brackets. The homozygous wildtype or wildtype allele has been used as the reference level.

Genotype	Control, n (%)	Case, n (%)	OR (95% CI)	P
IL-6 -174 G/C				
GG	55 (46.2)	4 (30.8)	1.00	
GC	42 (35.3)	7 (53.8)	2.29 (0.63 – 8.35)	0.21
CC	22 (18.5)	2 (15.4)	1.25 (0.23 – 7.32)	0.81
G allele	152 (63.9)	15 (57.7)	1.00	
C allele	86 (36.1)	11 (42.3)	1.30 (0.57– 2.95)	0.53
N-Ox 242 C/T				
CC	54 (45.4)	6 (46.2)	1.00	
CT	49 (41.2)	5 (38.5)	0.92 (0.26 – 3.20)	0.89
TT	16 (13.4)	2 (15.4)	1.13 (0.21 – 6.13)	0.89
C allele	157 (66.0)	17 (65.4)	1.00	
T allele	81 (34.0)	9 (34.6)	0.99 (0.42 – 2.33)	1.00
tPA -7351 C/T				
CC	46 (38.7)	6 (46.2)	1.00	
CT	62 (52.1)	6 (46.2)	0.74 (0.23 – 2.45)	0.62
TT	11 (9.2)	1 (7.7)	0.70 (0.08 – 6.40)	0.75
C allele	154 (64.7)	18 (69.2)	1.00	
T allele	84 (35.3)	8 (30.8)	0.82 (0.34 – 1.95)	0.83
tPA -4360 G/C				
GG	57 (47.9)	8 (61.5)	1.00	
GC	51 (42.9)	5 (38.5)	0.70 (0.22 – 2.27)	0.551
CC	11 (9.2)	0 (0.0)	-	-
G allele	165 (69.3)	21 (80.8)	1.00	
C allele	73 (30.7)	5 (19.2)	0.54 (0.20 – 1.48)	0.27
tPA 20324 C/T				
CC	85 (71.4)	8 (61.5)	1.00	
CT	29 (24.4)	5 (38.5)	1.83 (0.56 – 6.05)	0.32
TT	5 (4.2)	0 (0.0)	-	-
C allele	199 (83.6)	21 (80.8)	1.00	
T allele	39 (16.4)	5 (19.2)	1.22 (0.43 – 3.42)	0.78
eNOS -786 T/C				
TT	47 (39.5)	3 (23.1)	1.00	
TC	51 (42.9)	10 (76.9)	3.07 (0.80 – 11.85)	0.10
CC	21 (17.6)	0 (0.0)	-	-
T allele	145 (60.9)	16 (61.5)	1.00	
C allele	93 (39.1)	10 (38.5)	0.98 (0.34 – 2.24)	1.00
ET-1 +138A D/I				
DD	53 (44.5)	4 (30.8)	1.00	
DI	56 (47.1)	8 (61.5)	1.89 (0.54 – 6.66)	0.32
II	10 (8.4)	1 (7.7)	1.33 (0.13 – 13.13)	0.81
D allele	162 (68.1)	16 (61.5)	1.00	
I allele	76 (31.9)	10 (38.5)	1.33 (0.58 – 3.07)	1.33
PON1 -107 C/T				
CC	26 (21.8)	4 (30.8)	1.00	
CT	66 (55.5)	8 (61.5)	0.79 (0.22 – 2.84)	0.72
TT	27 (22.7)	1 (7.7)	0.24 (0.03 – 2.30)	0.22
C allele	118 (49.6)	16 (61.5)	1.00	
T allele	120 (50.4)	10 (38.5)	0.62 (0.27 – 1.41)	0.30

Table 6.20 – Univariate analyses for all polymorphisms with LA. Results are not available for some polymorphisms because the homozygous polymorphic genotype was not present in the patient group. aa denotes the homozygous wildtype, ab is the heterozygous genotype and bb is the homozygous polymorphism.

	aa vs ab/bb			aa/ab vs bb		
	OR	95%CI	p	OR	95%CI	p
IL-6 -174 G/C	1.93	0.56 – 6.63	0.29	0.80	0.17 – 3.88	0.78
N-Ox p22 242 C/T	0.97	0.31 – 3.06	0.96	1.17	0.24 – 5.77	0.85
tPA -7351 C/T	0.74	0.23 – 2.32	0.60	0.82	0.10 – 6.90	0.85
tPA -4360 G/C	0.57	0.18 – 1.86	0.36	-	-	-
tPA 20324 C/T	1.56	0.48 – 5.12	0.46	-	-	-
eNOS -786 T/C	2.18	0.57 – 8.32	0.26	-	-	-
ET-1 +138 D/I	1.81	0.53 – 6.19	0.35	0.91	0.11 – 7.72	0.93
PON1 -107 C/T	0.63	0.18 – 2.21	0.47	0.28	0.04 – 2.28	0.24

6.4.2.2 Multivariate Analyses

Multiple logistic regression was used to determine the relationship between the genotypes of individual polymorphisms with SVD. Again, only variables that caused a change of ±10% or more of the unadjusted odds ratio were included (**Appendix 3**).

Thus, when adjusting for all confounding variables, no significant associations were observed. OR for each polymorphism in both the dominant (aa vs ab/bb) and recessive model (aa/ab vs bb) are listed below in **Table 6.21**.

Table 6.21 – Multivariate analyses for each polymorphism with LA. Each model has been adjusted for confounding variables as determined using the tables in Appendix 3. The variables that each model has been adjusted for are indicated: a=age, c=hypercholesterolaemia, d=diabetes, f=family history, g=gender, h=hypertension and s=smoking.

	aa vs ab/bb			aa/ab vs bb		
	OR	(95%CI)	p	OR	(95%CI)	p
IL-6 -174 G/C	2.66 [ah]	0.69 – 10.20	0.15	2.00 [acd]	0.33 – 12.21	0.45
N-Ox p22 242 C/T	0.90 [ah]	0.26 – 3.15	0.87	1.05 [ah]	0.18 – 6.04	0.96
tPA -7351 C/T	0.67 [ad]	0.19 – 2.40	0.54	0.92 [ach]	0.08 – 10.28	0.95
tPA -4360 G/C	0.51 [ad]	0.14 – 1.85	0.31		-	-
tPA 20324 C/T	1.56	0.48 – 5.12	0.46		-	-
eNOS -786 T/C	2.82 [cdg]	0.70 – 11.38	0.15		-	-
ET-1 +138 D/I	1.59 [c]	0.45 – 5.57	0.40	0.77 [d]	0.09 – 6.75	0.81
PON1 -107 C/T	0.63	0.18 – 2.21	0.47	0.23 [ach]	0.02 – 2.17	0.20

6.4.3 Association Between Genetic Polymorphisms and Lacunar Infarction

6.4.3.1 Univariate Analyses

The genotype distribution for all participants based on the LI classification is shown in **Table 6.22**. Using Fisher's exact tests, univariate analyses show no significant results, except for tPA 20324 C/T (**Table 6.23**). Some analyses were not able to be performed as there were no participants with the required genotypes.

Table 6.22 – Genotype distribution based on LI, with the percentage in brackets. The homozygous wildtype or wildtype allele has been used as the reference level.

Genotype	Control, n (%)	Case, n (%)	OR (95% CI)	P
IL-6 -174 G/C				
GG	56 (44.4)	3 (50.0)	1.00	
GC	46 (36.5)	3 (50.0)	1.22 (0.23 – 6.32)	0.815
CC	24 (19.0)	0 (0.0)	-	-
G allele	158 (62.7)	9 (75.0)		
C allele	94 (25.4)	3 (25.0)	0.54 (0.15 – 2.12)	0.544
N-Ox p22 242 C/T				
CC	59 (46.8)	1 (16.7)	1.00	
CT	51 (40.5)	3 (50.0)	3.47 (0.35 – 34.41)	0.29
TT	16 (12.7)	2 (33.3)	7.38 (0.63 – 86.60)	0.11
C allele	171 (67.9)	7 (58.3)		
T allele	81 (32.1)	5 (41.7)	1.51 (0.46 – 4.90)	0.53
tPA -7351 C/T				
CC	48 (38.1)	4 (66.67)	1.00	
CT	67 (53.2)	1 (16.67)	0.18 (0.02 – 1.65)	0.129
TT	11 (8.7)	1 (16.67)	1.09 (0.11 – 10.74)	0.941
C allele	163 (64.7)	9 (75.0)		
T allele	89 (35.3)	3 (25.0)	0.61 (0.16 – 2.31)	0.55
tPA -4360 G/C				
GG	61 (48.4)	4 (66.7)	1.00	
GC	54 (42.9)	2 (33.3)	0.57 (0.10 – 3.21)	0.52
CC	11 (8.7)	0 (0.0)	-	-
G allele	176 (69.8)	10 (83.3)		
C allele	76 (30.2)	2 (16.7)	0.46 (0.10 – 2.17)	0.52
tPA 20324 C/T				
CC	91 (72.2)	2 (33.3)	1.00	
CT	32 (25.4)	2 (33.3)	2.84 (0.39 – 21.03)	0.31
TT	3 (2.4)	2 (33.3)	30.33 (3.13 – 294.34)	<0.01
C allele	214 (84.9)	6 (50.0)		
T allele	38 (15.1)	6 (50.0)	5.63 (1.73 – 18.39)	<0.01
eNOS -786 T/C				
TT	50 (39.7)	0 (0.0)	1.00	
TC	55 (43.7)	6 (100.0)	-	-
CC	21 (16.7)	0 (0.0)	-	-
T allele	155 (61.5)	6 (50.0)		
C allele	97 (38.5)	6 (50.0)	1.60 (0.50 – 5.10)	0.55
ET-1 +138A D/I				
DD	56 (44.4)	1 (16.7)	1.00	
DI	59 (46.8)	5 (83.3)	4.75 (0.54 – 41.90)	0.161
II	11 (8.8)	0 (0.0)	-	
D allele	171 (67.9)	7 (58.3)		
I allele	81 (32.1)	5 (41.7)	1.51 (0.46 – 4.90)	0.53
PON1 -107 C/T				
CC	29 (23.0)	1 (16.7)	1.00	
CT	70 (55.6)	4 (66.7)	1.66 (0.18 – 15.47)	0.66
TT	27 (21.4)	1 (16.7)	1.07 (0.06 – 18.04)	0.96
C allele	128 (50.8)	6 (50.0)		
T allele	124 (49.2)	6 (50.0)	1.03 (0.34 – 3.29)	1.00

Table 6.23 – Univariate analyses for all polymorphisms with LI. Results are not available for some polymorphisms because the homozygous polymorphic genotype was not present in the patient group. aa denotes the homozygous wildtype, ab is the heterozygous genotype and bb is the homozygous polymorphism.

	aa vs ab/bb			aa/ab vs bb		
	OR	95% CI	p	OR	95%CI	p
IL-6 -174 G/C	0.80	0.16 – 4.12	0.79	-	-	-
N-Ox p22 242 C/T	4.40	0.50 – 38.77	0.18	3.44	0.58 – 20.31	0.17
tPA -7351 C/T	0.31	0.05 – 1.74	0.18	2.09	0.22 – 19.53	0.52
tPA -4360 G/C	0.47	0.08 – 2.66	0.39	-	-	-
tPA 20324 C/T	5.20	0.91 – 29.67	0.06	**20.50**	**2.65 – 158.87**	**<0.01**
eNOS -786 T/C	-	-	-	-	-	-
ET-1 +138 D/I	4.00	0.45 – 35.23	0.21	-	-	-
PON1 -107 C/T	1.49	0.17 – 13.31	0.72	0.73	0.08 – 6.55	0.78

6.4.3.2 Multivariate Analyses

All the unadjusted models were then adjusted for the necessary risk factors, with the results shown in **Table 6.24**. How these risk factors were determined are shown in **Appendix 4.**

Table 6.24 – Multivariate analyses for each polymorphism with LI. Each model has been adjusted for confounding variables as determined using the tables in Appendix 4. The variables that each model has been adjusted for are also indicated: a=age, c=hypercholesterolaemia, d=diabetes, f=family history, g=gender, h=hypertension and s=smoking.

	aa vs ab/bb			aa/ab vs bb		
	OR	95%CI	p	OR	95%CI	p
IL-6 -174 G/C	0.77 [agh]	0.13 – 4.51	0.77	-	-	-
N-Ox p22 242 C/T	5.78 [h]	0.64 – 52.10	0.12	5.37 [fh]	0.77 – 37.46	0.09
tPA -7351 C/T	0.31 [ad]	0.05 – 1.90	0.20	1.82 [afh]	0.17 – 20.14	0.62
tPA -4360 G/C	0.50 [dh]	0.08 – 3.06	0.45	-	-	-
tPA 20324 C/T	5.20	0.91 – 29.67	0.06	12.96 [acdgh]	1.26 – 133.52	0.03
eNOS -786 T/C	-	-	-	-	-	-
ET-1 +138 D/I	4.00	0.45 – 35.23	0.21	-	-	-
PON1 -107 C/T	2.09 [hs]	0.22 – 20.16	0.52	0.93 [ah]	0.10 – 8.94	0.95

6.4.4 Statistical Power of Analyses

The post-hoc statistical power for each SNP in LA and LI is shown in **Table 6.25**. These values have been calculated using the homozygous polymorphic genotype of each candidate polymorphism observed in this study, thus power and sample size could not be calculated for some polymorphisms. Sample size is the estimated total number of people required to show a minimum OR of 1.5, α=0.05, power of 80% and a 1:1 case to control ratio.

