A HAPLOTYPE ANALYSIS OF THE ANGIOTENSIN CONVERTING ENZYME GENE IN ISCHAEMIC STROKE

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Background

Epidemiological studies lend some support for a genetic predisposition to human stroke. There is a growing body of evidence to suggest a role for Angiotensin II (ANGII) in vascular disease. The levels of Angiotensin converting enzyme (ACE), which converts ANGI to ANGII, are known to be under a significant degree of genetic control. The D allele of the *ACE I/D* polymorphism is associated with higher serum ACE levels and this allele has also been associated with ischaemic stroke. Recent identification of numerous *ACE* single nucleotide polymorphisms has allowed for a more powerful case control haplotype analysis of *ACE* in ischaemic stroke.

Patients and Methods

The validity of the published structure of the common *ACE* haplotypes was investigated and supported using long range allele specific PCR and DNA sequencing of a random sample of UK caucasian subjects. Using restriction fragment length polymorphism (RFLP) analysis of selected polymorphisms we generated *ACE* haplotypes for 359 ischaemic stroke patients and 328 unrelated controls.

Results

Age, hypertension, smoking, diabetes and hypercholesterolaemia were identified as significant clinical risk factors for ischaemic stroke. D allele frequencies showed no significant differences between cases and controls (0.55 vs 0.53 respectively). However a low frequency D allele haplotype (H9) was found to be an independent risk factor for ischaemic stroke (odds ratio 2.05, p=0.004).

Conclusion

This study has provided allele specific data to support the haplotype structure of the common *ACE* haplotypes in a UK caucasian population. For the first time, in a case control analysis, we have identified a significant and independent genetic association of a low frequency *ACE* haplotype, H9, with human ischaemic stroke. This result suggests an important role for *ACE* in ischaemic stroke which will require further study in other populations using a variety of control groups.

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The following tasks were performed almost exclusively by myself with the guidance of those stated:-

Study design and application for ethics approval.
Professor R Trembath

Clinical assessments, data collection, database management and risk group classification.
Dr. R.J. Abbott

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Abbreviations

3'	Three prime
4S	Scandinavian Simvastatin survival study
5'	Five prime
AA	Amino acid
ACE	Angiotensin converting enzyme
ADC	Apparent diffusion coefficient
AF	Atrial fibrillation
AHA	American Heart Association
ALLHAT	Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial
ANF	Atrial derived natriuretic factor
AngI	Angiotensin I
AngII	Angiotensin II
ApoE	Apolipoprotein E
ASO	Allele specific oligonucleotide
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BNF	Brain derived natriuretic factor
BP	Blood pressure
Bp	Base pairs
CABG	Coronary artery bypass graft
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy
CAT / CT	Computerised axial tomography
CAVATAS	Carotid and vertebral artery transluminal angioplasty study
CBF	Cerebral blood flow
CCF	Congestive cardiac failure
CEA	Carotid endarterectomy
CHD	Coronary heart disease
CM	Centi Morgans
CRP	C Reactive protein
CT	Computed axial tomography scan
CXR	Chest X ray
DBP	Diastolic blood pressure
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EAFT	European Atrial Fibrillation Trial
ECAS	European carotid artery surgery trial
ECC	Royal College of Physicians of Edinburgh Consensus Conferences
ECG	Electrocardiogram
Echo	Cardiac echocardiography
ELS	East Lancashire Study
eNOS	Endothelial nitric oxide synthase
ESR	Erythrocyte sedimentation rate
FBC	Full blood count
FH	Family history
GBS	Guillain Barre syndrome
GpIIb/IIIa, GpIb/IX, GpIa/IIa	Platelet glycoprotein receptors
HDL	High density lipoproteins
HPS	Heart protection study
IHD	Ischaemic heart disease

IMT	Intima-media thickness (carotid)
IST	International Stroke Trial
IWP	Intercollegiate Working Party
Kb	Kilobases
LACS	Lacunar syndrome
LDL	Low density lipoproteins
LFT	Liver function tests
LIFE	Losartan Intervention for Endpoint Reduction Trial
LIPID	Long-Term Intervention with Pravastatin in Ischaemic Disease Study
LVH	Left ventricular hypertrophy
Mb	Megabases
MELAS	Mitochondrial encephalomyelopathy, lactic acidosis and stroke like episodes
MI	Myocardial infarction
MODY	Maturity onset diabetes of the young
MRA	Magnetic resonance angiography
MRI / MR	Magnetic resonance imaging
MTHFR	Methylenetetrahydrofolate reductase
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NCBI	National Centre For Biotechnology Information USA
NNT	Numbers needed to treat
OCSP	Oxford Community Stroke Project
OR	Odds ratio
PACS	Partial anterior circulation syndrome
PAI1	Plasminogen activator inhibitor
PCR	Polymerase chain reactions
PFU	Pyrococcus furiosus
PICH	Primary intracerebral haemorrhage
PO	Per oral (By mouth)
POCS	Posterior circulation syndrome
PR	Per rectum
PVD	Peripheral vascular disease
RCT	Randomised control trial
RF	Risk factor
RFLP	Restriction fragment length polymorphism
RR	Relative risk
r-TPA	Recombinant tissue plasminogen activator
SAH	Sub-arachnoid haemorrhage
SBP	Systolic blood pressure
SHR	Spontaneously Hypertensive Rat
SIGN	Scottish Intercollegiate Guidelines Network
SLSR	South London Stroke Register
SNP	Single nucleotide polymorphism
SPSHR	Stroke Prone Spontaneously Hypertensive Rat
TACS	Total anterior circulation syndrome
Taq	DNA polymerase of the bacterium Thermus aquaticus
TFT	Thyroid function tests
TIA	Transient ischaemic attack
TOAST	Trial of ORG 10172 in Acute Stroke Treatment
U+E	Urea and electrolytes
µl	microlitre
UTR	Untranslated region
WT	Wild type

CONTENTS

Introduction

Stroke is an extremely common human disease causing significant worldwide morbidity and mortality, especially within elderly populations, in the developed world. It leads to a significant burden on individuals, families, communities and health care providers. Cerebral infarction is the commonest type of stroke and has well recognized clinical risk factors, many of which have successful and widely available preventative strategies. Risk factor management has lead to reductions in stroke incidence in many populations, however in an ageing population the burden on health care providers and society as a whole is likely to increase. This has major financial implications as preventative treatments are expensive and the numbers needed to treat (NNT) in each risk group to prevent one stroke are large.

This expense can be offset against the huge costs of stroke care, thereby making stroke prevention economically viable. At present prevention is targeted at clinically evident risk factors but many 'at risk' individuals may never have strokes but will be exposed to the side effects of drug treatments for many years. A much more efficient and economic approach would be to redefine and target the individuals who are at the highest risk of stroke thereby reducing the NNT.

Molecular biological techniques are now available to study and identify genetic risk factors which may independently have stronger predictive values than the clinical parameters. Acting in association with the known risk factors, however, genetic predisposition may signal even higher risks for stroke and so will allow much more aggressive, efficient and cost effective targeting of those individuals.

To date no studies have identified any consistent genetic risk factors for non-familial ischaemic stroke though good evidence is available to provide strong support for the presence of a genetic predisposition to cerebrovascular ischaemic disease. This evidence will provide the basis for the work that will be presented in this thesis, focusing on the search for genetic susceptibility factors in ischaemic stroke.

The thesis will begin by discussing the pathophysiology of stroke before moving on to the clinical and genetic epidemiological features and preventative management of cerebral infarction and its risk factors. I will then consider the principles of common disease genetics and their application to ischaemic stroke. At this point I will outline the rationale behind this 3 year case control study comparing the frequencies of haplotypes of the *ACE* gene in ischaemic stroke, introducing the practical issues of conducting small scale genetic research in this field. The chosen methodology and results will then be presented followed by discussions of their implications.

Due to the complexities of this type of common disease genetic study this work spanned an entire decade from its conception in 1998. Many important advances were made in the field during this period and these have been addressed in Chapter 8 which has provided an opportunity for this work to be put into a more contemporary context.

CHAPTER 1

AN INTRODUCTION TO STROKE

Chapter 1: List of Tables

This section will cover the definition, classification and pathophysiology of stroke. It will then move on to the epidemiology of stroke and its risk factors before introducing the evidence and guidelines for primary and secondary prevention.

1.1 Definitions

Stroke is a broad term for syndromes of rapidly developing symptoms and signs of cerebral dysfunction, often localized and sometimes global, lasting for longer than 24 hours (or causing earlier death) with no other cause than a sudden decrease in cerebral blood flow. Transient events lasting less than 24 hours and resulting in full recovery are generally termed Transient Ischaemic Attacks (TIAs).

1.2 Classification of Stroke

Stroke can be classified by its pathological type, aetiology, associated risk factors, or by its anatomical localization and resulting symptoms. There are two main pathological causes of stroke, cerebral ischaemia / infarction, and cerebral haemorrhage. Approximately 80% of first strokes are due to cerebral infarction, 10% primary intracerebral haemorrhage (PICH), 5% sub-arachnoid haemorrhage (SAH), and 5% of uncertain type (Bamford et al., 1990). Ischaemic stroke has a lower mortality than haemorrhagic stroke but those who survive often have significant morbidity (Table 1.1 & 1.3). Due to its high incidence and high prevalence of disabling morbidity this project will study cerebral infarction.

Table 1.1 Classification of Stroke (Bamford et al., 1990).

Stroke Classification	Mortality at one month (95% CI)	Mortality at one year (95% CI)
Cerebral infarction	10% (7-13)	23% (19-27)
Primary intracerebral haemorrhage	50% (38-62)	62% (43-81)
Sub-arachnoid haemorrhage	46% (29-63)	48% (24-72)
Uncertain type	77% (46-100)	84% (52-100)
ALL Strokes	19% (16-22)	31% (27-35)

1.2.1 Cerebral Ischaemia / Infarction

Even though the brain makes up only 2% of the total body weight, it receives about 15% of the resting cardiac output and accounts for approximately 20% of the oxygen consumption (Cotran and Pober, 1989). It can store neither oxygen nor glucose effectively and is therefore highly dependent on a constant blood supply (50-55ml/100g per minute at rest). Reversible cerebral dysfunction (ischaemia) is evident when flow decreases to 18ml/100g/min. In as little as 5 minutes following the reduction of blood flow (in the range of 8ml/100g/min) cell death can occur (infarction). (Heimer, 1995).

Acute reduction of cerebral blood flow is usually due to vessel occlusion or stenosis caused by either in-situ thrombosis or embolisation. Thrombosis often occurs as a consequence of atheroma (see section 1.3). Circulating debris from distant thrombosis within the heart or from unstable atheromatous plaques can lead to embolic occlusion. TIAs are thought to result from transient arterial embolisation from carotid plaques.

Identifying the sub-classes of ischaemic stroke is important for therapy and prognosis. To assist this there are two widely used classification systems, the Oxford Community Stroke Project (OCSP) (Bamford et al., 1991) and the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) (Adams et al., 1993).

1.2.1.1 The Oxford Community Stroke Project (OCSP) classification system

This system uses simple bedside clinical features, allowing anatomical localisation of the lesion. Patients are divided into 4 stroke syndromes based upon the site of cerebral infarction, Total anterior circulation syndrome (TACS), Partial anterior circulation syndrome (PACS), Posterior circulation syndrome (POCS) and Lacunar syndrome (LACS). (Table 1.2).

Table 1.2 The Oxford Community Stroke Project (OCSP) classification of sub-types of cerebral infarction (Bamford et al., 1991).