Table 6.25 – Post-hoc statistical power calculations and estimated sample sizes required for each candidate polymorphism based on the homozygous polymorphism to detect a minimum OR of 1.5 with α=0.05 and power of 80%.

	LA		LI	
SNP	Power	Sample Size	Power	Sample Size
IL-6 -174 G/C	2.5%	686	-	-
N-Ox p22 242 C/T	4.9%	1032	20.2%	1086
tPA -7351 C/T	4.1%	1594	7.0%	1698
tPA -4360 G/C	-	-	-	-
tPA 20324 C/T	-	-	63.2%	>5000 (6672)
eNOS -786 T/C	-	-	-	-
ET-1 +138 D/I	5.9%	536	-	-
PON1 -107 C/T	6.4%	534	3.0%	572

6.5 Results Summary

Overall

A total of 132 particpants were assessed in this study. According to the LA definition, there were 119 controls and 13 cases. As expected, cases were found to be significantly older and had higher systolic and diastolic BP. No other significant differences were detected using a two-tailed Mann-Whitney test.

Using the LI definition, there were 126 controls and 6 cases. Cases were also found to be significantly older and had higher systolic BP.

Age, hypertension and hypercholesterolaemia was identified as signficant risk factors for LA. No significant risk factors were identified for LI, although age and diabetes were marginally not significant (p=0.06 for both).

SVD and EF

Of these 132 participants recruited, 66 controls and 6 cases using the LA definition underwent ApT. Again, cases were significantly older and had a significantly higher systolic blood pressure. Baseline values and the final GTN and salbutamol AAIx were significantly different between cases and controls, however, there was no significant difference detected in the global EF (AAIx and PAIx) between cases and controls.

Of the 72 participants who underwent ApT, 70 were controls and 2 were cases according to the LI definition. Age and systolic BP were the only two variables that were significantly different between the cases and controls – cases were older and had higher systolic BP compared to the controls. There was no significant difference detected in the global EF (AAIx and PAIx) between cases and controls.

EF and genetics

All participants were gentoyped for eight candidate polymorphisms: IL-6 -174 G/C, N-Ox p22 phox 242 C/T, tPA -7351 C/T, tPA -4360 G/C, tPA 20324 C/T, eNOS -786 T/C, ET-1 +138A D/I and PON1 -107 C/T. Again using the cohort that underwent ApT (n=72), the tPA 20324 TT genotype was found to have significantly different global EF values ($p<0.01$) from the CC/CT genotypes, even after adjustment for age, gender, systolic BP, diastolic BP, total cholesterol, glucose levels, smoking, fish oil use and physical activity. The overall model with the variables included was significant ($p=0.05$).

When removing all partipants with LI or LA from this cohort (n=65), the tPA 20324 TT genotype was also found to have significantly different global EF values from the CC/CT genotypes in a univariate regression model ($p=0.02$). However, after adjustement for all risk factors listed previously, the coefficient remained significant ($p<0.01$), but the overall model was not found to be significant ($p=0.18$).

No significant associations were observed between EF and the other seven candidate polymorphisms. R^2 values for all linear regression models were poor, although the adjusted models showed improved R^2.

SVD and genetics

All 132 participants who were involved in the study were included to investigate the association between the eight candidate polymorphisms and SVD. Univariate analyses for LA detected no significant relationships between genotypes and SVD status. Multivariate

analyses for LA, adjusted for the necessary factors, were also non-significant for all dominant and recessive models.

Univariate analysis for the tPA 20324 TT with LI was significant, while multivariate analyses were also significant (p=0.03) when adjusting for age, hypercholesterolaemia, diabetes, sex and hypertension. However, this result does need to be considered with caution as there were only six LI cases.

CHAPTER 7 DISCUSSION

7.1 Introduction

This discussion will aim to address the results and the relevance of the results according to the three main foci of this study: the association between SVD and EF, the genetic predisposition of EF and the genetic predisposition of SVD. All results will be discussed with reference to the hypotheses and previous studies, whilst the discussion of genetic principles will be stratified according to individual polymorphisms. The study sample and limitations also be addressed in detail.

7.1.1 Prevelance of Cerebral Small Vessel Disease

7.1.1.1 Leukoaraiosis

The prevalence of LA in our sample (mean age of 55.3 ± 12.0 years) was 15.2%, while 9.8% of the sample was found to have a clinically relevent degree of LA (modified Fazekas grade 2 and 3). A Japanese study showed that 28.8% (of 1030 persons) of healthy persons had some degree of SVD, also in a relatively young (mean age 52.7 ± 9.6 years) cohort [516]. The Copenhagen Stroke Study had an LA incidence of 15% (163 out of 1084), although this was in an older sample with a mean age of over 70 years [71]. Since LA is associated with increasing age, it would be expected that the Copenhagen Stroke Study sample would have a higher incidence of LA than this study sample. The Austrian Stroke Prevention Study showed that in a group of 296 participants, 41 participants (13.9%) had a clinically relevant degree of LA (defined as early confluent or confluent) in a sample with a mean age of 59.9 years [4]. Although our study has a comparatively small sample, the overall prevelance of SVD is similar to that in the much larger Copenhagen Stroke Study, while the prevalence of a clinically relevant degree of LA is similar to that in the Austrian Stroke Prevention Study. The Austrian Stroke Prevention Study also has a sample with a mean age comparable to our study. With respect to SVD, this suggests that our sample is a reasonable representation of a normal sample even though our study is relatively small. The discrepancy in prevelance between the Japanese study and our study may be due to ethnic differences.

Overall, hypercholesterolaemia was identified as a significant risk factor with an OR of 7.0, while as expected, age and hypertension were also found to be risk factors for LA, with

significant OR of 3.4 and 4.9 respectively. This agrees with previous studies which identified age and hypertension to be important risk factors for LA [28, 64-66, 68, 70-72]. Diabetes was marginally not significant.

7.1.1.2 Lacunar Infarction

The prevalence of LI in our sample was 4.54% and the mean age of those with LI was 66.00 ± 9.78 years. In an Australian community-based study, 37 out of 477 (7.75%) participants aged between 60 and 64 years (mean age of those with LI was 62.51 ± 1.52 years) were found to have at least one LI [517]. In our study as well as the larger community-base study, all the participants with LI were Caucasian. The similarity in the descriptive statistics between the larger study and our study suggest that our sample, although small, is a reasonable representation of a normal population.

In a larger American study consisting of 3660 participants aged 65 years or over, 23% had more than one LI [83]. The mean age and ethnicity of the population were not indicated. The higher prevalence of LI in this American study may be accounted for by the older age group used compared to our study, where the mean age of the overall sample was 55.3 ± 12.0 years.

7.2 Association between Cerebral Small Vessel Disease and Endothelial Function

Hypothesis: *Impaired EF is associated with SVD. i.e. Individuals with SVD proven by MRI will have lower values of global EF (as determined by ApT) compared to those without SVD.*
Result: *EF was not significantly associated with SVD. Those with SVD did not exhibit lower values of global EF compared to those without SVD. Subgroup analyses stratified for SVD phenotypes revealed that LA risk is non-significantly altered by global EF values.*

The descriptive statistics of the ApT groups did not differ from the descriptive statistics of the overall study sample. Only age and systolic BP were significantly different between LA cases and controls, as would be expected when classifying participants based on LA.

Stratification for SVD phenotypes suggested that particiapnts with LI have better EF than those without, and the unadjusted OR of 15.66 (95% CI 0.06 – 4404.62, p=0.34) for EF with LI indicates that the risk of LI increases as the global EF AAIx ratio increases. However, this relationship did not reach significance. The relationship that our results suggest is contradictory to previous reports where LI cases had poorer EF [29, 58, 269, 399]. Our results may

be reflecting a Type I error generated from the small number of participants with LI (n=2) in the ApT sample.

Conversely, LA controls were found to have non-significantly higher values of global EF compared to LA cases. Although non-significant, this result agreed with the hypothesis that people with LA would have poorer EF compared to those without LA. The mean global EF AAIx for controls was higher than cases following adjustment for confounding influences, including age, gender, hypertension and diabetes, which indicated that the risk of LA decreases as global EF AAIx increases (adjusted OR 0.03, 95% CI 0.00008 – 9.52, p=0.23).

The statistical power for detecting a difference in the global EF AAIx using α=0.05 for LA was 12.2% and 15% for LI. Using these results, the estimated total sample size (1:1 case/control ratio) required to determine a statistically significant difference of α=0.05 and power of 80% is 444 for LA and 66 for LI. The estimated sample size based on the results of this study is far larger than the sample size recruited, and thus may explain why there is only a trend in the results and no statistically significant associations.

A search of the litereature revealed one study by Soiza et al. with similar results to our study [57]. The authors performed a small study using PWA to correlate EF with white matter lesions, but did not find any significant association between impaired EF and WMH. They also had a small sample size of 25 participants and used a similar global EF index. The major difference between the study of Soiza et al. and our study is the WMH rating scale. We used a 1 (no/mild WMH) to 3 (severe WMH) modified Fazekas scale. Soiza et al. used a 0 (no WMH) to 3 (severe WMH) Likert scale, but individually scored the frontal, parieto-occipital, temporal, infratentorial and basal ganglia regions then combined the score of each region to give a total WMH score out of 15. The authors reported the inter-observer reliability as low (κ<0.5) and that this, together with the small sample size, may have contributed to the lack of association between WMH and EF.

The deficiency in the amount of published evidence showing no association between SVD and EF could also be due to the minimal interest in publishing negative studies rather than an actual lack of association between SVD and EF.

Pretnar-Oblak et al. has previously supported the role of EF in LI [269], as did Chen et al. [399], while Hoth et al. showed an association between EF and LA [400]. These studies used brachial FMD to measure EF showed that subjects with LI or LA have lower EF compared to controls.

Hassan et al. also showed that EF may have a role in both LI and LA, but chose to use markers of endothelial activation rather than the endothelium-mediated vasodilation methods [58]. As endothelial activation is one of the three main processes of EF, the elevation of specific markers, such as ICAM-1, thrombomodulin, tissue factor and tissue factor pathway inhibitor, would indicate the endothelial activation. However, as the authors note in their discussion, the endothelial markers are not unique to brain events and are not time-specific. Thus the elevated markers may be due to injury in other vascular beds at any time and can not be attributed solely to brain injury.

The clinical evidence to support the role of EF in SVD is further supported by studies in vascular histopathology. Early animal studies have shown that the injection of plasmin, a plasma enzyme, into the brain parenchyma resulted in acute fibroid necrosis (i.e. lipohyalinosis) and increased blood-brain barrier permeability in the perforating artery [20], while leakage of plasma in the perforating artery wall initiated the development of lipohyalinosis [21]. A human study has suggested that the blood-brain barrier (or the cerebral endothelium) may be dysfunctional in LI, showing that post-constrast enhancement in the cerebrospinal fluid was greater in LI patients compared to cortical stroke patients [77]. These results suggested that the blood-brain barrier in LI patients were ‘leakier’.

Many of the published studies have used peripheral measures of EF to relate EF with SVD. This indicates that the studies have only drawn conclusions between SVD and systemic EF, rather than cerebral EF. Systemic EF and cerebral EF can only be said to be similar and not identical and this issue may need to be addressed before further cerebral studies using systemic EF can be undertaken. It is important to note that the correlation between peripheral EF and systemic FE has only been addressed recently (published in 2007, after recruitment for this study had started) [518]. This comparative study involving 62 subjects showed that L-arginine reactivity (a measure of cerebral EF [238-240]) was not closely correlated with FMD (a measure of systemic EF), although both were lower in patients with LI when compared to controls [518]. The authors concluded that their results suggest that there may not be a close association between cerebral and systemic (peripheral) EF. Further investigations are required to determine the association between cerebral and systemic EF, and to decide whether measuring systemic EF is a valid method of assessing cerebral EF.

7.3 Association between Genetic Polymorphisms and Endothelial Function

Hypothesis: *The candidate polymorphisms are independently associated with EF.*
Result: *Only the tPA 23024 TT polymorphism was found to be significantly associated with reduced EF following adjustment of confounding influences. No other candidate polymorphisms were shown to be signficantly associated with EF.*

Both uni- and multivariate linear regression analyses showed no significant associations between other candidate polymorphisms and EF. As indicated by the R-squared values, all models had poor predictive power. The equation for linear regression (i.e. Global EF = constant + coeff*genotype), is unable to accurately predict global EF based on genotype groups. The small sample size and differences in the cerebral vasculature (as compared to the coronary vasculature) may contribute to the poor predictive power.

The individual candidate polymorphisms will be discussed in relation to EF below.

7.3.1 Individual Candidate Polymorphisms

7.3.1.1 IL-6 G/C

SVD-free controls:
Dominant model: Global EF = 0.24 + (-0.03 x GC/CC) p=0.67, R^2<0.01
Recessive model: Global EF = 0.23 + (0.15 x CC) p=0.87, R^2<0.01

Data from previous studies showed that the G allele (wildtype) had higher IL-6 plasma levels, although other studies have found conflicting results [408, 412, 414, 415]. Saura et al. found that IL-6 had a direct effect on endothelial cells by inhibiting eNOS via the transcription factor Stat-3 [406]. Thus the hypothesis derived from these studies was that the participants with this polymorphism, which leads to decreased gene expression, would exhibit better EF compared to those participants without the polymorphism. The conflicting results observed in this study between the dominant and recessive models, high p-values and low R^2 values suggest that there is no association IL-6 genotypes and EF.

Brull et al. conducted a study where they investigated the effect of the SNP on EF in healthy volunteers [416]. They measured FMD in 248 healthy young adults (aged 20 to 28 years) and found that there was a non-significant trend (p=0.14) between absolute FMD values (defined

as the absolute change in vessel diameter after increased blood flow) and CC genotype, where the CC genotype was observed to have greater FMD compared to the other genotypes. Resting vessel size was an independent variable for which the regression model was adjusted.

A limitation of our study may be a lack of an objective measure of IL-6 activity, such as IL-6 concentrations. It would have been preferable to include an intermediate correlate to determine the effect of the SNP in EF. The logical pathway of this SNP would be to result in lower IL-6 concentrations and therefore should show better EF. The recessive model is suggesting that the CC genotype has higher values of global EF, which is according to the hypothesis, but the high p-value (p=0.87) indicates that this relationship is not statistically significant. If there were an intermediate correlate, such as IL-6 concentrations, it would provide a better interpretation of the relationship.

7.3.1.2 N-Ox p22 242 C/T

SVD-free controls

Dominant model: Global EF = 0.18 + (0.08 x CT/TT) p=0.27, R^2=0.02

Recessive model: Global EF = 0.20 + (0.16 x TT) p=0.08, R^2=0.05

Both the dominant and recessive models indicate that the participants with the homozygous polymorphic genotype have better global EF compared to the participants with the wildtype allele. This result is in accordance with the hypothesis for N-Ox p22, which was that the mutation would decrease superoxide production and therefore improve EF.

Although AIx is derived from PAIx using a transfer function, the N-Ox p22 model did not show significance when using AIx, but significance was found in the unadjusted model using PAIx. However, since the reproducibility substudy has shown that the PAIx results are not reproducible (r=0.18, p=0.82), the PAIx results can not be accepted in this study.

N-Ox p22 has been described previously to be a significant producer of superoxide, a contributor to ED by reducing NO bioavailability. One animal study has shown that elevated baseline values of FMD and reduced NO are mediated by NADPH oxidase-derived peroxides [519], while another study has shown that the inhibition of NADPH oxidase improved EF [358]. Previous studies have also demonstrated that carriers of the T allele have better EF than carriers of the C allele [448, 449], which would be in accordance with previous evidence that the T allele results in a histidine to tyrosine change affecting subunit stability [438] and therefore

reduce N-Ox function. The consequence would be reduced superoxide production and better EF.

Our results suggest a step-wise increase in global EF, with global EF values increasing as the number of T alleles increases, which would be in accordance with the biological pathway described. Although the result for the recessive model using AAIx is not significant, it is approaching significance (p=0.08) and the power of the model may be improved by using a larger sample. The percentage genotype distribution seen in this study was very similar to that seen in the study by Schachinger et al. [449], although the sample size in our study was slightly smaller than that of Schachinger et al. (n=72 vs 93, respectively).

In the cerebral vasculature, the reduction of superoxide also reduces the amount of H_2O_2 (via a reaction with SODs), which is a potent cerebral vasodilator. Effectively, cerebral vascular tone is in part modulated by the balance of NO (a vasodilator), superoxides (which attenuates the NO effect) and H_2O_2 (another vasodilator). As shown in a previous study, increased NADPH oxidase activity causing vasodilation in the cerebral artery is associated with chronic hypertension [445], which would normally be associated with poorer systemic EF. Thus using a measurement of EF in the systemic (peripheral) vasculature to analyse the effect of this mutation in a cerebral disorder may not be the most appropriate approach.

7.3.1.3 tPA -7351 C/T, -4360 G/C, 20324 C/T

tPA -7351 C/T

SVD-free controls

Dominant model: Global EF = 0.24 + (-0.02 x CT/TT) p=0.79, R^2<0.01

Recessive model: Global EF = 0.23 + (0.15 x TT) p=0.47, R^2<0.01

The results of this study have suggested that participants with the T allele have poorer EF compared to the homozygous CC participants. However, those with the TT genotype will have better EF than carriers of the C allele. The initial hypothesis was that the tPA -7351 C/T would have no effect on EF. The highly non-significant p values and the conflicting results would suggest that this hypothesis is correct. To our knowledge, there has been no study that has directly investigated this SNP in association with EF. This is a novel result.

Of the three tPA SNPs investigated in this study, the tPA -7351 C/T is the SNP with the most evidence for a plausible mechanism of effect. The T allele was shown to influence Sp1

binding and Sp1 is an important factor for transcription promotion, while CC subjects have been shown previously to have a two-fold higher tPA release rate compared to CT or TT subjects [466], although another paper showed that the SNP did not influence tPA release [468]. This evidence suggest that carriers of the T allele should have lower concentrations of plasma tPA, predisposing those with the T allele to altered fibrinolysis and its associated complications.

If we take the view that altered fibrinolysis is a result of ED, rather than a cause of ED, the effect(s) of the SNP may not be evident until ED is present. It could be hypothesised that in the presence of ED, there is reduced tPA activity (as shown in a Japanese study [520]) and therefore those with the SNP (with further reduced tPA release [466]) would be at a higher risk of events. However, the SNP itself would not affect EF directly. Therefore, our results may be plausible in showing no association between the SNP and EF if our sample has normal EF (since the fibrinolytic pathway has not been activated). This would need to be verified in a larger sample, where various degrees of EF are present and the individual genotypes could be compared with the different degrees of EF. A measurement of plasma tPA would also facilitate this investigation.

tPA -4360 G/C

SVD-free controls

Dominant model: Global EF = 0.24 + (-0.02 x GC/CC) p=0.73, R^2<0.01

Recessive model: Global EF = 0.23 + (0.15 x CC) p=0.47, R^2<0.01

The conflicting results between the dominant and recessive models indicate that this SNP does not affect EF, and this is highlighted by the high p-values and poor R^2 values. To our knowledge, there has been no study that has directly investigated this SNP in association with EF. As there is no biological data for this SNP, a hypothesis about the effect of this SNP on EF was difficult to generate. This is a novel result.

tPA 20324 C/T

SVD-free controls

Dominant model: Global EF = 0.23 + (0.004 x CT/TT) p=0.95, R^2<0.01

Recessive model: Global EF = 0.22 + (0.63 x TT) **p=0.02**, R^2=0.08

In contrast to the -7351 C/T and -4360 G/C polymorphisms, only the tPA 20324 C/T has shown agreement between the dominant and recessive models. The results from this study

show that the participants with the TT genotype have better EF than those with the CC or CT genotypes.

The association between the tPA 20324 TT genotype with LI (will be discussed in detail in Chapter 7.4.2.1) suggests that the SNP alters haemostasis to promote impaired fibrinolyis. Fibrinolysis is a pathway of EF, and impaired fibrinolysis would theoretically impair EF, as was demonstrated in a small, early hypertensive Japanese population (50 cases and 10 controls) [520]. Their results showed that EF, as measured using brachial artery diameter following hyperaemia, was significantly negatively correlated with tPA activity ($r=-0.52$, $p<0.01$). However, this is not reflected in our results, where carriers of the TT genotype have higher values of global EF than CC/CT carriers. The following are reasons as to why our results may not conform to the hypothesis:

1. the TT genotype does impair fibrinolysis and the association with EF is false. LI is triggered by a pathway other than EF;
2. the TT genotype improves tPA function or activity and the association with LI is false;
3. the tPA 20324 TT genotype does not affect tPA function or activity and both the associations with LI and EF are false.

It is acknowledged that a small sample size has been used in these analyses, thus the most likely explanation for the association between the TT genotype and EF is that it is a false positive, or Type I error, due to the sample size and genotype distribution. In the sub-cohort of SVD-free controls (n=65), the genotype distribution for this SNP was: CC=46, CT=18 and TT=1, indicating that the association could be heavily influenced by the EF of the single TT carrier. This investigation needs to be repeated in a much larger sample to validate this result and the observed association needs to be considered with caution.

An extensive literature search has not revealed any studies that have directly investigated this SNP in association with EF. This is a novel result.

7.3.1.4 eNOS -786 T/C

SVD-free controls:

Dominant model: Global EF = 0.21 + (0.03 x TC/TT) $p=0.64$, $R^2<0.01$

Recessive model: Global EF = 0.22 + (0.06 x TT) $p=0.53$, $R^2<0.01$

eNOS is a key enzyme in maintaining normal EF, thus any changes in eNOS and NO production would affect EF. The eNOS -786 T/C is located in the promoter region and the C alleles have been reported to reduce gene transcription by up to 50% [285]. An essential binding protein (RPA1) was found to bind specifically to the C allele, and acted as a repressor protein to inhibit gene transcription [476]. From these studies, it would be hypothesised that carriers of the C allele would have less eNOS and therefore poorer EF. Both the dominant and recessive models, although unadjusted and are not significant, suggest that carriers of the C allele have slightly better EF compared to carriers of the T allele.

Other studies have shown either a result in accordance with the hypothesis [521], or a result that agreed with those of ours (i.e. no effect) [325]. Both other studies used healthy, young males, although ethnicities varied (Japanese [521] and Caucasian [325]), and both studies had relatively small sample sizes (60 and 100 subjects, respectively).

7.3.1.5 ET-1 +138A D/I

SVD-free controls:

Dominant model: Global EF = 0.25 + (-0.05 x DI/II) p=0.50, R^2<0.01

Recessive model: Global EF = 0.25 + (-0.17 x II) p=0.14, R^2=0.03

This polymorphism is an deletion (D)/insertion (I) polymorphism where the polymorphism results in an extra adenosine being added to the sequence. II carriers were shown in a past study to have significantly higher mRNA levels compared to DI and DD carriers, which was suggested to be due to enhanced mRNA stability and increased mRNA half-life [491]. As a consequence, it would be expected that II carriers would have higher ET-1 protein levels. This was also shown by Popowski et al., where it was demonstrated that II carriers had significantly higher ET-1 protein levels compared to ID and DD carriers [491].

ET-1 has been demonstrated previously to decrease NO production by upregulating caveolin-1, a major negative regulatory protein of eNOS [522, 523]. ET-1 was also shown to increase eNOS/caveolin-1 binding [524]. ET_A [525] and ET_B [428] receptors have also been shown to increase ROS, which negatively impact on EF as previously discussed. Thus, based on these previous studies, it would be expected that those with the II genotype would have lower global EF values compared to those with the ID or DD genotypes.

Our results appear to agree with this hypothesis although the associations are non-signficant. A step-wise decrease in global EF as the number of I alleles increased was also observed. To our knowledge, there are no other studies that have directly investigated the role of this polymorphism in EF.