Total anterior circulation syndrome (TACS)	A combination of new higher cerebral dysfunction (e.g. dysphasia), homonymous visual field defect and ipsilateral motor and / or sensory deficit of at least two areas (out of face, arm and leg).
Partial anterior circulation syndrome (PACS)	Only two of the three components of a TACS, or with higher cerebral dysfunction alone, or with a motor / sensory deficit more restricted than those classified as LACS (e.g. confined to one limb).
Posterior circulation syndrome (POCS)	Any of: ipsilateral cranial nerve palsy with contralateral motor and/or sensory deficit; bilateral motor and / or sensory deficit; disorder of conjugate eye movement; cerebellar dysfunction; isolated homonymous visual field defect.
Lacunar syndrome (LACS)	Pure motor stroke (Fisher and Curry, 1965) , Pure sensory stroke (Fisher, 1965), Ataxic hemiparesis (Fisher and Cole, 1965), (Fisher, 1967) and Sensorimotor stroke (Mohr et al., 1977).

1.2.1.1.1 Anterior Circulation Syndromes (TACS, PACS)

These stroke syndromes result from thromboembolic occlusion of the major anterior cerebral vessels (carotid, anterior or middle cerebral arteries). The final clinical outcome will be highly dependent upon the collateral circulation from the other cerebral arteries (Ringelstein et al., 1992). Patients with TACS present with a triad of symptoms, higher cerebral dysfunction (dysphasia, dyscalculia or visuospatial disorder), homonymous hemianopia and ipsilateral motor and or sensory deficit in at least two areas (face, arm, leg). PACS patients present with any combination of the two components of the TACS triad.

1.2.1.1.2 Posterior Circulation Syndromes (POCS)

These syndromes often result from brainstem, cerebellar and occipital lobe dysfunction causing cranial nerve deficits, ataxia and field defects. At their most severe brainstem dysfunction can be rapidly fatal.

1.2.1.1.3 Lacunar Syndromes (LACS)

The classification of these syndromes has been the subject of much debate. One difficulty is diagnostic uncertainty as highlighted by Toni et al. 1994, who found only 56% (123/219) of patients with clinically defined lacunar strokes actually had anatomically defined lacunes, while 27% (47/170) of patients with anatomical lacunar strokes did not have clinical lacunar syndromes (Toni et al., 1994). It is however generally accepted that lacunar syndromes result from intrinsic disease of small deep perforating vessels in the basal ganglia or the pons this is supported by identification of small (< 1.5 cm in diameter) deep infarcts (lacunes) in these areas (Bamford and Warlow, 1988). The pathology of lacunar infarcts remains poorly understood however it is thought to result from either lipohyalinosis or microatheroma (Fisher, 1979).

The OCSP classification has proven prognostically useful in the clinical setting with LACS having the best outcome and TACS the worst (Table 1.3)

Table 1.3 Prognosis comparisons of the OCSP stroke sub-types (Bamford et al., 1991).

Sub-type	Case fatality (%)			Functionally dependent (Rankin 3-5) (%)		
	1 month	6 months	12 months	1 month	6 months	12 months
TACS	39	56	60	56	39	36
PACS	4	10	16	39	34	29
POCS	7	14	19	31	18	19
LACS	2	7	11	36	26	28
All	10	18	23	39	29	28

1.2.1.2 The TOAST Classification System (Adams et al., 1993)

The TOAST system was designed to be applied in large multi-centred treatment studies and focuses on the aetiological mechanism of stroke. It incorporates the results of clinical data and ancillary investigations (CT / MRI, ECG, ECHO, carotid doppler, arteriography, and clotting studies) and provides a uniform pathologically based classification which allows for more focused treatments and genetic analysis. The system includes five categories (Table 1.4) : Large-artery atherosclerosis, Cardioembolism, Small-artery occlusion (lacunar), Stroke of other determined aetiology, and Stroke of undetermined aetiology. An important part of the classification is the ability of the physician to categorize a specific subtype diagnosis as probable or possible based on the degree of certainty. A "probable" diagnosis is made if the clinical findings, neuroimaging data, and results of diagnostic studies are consistent with one subtype and other aetiologies have been excluded. A "possible" diagnosis is made when the clinical findings and neuroimaging data suggest a specific subtype but other studies are not done. Because many patients will have a limited number of diagnostic tests, the probable and possible sub categorizations allow the physician to make as precise a subgroup diagnosis as can be achieved.

Table 1.4 The TOAST classification system (Adams et al., 1993).

TOAST Class	Description
1	Large-artery atherosclerosis (embolus/thrombus)
2	Cardioembolism (high-risk/medium-risk)
3	Small-vessel occlusion (lacunar)
4	Stroke of other determined aetiology
5	Stroke of undetermined etiology 2 or more causes identified Negative evaluation c) Incomplete evaluation

1.2.1.2.1 Large Artery atherosclerosis.

These patients will have clinical and brain imaging findings of either significant (>50%) stenosis or occlusion of a major brain artery or branch cortical artery. Diagnostic studies should exclude potential sources of cardiogenic embolism. Cortical or cerebellar lesions and brain stem or sub-cortical hemispheric infarcts greater than 1.5 cm in diameter on CT or MRI are considered to be of potential

large-artery atherosclerotic origin. Clinical findings include those of cerebral cortex, brain stem or cerebellar dysfunction. A history of intermittent claudication, TIAs in the same vascular territory, a carotid bruit provide supportive evidence.

1.2.1.2.2 Cardioembolism

This category includes patients with arterial occlusions due to cardiac emboli. Potential large-artery atherosclerotic sources of thrombosis or embolism should be eliminated. Cardiac sources are divided into high-risk and medium-risk groups based on the evidence of their relative propensities for embolism (Table 8.1). At least one cardiac source for an embolus must be identified for a possible or probable diagnosis of cardioembolic stroke. Clinical and brain imaging findings are similar to those described for large-artery atherosclerosis. Evidence of a previous stroke in more than one vascular territory or systemic embolism supports a clinical diagnosis of cardiogenic stroke.

Table 1.5 Classification of high and medium-risk sources of cardioembolism

High risk sources of cardiac emboli	Medium risk sources of cardiac emboli
Mechanical prosthetic valves	Mitral valve prolapse
Mitral stenosis with AF	Mitral annulus calcification
AF (other than lone AF)	Mitral stenosis without AF
Left atrial or appendage thrombus	Left atrial turbulence
Sick sinus syndrome	Atrial septal aneurysm
Recent MI (<4/52)	Patent foramen ovale
Left ventricular thrombus	Atrial flutter
Dilated cardiomyopathy	Lone AF
Akinetic left ventricular segment	Bioprosthetic cardiac valve
Atrial myxoma	Nonbacterial thrombotic endocarditis
Infective endocarditis	Congestive heart failure
	Hypokinetic left ventricular segment
	Myocardial Infarction (>4 /52, <6/12)

1.2.1.2.3 Small Artery Occlusion (Lacunar Stroke)

The patient should have one of the traditional clinical lacunar syndromes and should not have evidence of cerebral cortical dysfunction. Potential cardiac sources of emboli and >50% carotid artery stenosis should be excluded. A history of diabetes mellitus or hypertension supports the clinical diagnosis. The patient should have a relevant brain stem or sub-cortical hemispheric lesion with a diameter less than 1.5 cm on cerebral CT / MRI studies however a normal cerebral image is compatible with the diagnosis.

1.2.1.2.4 Acute Stroke of Other Determined Aetiology

This category includes individuals with strokes of rare aetiology such as haematological disease. Diagnosis should be supported by clinical and CT or MRI findings of an acute ischemic stroke and other diagnoses should be excluded.

1.2.1.2.5 Stroke of Undetermined Aetiology

In several instances the cause of a stroke cannot be determined with any degree of confidence despite extensive evaluation. In purely clinical settings no cause may be found but the evaluation may be curtailed for various reasons. This category also includes patients with two or more potential causes of stroke.

1.3 The Pathophysiology of Cerebral Ischaemia / Infarction

Healthy large arteries consist of three distinct layers, the intima, media and adventitia. The thin luminal layer, the intima, consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The intima is bounded by a monolayer of endothelial cells on its luminal side and a sheet of elastic fibres, the 'internal elastic lamina', on the outer side. The media, (middle layer), consists of smooth muscle cells and the adventitia, (outer layer), consists of connective tissue interspersed with fibroblasts and smooth muscle cells.

Vessel occlusion occurs due to either disease of the vessel wall, embolisation or hypercoagulability (Table 1.6).

Table 1.6 The causes of cerebral ischaemia / infarction

Vessel Wall Disorders	Embolism from the heart	Haematological Disorders	Miscellaneous conditions
Atherosclerosis (Atherothromboembolism)	**High risk sources of cardiac emboli**	**Cellular disorders:**	Oral contraceptives
Intracranial small vessel disease	Mechanical prosthetic valves	(a) Myeloproliferative	Drug abuse
Trauma	Mitral stenosis with AF	Polycythaemia rubra vera / Essential thrombocythaemia	Cancer
Dissection	AF (other than lone AF)	(b) Sickle cell disease	Migraine
Fibromuscular dysplasia	Left atrial or appendage thrombus	(c) Paroxysmal nocturnal haemoglobinuria	Inflammatory bowel disease
Congenital arterial anomalies	Sick sinus syndrome	(d) Thrombotic thrombocytopoenic purpura (TTP)	Fabry's disease
Moyamoya syndrome	Recent MI (<4/52)	(e) Leukaemia	Mitochondrial cytopathy
Embolism from arterial aneurysms	Left ventricular thrombus	(f) Intravascular lymphoma	Hypercalcaemia
Inflammatory vascular diseases	Dilated cardiomyopathy	**Disorders of coagulation/fibrin:**	Hypoglycaemia
Irradiation	Akinetic left ventricular segment	**(a) Congenital:**	
Infections	Atrial myxoma	(i) Natural anticoagulant disorders	
	Infective endocarditis	Proteins C & S deficiency	
	Medium risk sources of cardiac emboli		
	Mitral valve prolapse	Antithrombin III deficiency	
	Mitral annulus calcification	(ii) Fibrinolytic system disorders:	
	Mitral stenosis without AF	Hyperfibrinogenaemia	
	Left atrial turbulence	Plasminogen deficiency	
	Atrial septal aneurysm	(iii) Other	
	Patent foramen ovale	Hyperhomocysteinaemia	
	Atrial flutter	Factor V Leiden, VII, XIII Mutations	
	Lone AF	Antiphospholipid antibody syndrome	
	Bioprosthetic cardiac valve	**(b) Acquired:**	
	Nonbacterial thrombotic endocarditis	Disseminated intravascular coagulation (DIC)	
	Congestive heart failure	Lupus anticoagulant/anticardiolipin syndrome	
	Hypokinetic left ventricular segment	Pregnancy and the puerperium	
	Myocardial Infarction (>4 /52, <6/12)	Oral contraceptive pill	
		Paraproteinaemias	
		Polycythaemia	
		Elevated C Reactive Protein (CRP)	

1.3.1 Vessel Wall Disease

1.3.1.1 Medium and Large Vessel Disease–Atherosclerosis/Atheroma

Atherosclerosis is the commonest arterial disease of the Western world with incidence increasing with increasing age. In situ thrombosis, vessel stenosis and distal embolisation are common sequelae. The presence of atheroma does not inevitably lead to vessel obstruction. Severe atheroma can be asymptomatic while fatal stroke and myocardial infarction (MI) can occur when atheroma is mild. Vessel stenosis is therefore likely to be an interplay between vessel and haematological pathology.