7.3.1.6 PON1 C/T

SVD-free controls:

Dominant model: Global EF = 0.24 + (-0.01 x CT/TT) p=0.89, R^2<0.01

Recessive model: Global EF = 0.22 + (0.02 x TT) p=0.77, R^2<0.01

The non-significant results combined with the conflicting results between the dominant and recessive models suggest that this PON1 polymorphism has no effect on EF.

PON1 has been shown in the past to reduce oxidative stress and improve endothelial cell function in mice with pre-existing atherosclerosis [526]. The -107C allele was shown to double the transcriptional activity of the PON1 gene compared to the T allele, and the -107 C/T accounted for approximately 25% of the variance seen in serum PON1 levels [501, 502]. Increased PON1 activity has also been directly related to greater brachial FMD, a measure of EF (r=0.46, p=0.004) [500]. Thus, this evidence suggested that the T allele would show lower values of EF compared to the C allele.

There are very few studies that have directly investigated the association between this polymorphism and EF. This may be because the -107 C/T SNP is in strong linkage disequilibrium (p<0.001) with another polymorphism, the PON1 L54M (leucine to methionine substitute in position 54) [502]. The PON1 L54M was not found to be significantly associated with coronary EF [527], and from the strong linkage disequilibrium, it could be inferred this result could be extended to the -107 C/T SNP. If so, the results of this study are in agreement with the Lavi, et al. study, suggesting that the PON1 -107 C/T does not modulate EF.

7.4 Association Between Genetic Polymorphisms and Cerebral Small Vessel Disease

Hypothesis: *SVD has a genetic predisposition. Cases and controls will exhibit different genotypes.*
Result: T*he tPA 23024 TT polymorphism was found to be a significant determinant of LI. No other candidate polymorphisms were found to be associated with SVD.*

SVD, like large-vessel disease and most other chronic diseases, would be a polygenic disorder with multiple weakly significant polymorphisms and genetic aberrations combining synergistically to contribute to the overall risk.

In this study, no significant associations were detected between SVD and seven of the eight candidate genetic polymorphisms: tPA -7351 C/T, tPA -4360 G/C, IL-6 -174 G/C, N-Ox p22 phox 242 C/T, eNOS -786 T/C, ET-1 +138 D/T and PON1 -107 C/T. Only the tPA 23024 C/T polymorphism was observed to have a significant association with SVD. The frequency of all the homozygous polymorphic genotypes in the control sample was similar to those seen in the literature (see **Table 3.2**).

The increased risk of SVD with the mutant alleles (dominant model) for IL-6, tPA 20324, eNOS and ET-1 are in accordance with previous studies. Both NAPDH and PON1 showed a decreased risk of SVD, as was hypothesised based on biological and functional data. However, none of these associations were statistically significant. The results for each candidate polymorphism are discussed below.

7.4.1 Hardy-Weinberg Equilibrium

All polymorphisms were in Hardy-Weinberg (H-W) equilibrium except for IL-6 (p=0.024). H-W disequilibrium usually has two causes: (i) ethnic diversity and (ii) genotyping errors. Kocsis et al. suggested that strong deviations from H-W equilibrium, especially in controls, are the result of genotyping errors [528]. Controls in this sample had an H-W equilibrium of p=0.01 and cases were p=1.0, which fits the situation described by Kocsis et al.. Banding on the gel was defined and the repeated genotyping for 13 (out of a possible 132 participants) randomly selected participants per polymorphism showed no errors with the initial result. Hence, it is unlikely that H-W disequilibrium in IL-6 is due to genotyping errors. 128 (97%) of all participants were Caucasian, with the remaining four being Asian (n=3) and Aboriginal

(n=1), thus, ethnic diversity is also unlikely to be the reason for the observed H-W disequilibrium.

Another possible reason for deviations are null alleles, which occur when there is a mutation in the primer binding sites, thus resulting in the non-amplification of product [529]. Thus it may be that a mutation in the binding site has caused a product not to appear even if the mutation of interest is present. It is unlikely that this is the reason for the disequilibrium in IL-6 as all primers were checked using a BLAST search and were also checked against known sequences (and SNPs) in Entrez Nucleotide.

Thus, the reason for IL-6 H-W disequilibrium in this sample is unknown, although the small sample size may have had a role.

*7.4.2 **Individual Candidate Polymorphisms***

7.4.2.1 tPA -7351 C/T, -4360 G/C, 20324 C/T

A significant association was observed between tPA 20324 TT and LI (adj OR 12.96, p=0.03). No other associations were observed between the other tPA SNPs and SVD. The initial reason for selecting these three SNPs was to validate results seen in other small studies.

tPA -7351 C/T

Our study has not observed an association between the tPA -7351 C/T polymorphism and LA or LI. As discussed earlier in Chapter 7.3.1.1, this SNP is the only tPA SNP out the three investigated that has a plausible pathway for the mechanism of effect. One study has found an association with LI [107], although the result was not repeated in other studies [108, 109]. However, all three previous studies found OR greater than 1 (indicating an increased risk), and likewise, this study showed a non-significant OR of 1.82 for the recessive model with LI. For LA, a non-significant OR of 0.86 was observed (p=0.90).

In studies that investigate SVD, the ability to distinguish and correctly identify phenotypes is of utmost importance. Some studies have used clinical classification systems while others have used a radiologically-based method. The OCSP classification system [7], as adopted by Jannes et al. [107], classifies a stroke based on the presenting symptoms. Thus, it is possible that

there may have been classification errors – a large-vessel stroke could have been misclassified as a small-vessel stroke because of the presenting symptoms, and vice versa.

Studies using the TOAST criteria [108] may also have these issues, although the likelihood of misclassification with TOAST is reduced due to the corroboration of symptoms with CT/MRI. However, both OCSP and TOAST are clinical classifications aimed at subtyping ischaemic stroke and therefore overlook LA burden. The severity and/or presence of LA can not be determined clinically. Studies that use MRI [109] as the basis for determining SVD load are less likely to result misclassification, while also being able to measure LA, even if only semi-quantitatively using a visual rating scale. Our study used MRI as the basis of SVD classification and therefore we were able to assess LA burden and better identify the presence or absence of LI.

It is also possible that because TOAST and OCSP rely on presenting symptoms for stroke classification, there is a bias towards detecting the larger, more symptomatic LI caused by microatheroma. The theory is that the large, symptomatic LI have different pathophysiologies to the smaller, less symptomatic LI, which are caused by arteriolosclerosis and lipohyalinosis [39]. In our study, five of the six LI cases had LI with diameters less than 5mm, meaning that these LI were classified as 'small'. We therefore had an LI cohort that was phenotypically similar, thus our results are more reflective of the small LI with an ateriolosclerosis/lipohyalinosis basis. The previous studies using TOAST and OCSP are relevant for the large LI with an occlusive cause. The different pathophysiologies in the LI subtypes may partly explain the differences in results between the original positive Jannes et al. study and this study.

<u>tPA -4360 G/C</u>

The results for the relationships for tPA -4360 G/C with a SVD sample is novel. The only previous study analysing this SNP in CVD was in a generic ischaemic stroke cohort that was not stratified into stroke sybtypes [470]. Our results showed a non-significant adjusted OR of 0.5 for LI (p=0.45) and 0.44 for LA (p=0.24). Both results are for the dominant model only as there were no cases with the CC genotype.

A hypothesis for the effect of this SNP in SVD was difficult to formulate because: (i) this is a novel investigation and (ii) there is no obvious pathway for the SNP effect due to the lack of molecular data available. Saito et al. showed that the increased risk of ischaemic stroke due to tPA -4360 and tPA 20324 was only significant when both homozygous polymorphisms were

present together [470]. Because the tPA -4360 CC and tPA 20324 TT genotypes were not found together in any of the cases, the increased risk observed by Saito et al. was not able to be verified in this study, and it may be that the significant observation is only evident when this SNP is in combination with the tPA 20324 TT genotype.

tPA 20324 C/T

This study has shown a significant association between tPA 20324 TT and LI, with an OR of 12.96 (95% CI 1.26 – 133.52, p=0.03), adjusting for age, gender, hypertension, hypercholesterolaemia and diabetes. A gene-dose effect was seen with the polymorphism, with the OR increasing with the number of T alleles present. The recessive model also showed a higher OR compared to the dominant model (OR 5.2, p=0.06).

The heavy chain of tPA has two triple disulfide bonds, otherwise known as 'kringles' [462]. It is thought that these kringles are important in fibrin binding as kringles are also found in prothrombin, plasminogen and urokinase. tPA 20324 C/T is located in intron F and this intron divides one of the kringle codon regions into two exons. It may be possible that because of the close proximity between the SNP and an important binding region, the 20324 C/T SNP affects the kringle coding, hence affecting protein structure and function. If the SNP increases stroke risk, it is possible that the SNP decreases tPA function. So, hypothetically, it could be that the SNP affects the kringle structure (such that it does not bind to fibrin as effectively, thus resulting in reduced function), therefore leading to a decreased ability to dissolve fibrin and increasing the risk of LI.

Currently, there is very little published information regarding this polymorphism. tPA 20324 is located in an intron that neighbours an exon encoding a vital area, but any insight into the mechanism of how it affects stroke risk is purely hypothetical with the evidence available. Mechanisms are also yet to be suggested in the literature. Saito et al. suggested that tPA 20324 C/T may be in linkage disequilibrium with another SNP within the gene because of the intronic location of the SNP [470].

Saito et al. also investigated this SNP in ischaemic stroke, but did not observe any associations. There may be two reasons for the differences in results between our study and that of Saito et al.: (1) samples are genetically different – the Saito et al. sample is Japanese, where as the sample in this study is predominantly Caucasian; and (2) Saito et al. did not divide their patient group into the subtypes of ischaemic stroke, so if the 20324 T/T risk is

only associated with LI, the relationship may not have been evident in a generic ischaemic stroke cohort.

Our findings support the role of altered fibrinolysis in the pathogenesis in LI. Fibrinolytic acitivity (as measured using the euglobulin lysis time) has been shown to be lower in a larger number of patients with LI compared to those with a large-vessel infarction (67% and 20% respectively) [530], while animal brain studies show that tPA expression is limited to the endothelial cells of the brain capillaries with no expression of tPA in the larger arteries under normal circumstances [458-460]. Fibrinogen and prothrombin levels have also been found previously to be elevated in LI [531, 532].

Acute tPA release from the endothelium is considered to be the one of the main mechanisms in facilitating the dissolution of arterial thrombi [463]. The normal plasma tPA concentration is approximately 3 to 10 ng/mL, but only a fraction of this is active, as tPA is deactivated by serine protease inhibitors, such as PAI-1 [533]. Concentrations of active tPA are inversely correlated with plasma PAI-1 concentrations [534], and the kinetics of the interaction between PAI-1 and tPA indicate that the acute release of local active unbound tPA (from the endothelium) is vital for the dissolution of the thrombus [535]. tPA expression has been observed to be more intense in the white matter of the brain compared to the grey matter in human autopsy brains [536], suggesting that the role of tPA and fibrinolysis may be more important in small vessels compared to the larger vessels, and why the small vessels would be more susceptible to changes in tPA than the larger vessels.

It is acknowledged that this signficant association was observed in a small sample size with only 6 cases and the statistical analyses showed large 95% confidence intervals. Post-hoc power calculations (**Table 6.25**) show that the study is under-powered at 63%. Small sample sizes are also more prone to Type I errors (false positives), which may be the situation here. Nevertheless, this result is hypothesis-generating and because there have been very few studies incorporating this SNP, this result may provide useful information for future studies.

Although there is the possibility that this is a Type I error, this result can not be completely disregarded when looking at the results as a whole, instead of specifically with LI. tPA is a gene involved in fibrinolysis so it wold be logical to hypothesise that any polymorphisms (if it had a clinical outcome) would affected fibrinolysis and thrombus formation. Altered fibrinolysis and thrombus formation are involved in LI, but are not part of the LA pathophysiology. Therefore, if a positive result were also seen between this SNP and LA, it

would be nonsensical and we would be more inclined to disregard the result as a false positive.

This study also observed an association between the tPA 20324 C/T and EF, where the carriers of the TT genotype had a higher global EF ratio compared to CC/CT carriers. This observation has been discussed previously in Chapter 7.3.1.3. Considering these two significant but under-powered results (tPA 20324 C/T with EF and LI) together suggests that this SNP may be altering the LI risk using a pathway other than altering EF. Again, knowing that tPA is involved in fibrinolysis, it is plausible that a tPA SNP may be exerting its effect by modulating haemostasis directly rather than via EF. However, both results and the hypothesis generated here need to be considered with caution as all analyses have involved a small sample.

7.4.2.2 IL-6 -174 G/C

The results of this study do not support the involvement of the IL-6 -174 G/C polymorphism in LI or LA (dominant model: adjusted OR 0.77, p=0.77 for LI and 2.66 p=0.15 for LA). The results of this study suggests that the C allele (as represented in the dominant model) is protective for LI as was predicted, although the result is non-significant and must therefore be considered carefully. The C allele was also not significantly associated with a higher risk for LA. There could be two explanations for this result: (i) the IL-6 -174 G/C polymorphism was not found to be in Hardy-Weinberg equilibrium, thus the results are not accurate; and (ii) there were only six LI cases, none of which had the homozygous polymorphic genotype.

Revilla et al. showed that the CC genotype was significantly better represented in people with LI compared to controls, with a reported OR of 4.29 when adjusting for hypertension, diabetes, hyperlipidaemia and smoking [418]. An OR of 3.22 was reported by Chamorro et al. for the same relationship [419]. A common factor of both studies is that neither study subtyped LI into single, large LI and multiple, small LI. As discussed previously, it has been proposed and increasing evidence supports the theory that the two subtypes of LI have different pathophysiologies [13-15, 29, 39].

It is interesting that these two studies have found the CC genotype to be associated with LI. According to the molecular data of the SNP, the G to C change creates a potential NF-1 binding site, and NF-1 has been shown to be a repressor of gene expression. Theoretically, if the C polymorphism is present, there should be reduced gene expression (due to NF-1

binding), thus less IL-6 being produced leading to decreased inflammation. Since inflammation in implicated in the progression of SVD, the C allele should hypothetically reduce the risk of SVD. Although our results are non-significant, they suggest that the C allele increases the risk of LA (adjusted OR 2.66, p=0.15) while it decreases the risk of LI (adjusted OR 0.80, p=0.79). The results for LI are in accordance with the hypothesis, while the results of LA agree with previous studies. However, these results must be considered with caution as neither has reached signifance and associations are based on a small number people with the C allele.

Elevated IL-6 levels have been implicated previously in increasing SVD risk. Several studies have detected assocations between elevated IL-6 concentrations and LI in acute [418, 419, 537, 538] LI populations. In a non-acute LI population, IL-6 levels have also been associated with increasing WMH risk [410]. The same study also found the G allele increased the risk of WMH (OR 1.14, 95% CI 1.14 – 1.28), which is in accordance with the hypothesis for the SNP.

A study by Baune et al. showed that no single cytokine (of the ones investigated) was associated with WMH or LI, thus suggesting that the effects of cytokines in LA and LI are due to a combination of cytokines rather than a single cytokine [539]. The results of that study suggest that the investigation of cytokines in SVD should not be limited to individual cytokines, and should incorporate several cytokines and haplotype analyses. Thus, it may be that the results of our study do not agree with the hypothesis, but if undertaken in combination with other cytokines in the future, results could be more promising.

7.4.2.3 N-Ox p22 phox 242 C/T

The results of this study suggest that the N-Ox p22 phox 242 TT genotype may increase the risk of LI (adjusted OR 5.37, 95% CI 0.77 – 37.46, p=0.09), but does not alter LA risk (recessive model adjusted OR 1.05, 95% CI 0.18 – 6.04, p=0.96). The hypothesis, based on a previous study, was that the polymorphism would increase LI risk. The effect of the polymorphism on LA was unclear, but was hypothesised to have a similar effect on LA as it would on LI. This polymorphism was found to be Hardy-Weinberg equilibrium.

The role of this polymorphism in SVD is unclear, with conflicting results in non-Caucasian populations [450-452]. Theoretically, the mutation creates less superoxide, thereby improving EF. However, by reducing superoxide, there is a reduction in H_2O_2, which reduces cerebral blood flow since H_2O_2 is a vasodilator and therefore increases the risk of ischaemia. A study by

Kuroda et al. showed that there was no difference in superoxide production between the C and T allele [452].

Khan et al. investigated the role of this SNP in SVD in a Caucasian population [453]. They reported that there were no significant associations with either the dominant or recessive models with SVD (determined by TOAST), isolated LI (defined as LI with absent/mild LA) or ischaemic LA (defined as LI with moderate/severe LA). Khan et al. show a highly non-significant neutral OR for isolated LI vs controls, whereas our results for LI show a trend suggesting a five-fold increased risk of LI associated with the TT genotype (p=0.09). The difference in results may be arising from the genotype distribution, where in our cohort of six cases, only one had the homozygous wildtype (CC) genotype, two had the homozygous polymorphism (TT) and the rest were heterozygous. Our genotype distribution and small sample size suggests that the trend observed is more likely due to a Type I error rather than a reflection of a true association.

A possible explanation accounting for the discrepancy in associations between our study and that of Khan et al. for this polymorphism in LI and in LA may relate to the phenotype classification used. The TOAST subtyping of strokes is based on both the clinical presentation and MRI/CT. The TOAST definition of SVD is the presentation of a lacunar syndrome without cortical or cerebellar dysfunction with a subcortial or brain stem infarct less than 1.5cm [38]. As discussed earlier in Chapter 3.3, the definition has a bias towards the larger LI caused by occlusion as these tend to be more symptomatic than the smaller LI caused by ateriolosclerosis and lipohyalinosis [14]. The definition also does not include LA. Thus, this may be the reason why Khan et al. also included the isolated LI and ischaemic LA defintions with separate analyses. Their study also has greater statistical power as they have a far larger population, with 316 cases and 638 age- and sex-matched controls. The authors specify that their power calcuations show that they are statistically powered to detect associations with an OR of 1.5 and above.

The results of our study partially agree with the hypothesis, with the SNP showing a tendency to increase LI risk, but not for LA. Our results also do not fully agree with a previously published SVD study [453], and this can partially be attributed to the different SVD defintions used in their study compared to ours, as well as the difference in sample size. As there has only been one substantial study published regarding the relationship between this SNP and SVD in a Caucasian population, further investigations need to be undertaken to verify the results seen in previous studies as well as this study.

As discussed in Chapter 3.3, vascular N-Ox has different isoforms with Nox4 being of particular interest in the cerebral vasculature. p22 phox is associated with the Nox1/Nox2 and Nox4-containing isoforms, and thus the polymorphism of interest (242 C/T) would theoretically affect all three isoforms. For future studies, it would be interesting to see whether genetic polymorphisms specific to individual isoforms would have an effect on SVD or EF. Further investigations to elicit the role of individual isoforms in the cerebral vasculature clearly are also required.

7.4.2.4 eNOS -786 T/C

No significant association was observed between the eNOS -786 T/C polymorphism and LA (dominant model adjusted OR 2.82, p=0.15). Associations between this polymorphism and LI were unable to be investigated due to the genotype distribution. This polymorphism was found to be in Hardy-Weinberg equilibrium. Associations between this polymorphism and LI were unable to be investigated as there were no LI cases with homozygous genotypes (wildtype or polymorphic).

Knock-out mice lacking the eNOS gene have been shown to exhibit brain lesions similar to leukoaraiosis [303]. The eNOS -786 T/C genotype has also been shown to affect NO levels in the body, but the effect was only seen when the eNOS intron 4ab genotype was also present [477]. Based on physiological mechanisms, it is possible that alterations in endothelial NO would affect the risk of SVD as endothelial dysfunction would lead to: decreased cerebral blood flow (as vessels would not be dilating as effectively), breakdown of the cerebral endothelium (ie weakening the blood-brain barrier) thus resulting in cerebrovascular remodelling (arteriolosclerosis and lipohyalinosis). Therefore it would be more likely that changes in endothelial NO would affect the small, multiple LI subtype and LA rather than the single, large LI subtype.

The role of eNOS SNPs in SVD have been demonstrated in past studies, with the eNOS -786 C/C genotype being more prevalent in SVD cases. Although the relationship between eNOS and SVD in this study has not been significant, the results show a trend that agrees with the hypothesis and is in accordance with the results from previous larger studies. For LA, the OR was 2.82 (95% CI 0.70 – 11.38, p=0.15) after adjusting for hypercholesterolaemia, diabetes and gender. The OR associated with LI was not able to be calculated due to the genotype distribution among the LI cases.

Our results are in accordance with those published by Hassan et al. [477], who performed a similar study investigating the role of the three main eNOS SNPs in SVD (both LI and LA individually and combined). For the -786 T/C, Hassan et al. found no significant differences in the OR for all SVD, LI and LA. This group had a much larger sample size of 600 controls and 297 SVD cases. All OR reported for this SNP was approximately 1, suggesting that the eNOS SNP does not affect SVD risk. Our results, although non-significant, agree with those of Hassan et al. in suggesting that the -786 T/C polymorphism does not independently play an important role in SVD.

7.4.2.5 ET-1 +138A D/I

This study does not support the role of the ET-1 +138A D/I polymorphism in LI (dominant model adjusted OR 4.00, 95% CI 0.45 – 35.23, p=0.21) or LA (dominant model adjusted OR 1.59, 95% CI 0.45 – 5.57, p=0.40). This polymorphism was found to be in Hardy-Weinberg equilibrium.

Although the role of ET-1 has been well documented in ED, the evidence for the involvement ET-1, especially the +138A D/I polymorphism, in SVD is less well defined. Gormley et al. showed no relationship between SVD and four polymorphisms in the endothelin receptors A and B [540]. To our knowledge, there are no studies that have directly investigated the +138 D/I polymorphism in SVD.

The hypothesis for the involvement of ET-1 in SVD is based on the insertion polymorphism leading to higher concentrations of ET-1 by enhancing protein and mRNA stability [491], resulting in increased vasoconstriction by ET-1. Increased cerebral vasoconstriction then leads to chronic ischaemia resulting in LA and LI. Thus we hypothesised that the polymorphic genotype (the insertion) would be more prevalent in cases than controls.

The results for ET-1 in LA are conflicting. The analyses for the dominant model suggest that the polymorphism increases the risk (adj OR 1.59, p=0.40), but the recessive model analyses indicate that the polymorphism decreases the risk (adj OR 0.77, p=0.81), although no analyses were significant. These observations may be attributed to the low number of cases with the homozygous polymorphic genotype (n=1).

For LI, there were no cases with the homozygous polymorphic genotype and thus analyses for the recessive model were not possible. The dominant model analysis agrees with the hypothesis and indicates that the polymorphic allele increases the risk of LI (adj OR 4.00, p=0.21), although again the association is not significant. It may be that the association would become clearer with a larger sample size.

7.4.2.6 PON1 -107 C/T

The results of this study do not support the involvement of the PON1 -107 C/T in LI (dominant model adjusted OR 0.63, p=0.47) or LA (dominant model adjusted OR 2.09, p=0.52). PON1 is involved in lipid metabolism and previous studies have shown that the -107 C/T SNP alters gene transcription [501, 502]. The initial hypothesis based on these studies is that carriers of the T allele would have a higher risk of SVD due to increased LDL.

As mentioned before, this SNP is in linkage disequilibrium with the PON1 L54M [502]. The PON1 54L allele has been previously shown to be significantly associated with a greater extent and progression of white matter lesions over three years in a population with a mean age of 59.9 years [111]. As the two SNPs are in linkage disequilibrium ($p<0.001$) [502], the effect of the -107 C/T SNP may have a similar trend to the L54M and the association between -107 C/T and LA or LI may not be evident until lesion progression can be assessed in our sample.

7.5 Study Limitations

7.5.1 Recruitment

The study recruited participants who potentially already have an underlying neurological disorder, hence the reason for an MRI. Due to limited funding, participants were recruited from patients who already had undergone a MRI brain. Eligible participants had scans for: stroke/TIA (although no radiological evidence), headache/migraine, dizziness/vertigo, tinnitus, epilepsy and a variety of other reasons. The effect of these conditions on EF or SVD has not been clearly defined and thus these conditions may have an unknown impact on the final results. If we were to exclude participants with any of these conditions, then the final sample would be severely depleted and our recruitment method would not be suitable.

A solution to this would be to recruit all participants from the community and perform an MRI on all eligible participants. The chances of selecting people with no neurological or

cranial issues would be much higher, as would the number of healthy controls, which would improve the power of study. However, recruiting community volunteers who do not have any previous issues and has not had a MRI has two main issues:

(i) the cost of the study: each MRI is approximately $600 per participant depending on the sequences required. The current methodology means that participants already have a recent MRI and we did not need to pay for any extra radiological testing;

(ii) requiring participants to undergo a potentially uncomfortable procedure that they would normally not require. Upon discussion with the participants of our study, many highlighted the discomfort they felt while undergoing the MRI. Noise and claustrophobia were the most common problems mentioned.