Atherosclerosis is fundamentally an inflammatory process (Lusis, 2000; Poole and Florey, 1958; Stratford et al., 1986). The process begins with the accumulation of cholesterol engorged macrophages, (foam cells) and fatty streaks within the sub endothelial regions of the intima. Areas affected most are those with greater flow velocities such as the carotid artery bifurcation. Fatty streaks mature by the accumulation of lipid rich necrotic debris and smooth muscle cells (SMCs) which can then fibrose and calcify. Unstable plaques are prone to internal haemorrhage from friable infiltrating vessels leading to thrombosis and vessel occlusion. Atherosclerosis is therefore a response to injury, with glycoproteins acting as the injurious agents (Ross, 1993) (Libby, 2000).

1.3.1.2 Small Vessel Disease

Various terms have been used to describe small vessel disease (lipohyalinosis, fibrinoid necrosis, hyalinosis and angionecrosis) (Fisher, 1991). The result is small areas of cerebral infarction (lacunar infarction, lacune = hole) which accounts for up to 25% of all clinically evident cases of ischaemic stroke (Bamford et al., 1987; Hankey and Warlow, 1991) and an unknown proportion of TIAs. (Kappelle et al., 1991). Small vessel disease often goes unnoticed producing 81% of the total number of infarcts seen in the largest post-mortem study published to date (Byrom and Dodson, 1948). Mortality is lower than other forms of stroke.

The lenticulostriate and thalamo perforating arteries (diameter 40 - 800 micrometers) are most often affected (Bamford and Warlow, 1988). These arteries are end arteries with no collateral

circulation and so occlusion invariably results in infarction. Occlusion of these small vessels is most likely to be due to thrombosis rather than embolisation as fewer sources of emboli are seen in lacunar infarct victims (Olsen et al., 1985) (Kappelle and van Gijn, 1986) (Bamford et al., 1990; Hankey and Warlow, 1991). Using newer more sensitive ultrasound equipment microemboli are now being recognized which may also play an important role (Laloux and Brucher, 1991) (Lund et al., 2000; Wong et al., 2002)).

Histologically the muscle in the walls of small vessels is replaced with collagen, with resultant tortuosity of the vessel and growth of small friable aneurysms (Fisher, 1982). Hypertension is a common feature in small vessel disease although this association is not exclusive. Two large post mortem studies identified hypertension in only 58% of patients with lacunar infarction (Dozono et al., 1991; Tuszynski et al., 1989).

1.3.2 Cardiac Embolism

The intracardiac sources of emboli are outlined in Table 1.5 Cardiac embolism causes approximately 20% of all ischaemic stroke (Broderick et al., 1992). Emboli can be recognized relatively easily with ultrasound techniques however in the presence of other stroke risk factors such as hypertension and downstream atheroma it is often unclear whether cardiogenic emboli are causative. Examining ischaemic stroke patients in AF with transthoracic ultrasound, Daniel et al (1993) were only able to identify cardiac thrombus in 15%. (Daniel et al., 1993). Atrial fibrillation (AF) is the commonest cause of cardioembolic stroke accounting for up to 20% of all cases (Sandercock et al., 1992).

1.3.3 Hypercoagulability

Unlike venous thromboembolism which is often associated with clotting irregularities (Martinelli et al., 1998) the relationship between thrombophilia and arterial stroke remains uncertain. Hypercoagulability disorders are relatively uncommon although they are often over represented in young stroke victims. Recognised pro-thrombotic disorders include the factor V Leiden mutation (Wu and Tsongalis, 2001), hyperfibrinogenaemia (Heinrich et al., 1994; Meade, 1981),

factor VII , factor XIII (Elbaz et al., 2000) Protein C, S and antithrombin III deficiencies (Hossmann et al., 1983; Nagaraja et al., 1999; Tiong et al., 2003), use of the oral contraceptive pill, systemic inflammatory disorders, malignant disease, hyperhomocystinaemia, the antiphospholipid antibody syndrome, and the haemoglobinopathies. These conditions may all play a role in the development of ischaemic stroke with or without underlying vessel wall disease (Markus and Hambley, 1998) (Table 1.5).

1.3.4 Molecular Effects of Ischaemia

The molecular pathology of cerebral infarction is complex and not yet completely understood. All subtypes of cerebral infarction may have common downstream consequences irrespective of the stimulus. The consequences of cerebral ischemia depend on the degree and duration of reduced cerebral blood flow (CBF). Neurons can usually tolerate ischemia for 3-6 hours with CBF between 12 and 18 ml/100g/min. This ischaemic tissue has been called the "ischemic penumbra" (Astrup et al., 1981) and is seen as the target for therapeutic intervention. The duration of this reversible period is unknown and depends on many factors including the extent of collateral blood flow to the ischaemic area. Estimates of up to 17 hours have been postulated (Marchal et al., 1996).

Excitatory amino acids such as glutamate also play a destructive role. In 5 minutes of experimental ischemia, extracellular concentration of glutamate in vivo increases approximately 8 fold (Benveniste et al., 1984) partly due to increased release from injured neurons and partly to impaired re-uptake systems.

Glutamate triggers two distinct types of injury (Choi and Rothman, 1990). The first is a reversible, acute neuronal swelling which depends on the influx of extracellular Na^+, Cl^-, and water (Rothman, 1985). The second, irreversible step, is dependent on extracellular Ca^{2+} (Choi, 1987) which influxes through glutamate and voltage operated Ca^{2+} channels. This leads to excessive intracellular enzyme activation (protein kinase C, calmodulin-dependent protein kinase II, proteases, nitric oxide synthase (Choi, 1988) (Dawson et al., 1991) and endonucleases (Altman, 1992) consuming cellular energy and depleting ATP. Repolarization is prevented by the

Ca^{2+} influx leading to a spreading depression which depletes the energy of the surrounding neurons allowing concentric migration of severe ischemic damage from the core of the infarct to the potentially viable periphery, thus increasing the final size of the infarct. Autolysis (and possibly apoptosis) (Altman, 1992) by the activated enzymes perpetuates the loss of viable tissue. The final insult is the release of pathological free radicals (OH^-, H_2O_2, NO^-) from ischaemic mitochondria which enhance the autolytic process (Dawson et al., 1991) (Radi et al., 1991).

The toxicity of glutamate has been established for many decades (Lucas and Newhouse, 1957) and antagonists of glutamate are known to reduced hypoxic damage both in vitro (Rothman, 1984) and in vivo (Simon et al., 1984). Glutamate interacts with at least three different types of postsynaptic receptors on the cell membrane: N-methyl-D-aspartate (NMDA), quisqualate, and kainate. The most well studied receptor and also possibly the most relevant in stroke is the NMDA receptor. Activation of the receptor by either glutamate or NMDA opens the channel and allows Ca^{2+} and Na^+ to enter the cell setting off the destructive cascade.

Over recent years with the acquisition of such knowledge the treatment of stroke has received much attention. Statins are aimed at reducing the accumulation of intravascular lipid. Angiotensin converting enzyme inhibitors (ACE inhibitors) have been used to alter endothelial function, reduce smooth muscle proliferation and overturn the noxious effects of nitric oxide. Thrombolysis has focused on salvaging the ischaemic penumbra and neuroprotection has attempted to reduce the toxic effects of glutamate. The role of the Renin – Angiotensin system (RAS), in particular the *ACE* gene, in ischaemic stroke will be covered in Chapter 3 and a summary of the stroke treatments can be found in Chapter 9 (Appendix).

1.4 Epidemiology and the Burden of Stroke

Stroke is the third most common cause of death in the Western world. (WHO, 2002). It accounts annually for 11% of all deaths in the UK (HMSO, 2000) and is the most common cause of adult physical disability (Harris, 1971) which has been acknowledged by two recent UK government white papers (HMSO, 1999) (HMSO, 2001).

1.4.1 Incidence

The average annual incidence of stroke in the UK is about 3/1000 population (Bamford et al., 1988) rising steeply with age (Table 1.7) and is more common in men. Worldwide incidence rates vary between 0.61-6.27/1000 (Sudlow and Warlow, 1997) (Thorvaldsen et al., 1995). TIA incidence and prevalence rates vary between 0.3-1.9/1000 (Dennis et al., 1989) (Brown et al., 1998) (Sempere et al., 1996) (Gibbs et al., 2001) and 14-30/1000 (Bots et al., 1997) (Toole et al., 1996) respectively. Locally approximately 1,000 new stroke patients are admitted to the three acute Leicestershire hospitals annually (Dennis, 1999) unpublished data).

Table 1.7 Age-specific annual incidence of first stroke per 1000 population.

Comparison of the OCSP (Bamford et al., 1988), the East Lancashire Study (ELS) (Du et al., 1997) and the South London Stroke Register (SLSR)(Stewart et al., 1999) (95% confidence intervals)

Age group (yrs)	OCSP	ELS	SLSR
<15	0.03		0.01 (0.00-0.04)
15-24	0.06		0.03 (0.00-0.07)
25-34	0.08		0.12 (0.08-0.18)
35-44	0.23		0.30 (0.21-0.42)
<45	0.09 (0.06-0.13)		
<50		0.09 (0.06-0.13)	
50-54		0.88 (0.49-1.26)	
45-54	0.57 (0.35-0.79)		0.87 (0.68-1.10)
55-64	2.91 (2.37-2.45)	1.69	2.19 (1.88-2.53)
65-74	6.90 (5.93-7.87)	4.67	4.96 (4.44-5.51)
75-84	14.34 (12.49-16.19)	10.64	9.34 (8.41-10.34)
>85	19.87 (15.78-23.95)	20.86	19.72 (17.08-22.65)
Total Samples	1.60 (1.48-1.72)	1.58 (1.46-1.71)	1.33 (1.26-1.41)

1.4.2 Mortality

Mortality rates are often inversely proportional to degrees of affluence, both in the UK (National Institute of Epidemiology (NationalInstituteEpidemiology, 1997) (Table 1.8) and abroad (Sarti et al., 2000). In Europe mortality figures (/100,000 males age 35-74) range from 34 in Switzerland to 162 in Portugal with the UK in 9th place at 65 (Sarti et al., 2000) .

Table 1.8 Case fatality after first stroke Comparison of the OCSP (Bamford et al., 1988), the East Lancashire Study (ELS) (Du et al., 1997) and the South London Stroke Register (SLSR)(Stewart et al., 1999) (95% confidence intervals).

Days after stroke	OCSP	ELS	SLSR
28	19%	34%	26%
90		40%	33%
180			37%
1 year	31%		

Mortality is also higher in minority ethnic groups in both the UK (Wild and McKeigue, 1997) (Balarajan, 1991; Wolfe et al., 2002). (Table 1.9) and USA (Broderick et al., 1998) with highest rates seen in West Africans and Afrocaribeans.

Table 1.9 Standardised mortality ratios (95% confidence intervals) for stroke in England and Wales by country of birth, 1989-92 (Wild and McKeigue, 1997)

Country of birth	Men	Women
Total population	100	100
West Africa	271 (210-344)	181 (118-265)
Caribbean	168 (151-186)	157 (136-179)
South Asia	155 (143-168)	141 (127-157)
Ireland	138 (128-148)	123 (113-133)
Scotland	125 (115-136)	125 (113-137)
East Africa	114 (86-147)	122 (88-164)

In recent years there has been a fall in stroke incidence as well as age specific mortality. Mortality rates have declined by 42% in men and 32% in women, (HMSO, 1988; HMSO, 2000; Thorvaldsen et al., 1997). Despite this, a steady rise in the average age of the population is likely to ensure that stroke will become an increasing burden on health services in the years to come.

1.4.3 Economic Burden

Between 4% (OHE, 1988) and 6% (HMSO, 1992) of the annual National Health Service budget is spent on stroke. In 1995-96 this accounted for £2318 million (£758 million for new patients, £1560 million for long-term care, community support and treatment of recurrent strokes). This figure rises to almost £3000 million if the costs of informal care are also taken into account (Bosanquet and Franks, 1998).

In order to keep these costs under control stroke prevention should play a central role in future health care provision.

1.4.4 Effects of Stroke

1.4.4.1 Incidence of disability following stroke

The true extent of disability caused by stroke is unknown however the majority of stroke survivors require care. A summary of the available information is found in Table 1.10 (Wade, 1994)

Table 1.10 Disability rates six months after stroke (Wade, 1994; Wade and Hewer, 1985; Wade and Hewer, 1987; Wade et al., 1986; Wade et al., 1989).

Disability	%	Level Of Dependence	%
Motor dysfunction (partial or complete)	53%	Physically independent	47%
Help washing	49%	Mildly dependent	32%
Help with feeding	33%	Moderately dependent	12%
Help dressing	31%	Severely dependent	9%
Disoriented (or unable to talk)	27%		
Help with toileting	20%		
Help transferring	19%		
Unable to walk independently indoors	15%		
Aphasia	15%		
Help with grooming (teeth, hair, face)	13%		
Urinary incontinence	11%		
Faecal incontinence	7%		

1.5 The Multi-factorial Aetiology of Stroke / Stroke Risk Factors

Much of our current knowledge about the numerous risk factors for ischaemic stroke has been obtained from the Framingham heart study. (Table 1.11) Risk factors can be seen as either modifiable or unmodifiable and their importance varies with each pathological subtype.

Table 1.11 Factors associated with an increased risk of vascular disease

Unmodifiable	Modifiable
Age Male sex Family history of stroke Race	Blood pressure (BP) Atrial Fibrillation (AF) Transient Ischaemic Attacks (TIA) Diabetes Mellitus (DM) Ischaemic Heart Disease (IHD), Cardiac Failure Hyperlipidaemia Peripheral Vascular Disease (PVD) Smoking Alcohol Obesity and diet Plasma fibrinogen Raised C Reactive Protein Hyperhomocystinaemia Factor VII coagulant activity Oral contraceptives Impaired Ventilatory Function Physical inactivity Social deprivation

Most modifiable risk factors are based largely on epidemiological associations, mostly without direct explanation of their pathological effects and some of these links may simply be due to confounding. (Ebrahim and Harwood, 1999; Goldstein et al., 2001; Warlow, 1998). Most patients have been recognised as being 'at risk' prior to the onset of stroke (Shaper et al., 1991) but although there have been attempts to assign risk categories they remain unvalidated and are largely unused in clinical practice (Wolf et al., 1991). The evidence in favour of modification of the more important risk factors is detailed in Table 1.12. While some of these risk factors are dichotomous (present / absent e.g. AF), others, such as hypertension and obesity, are continuous variables with arbitrary cut-offs between 'normal' and 'abnormal'.

Table 1.12 Relative risks (RR) and prevalence of modifiable risk factors (RF) for stroke

RF	RR of stroke when RF present	Reference	UK Prevalence of RF	Reference
Good evidence that treatment / avoidance can lower stroke risk				
Hypertension	1.5 for every 5 mmHg increase in diastolic blood pressure	(MacMahon et al., 1990)	BP>140/90 mmHg Men = 38% Women = 30%	(Erens and Primatesta, 1999)
	7, 160/95 vs 120/80	(Markus, 2003)		
	Between age 40-69 yearsa rise of 20 mm Hg of usual SBP or 10 mm Hg DBP = > 2X increase in stroke mortality	(Lewington et al., 2002)		
AF	4-5	(Brand et al., 1985; Kopecky et al., 1999)	5% of 65+ yrs	(Sudlow et al., 1998; Wheeldon et al., 1998)
Smoking	1.5	(Shinton and Beevers, 1989)	Men = 28% Women = 27%	(Erens and Primatesta, 1999)
Diabetes mellitus	2-3	(Ebrahim and Harwood, 1999) Kannel and MacGee 1979	Men = 2-3% Women = 1.5-2.5%	(Erens and Primatesta, 1999; Gatling et al., 1998)
Undiagnosed Diabetes (glycosylated haemoglobin >5.2%)			Men = 1% Women = 1%	(Bennett et al., 1995)
Ischaemic heart disease (Angina or MI)	2.5	(Ebrahim and Harwood, 1999)	Men = 7% Women = 4.5%	(Erens and Primatesta, 1999)
Previous stroke	15 in first year after stroke, dropping to 2 after 5 years	(Burn et al., 1994)	1.5-1.75% of total population	(Geddes et al., 1996; O'Mahony et al., 1999)
TIA	80 within first month, 13 within first year, 7 overall	(Dennis et al., 1990)	1.5% of > 55yrs report symptoms of TIA in preceding 3 years	(Bots et al., 1997)
Hyperlipidaemia	1.4 between lowest & highest quartiles of total cholesterol	(Benafante et al 1994)		
Weaker evidence that treatment / avoidance can lower stroke risk				
Obesity (BMI >30kg/m2) Overweight (BMI 25-30 kg/m2)	1-2	(Ebrahim and Harwood, 1999)	Men = 17% Women = 21% Men = 45% Women = 32%	(Erens and Primatesta, 1999)
Physical inactivity	2.5	(Wolfe et al., 1996)	< 30 mins of moderate exercise / week Men = 35% Women = 41%	(Erens and Primatesta, 1999)
Excessive alcohol consumption	1.5-2 for ≥5 units/day	(Hart et al., 1999a; Sacco et al., 1999)	35 units per week Men = 15% Women = 3%	(Erens and Primatesta, 1999)
Oral contraception (OCP)	3, 2 if low oestrogen preparation	(Gillum et al., 2000)	27% of women aged 16-54 current users	(Erens and Primatesta, 1999)

Unmodifiable Risk Factors

1.5.1.1 Age

The Oxford Community Stroke Project identified age as the strongest risk factor for cerebral infarction with a 25 fold increase in events between groups aged 45-54 and 75-84 (0.57/1000 vs 14.3/1000) (Bamford et al., 1990).

1.5.1.2 Gender

Males are at increased risk but to a much smaller degree than seen in other vascular diseases. The effect is most prominent in middle age becoming less so at the extremes of the age range (Haberman et al., 1981; Thorvaldsen et al., 1995).

1.5.1.3 Family History and Race

These will be covered in the stroke genetics section.

1.5.2 Modifiable Risk Factors

1.5.2.1 Hypertension

This is the most important treatable and independent risk factor for stroke. In the Framingham Heart Study the age standardized incidence of cerebral infarction was approximately 3 times greater in people with systolic blood pressure (SBP) >160 mmHg and 2 times higher in those with SBP 140-159 mmHg compared with patients with SBP <139 mmHg (Wolf, 2003).

For every 7.5 mmHg increase in DBP above 85 mmHg the risk of stroke doubles (Collins et al., 1990; MacMahon et al., 1990; MacMahon and Rodgers, 1994). However even modest elevations of blood pressure confer significant risk. Approximately 50% of the first strokes identified in the Framingham study occurred in subjects with mild hypertension or normal blood pressure (Wolf, 2003). Overall approximately 75% of all stroke deaths occurred in patients who have DBP less than 110 mmHg. Most studies include BP measured at the time of the study however persistently

elevated BP during the preceding 10 years increases the relative risk (RR) of stroke by 1.68 in women and by 1.92 in men (Seshadri et al., 2001). This data is supported by treatment trials of BP reduction which show reductions in stroke incidence (see section 1.6.2.2).

1.5.2.2 Atrial Fibrillation (AF)

AF is recognised as the greatest risk factor for a cardio-embolic stroke. It is often associated with underlying cardiac dysfunction (Kannel et al., 1998) (Psaty et al., 1997) (Levy et al., 1999; Lip et al., 1997). AF confers a 4-5 fold increase in RR of stroke (Brand et al., 1985; Kopecky et al., 1999). This risk may not be entirely independent as AF is often associated with underlying cardiac dysfunction, increasing age, DM, left ventricular dysfunction and hypertension and the likelihood of embolisation increases in the presence of these risk factors (van Latum et al., 1995). The prevalence of AF has risen over time, from 3.2% in men aged 65-84 in 1968 to 9.1% in 1989 (Kannel et al., 1998). Non-rheumatic AF (lone AF) also carries a 5% risk of ischaemic stroke per annum (Hart and Halperin, 1994).

Four UK prevalence surveys of AF are summarised in Table 1.13. Approximately 25% of AF is paroxysmal and the rest chronic (Lip et al., 1997; O'Connell and Gray, 1996).

Table 1.13 The prevalence of AF A Summary of 4 UK prevalence surveys.

Study	Population	Results
(Sudlow et al., 1998)	Random sample of 4,843 people aged ≥65 from 26 practices in Northumberland	Overall prevalence: = 4.7% 65-74 yrs: 3.5% men, 2.4% women 75+ yrs: 10% men, 5.6% women
(O'Connell and Gray, 1996)	Single practice in Gateshead (n = 9,162)	Overall prevalence: = 1% 76% chronic, 24% paroxysmal
(Wheeldon et al., 1998)	Single practice in Sheffield: all patients aged ≥65 (n = 1,422)	Overall prevalence: = 5.4% 75+ yrs: 6.6%
(Lip et al., 1997)	2 Birmingham practices (n = 16,519)	Prevalence: 50+ yrs = 2.4% 73% chronic, 27% paroxysmal

1.5.2.3 Transient Ischaemic Attack (TIA)

TIAs are warnings of future stroke conferring a 5-10x RR. After TIA the risk of stroke is 4.4% (95% CI: 1.5-7.3% (RR=80)) in the first month, 11.6% (95% CI: 6.9-16.3% (RR=13)) in the first year and 29% (95% CI: 21.3-37.3%) over 5 years. The average annual risk of stroke after a TIA is 6% and MI is 2.4% (Dennis et al., 1990). TIA is often associated with carotid atheroma which is implicated in 9% of all ischaemic stroke (Kistler and Furie, 2000). When associated with symptoms of TIA, severe carotid artery stenosis (70-99% reduction of luminal diameter) is associated with a 20% risk of major stroke in three years ((ECAS), 1991; (NASCET), 1991).

1.5.2.4 Asymptomatic carotid artery stenosis

Identification of carotid artery stenosis in the absence of a stroke (asymptomatic stenosis) carries a significant but lower risk of stroke than symptomatic stenosis. In the Asymptomatic Carotid Artery Stenosis trial the risk of ipsilateral stroke or death after 5 years in the control group was 11% (AsymptomaticCarotidAtherosclerosisStudy, 1995) which compares favourably with a 29% 5 year risk of stroke after a previous TIA (Dennis et al., 1990). High grade asymptomatic stenosis (60-99%) still conferred a 50% greater risk of a first stroke compared with lower grade stenoses (< 60%) (Inzitari et al., 2000) but only 55% of these strokes were classified as attributable to the carotid stenosis thereby diluting the overall relative risk of stroke (Barnett et al., 2000).

Carotid stenosis implies atheroma of other vessels. In a cohort study of men born in1914 those with carotid stenosis had a higher death rate from IHD but not ischaemic stroke. As stroke often occurs later in life the earlier heart related deaths of these men may have reduced the incidence of cerebrovascular events (Ogren et al., 1995).

1.5.2.5 Diabetes Mellitus (DM)

DM confers a 2x increase in stroke risk (Kannel and McGee, 1979) and is an independent marker of poor prognosis (Barrett-Connor and Khaw, 1988; Fuller et al., 1983; Rosengren et al., 1989; Stout, 1989).