The other option would be to expand the inclusion criteria to include those with dementia, previous strokes and the use of vasoactive drugs. This would also produce a sample of patients more representative of the general population and allow the results to be better extrapolated into a clinical situation. The reason why these where originally exluded were to ensure that there were no variables that may potentially affect endothelial function and SVD other than those being investigated. However, in the attempt to achieve a clean sample, recruitment was severly hindered thus limiting sample size.

Because of the recruitment process, it is possible that selection biases may have arisen. For example, our recruitment process has favoured English-speaking participants and those who were able to travel to the hospital. We are thus biasing ourselves towards participants that are less likely to have severe disease.

Also, because our recruitment was targeted at people who already had had an MRI, our recruited sample may not be truly representative of the general population, even though our LA and LI prevalences are similar to those seen in larger studies.

7.5.2 Sample size

The main limitation to this study is the sample size being much less than expected. Only 38.8% of MRI patients were eligible after Screening #1, with age being the main exclusion criterion (23.8% were excluded based on age). Of the eligible people, only 52.1% responded to the invitation, and of these people, only 65% agreed to participate. The main reasons for declining were that people were not interested in the study, and that the distance required to travel was an issue. Because of the location of the hospitals involved, the catchment area for

the study covered most of metropolitan Adelaide. However, due to availability of personnel and logistical issues, clinical assessment was only available at TQEH in western metropolitan Adelaide. Participants were also not reimbursed for travel costs because of limited funding.

According to sample size calculations, the minimum number of participants required for the SNP with the highest polymorphic prevalence (32%) is 96. The prevalences of the other polymorphisms range from 1% to 18%, requiring a sample size between 231 and 5415 (cases and controls combined with a 2:1 case to control ratio). The number of participants required increases as the polymorphic prevalence decreases. A study sample with fewer people is simply not powerful enough to detect any true significant relationships. At the start of the study, we understood that a sample of over 600 was not possible, but a sample of approximately 300 was feasible and the sample size estimations for both EF and genetics showed that a sample of 300 participants would be sufficient for most polymorphisms. The literature showed that tPA 20324 T/C and tPA -4360 G/C had SNP frequencies of 1% and 1.7%, respectively, in healthy populations. Because of the huge sample sizes required for sufficient power, this study was a pilot study for the role of these SNPs in SVD and EF, and to determine the SNP frequency in an Australian population.

For SVD and EF, a sample size can be estimated based on these results. A minimum of 222 participants per group (total 444 participants) would be required to detect a significant difference (α=0.05, power=0.8) in global EF AAIx between LA cases and controls. The power using the current sample size of 66 controls and 6 cases is 0.122 (12.2%).

The post-hoc statistical power calculations and estimated sample sizes for SVD and genetics are shown in **Table 6.25**. All the SNPs have low statistical power, and all require sample sizes much larger than the one available in this study. Thus any assocations identified in this study can not be accepted as true associations, but can be a basis for further investigation.

A solution to the sample size issue would be to expand the target population as discussed in Chapter 7.5.1. Other solutions include multi-centre testing or merging samples with other established control and SVD groups. Although this was not possible within this study, this may be an option for future studies.

7.5.3 SVD Classification

A modified Fazekas scale was chosen to classify SVD, where mild LA cases were grouped with LA-free controls. The reason that this scale was chosen was so that the results would be able to reflect a clinically relevant situation. There will inevitably be some degree of LA in a healthy elderly person [25] and a large proportion of people over 50 years old will have LA [72]. Because of the high prevelance of LA, it is important to distinguish between a clinical and non-clinical level of LA. Although there is no defined volume of WMH at which the LA becomes clinically relevant, the Austrian Stroke Prevention Study (ASPS) showed that early confluent and confluent lesions are more likely to progress [4] and are therefore more likely to have a clinical outcome. The early confluent and confluent definitions used in the ASPS are similar to the modified Fazekas grade 2 and grade 3 used in our study. Our modified Fazekas grade 1 included participants: absent of any WMH, with punctate foci (as these are not believed to be ischaemic in nature), with halo caps or WMH with up to 20% of the WM affected. Thus it is possible that some of our participants, who were classed as modified Fazekas grade 1, may be classified as early confluent by the ASPS. This suggests that our scale is more conservative than that of the ASPS, and would require a greater number of SVD cases to show an association.

7.5.4 Pathophysiology

Due to the small number of cases in this study, it was not possible to subdivide the analyses into: isolated lacunar stroke and ischaemic LA, which is defined as a clinical lacunar stroke with LA; stratify the results according to SVD grade; or subdivide the lacunar sample based on infarct size. Previous studies, such as those by Hassan et al., have subdivided their population into isolated LI and ischaemic LA (LA with lacunar syndromes) to distinguish between LA caused by chronic hypoperfusion and non-ischaemic LA [58, 541]. LI is the result of acute ischaemia, whereas LA arises when chronic ischaemia is present. It is difficult to determine whether our results reflect the differences in pathophysiology between LI and LA due to the small sample involved (only two LI cases involved in the EF analyses).

It has also been proposed that single, large LI are caused by microatheroma, which is different to the arteriolosclerosis and lipohyalinosis of multiple, smaller infarcts [13, 39]. Thus, it may be that impaired EF or genotypes are associated only with one subtype and not another. In the same study, Hassan et al. also found that hyperhomocysteinaemia was only associated with large focal lesions (>5mm) and not small focal lesions (≤5mm) [29]. The authors suggested that

homocysteine may be more important in the development of arteriolosclerosis than microatheroma, which again supports the hypothesis that large and small infarctions have different pathophysiologies.

The advantage of this study is that our LI results reflect a specific LI subgroup, particularly, the small LI that are more likely to be caused by arteriolosclerosis and lipohyalinosis rather than the large infarcts that tend to have an occlusive cause [13, 14]. Five of the six LI cases used in the genetic/SVD substudy had LI that were classified as 'small', i.e. infarcts less than 5mm in diameter; only one case had a large LI (6-14mm in diameter). We therefore have an LI cohort that is phenotypically similar, avoiding the issue of different pathophysiologies between the LI subgroups. However, we can not be absolutely certain of this phenotypic similarity without confirmation, which is only possible via a histological analysis of the affected brain tissue. It would be interesting to see whether the tPA 20324 C/T polymorphism would be associated with specific infarct subgroups, as usually only the larger infarcts have an occlusive cause.

Homocysteine

This study is lacking a measure of homocysteine in participants. Homocysteine has previously been indicated as a risk factor for SVD and not large-vessel disease [29], and previous studies have observed elevated homocsyteine levels in patients with SVD [542, 543]. Other studies have shown that hyperhomocysteinemia impacts on EF [325, 369]. Although considered, a measure of homocysteine was not included into the study due to budgetary reasons. Because our study did not measure homocysteine levels, it is one risk factor that we have not been able to adjust for in our statistical analyses for EF and SVD. Thus our associations between genetics and SVD or EF have not taken into consideration the effect of homocysteine levels.

7.5.5 Endothelial Function: Systemic Versus Peripheral

One assumption made in this study is that a peripheral measurement can accurately reflect the situation inside the small vessels of the brain. Because ED is considered a systemic disorder [236, 375, 544], Targonski et al. speculated that systemic (peripheral) ED can reflect cerebral ED [226]. Many studies that have investigated the relationship between EF and SVD have accepted this and have used techniques that measure systemic (peripheral) EF in their protocol [57, 255, 269, 395, 399, 400].

The advantage of peripheral studies is that the arteries are readily accessible, and the non-invasive nature of peripheral studies was a major factor in selecting ApT as our technique of preference. FMD and PWA both rely on the fact that the radial and brachial arteries are relatively superficial and therefore easy and safe for a measurement of a response. Taking measurements directly from the aorta is more difficult and obtaining aortic EF via the carotid arteries may be uncomfortable for the subject during the testing.

Evidence unavailable at the time of planning has shown that systemic and cerebral measures of EF are not closely associated [518]. In light of this evidence, it would have been preferable to select a method that could measure EF directly in the cerebral arteries. Direct insertion of Doppler wires and infusions into the cerebral microcirculation would not only be costly, but also dangerous. The alternative would be to use transcranial Doppler sonography, which has been used in previous studies to determine cerebral EF [336]. However, the sonography in previous studies has been performed on the carotid arteries, which are again different to the cerebral small vessels, although it is at least part of the cerebral vasculature. The exclusive nature of the cerebral small vessels makes it difficult to assess them directly. Similar issues are encountered in the cardiology field where the investigation of the coronary microvasculature is limited by the ability to access these small vessels directly [545].

Superoxide and peroxynitrites, as described before, are contributors to ED by creating oxidative stress. However, in the cerebral microvasculature, superoxide and hydrogen peroxide (H_2O_2) are strong vasodilators [546, 547]. H_2O_2 and superoxide generate a hydroxyl radical, which then inactivates guanylate cyclase [547, 548]. The non-significant results of this study may be partially explained by the vasodilatory effects of H_2O_2 and superoxide, in addition to the small sample size.

7.5.6 Applanation Tonometry

Putting aside the systemic versus cerebral EF argument, ApT is a validated method of measuring arterial reactivity, an indicator of EF [256]. ApT does not measure total EF – additional tests would be required – but it does allow us to to determine the degree of endothelium-dependant vasodilation, which reflects overall EF [189].

As FMD is generally measured using the brachial artery, FMD only gives an indication of EF in the larger arteries. However, ApT gives an indication of EF in both smaller and large arteries as a transfer function is used to convert the PAIx into AAIx. Since this study focuses

on the smaller arteries in the brain, we decided that ApT would be a more suitable method of estimating EF than FMD.

The disadvantage of ApT is that it requires some degree of patient cooperation, even though the procedure is non-invasive. The test requires the patient to lie supine and be relaxed for up to two hours. Some participants from this study were not able to lie still for this duration of time, even when they were given a short break between the GTN and salbutamol halves of the procedure (approximately 30 minutes per half). Fidgeting tended to occur more during the salbutamol section of the testing, as this was later in the protocol. Theoretically, if a person is not totally relaxed, the vessel will dilate as a sympathetic response, irrespective of the salbutamol. Thus the response seen while the patient is fidgeting may not be due to the salbutamol, therefore giving a falsely higher global EF. Whether or not this pushes the trend to agree or disagree with the hypothesis, the issue is that the results from ApT may not be accurate. A small reproducibility study was performed using healthy researchers of varying ages independent of the study. Although the reproducibility study may show that the results of ApT are reproducible under ideal situations (as the researchers understood the importance of remaining still), this may not fully extend into the study cohort due to poor cooperation with some patients. There is also the issue of whether the indication for MRI investigation may impact on EF reproducibility.

7.5.6.1 AAIx vs PAIx

Although both the AAIx (aortic) and PAIx (peripheral) are presented in this study, most published studies using PWA report only the AAIx becase the majority of studies are cardiac studies. The differences between the two indices have been explained previously, but to summarise, PAIx is the augmentation index calculated directly from readings taken at the radial artery and AAIx is the augmentation index calculated using a transfer function on the peripheral waveform to obtain a reading as though it was measured from the aorta. Both indicies are closely correlated [549]. A study by Stenborg et al. [255] reported the AAIx in a study involving CADASIL patients. Another stroke study by the same group did not use AAIx, but reported the reflective index using the aortic waveform [395].

Although AAIx and PAIx are closely correlated, the concern about using AAIx for a cerebral small vessel study is that AAIx is a measurement with reference to a large artery, whereas the PAIx is from a smaller artery, and is more likely to be similar to the cerebral small vessels. However, the sub-study performed for determining ApT repeatability has shown that AAIx is

more reproducible than PAIx, thus indicating that AAIx is a more reliable estimation of EF than PAIx in this study. Therefore, although both AAIx and PAIx values and analyses are presented in this study, only the AAIx values and analyses can be accepted as valid. It could be argued that because only the values with reference to a large artery (AAIx) are valid, then, in this situation, ApT is no more advantageous than FMD using the brachial artery.

7.6 Conclusion

In summary, this study does not support the involvement of EF in SVD. The only significant genetic associations involved the tPA 20324 TT genotype, which was found to be significantly associated with better EF (as determined using ApT) and LI when compared to the CC/CT genotypes. This was a novel finding.

The tPA 20324 TT genotype was also the only polymorphism to have a nearly significant association with LI ($p=0.06$), while no other significant relationships were observed between SVD and the eight candidate polymorphisms. These results support the role of altered fibrinolysis in the development of LI. However, it is acknowledged that a small sample size was used in the analyses and that the findings may be a Type I error rather than a reflection of a true association. This study needs to be performed in a larger sample to validate the findings.