1.5.2.6 Ischaemic Heart Disease (IHD) and Peripheral Vascular Disease (PVD)

CHD is unequivocally associated with an increased risk of stroke (Friedman et al., 1968); (Kannel et al., 1983). Within two weeks of MI 0.7-4.7% of patients suffer stroke (Loh et al., 1997). Co-incident PVD reflects the presence of widespread atherosclerosis and as such confers a three fold risk of stroke mortality. (Smith et al., 1990)

1.5.2.7 Hyperlipidaemia

High levels of total plasma cholesterol and low density lipoproteins (LDL) as well as low levels of high density lipoproteins (HDL) are well established risk factors for IHD (Martin et al., 1986; Shaper et al., 1991). Conversely a number of large studies have failed to find an independent association of stroke and serum cholesterol (ProspectiveStudiesCollaboration, 1995) or triglycerides (Hulley et al., 1980; Shahar et al., 2003). There have however been 2 notable exceptions. The Honolulu Heart Study of Hawaiian men of Japanese ancestry (Benfante et al., 1994) showed a historic elevation in total cholesterol correlated with an increased incidence of thromboembolism with RR of stroke of 1.4 between the lowest and highest quartiles of serum cholesterol. The Multiple Risk Factor Intervention Trial (MRFIT) study (Iso et al., 1989) showed that high levels of total serum cholesterol measured six years before death significantly correlated with a higher incidence of ischaemic stroke however cholesterol levels <4.14 mmol/L conferred a 3 fold higher risk of haemorrhagic stroke. Further support of the candidacy of high serum lipids as risk factors comes from treatment trials with statin therapy reducing the risk of stroke particularly in patients with IHD (Herbert, 1997).

Extra-cranial carotid artery atherosclerosis, a surrogate marker for large vessel stroke, has been correlated with high levels of total serum cholesterol and LDL cholesterol, with high HDL cholesterol showing a protective effect (Fine-Edelstein et al., 1994; O'Leary et al., 1996; Wilson, 1995). Statin therapy can slow progression and occasionally promote regression of carotid artery plaques (Furberg et al., 1994). The extent to which the hyperlipidaemia are risk factors for stroke remains unclear. Statins may be working in ways other than reducing the extent of atheroma possibly by reducing the tendency for plaque rupture by altering lipid composition. (HPS, 2002; Sever et al., 2003).

Possible explanations for these contradictory results include a masking effect of low cholesterol levels associated with haemorrhagic stroke, (Iso et al., 1989; Tanaka et al., 1982) underpowered studies with stroke as a secondary rather than primary end-point and beneficial effects of statins independent of their cholesterol lowering properties. This latter point is emphasized by the fact that the extent of lipid lowering in statin trials is relatively small in relation to the large effects on relative risk reduction.

1.5.2.8 Smoking and Alcohol Consumption

Smoking increases the risk of stroke by approximately 1.5, with risk increasing with increasing dose (Shinton and Beevers, 1989). Passive smoking also increases the risk (Donnan et al., 1989). Direct pathological data concerning the tissue effects of smoking is lacking however smoking has been strongly associated with an increase in carotid atheroma (Homer et al., 1991). Studies of the effects of alcohol use are complicated by inaccuracies in measuring intake, difficulties in identifying control populations and the possible synergistic effects of smoking with other risk factors. Minimal as well as heavy alcohol consumption is associated with a greater risk compared to moderate use (Djousse et al., 2002; Doll et al., 1994; Gill et al., 1986; Gordon and Kannel, 1983; Kozararevic et al., 1980). Alcohol may also cause stroke by promoting other risk factors such as hypertension (Friedman et al., 1988; Malhotra et al., 1985), hyperlipidaemia (Gordon and Kannel, 1983) and AF (Ettinger et al., 1978)

1.5.2.9 Obesity

Ischaemic stroke has been linked with obesity (Welin et al., 1987) however this association is complicated by stronger risk factors such as hypertension, hyperlipidaemia and diabetes which are known to have a higher prevalence in obese individuals (VanItallie, 1990). Insufficient data is available to confirm obesity as an independent risk factor.

1.5.2.10 Haematological Disorders

Cerebral infarction is a consequence of a number of haematological disorders (Table 1.6).

1.5.2.10.1 Fibrinogen

Plasma fibrinogen is consistently found as an adverse risk factor for stroke with higher risk when combined with smoking and hypertension, possibly due to accelerated thrombosis (Cook and Ubben, 1990; Kannel et al., 1987; Meade et al., 1987; Qizilbash et al., 1991; Rosengren et al., 1990; Wilhelmsen et al., 1984). A large meta-analysis conducted by the Fibrinogen studies collaboration in 2005 identified moderately strong associations between plasma fibrinogen and the risks of coronary heart disease, stroke and other vascular diseases in middle-aged adults. The age and sex adjusted hazard ratio per 1 g per litre increase in usual fibrinogen level for stroke was 2.06 (95% CI 1.83 - 2 .33). (Danesh et al., 2005)

1.5.2.10.2 Elevated CRP

In the original Framingham cohort elevated CRP was found to be an independent risk factor for stroke and TIA (Rost et al., 2001). Male subjects with CRP levels within the top 25% were found to have twice the risk of stroke and TIA as compared with men in the lowest 25%. For women in the top 20% of the risk was almost threefold. A meta-analysis of the available data by Kuo et al (Kuo et al., 2005) identified nearly 70% increase in risk of stroke for those individuals with CRP concentrations in the highest quartile compared with those in the lowest quartile. Although there appears to be an association between CRP and risk of stroke there is currently no strong evidence suggesting that the identification and primary preventative treatments improve health outcomes. In secondary prevention elevated CRP appears to increase the risk of future stroke however at present there is no specific therapy which reduces a nonspecifically elevated CRP and so high CRP should merely highlight the need for well-established secondary preventative therapies.

1.5.2.10.3 Hyperhomocysteinaemia

Increased incidence of stroke (Boushey et al., 1995) and carotid intima-media thickness (IMT) (Malinow et al., 1993; Selhub et al., 1995) has been linked with hyperhomocysteinaemia. The Framingham Heart Study identified a RR of 1.82 of stroke in patients with serum homocysteine

in the top 25% compared with the bottom 25%. Homocysteine was found to be an independent risk factor for stroke in the British Regional Heart study with risk rising with increasing levels of serum homocysteine (Perry et al., 1995). Individuals with greater than 15.4 μmol/L of homocysteine had a 4.7 times RR compared with those with levels of less than 10.3 μmol/L. The ARIC study, Physicians Health study and MRFIT have failed to reproduce these results for stroke (Evans et al., 1997; Folsom et al., 1998; Stampfer et al., 1992; Verhoef et al., 1994).

The metabolism of homocysteine is complex and closely related to folic acid and vitamins B12 and B6 levels. Fasting homocysteine levels may give a spuriously normal result as 40% of individuals with normal fasting levels are demonstrated to have elevated homocysteine levels in response to a methionine challenge. In the presence of low folate and B12, elevation of homocysteine is more significant. As a consequence folic acid, B6 and B12 supplementation has been used in a few studies however they have shown no benefit in preventing stroke to date (Stampfer and Malinow, 1995; Stampfer and Rimm, 1996).

1.6 Management Of Stroke: Preventative Strategies

This section will consider the prevention of first stroke in at risk individuals (primary prevention) and second stroke in stroke victims (secondary prevention). The diagnosis of stroke as well as the acute, medium and long term treatments are considered in Chapter 8 (Appendix).

1.6.1 Primary Prevention

1.6.1.1 High risk individuals

Table 1.14 summarises the evidence for the effects of the preventative strategies. The greatest RR reduction of up to 68% is seen when patients with AF are treated with warfarin (AtrialFibrillationInvestigators, 1994; Hart et al., 1999b) which far outweighs the risk of serious haemorrhage. Patients less than 65 years old with lone AF are the only group who do not benefit. Despite this, in the UK and USA only 21-36% of eligible patients are on warfarin (Lip et al., 1997; O'Connell and Gray, 1996; Sudlow et al., 1998; Wheeldon et al., 1998) (Stafford and Singer, 1998). Aspirin produces 32% less reduction in stroke risk compared to warfarin (Benavente et al., 2000a; Hart et al., 1999b) however some authors advocate its use as first line therapy due to its relative safety (Taylor et al., 2001). The treatment of AF therefore, especially in the elderly, remains a difficult area and a RCT (The Birmingham Atrial Fibrillation Treatment of the Aged Trial (BAFTA)) is currently addressing this issue. (Fitzmaurice et al., 2000)

Modest reductions in blood pressure can lower the stroke risk by up to 40% (Collins, 1994). Risk reductions in the region of 30% are seen by treating vascular disease with aspirin (AntiplateletTrialists'Collaboration, 1994) or statins (Herbert, 1997) and asymptomatic carotid stenosis with carotid endarterectomy (Benavente et al., 1998; Chambers et al., 2000).

Table 1.14 Effectiveness of primary stroke prevention strategies in high risk individuals

Risk group	Intervention	Reduction in stroke risk	Comments	Reference
Hypertension	Lowering BP	35-40%	- mean reduction of 5-6 mmHg in diastolic blood pressure	(Collins, 1994)
AF	Warfarin	68%	- independent of age	(AtrialFibrillationInvestigators, 1994)
		59%,	- with mean achieved INR from 2.0-2.6	(Hart et al., 1999b)
	Aspirin (50 mg-1.3 g / day)	20-22%,	10 strokes prevented for every 1000 treated	(Benavente et al., 2000a; Hart et al., 1999b)
	Warfarin versus aspirin	32-36%	reduction if treated with warfarin in preference to aspirin	(Hart et al., 1999b; Taylor et al., 2001)
Non Valvular AF (Lone AF)	Warfarin		INR of 2-3 reduces stroke but significant bleeding risk 25 strokes and 12 disabling / fatal strokes prevented yearly for every 1000 treated.	(Benavente et al., 2000b)
Carotid artery stenosis (asymptomatic)	Carotid endarterectomy	27-38%	reduction in risk of ipsilateral stroke or peri-operative stroke or death after mean of 3 years follow-up	(Benavente et al., 1998; Chambers et al., 2000)
High vascular risk (PVD, IHD, Cerebrovascular Disease)	Aspirin	27%	- reduction in risk of MI, stroke or vascular death;	(Johnson et al., 1999)
		31%	- reduction in risk of non-fatal stroke	(AntiplateletTrialists'Collaboration, 1994)
	Thienopyridine derivatives (Clopidogrel, Ticlopidine) vs Aspirin	12%		(Hankey et al., 2000a; Hankey et al., 2000b)
	Statin therapy	32%	Pravastatin vs placebo in 4159 MI survivors (NNT = 40).	Cholesterol and Recurrent Events (CARE) (Plehn et al., 1999)
		30%	Simvastatin 40 mg vs placebo 20,536 vascular disease subjects	Heart Protection study ((HPS, 2002)
		73%	10,305 high risk hypertensives Stopped early 121 strokes in placebo group vs 89 in treatment group	Anglo Scandinavian Cardiac Outcomes Trial - Lipid Lowering Arm (ASCOT-LLA). (Sever et al., 2003)

1.6.1.2 Lower Risk Individuals

The American Heart Association and Scottish Intercollegiate Guideline Network guidelines (SIGN) are summarised in Table 1.15. Lifestyle interventions (e.g. smoking cessation, weight loss, healthy diet, exercise and control of alcohol consumption) are not supported by randomised control trial (RCT) evidence however observational studies demonstrate associations between the risk factor and stroke. Changes in risk factor status do correlate with better outcome (Ness and Powles, 1997).

Table 1.15 Guidelines for the primary prevention of stroke (AHA unless stated otherwise).

Hypertension *	Adult Screening at least every two years. Appropriate management. Closer observation of hypertension in diabetes. (Neal et al., 2000)
Smoking *****	Cessation recommended.
Diabetes *****	Glycemic control is recommended to reduce microvascular complications.
Asymptomatic carotid stenosis*	Endarterectomy considered in high grade asymptomatic carotid stenosis performed by a surgeon with <3% morbidity/mortality rate.
AF *****	Warfarin or aspirin should be considered for non-valvular atrial fibrillation whilst considering risk of embolism and bleeding complications. Higher risk patients considered for warfarin (INR of 2.5). Aspirin possibly a safer alternative to warfarin in some. (SIGN)
Hyperlipidaemia *****	Known coronary heart disease with elevated LDL cholesterol should be considered for statin treatment.
Obesity	Weight reduction recommended.
Physical Inactivity	>/= 30 minutes of daily moderate intensity activity recommended.
Poor diet/nutrition	>/= five daily servings of fruit and vegetables recommended.
Alcohol abuse	Men - 2 drinks per day Women (non pregnant) - 1 drink per day
Hyperhomocysteinaemia	Folic acid and B vitamins should be considered.
Oral contraceptive use	Avoid in women with additional risk factors (e.g. cigarette smoking or prior thromboembolic events).

AHA: American Heart Association(Goldstein et al., 2001) ; SIGN: Scottish Intercollegiate Guidelines Network. (SIGN, 1999) ;

* Randomised control trial (RCT) evidence

1.6.1.3 Population Strategies

This topic was reviewed by Rees et al in 2000 (Rees, 2000). Although dietary improvements for individuals are associated with reductions in stroke risk (Ness and Powles, 1997) this is unfortunately not supported by trial evidence in large populations. Promoting less cigarette use by raising the cost (Chaloupka and Wechsler, 1997), focused mass media campaigns (Sowden, 2000) reducing advertising (Smee, 1992) and smoke free workplaces (Chapman et al., 1999) are all effective especially for women and younger people. Increases in exercise as part of the everyday life of the population by encouraging walking and cycling is thought to be more effective than the more artificial measure of promoting the use of exercise facilities (Hillsdon and Thorogood, 1996) however no randomized evidence is available.

1.6.2 Secondary Prevention

Table 1.16 provides a summary of the main features of the secondary prevention guidelines. There is general agreement that all stroke patients should have their BP monitored and be prescribed an antiplatelet agent. There is also good evidence to support the treatment of symptomatic carotid stenosis and AF and hypercholesterolaemia.

Table 1.16 Summary of guidelines for secondary prevention of stroke

Hypertension	Blood pressure monitoring for all patients. Treat hypertension persisting for > 1 month (IWP, 2000) Control of hypertension … should be advocated once the initial event has stabilized. (SIGN, 1997)
Antiplatelet therapy	Aspirin therapy (50-300 mg od) for all (unless anticoagulated) Aspirin failure - aspirin + dipyridamole MR (200 mg bd). Aspirin intolerant - clopidogrel 75 mg od or dipyridamole MR (200 mg bd) (IWP) (SIGN) (ECC) Insufficient evidence in view of cost to justify clopidogrel or dipyridamole as first line treatment. (ECC)
Anticoagulation	AF, mitral valve disease, prosthetic heart valves or within 3 months of MI, anticoagulation should be considered for all patients who have ischaemic stroke. (IWP) (SIGN) (Target INR 2.5). (ECC) Cochrane Stroke Group(Koudstaal, 2000a; Koudstaal, 2000b) No clear benefit from long-term anticoagulant therapy in people with non-embolic ischaemic stroke or TIA(Liu, 2000) More intense anticoagulation (INR 3.0 - 4.5) is not safe.(Algra, 2001)
Carotid endarterectomy (CEA)	Any patient with a carotid artery area stroke, and minor or absent residual disability, should be considered for carotid endarterectomy. (IWP) Surgery should be targeted at patients at highest risk of further stroke, (severe stenosis of the ipsilateral carotid artery and performed as soon as possible after the initial event (within 6 months). (ECC) CEA reduced the risk of disabling stroke or death for patients with stenosis exceeding ECST-measured 70% or NASCET-measured 50%. Result is only generalisable to surgically fit patients operated on by surgeons with complication rates less than 6%).(Cina, 2000)
Other risk factors	All patients should be assessed and treated for other vascular risk factors and advised about lifestyle factors. (IWP)

Intercollegiate Working Party for Stroke National Clinical Guidelines (IWP, 2000)
Scottish Intercollegiate Guidelines Network (SIGN) (SIGN, 1997)
Edinburgh Consensus Conferences on stroke (ECC) (RCPE, 2000)
Cochrane Stroke Group Review of Secondary Prevention of Stroke

1.6.2.1 Antiplatelet agents

There are three antiplatelet agents currently in common use for secondary stroke prevention, aspirin, dipyridamole and clopidogrel. The size of the risk reduction with aspirin therapy is similar to that achieved in primary prevention (Table 1.14) although there is a small increase in risk of haemorrhagic stroke which is proportional to dose (AtrialFibrillationInvestigators, 1994). There is no evidence that 300 mg/day is any more effective than 75 mg/day (Johnson et al., 1999). Clopidogrel and ticlopidine are effective alternatives in patients who are intolerant of aspirin (Hankey et al., 2000a).

Combination of aspirin and dipyridamole has been shown by one trial (European Stroke Prevention Study (ESPS-2), (Diener et al., 1996) to be beneficial and this combination is currently under review by the Antithrombotic Trialists Collaboration (Sudlow, 1998). In acute coronary syndromes and during coronary endovascular procedures the combination of clopidogrel and aspirin has been shown to be more effective than aspirin alone in preventing vascular events, (Mehta et al., 2001; Yusuf et al., 2001) however the MATCH trial has shown no significant reduction in the rates of stroke and a greater rate of haemorrhage with this combination and so this is unlikely to become established practice for long term stroke preventative therapy (Diener et al., 2004).

1.6.2.2 Blood Pressure Reduction

The control of SBP and DBP is the cornerstone of stroke prevention. Results of the important trials are detailed in Table 1.17. Reduction in the recurrence of stroke is in the region of 28-73% with the greatest effects seen in those at the highest risk such as severely hypertensive, elderly and diabetic patients. The PROGRESS trial has shown that there are also significant advantages of lowering blood pressure from levels previously thought to confer no increased risk of recurrent stroke.

Table 1.17 Secondary prevention of stroke by blood pressure reduction

Date of Publication	Trial	Details	BP Reduction	Risk Reduction (95% CI)
1990	(Collins et al., 1990) (MacMahon et al., 1990)	37,000 elderly diabetics	5.8 mm Hg DBP	42%
1991	Systolic Hypertension in the Elderly (SHEP, 1991)	4736 patient>60 yrs. SBP >160 mmHg DBP<90 mmHg 4.5 yrs follow up		36%
1997	(Staessen et al., 1997)	4699 patients with isolated systolic hypertension 2 yrs follow up		42% 73% in diabetics
1997	Meta-analysis (Gueyffier et al., 1997).			28% (61-85)
2001	(PROGRESS, 2001)	6105 patients with stroke or TIA	9/4 mmHg	28% (17-38)

The studies comparing the effects of different antihypertensives are summarised in Table 1.18. All antihypertensives reduce stroke risk and the size of the benefit is proportional to the level of the blood pressure control. The HOPE and PROGRESS trials show additional benefits of ACE

inhibitors and LIFE shows similar benefits of an angiotensin II receptor antagonist, independent of their effects on BP. This may be related to beneficial effects on endothelial function, fibrinolysis and smooth muscle proliferation (Lonn et al., 2001)

Table 1.18 Comparison of antihypertensives used in secondary prevention.

Date Of Publication	Trial	Details	Patient Numbers	Results
2000	HOPE (Yusuf et al., 2000)	10 mg Ramipril vs placebo	9297 with vascular disease or diabetes	3 mmHg BP reduction with Ramipril conferring 32% reduction in stroke
2001	(PROGRESS, 2001)	ACE inhibitor (Perindopril) + Thiazide diuretic (Indapamide) vs Peridopril	6105 patients with stroke or TIA, hypertensives and normotensives	Combination therapy : mean BP reduction = 12/5 mmHg, Stroke risk reduction = 43% Perinopril therapy : mean BP reduction 5/3 mmHg, Stroke risk reduction = 5%.
2002	Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Study ALLHAT (ALLHAT, 2002)	Ca Blockers, vs ACE inhibitors, vs Thiazide diuretics vs B blockers		All drugs showed similar benefit proportional to degree of BP reduction
2002	Losartan Intervention for Endpoint (LIFE)(Kjeldsen et al., 2002)	Angiotensin II receptor antagonist (Losartan) vs B Blocker (Atenolol)	9193 hypertensives and left ventricular hypertrophy	Equivalent mean BP reduction 32/18.5 mmHg in Losartan group, 29.1/19.2 mmHg in Atenolol group. Risk of stroke 25% lower with Losartan

1.6.2.3 AF Therapy

There is general agreement that stroke in the context of AF, recent MI and valvular heart disease requires warfarin therapy. Similar stroke reduction is achieved as with primary prevention (68%) (Hart et al., 1999b) however as previous stroke confers a higher future risk the benefits of warfarin are greater than for primary prevention. The use of aspirin in post stroke AF is clearly inferior to warfarin. (European Atrial Fibrillation Trial, (EAFT, 1993)

1.6.2.4 Symptomatic Carotid Artery Stenosis Therapy

1.6.2.4.1 Carotid endarterectomy (CEA)

There have been two large trials of CEA in symptomatic carotid artery stenosis (European Carotid Surgery Trial (ECST, 1998) and North American Symptomatic Carotid Endarterectomy Trial ((NASCET), 1991)). The methodology of the trials is slightly different however the results were broadly similar. Overall benefit is dependent upon surgical skill however as long as the

surgical complication rate is less than 6% the benefits outweigh the risk (Cina, 2000). In patients with severe carotid artery stenosis (>80% ECST; >70% NASCET) surgery reduces the risk of disabling stroke or death by 48% (95% CI: 27-73%). For moderate stenosis (70-79% ECST; 50-69% NASCET) surgery reduces risk of stroke or death by 27% (95% CI: 15-44%). Conversely for less severe stenoses, surgery increases the risk of stroke or death by 20% (95% CI: 0-44%) (Cina, 2000) . The results of NASCET however found that elderly patients may have more benefit from surgery overall and also at lesser degrees of stenosis (50-69%) (Alamowitch et al., 2001).

1.6.2.4.2 Endovascular treatment

Percutaneous transluminal balloon angioplasty and intravascular carotid stent insertion is an alternative to carotid endarterectomy. After three years of follow up the CAVATAS trial found similar rates of recurrent ipsilateral stroke with this method compared with carotid endarterectomy (hazard ratio: 1.04; 95% CI: 0.63-1.70) (CAVATAS, 2001). However, severe (70-99%) ipsilateral stenosis was more common after 1 year in the endovascular group (14% versus 4%). Since CAVATAS endovascular techniques have evolved and data from ongoing trials (CREST (Carotid Revascularisation Endarterectomy versus Stenting Trial) and International Carotid Stenting Study (ICSS)) is awaited (Spence and Eliasziw, 2001).

The early use of carotid endarterectomy after stroke (if disability is not severe) or TIA in the presence of significant (>70%) ipsilateral internal carotid artery stenosis should be advocated.