CHAPTER 8 FUTURE DIRECTIONS

8.1 The Ideal Study

The main disadvantage of our study is the small sample size. However, our study does have the advantage of a MRI-based classification system. Based on the advantages and disadvantages of our study as well as those published in the literature, the following briefly disucsses the features of the ideal SVD study, and these features are summarised in **Table 8.1** at the end of the section.

1. *The ideal study should have a large enough sample size to allow for subgroup analyses as well as be large enough to detect significant associations with sufficient statistical power.*

LA and LI have different pathophysiologies and therefore should not be merged into a single SVD group. There is also evidence to suggest that the subtypes of LI have different pathophysiologies [13-15, 29, 39], and thus subdividing the LI group into the small/multiple infarct and large/single infarct is necessary to ensure uniformity in phenotype within the sub-categories.

Using the results from this study, the estimated sample sizes required to detect significance between means in global EF AAIx values was 444 for LA and 66 for LI (α=0.05 and power of 80%, 1:1 case/control ratio). If the minimum sample size for a simple yes/no comparison is 450, then the total sample required for sub-group analyses that are sufficiently powered would need to be much larger.

Recruiting from the general population (e.g. by raising public awareness via the media) may facilitate in obtaining a larger sample size, as would collaborations with other research groups to merge patient samples.

Sample sizes are also the major limitation of many genetic association studies and also the cause of many false positive/negative associations with a disease. Many studies simply do not have an adequate sample size to identify a significant association with sufficient power. Sample size will depend on the SNP frequency as well as the phenotype with which it is being associated.

2. *A quantitative method of determining SVD load removes the subjectivity of visual rating scales.*

It is evident that there are many different types of visual rating scales established for determining SVD load. However, all the visual rating scales have a high degree of subjectivity that may depend on the assessors and their experience [61]. A method that removes the subjectivity of assessment and is able to output data as a continuous variable, rather than an ordinal variable of the visual rating scales, provides a greater degree of flexibility in how the investigators can use the data. An objective assessment also enables greater ease when comparing results between different studies and different samples.

In comparison with the rating scale used in this study (the modified Fazekas scale), a quantitative measure would be better for a longitundinal study as it would be more sensitive in detecting disease progression. Disease progression using the current scale can only be described in broad terms (e.g. from 'mild' to 'moderate'), which is not sensitive enough to detect minor changes.

The advantage of using MRI over clinical classification systems such as the OCSP and TOAST is that the presence or absence of SVD can be determined definitively. For example, the OCSP stroke classification is based on the presenting symptoms and syndromes of the stroke sufferer. Thus there is potential for stroke subtype misclassification if using OCSP. The TOAST criteria, as discussed previously, does not incorporate WMH burden into the classification, and therefore would be inappropriate to classify LA.

3. *A longitudinal study enables changes to be studied over a longer period of time, which is important for slow-developing diseases such as SVD.*

Case-control studies are currently the most efficient way of investigating associations of SVD with EF or genetic polymorphisms since SVD is a disease with slow progression. Because of this, a 'one-off' study, such as our study and other published studies [57, 58, 269, 399], can only provide limited information as the data collected is a 'snap-shot' of a single moment. Extending the study into a longitudinal study can then provide information that is collected over a longer period of time. With results from two or more time points, the progression of disease as well as the rate of decline in EF can be determined and any association with genetics or disease would be more informative than an association with a single time point. A quantitative measure of WMH, as described previously, would also facilitate a longitudinal

study. Volumetric measurements have already been found to be more reliable and sensitive in measuring WMH changes than visual rating scales [550]. If volumetric measurements are unavailable, then a visual rating scale with detailed categories, like the Longstreth [52] or Wahlund [54] scales, should be used in preference to broad categories (e.g. the modified Fazekas scale used in this study) so that small changes in SVD burden can be identified. However, adequate training is required to ensure that inter-rater reliability is high, as scales with detailed categories tend to show poor inter-rater reliability.

4. *The selection and definition of healthy controls is important.*

This study has used participants who potentially have an undiagnosed medical condition, thus the reason for a MRI. How this undiagnosed condition may impact SVD and/or EF is unknown, and therefore there is the possibility that the results may be affected by this. Some participants were scanned because they were healthy but had 'unexplained dizziness', while others had scans for tinnitus and vertigo. If the participant is experiencing these broad, non-specific symptoms, while being healthy otherwise, it is difficult to determine what the underlying condition is and whether or not it will affect EF or SVD.

Certain other neurological disorders have been associated with changes in EF. For example, there is the suggestion that decreased endothelium-dependant vasodilation is associated with migraines [551], although the relationship is yet to be fully investigated [552, 553]. Specific migraine subtypes have also been associated with increasing stroke risk [554]. EF is also suggested to have a role in epilepsy as a leaky endothelium is known to increase neuronal excitability [555]. The degree of influence that EF has in these disorders is still yet to be determined.

The advantage of using healthy controls is that the potential for the results to be influenced is minimised. Ideally, controls should also be selected randomly and age-matched to cases.

5. *The method of measuring EF needs to be validated in multiple populations and shown to be reproducible in those populations as well as the population of interest.*

There are a variety of methods to measure EF, most of which rely on endothelium-dependant vasodilation as an indicator of EF and this is an accepted practice [221, 222]. The problems associated with these techniques are that they are time consuming and also have some degree of operator variability, which makes the comparison of results between studies difficult.

Although previous studies have shown associations between systemic (peripheral) EF and SVD, the ideal method of investigating EF in SVD should include a measure of cerebral EF, as ultimately, SVD is a cerebral disease. The difficulty arises from the inability to assess EF in the cerebral small vessels and the lack of evidence showing the relationship between small vessel EF and cerebral large vessel (e.g. carotid artery) EF, in the hope that cerebral large vessel EF could be a satellite measure of small vessel EF. Systemic and cerebral EF have also been shown not to be closely associated [518]. It may be that further studies are required to investigate the relationship between systemic and cerebral EF, and cerebral small and large vessel EF.

Ideally, the methodology would also include a blood profile of endothelial activation and inflammatory markers. We had intended to include markers of endothelial activation (e.g. vWF) and inflammation (e.g. C-reactive protein and ICAM-1) as part of the EF measurement in this study, but we did not have access to the equipment nor the budget required for these assessments. The non-invasive measures of EF are based on endothelium-dependant vasodilation. Platelet activation and endothelial cell adhesion molecule expression also have a role in EF and studies have rarely included these two aspects of ED, as it has become accepted that endothelium-dependant vasodilation is the standard test of EF [221, 222]. It may be that different disease states have ED with a different balance of endothelium-mediated vasodilation, platelet activation and endothelial cell adhesion molecule expression. Hence, accuracy in determining EF can be increased by incorporating a blood profile of endothelial and inflammatory markers and analysing these together with endothelium-dependant vasodilation techniques.

6. *Genetic studies need to be cost-effective and be able to analyse more than one SNP simultaneously. The candidate SNPs or polymorphisms also need to have some biological plausibility to maximise cost-effectiveness.*

Chronic diseases are usually polygenic and thus highlight the importance of haplotype analyses. Phenotypes may not be evident unless polymorphisms interact with the environment or with each other and a certain threshold is exceeded, as explained in the ‘threshold model’. This highlights the necessity of haplotype analyses, and the advantage of genome-wide association studies, where combinations of polymorphisms can be analysed to assess the concurrent effect of several polymorphisms on disease states. Again, the importance of sample size arises as the potential for haplotype analyses will depend on having a large sample size.

Table 8.1 – Summary of the features of the ideal SVD study

1. Have a large enough sample size to perform sub-analyses on LI and LA separately, LI size, and LA grades (if using a visual grading scale). The sample size should also be large enough to detect associations with sufficient statistical power.
2. Use a quantitative method of determining SVD load (eg volumetric software) from MRI.
3. Be a case-control study but be able to extend into a longitudinal study, so that participants can be followed up in five to ten years and progression of SVD can be correlated with changes in EF.
4. Use healthy controls who have been assessed radiologically. Participants should also be free of other neurological diseases.
5. Use a validated, reproducible non-invasive method of measuring EF while also including a full blood profile for markers of endothelial activation and inflammation.
6. Be cost-effectivenes and be able to analyse more than one SNP at a time.
7. Include haplotype analyses (dependant on sample size).
8. Have clear biological plausibility for all SNPs.

8.2 Genetics

Single SNPs rarely have a significant effect on disease risk, unless the SNP is positioned in a vital area. It is more likely that a combination of multiple SNPs within several different genes, while also interacting with the environment, will significantly alter disease risk. Thus, the candidate gene approach used in this study is highly inefficient for identifying important SNPs and does not provide the best cost-to-benefit ratio. Newer studies are now using genome-wide strategies to identify important SNPs and SNP combinations. In 2007, the genome-wide approach was used to identify a genetic locus for CarVD [556], and the same techniques have been applied in multiple sclerosis [557], as well as type 2 diabetes [558].

There is also preliminary data from a genome-wide study for ischaemic stroke [559]. The data from this study did not reveal any single locus with a large effect on the risk of ischaemic stroke. However, sample size was relatively small (n<600, cases and controls combined). Research into the genetics of SVD needs to follow the same approach to effectively and efficiently identify genes and SNPs of interest.

The benefits of genome-wide analyses are that multiple genes and SNPs can be analysed concurrently and the computerised nature of the analyses allow a high throughput of samples. Current disadvantages for genome-wide studies are that it is still relatively expensive

(compared to the candidate gene approach), although costs are decreasing due to its rising popularity, and that very large study samples are required, especially if haplotype analyses are to be performed. Because of the large number of genes that can be analysed, relationships such as linkage disequilibrium can also be investigated easily.

It is known that ischaemic stroke is a polygenic disease and SVD would be no different, thus the candidate gene approach to determining genes and SNPs related to SVD is a very slow and inefficient way of doing so. If genome-wide analyses could be applied to a large enough sample, genes that modulate SVD risk could be identified in a time-efficient manner. In the future, pharmacogenomics could be applied to generate SVD or stroke-specific pharmacotherapy for at-risk individuals.

8.3 Imaging

The majority of published studies involving WMH have used neuroradiologists and visual rating scales to assess the degree of SVD. Although the results are usually reliable, there is always some degree of subjectivity as demonstrated through kappa (κ) values as inter-rater variability studies rarely report $\kappa>0.9$. Some studies are now reporting WMH as a volume (a continuous variable). The volume of WMH is calculated using MRIs and specialised software, where hyperintensities are identified and isolated. Each voxel (a 3D pixel) has a known volume, thus if all the voxels with an intensity greater than a prespecified magnitude are counted, the total volume of WMH can be calculated using several slices of MRI.

Statistically, a continuous variable for WMH would be much more powerful variable compared to a categorical variable from visual rating scales. It would also provide a better platform to compare different studies, as it would be a uniform measure. Currently, different studies may use different rating scales, thus making it difficult to compare the results of one study with those of another.

Qquantitative measures have not been fully embraced yet due to the complexity of the algorithms required. Quantitative analysis using SPM2 were considered for this study and a small number of scans were experimented with using this method. Our study witnessed first-hand the effort required to firstly convert the MRI scans into acceptable formats, and secondly to calibrate the program to analyse the areas of interest. It is accepted that the process our study experimented with may be different to those used in the other studies with quantitative measures. With the equipment and personnel available to us, the time and effort required to

process the 132 participants in this manner would have been far too inefficient. In the future, if specialised programs could be developed to include WMH specifically without too much program calibration, then the use of quantitative measures may become more attractive.

8.4 Other Related Studies

The degree of WMH is irrelevant until it is clinically related. It has been established that WMH are associated with cognitive decline, resulting in dementia, and it has also been suggested that SVD and dementia have a common pathology [22]. Thus, it is only logical that the next step for this study is to incorporate a cognitive aspect where cognitive function can be studied concurrently with WMH, EF and genetics. As mentioned briefly earlier in Chapter 5, particpants from this study were invited to participate in a separate study investigating EF, WMH and cognitive function, if they were eligible according to the criteria of that study. No significant associations were detected between EF and cognition [509].

Also briefly mentioned was a retinal nerve study, which involved a small number of participants from our study [510]. This eye study was investigating whether changes in the retinal nerve fibre layer was associated with LA or LI. The significance of this study was that SVD could be assessed easily, non-invasively and quickly if retinal nerve fibre layer changes were associated with SVD. However, no significant correlations were detected between retinal nerve fibre layer thickness and LA/LI.

Because of the increased life-span and ever-growing ‘older’ population, the burden of dementia and other non-fatal chronic diseases on community resources is increasing. SVD itself is often not fatal, but it is strongly associated with dementia. Studies investigating the prevention and treatment of SVD may not be ‘life-saving’ like those of large-vessel ischaemic stroke, but they are important for preventative strategies.

Final Considerations

This study has found a significant association between the tPA 20324 C/T and EF and a significant association between the same SNP and SVD. In this sample, this polymorphism has been associated with higher values of global EF, but also a 13-fold increase in the risk of SVD. These results suggest that the effect of the SNP on LI is not via EF, but rather through altered fibrinolysis. However, the associations observed may represent Type I errors from the small sample size, thus further studies need to be performed on this SNP to elicit the mode of action and to validate the results from this study.

To investigate this association further, the sample needs to be limited to LI only and include careful subtyping of LI using MRI. The inclusion of intermediate correlates, such as plasma tPA concentrations and other prothrombotic mediators, would be useful in determining the effect of the SNP on SVD and EF.

Findings between SNPs or EF and SVD provide important information into the pathogenesis of SVD. Using EF as an estimator of SVD risk would be beneficial as changes in EF could be detected easily prior to the onset of clinical symptoms.

SNP analysis is a relatively simple procedure and advances in technology allow investigators to analyse several thousand SNPs in a cost-effective manner. Identification of genetic mutations associated with disease enables both primary and secondary preventative therapies to be targeted at reversing any negative effects caused by the mutation. If the genetic mutations specific to SVD can be identified, then therapies fot reducing SVD and its sequelae, like dementia, could be developed and administered with minimal adverse effects due to its specificity.

Due to the limited sample size and subsequent low statistical power, the study may therefore be perceived as a pilot study and the results can only be considered as hypothesis-generating. The next step is to address the limitations and issues of the study as discussed previously and to validate these associations in larger sample sizes with greater statistical power. With the continuing advances in technology and the better understanding of the mechanisms of SVD, the possibility of conducting the ‘ideal study’ is feasible. The methods and techniques are now available – the aim for the future would be to apply these appropriately to produce valid results in association studies of SVD, EF and genetics.

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Appendix 1: Publication: Cerebral Small Vessel Disease – Genetic Risk Assessment for Prevention and Treatment

Lam, A., Hamilton-Bruce, M.A., Jannes, J. and Koblar, S. (2008) Cerebral Small Vessel Disease: Genetic Risk Assessment for Prevention and Treatment. Molecular Diagnosis & Therapy, v. 12 (3), pp. 145-156, May 2008

Appendix 2: DNA extraction protocol

A stock solution of Dulbecco's Phosphate Buffered Saline (Calcium and Magnesium free) (PBS) was made using the following formula and pH adjusted to 7.4:

Sodium chloride	8 g
Potassium chloride	0.2 g
Disodium hydrogen orthophosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Deionised water (milliQ)	to 1 L

Lymphocytes were separated using LSM Lymphocyte Separation Medium (MP Biomedicals Australia LLC) within four hours of blood collection. The protocol for lymphocyte separation was as follows:

1. 2 mL of whole blood was mixed with 5 mL PBS in a 10mL tube.
2. 2 mL of LSM was carefully inserted under the blood/PBS mixture to create two distinct layers.
3. This was centrifuged at 400 g (1500 rpm) for 20 minutes.
4. The buffy layer was removed by aspiration and transferred into a clean 10 mL tube.
5. The tube was filled to 10 mL with PBS. This was centrifuged again at 1500 rpm for 10 minutes.
6. The PBS was decanted and the pellet was resolubilised in the remaining PBS.

DNA was extracted using DNAzol (Molecular Research Centre Inc). The following procedure was used:

1. 100 µL of the resolubilised pellet was added to 1 mL of DNAzol in a 2 mL centrifuge tube to lyse the cells. This was mixed by shaking.
2. 500 µL of 100% ethanol was added to the tube. This was mixed by inversion, and then centrifuged briefly.
3. The supernatant was carefully removed and 1 mL of 75% ethanol was added. The tube was again inverted before briefly centrifuging again. This step was repeated once more.
4. All ethanol was removed before 200 µL of 8mM NaOH was added to resolubilise the DNA pellet overnight at room temperature. A fresh batch of 8 mM NaOH was made up every month as directed by the DNAzol protocol.

Appendix 3: Logistic regression model adjustment (LA)

The following tables show the calculations required to identify the variables required to be included in the regression model between LA and the candidate polymorphism. The '% change odds ratio' is calculated based on the unadjusted ('none') OR.

IL-6 -174 G/C

Variable	Odds Ratio		% change in odds ratio	
	GG vs GC/CC	GG/GC vs CC	GG vs GC/CC	GG/GC vs CC
None	1.934	0.802	-	-
Age	2.531	1.514	31	89
Gender	1.827	0.812	-6	1
Family History	1.905	0.796	-1	-1
Hypertension	2.287	0.830	18	3
Hypercholesterolaemia	2.071	0.904	7	13
Diabetes	1.911	0.694	-1	-13
Smoker	1.917	0.765	-1	-5

NADPH p22 phox C/T

Variable	Odds Ratio		% change in odds ratio	
	CC vs CT/TT	CC/CT vs TT	CC vs CT/TT	CC/CT vs TT
None	0.969	1.171	-	-
Age	0.861	1.005	-11	-14
Gender	0.983	1.080	1	-8
Family History	0.979	1.234	1	5
Hypertension	1.131	1.312	17	12
Hypercholesterolaemia	1.028	1.272	6	9
Diabetes	0.965	1.152	0	-2
Smoker	0.991	1.209	2	3

tPA -7351 C/T

Variable	Odds Ratio		% change in odds ratio	
	CC vs CT/TT	CC/CT vs TT	CC vs CT/TT	CC/CT vs TT
None	0.735	0.818	-	-
Age	0.623	0.594	-15	-27
Gender	0.710	0.772	-3	-6
Family History	0.730	0.848	-1	4
Hypertension	0.741	0.580	1	-29
Hypercholesterolaemia	0.719	0.913	-2	12
Diabetes	0.885	0.799	20	-2
Smoker	0.708	0.842	-4	3

tPA -4360 G/C

Variable	Odds Ratio		% change in odds ratio	
	GG vs GC/CC	GG/GC vs CC	GG vs GC/CC	GG/GC vs CC
None	0.575	-	-	-
Age	0.492	-	-14	-
Gender	0.570	-	-1	-
Family History	0.557	-	-3	-
Hypertension	0.521	-	-9	-
Hypercholesterolaemia	0.567	-	-1	-
Diabetes	0.654	-	14	-
Smoker	0.564	-	-2	-

tPA 20324 C/T

Variable	Odds Ratio		% change in odds ratio	
	CC vs CT/TT	CC/CT vs TT	CC vs CT/TT	CC/CT vs TT
None	1.563	-	-	-
Age	1.423	-	-9	-
Gender	1.558	-	0	-
Family History	1.601	-	2	-
Hypertension	1.458	-	-7	-
Hypercholesterolaemia	1.647	-	5	-
Diabetes	1.534	-	-2	-
Smoker	1.549	-	-1	-

eNOS -786 T/C

Variable	Odds Ratio		% change in odds ratio	
	TT vs TC/CC	TT/TC vs CC	TT vs TC/CC	TT/TC vs CC
None	2.176	-	-	-
Age	2.157	-	-1	-
Gender	2.387	-	10	-
Family History	2.234	-	3	-
Hypertension	2.018	-	-7	-
Hypercholesterolaemia	2.547	-	17	-
Diabetes	2.411	-	11	-
Smoker	2.146	-	-1	-

ET-1 138A D/I

Variable	Odds Ratio		% change in odds ratio	
	DD vs DI/II	DD/DI vs II	DD vs DI/II	DD/DI vs II
None	1.807	0.908	-	-
Age	1.784	0.949	-1	-5
Gender	1.879	0.974	4	7
Family History	1.802	0.908	0	0
Hypertension	1.815	0.935	0	3
Hypercholesterolaemia	1.590	0.936	-12	3
Diabetes	1.760	0.769	-3	-15
Smoker	1.759	0.904	-3	0

PON1 -107 C/T

Variable	Odds Ratio		% change in odds ratio	
	CC vs CT/TT	CC/CT vs TT	CC vs CT/TT	CC/CT vs TT
None	0.629	0.284	-	-
Age	0.622	0.357	-1	26
Gender	0.594	0.287	-6	1
Family History	0.636	0.278	1	-2
Hypertension	0.688	0.326	9	15
Hypercholesterolaemia	0.664	0.224	6	-21
Diabetes	0.604	0.291	-4	2
Smoker	0.653	0.291	4	2

Appendix 4: Logistic regression model adjustment (LI)

The following tables show the calculations required to identify the variables required to be included in the regression model between LI and the candidate polymorphism. The '% change odds ratio' is calculated based on the unadjusted ('none') OR.

IL-6 -174 C/T

Variable	Odds Ratio		% change in odds ratio	
	GG vs GC/CC	GG/GC vs CC	GG vs GC/CC	GG/GC vs CC
None	0.800	-	-	-
Age	0.882	-	10	-
Gender	0.690	-	-14	-
Family History	0.759	-	-5	-
Hypertension	0.971	-	21	-
Hypercholesterolaemia	0.800	-	0	-
Diabetes	0.763	-	-5	-
Smoker	0.787	-	-2	-

NADPH p22 phox C/T

Variable	Odds Ratio		% change in odds ratio	
	CC vs CT/TT	CC/CT vs TT	CC vs CT/TT	CC/CT vs TT
None	4.402	3.438	-	-
Age	4.327	3.349	-2	-3
Gender	4.526	3.145	3	-9
Family History	4.534	4.053	3	18
Hypertension	5.781	4.429	31	29
Hypercholesterolaemia	4.442	3.455	1	0
Diabetes	4.534	3.500	3	2
Smoker	4.622	3.670	5	7

tPA -7351 C/T

Variable	Odds Ratio		% change in odds ratio	
	CC vs CT/TT	CC/CT vs TT	CC vs CT/TT	CC/CT vs TT
None	0.308	2.091	-	-
Age	0.271	1.839	-12	-12
Gender	0.289	1.938	-6	-7
Family History	0.302	2.324	-2	11
Hypertension	0.302	1.333	-2	-36
Hypercholesterolaemia	0.308	2.102	0	1
Diabetes	0.390	2.086	27	0
Smoker	0.286	2.196	-7	5

tPA -4360 G/C

Variable	Odds Ratio		% change in odds ratio	
	GG vs GC/CC	GG/GC vs CC	GG vs GC/CC	GG/GC vs CC
None	0.469	-	-	-
Age	0.435	-	-7	-
Gender	0.464	-	-1	-
Family History	0.440	-	-6	-
Hypertension	0.408	-	-13	-
Hypercholesterolaemia	0.469	-	0	-
Diabetes	0.572	-	22	-
Smoker	0.456	-	-3	-

tPA 20324 C/T

Variable	Odds Ratio		% change in odds ratio	
	CC vs CT/TT	CC/CT vs TT	CC vs CT/TT	CC/CT vs TT
None	5.200	20.500	-	-
Age	4.972	18.328	-4	-11
Gender	5.200	24.948	0	22
Family History	5.571	19.556	7	-5
Hypertension	4.900	11.000	-6	-46
Hypercholesterolaemia	5.211	23.804	0	16
Diabetes	5.277	16.029	1	-22
Smoker	5.147	19.997	-1	-3

eNOS -786 T/C – no results available

ET-1 138A D/I

Variable	Odds Ratio		% change in odds ratio	
	DD vs DI/II	DD/DI vs II	DD vs DI/II	DD/DI vs II
None	4.000	-	-	-
Age	3.974	-	-1	-
Gender	4.259	-	6	-
Family History	3.981	-	0	-
Hypertension	4.079	-	2	-
Hypercholesterolaemia	4.048	-	1	-
Diabetes	3.883	-	-3	-
Smoker	3.890	-	-3	-

PON1 -107 C/T

Variable	Odds Ratio		% change in odds ratio	
	CC vs CT/TT	CC/CT vs TT	CC vs CT/TT	CC/CT vs TT
None	1.495	0.733	-	-
Age	1.609	0.949	8	29
Gender	1.395	0.749	-7	2
Family History	1.540	0.703	3	-4
Hypertension	1.765	0.950	18	30
Hypercholesterolaemia	1.499	0.725	0	-1
Diabetes	1.450	0.782	-3	7
Smoker	1.638	0.801	10	9