1.6.2.5 Hypercholesterolaemia Therapy

1.6.2.5.1 Statins

There are a number of clinical trials aimed at reducing serum cholesterol levels using statins. (4SStudy, 1994; HPS, 2002; Plehn et al., 1999). Overall reduction in risk of stroke was in the order of 20-30%. The CARE trial (Cholesterol and Recurrent Events Trial) randomised 4159 survivors of MI to Pravastatin or placebo. Fifty four patients in the Pravastatin group (2.6%) and 78 placebo patients (3.8%) suffered ischaemic stroke during the study period corresponding with a relative risk reduction of 31% (NNT=40 to prevent one stroke) (Plehn et al., 1999) The LIPID trial (LIPID, 1998) (Longer Term Intervention with Pravastatin in Ischaemic Disease) showed

equivalent benefits on stroke risk as the CARE study. The Heart Protection Study randomised 20,536 high risk individuals to receive either simvastatin (40 mg) or placebo. A 30% relative risk reduction of stroke was seen (HPS, 2002).

1.6.2.6 The Uptake of Secondary Prevention Strategies

Although there is good evidence for the use of the above strategies in secondary stroke prevention, the uptake of preventative strategies in everyday clinical practise is not universal (McCallum et al., 1997; O'Connell and Gray, 1996; Stafford and Singer, 1996; Stafford and Singer, 1998; Sudlow et al., 1998; Velasco, 1999) suggesting that patients could receive far greater benefits from health care than they are currently getting.

1.7 Summary

This section has covered some of the evidence for the processes underlying cerebral infarction. It has outlined the significant burden of the problem and the extent to which recognized clinical factors increase the risk of events. The chapter was concluded with a review of the good evidence for risk factor management which can play a significant role in reducing the risk of the development of ischaemic stroke.

CHAPTER 2

COMMON DISEASE GENETICS

Chapter 2: List of Tables

2.1 From Mendel To Today

Much has been learnt since Mendel's experiments on the garden pea which initiated the modern era of genetics. Pre-Mendel, inheritance theories were based upon a notion that parental material was blended and passed on to the offspring. Mendel developed the alternative concept of the 'gene', inherited in its entirety from a single parent. This idea has evolved into the contemporary belief that many common human traits are believed to be due to additive effects of allelic variations in numerous genes with no gene having an independently large effect.

The genetic and technical revolution of the last decade has promised much, although real success has been limited. The clear phenotypic variation of Mendel’s pea samples is not mirrored by multi-factorial common diseases. Although inheritance patterns exist, there are clearly more complex protein-protein and protein-environment interactions. Success has been mainly limited to the uncommon single gene disorders which may give hints about pathological mechanisms but do not always correlate with the more common, oligogenic / polygenic, forms of the disease. Tools such as automated large scale genotyping, powerful statistical techniques, multinational DNA analysis such as the Human genome and HapMap projects whose results are all freely accessible on the internet, should all improve the future prospects for successfully understanding the genetic basis of common human disease.

This chapter will cover the definition of the common traits and the reasons behind their genetic complexity. It will address the strategies of gene detection and the refinements required to improve the chances of success. Finally, it will introduce some of the methods involved in establishing a role of candidate genes in specific disease pathogenesis.

2.2 Complex Genetic Traits

A complex genetic trait has evidence of heritability from a parent but the inheritance pattern is not entirely compatible with Mendelian laws i.e., the inheritance is multi-factorial and is due to a number of genetic effects and environmental interactions, thus leading to the breakdown of the simple link between genotype and phenotype.

2.2.1 Complexities Of Identifying Complex Disease Genes

Identification of perfect co-segregation of a gene with a complex disease is a difficult task due to a number of reasons:-

2.2.1.1 Phenotypic Heterogeneity

Identical genotypes at disease loci can produce a variety of different clinical appearances. In sickle cell anaemia, identical beta globin mutations can produce a variety of phenotypes ranging from childhood mortality to near normal life expectancy (Huisman, 1979).

2.2.1.2 Phenocopy

Environmental exposure to a disease causing agent produces a disease phenotype in the absence of a genetic predisposition.

2.2.1.3 Genetic Heterogeneity

Identical phenotypes may be produced by mutations in a variety of genes which hampers genetic mapping as chromosomal regions may cosegregate with a disease in some patients but not in others. Some traits may require the simultaneous action of mutations in multiple genes (epistatic interaction). Such traits may be classified as either discrete traits, measured by a specific outcome (i.e., retinitis pigmentosa (Kajiwara et al., 1994)), or quantitative traits, measured by a continuous variable (i.e. BP) whose level may be set by the combined action of individual quantitative trait loci (QTL).

2.2.1.4 Late Onset of Disease

Family based studies are hampered because relatives may be unavailable. Prospective studies require long and expensive follow up periods and case control studies may incorrectly label controls too early, prior to disease onset.

2.2.1.5 Incomplete Penetrance

The inheritance of a disease predisposing allele does not lead to disease due to the absence of an environmental trigger, or inaction of interacting genes.

2.2.1.6 Confounders

Risk factors for similar traits may all interact with the genes which have a direct action on the disease being studied making independent gene effects much more difficult to identify.

2.2.1.7 High frequency of disease-causing alleles

Under these circumstances the expected Mendelian inheritance pattern of disease will be confounded by multiple independent copies of high frequency alleles segregating in the pedigree. This will make even a simple trait difficult to map. One example is late onset Alzheimer's disease (AD). Early linkage studies found weak evidence of linkage of AD to chromosome 19q, but results were dismissed (Pericak-Vance et al., 1991). When the apoE gene was discovered on 19q it was apparent that the linked allele was found in a relatively high proportion (16%) of most populations which had interfered with the traditional linkage analysis (Corder et al., 1993)

2.2.1.8 Other Forms of Non-Mendelian Inheritance

Mitochondrial inheritance, imprinting and trinucleotide repeat expansions are relatively easy to identify in their purest forms however they can all complicate gene detection studies.

2.2.2 Ways of Improving Gene Detection

Accurate disease definition, assessment of disease severity and age of onset, family history and studies of isolated populations may help to identify genes with the greatest effects on the trait.

2.2.2.1 Disease Definition

Identifying affected individuals with the same clinical and pathological phenotype brings a common disease as close to a Mendelian disease as possible.

2.2.2.2 Disease Severity, Early Age of Onset and Intermediate Phenotypes

Using the most severe end of a continuous trait or patients with an early age of onset can enhance disease gene identification as seen in the studies of hypertension (Carey and Williamson, 1991; Lander and Botstein, 1989), Alzheimer's disease and breast cancer (Hall et al., 1990). Where clinical phenotyping is difficult, studying intermediate phenotypes can enhance disease gene detection. In relation to stroke disease such intermediates include carotid intima-media thickness (IMT) for large vessel disease and white matter hyperintensities seen on MRI for small vessel disease.

2.2.2.3 Family history

Successful genetic mapping was achieved in Hereditary non-polyposis coli by only including patients who had at least two other affected relatives (Fishel et al., 1993)

2.2.2.4 Population Selection

There is less genetic and allelic heterogeneity in geographically isolated populations. This can be harnessed in linkage studies to positionally clone disease causing alleles using the power of linkage disequilibrium (LD) (see section 2.3.2).

2.3 Gene Identification Strategies

Relatively little is known about the underlying pathobiology of complex diseases. Genetic mapping and positional cloning do not require such knowledge and have therefore been used extensively. This approach is completely generic across the spectrum of all known medical disease. Prior to outlining the detail of the various gene identification methods it will be instructive to discuss two key concepts which are intrinsic to their understanding; genetic markers and linkage disequilibrium (LD).

2.3.1 Genetic Markers

A genetic marker is a specific segment of DNA which varies between individuals; it does not usually cause disease itself but may but may be linked to other pathogenic variants. The recognition of DNA variation has formed the basis of the revolution in medical genetics. When studied in the context of a population, these differences in DNA sequences are called polymorphisms and are found throughout the genome in coding (exons) and non-coding regions (introns). Polymorphisms can take the form of chromosomal deletions, insertions and translocations, minisatellites, microsatellites (simple tandem repeats (STR)), alu repeat insertions and deletions and single nucleotide polymorphisms (SNPs). SNPs and microsatellites have been most extensively used in gene mapping experiments. It is likely that SNPs will continue to have an important role in future studies.

Interest in single base variation in the human genome began in the early 1980s with the generation of a human linkage map using bacterial restriction endonucleases which recognized and cut specific DNA sequences (Botstein et al., 1980). Single nucleotide variation in the DNA sequence can potentially create or remove an enzyme recognition sequence (restriction site). Hence the genotype at a particular single base pair locus can be identified by the length of the resulting DNA fragment, named a restriction fragment length polymorphism (RFLP).

Use of RFLP analysis was largely replaced in the 1990s by STRs (di-, tri- or tetranucleotide repeats) as the marker of choice for linkage studies. STRs are highly abundant and evenly distributed across the human genome, they have high mutation rates and so show high degrees of allelic variation in the number of repeat units (Chakraborty et al., 1997). Furthermore, too few

STR loci exist across the genome to allow identification of areas of linkage disequilibrium (LD) (see section 1.3.2). STRs are therefore inferior to SNPs for large scale population studies.

In contrast to STRs, SNPs are highly abundant, have low mutation rates and consequently are less polymorphic and more stable. Furthermore SNPs can have functional relevance if located in coding or regulatory regions of a gene. This has resulted in the generation of high-density SNP maps which have gained increasing popularity in large scale common disease studies (Chappell et al., 2004; John et al., 2004; Lappalainen et al., 2004; Tayo et al., 2005)

2.3.2 Linkage Disequilibrium (LD)

Linkage Disequilibrium, (or population allelic association), occurs when two DNA loci on the same chromosome lie close enough (or within an area of low recombination) to have been transmitted together, by avoiding separation during random meiotic recombination through multiple generations. Due to LD, identifiable DNA markers are transmitted along with pathogenic mutations. LD is therefore an indirect measure of associating a putative disease locus with a phenotype by means of a surrogate marker which is associated independently with both.

With each subsequent generation the likelihood of recombination events between two loci increases, thus in young populations LD spans greater genetic distances. Mohlke et al identified 100% of 43 microsatellite markers spaced 1cM apart on chromosome 20 in complete linkage disequilibrium in a sample of 1223 Finish individuals. LD was identified in 78% of the markers spaced 1-3 cM apart (Mohlke et al., 2001). Similarly Jorde et al (2000) identified greater LD at the Neurofibromatosis 1 (NF1) locus in Finnish populations compared with African, Asian and other northern European groups (Jorde et al., 2000).

True associations due to LD can produce confusing results when comparing different populations. For example a trait might show positive association with allele A in one isolated population, with allele B in a more isolated population, and with no allele in a mixed population. This is due to differential sites of meiotic recombination separating the disease causing allele from the marker allele. Genomic areas where LD exists within a human population are almost analogous to animal model systems. Over the region of LD, unrelated individuals possess similar genotypes and can be treated as if they belong to the same pedigree. The principle behind LD

mapping is to observe large numbers of affected individuals, preferably within a homogeneous population, who have inherited the same disease-causing haplotype from a common ancestor.

2.3.3 Types of Gene Identification (Mapping) Studies

Animal studies, family studies and case control association studies are the three main approaches to gene identification.

2.3.3.1 Animal models

Study of animal common disease model systems has been more successful than in humans as large pedigrees can be bred from the same parents, overcoming the problem of genetic heterogeneity. Translation of the results from animal study to human disease can be troublesome. Nevertheless, they are useful at identifying key genes acting in common physiological systems. Much success has been achieved with quantitative trait locus (QTL) mapping.

2.3.3.1.1 Quantitative Trait Locus (QTL) Mapping

The principle of QTL mapping is to use phenotypic and genetic marker information to estimate the effects of genotype on the quantitative trait at every point in the genome by means of maximum likelihood linkage analysis. Studies using the spontaneously hypertensive rat have used the QTL approach to identify the *Bp1* gene on rat chromosome 10 exerting a major effect on blood pressure with a LOD score of 5.10 (Jacob et al., 1991). The *Bp1* gene is closely linked to the *Angiotensin converting enzyme* gene and has lead to many investigations of the Renin Angiotensin System (RAS) in humans with many interesting results which will be covered later. Other examples of QTL studies in the rat include the identification of loci having roles in ischaemic stroke on chromosomes 1, 4 and 5 (Jeffs et al 1997, (Ikeda et al., 1996). A recent review of this area in the field of pig genetics has recognised 110 published studies which have identified 1,675 QTLs with roles in traits such as leanness, growth rate, litter size and disease resistance (Rothschild et al., 2007).

2.3.3.2 Family studies

Family studies use two main approaches, linkage analysis and allele sharing methods. Family studies make no assumptions about the location of candidate genes. They incorporate affected and unaffected family members and compare genotypes at multiple loci over variable genomic distances in order to identify genomic areas (haplotypes) shared only by affected individuals. Such haplotypes may house putative disease causing genes.

2.3.3.2.1 Linkage

Linkage studies rely on the co-segregation of polymorphic genetic markers with affected individuals. They are effective with single gene disorders as long as phenotyping is accurate, the pedigree is large and the number of affected individuals is high. Application of linkage to complex traits can be more problematic, due mainly to the difficulties with identifying precise inheritance patterns (Eaves, 1994).

2.3.3.2.2 Population Based Case Control Association Studies of Candidate Genes

These are the most common studies of human stroke. They investigate candidate genes whose physical location is known, markers have been identified within (or linked with) the gene and whose known biological function / dysfunction is compatible with the pathological processes known to occur in the disease under study. Frequency comparisons of the candidate gene allele(s) are made between unrelated affected (case) and unaffected (control) individuals from a given population. A genetic allele is said to be associated with the trait if it occurs at a significantly higher frequency among affected compared with control individuals. Control group selection is crucial to the specificity and sensitivity of studies of this type. Association studies rely on LD between the genetic markers and putative disease causing loci. Such genetic markers can be random DNA polymorphisms but studies are most meaningful when applied to functionally significant variations in genes having a clear biological relation to the trait.

Interpretation of association studies can be a difficult task as positive associations can arise for three reasons:-

1) The associated allele is aetiologically significant.
2) The associated allele it not disease causing but is in LD with the functional variant.
3) Artifactual positive association due to population admixture. Studies incorporating mixed ethnic groups are likely to be more genetically heterogeneous. Traits with high incidence in particular ethnic groups are more likely by chance alone to be positively associated with the group's high frequency alleles and failure to control for this ethnic variability may lead to false positive results. It has been proposed that the HW equilibrium (HWE) test should be routinely performed in association studies as a method for assessing population admixture (Tiret and Cambien, 1995).

Artifactual associations arising from admixture can be prevented by choosing homogeneous populations and recruiting family members to verify ethnic ancestry. If parents are available, non inherited parental alleles are a useful means of providing an internal control group (family-based control or haplotype relative risk method (Falk and Rubinstein, 1987). Parental alleles can also be incorporated into the Transmission disequilibrium test (TDT) (Spielman et al., 1993). The TDT test has the premise that a parent heterozygous for an associated allele A1 and a non-associated allele A2 should more often transmit A1 than A2 to an affected child.

Historically, association studies have not been well suited to whole-genome searches in mixed well established populations because LD was considered to extend over very short distances and so negative results could have been merely due to missing disease causing loci due to suboptimal marker density. This method remains many times more powerful than linkage analysis when applied to complex disease as outlined by Risch (2000) (Risch, 2000). Furthermore recent advances in SNP detection and storage on public databases (such as dbSNP) and identification of the haplotype structure of the human genome (from data generated from the HapMap project – see Chapter 8) has made them a popular choice for common disease geneticists.

2.3.3.2.2.1 The Choice of SNPs

The choice of the best SNPs to use in association studies has been a topic of much debate. Risch and Merikangas (Risch and Merikangas, 1996) favored the use of coding or promoter variants with potential functional significance. However there is some merit in the use of high density, non-coding SNPs to track disease associated haplotypes (Collins et al., 1997).

SNPs are known to affect the resultant amino acid codes in a number of ways. They have been categorised into 6 subgroups Table 2.1. Types I-III SNPs are found in coding regions, types IV-VI are non-coding.

Table 2.1 Classification of SNPs

Class	Definition
Type I	Non-synonymous, non-conservative
Type II	Non-synonymous, conservative
Type III	Synonymous
Type IV	5' untranslated SNPs
Type V	3' untranslated SNPs
Type VI	Other non coding SNPs

Synonymous SNPs change the three base amino acid codon to a sequence which encodes the same amino acid and so the protein remains unchanged. Non synonymous SNPs change the amino acid code, this transition can be conservative if the amino acid group is conserved or non-conservative if the substituted amino acid is from a different group.

In 1999 two groups searched for SNPs within many known candidate genes and provided estimates of over 2 million SNPs throughout the genome (Cargill et al., 1999; Halushka et al., 1999). They identified a relative deficiency of non-synonymous and 5' untranslated region (UTR) SNPs which implies that they are subject to selection and hence have functional and phenotypic significance. The same would be true for any non-coding variants creating or deleting a splice site (Halushka et al., 1999). These figures have proven to be hugely underestimated. Since 1998 the dbSNP database has provided a central worldwide repository for recognised sequence variation in humans and other species (Sherry et al., 2001). Up until March 2007 11,751,216 human SNPs had been submitted to the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi) and as millions are being deposited each

year many more SNPs could be identified in the near future to the benefit of future common disease genetic research.

2.3.3.2.2.2 Population variation and replication of results

As mentioned in section 2.3.2, association study results may not be reproduced across populations (see tables 9.5, 9.6, 3.5 & 3.6). Rare variants may be population specific and are more readily identified by LD mapping. The lack of statistical association in other populations does not however rule out these regions. It may merely suggest the absence in other populations of LD between the particular marker and the disease gene. Cloning of the putative disease gene in the initial population will allow the wider application of mutation detection studies which may identify separate population specific markers linked to the same disease gene. Lack of pan-ethnic reproducibility could also be explained by a specific pathogenic mutation in one population which is not pathogenic in others and so genotype-phenotype association would not be expected. This situation is seen in studies of human Alzheimer's disease where the association of the apoE e4 allele is much stronger in Caucasian and Asian populations than in African Americans and Hispanics (Farrer et al., 1997). In contrast Ionnidis et al have identified data that suggest that although genetic markers for proposed gene-disease associations vary in frequency across populations, their biological impact on common disease aetiology may be consistent across 'racial' boundaries. They examined the genetic effects of 43 validated gene-disease associations across 697 racially diverse study populations. Control population frequencies of genetic markers of interest often showed heterogeneity between 'races' of up to 58% but large heterogeneity in the genetic effects between 'races' was seen in only 14% of cases (Ioannidis et al., 2004). These data suggest that once candidate genes have been identified validation studies using different ethnic groups has a reasonably high chance of identifying meaningful genotype / phenotype correlations.

2.3.3.2.2.3 Confirmation of a Pathological Role For Candidate Genes

The Human Genome Project has become essential for the successful positional cloning of genes for complex traits by potentially providing a complete catalog of all genes in a relevant region. With such information, systematic candidate gene analysis will become far more manageable. Once identified a candidate gene must always be subjected to rigorous evaluation before it is accepted as having a role in the development of the trait or disease in question. The necessary

tests include association studies demonstrating a clear correlation between functionally relevant polymorphisms and human disease as well as transgenic animal studies demonstrating gene addition or gene knockout produces phenotypic effects.

2.3.3.2.2.4 The Problem of Statistical Significance

This is one of the most controversial aspects of complex disease genetics. Many associations of complex traits to genetic loci are not replicated and this may in part be due to increases in chance findings due to multiple testing. A positive LOD score of 3 (which is often accepted as significant in Mendelian diseases) and a p value of 0.05% both include a chance element of 5% in their design. Thus for every 100 tests conducted 5 would be expected to be positive by chance alone. Modern association studies test thousands of markers in large numbers of patients which merely serves to increase the chances of artifactual results. To control for this potential error the Bonferroni correction is often used (Tukey, 1977) (Bland and Altman, 1995; Greenhalgh, 1997) . The correction states that if an experiment is testing a new independent hypothesis on a set of data, then the statistical significance level that should be used for each hypothesis separately is 1/n times what it would be if only one hypothesis were tested. This then produces more stringent levels of significance. The theory behind correction for multiple testing protects against excessive analysis of data in the same population group in order to find any positive result and has a role particularly when numerous genetic markers are under evaluation.

The Bonferroni correction however has been disputed by some epidemiologists (Perneger, 1998). on the grounds that the universal null hypothesis, which assumes that the groups tested are identical on all tests, rarely occurs in practice. Bonferroni adjustments will reduce type 1 errors (false positive results) however they will increase type II errors (the probability of accepting the null hypothesis when the alternative is true). Type II errors are no less false than type I errors. Meta-analyses which are re-evaluation of previous published data incorporating numerous tests would strictly require a Bonferroni correction which would seem excessive and potentially reduce the chances of identifying any positive findings -- the exact opposite of the purpose of such studies.

Perneger (1998) argues that clearly stating which tests have been conducted and the reasons behind them and discussing the possible interpretations of each result should enable the reader to reach a reasonable conclusion without the help of Bonferroni adjustments. What is also

imperative is that positive results in any trial should be re-evaluated in separate populations and ideally by separate groups of researchers before the possibility of a type I error is excluded.

2.4 Summary

This chapter has covered the definitions and complexities of common traits and some of the four the strategies available for disease gene detection and confirmation. The following section will focus on the genetics of stroke.

CHAPTER 3

STROKE GENETICS

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In the previous chapters, I have outlined the significant social and economic burden caused by stroke making it an important modern-day health priority. The available evidence suggests that stroke has a multi-factorial aetiology contributed to by an individual's unique DNA structure. At the present time there are few successful acute ischaemic stroke treatments available, thereby elevating the importance of prevention. Delineating the genetic contribution to stroke disease may go some way to improving preventative and treatment measures.

The next section will address the genetic evidence applicable to the aetiology of ischaemic stroke. It will begin by considering the epidemiological perspective and then move on to discuss the contribution made by studies on animal models. Following this the single gene disorders linked with ischaemic stroke phenotypes will be considered. The chapter will be concluded by a review of the candidate gene studies before introducing the rationale of performing a novel association study of the Angiotensin Gene in a large sample of caucasian subjects from the East Midlands of the UK.

3.1 Introduction

Most modern day health care focuses on diseases with high population prevalence but without simple Mendelian inheritance patterns. Nevertheless common disease phenotypes do occasionally occur within single Mendelian syndromes. Stroke is no exception with at least two syndromes Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) and Mitochondrial encephaolmyelopathy, lactic acidosis and stroke-like episodes (MELAS) known to be caused by point mutations in genomic and mitochondrial DNA respectively. Study of single gene disorders provides insight into pathophysiological mechanisms of the specific defects in these conditions though their contribution to common multifactorial stroke is likely to be small. From a population perspective sporadic non-Mendelian forms of disease have a more important role and although the aetiology in these cases is multifactorial, the evidence strongly suggests the importance of genetic factors.

Cardiovascular and cerebrovascular diseases are strongly correlated, sharing many of the same risk factors but with differing importance. Hunt *et al* (1989) compared the relative importance of genetic and environmental factors determining blood pressure, lipid levels and BMI in over 300 twins and 1102 healthy adults. They found the genetic background had greater influences on these risk factors than environmental components (Hunt et al., 1989). Genetic influences are estimated to account for over half of the risk for cardiac disease (Berg, 1985) especially in younger individuals (Nora, 1980) and they may have similar effects on stroke.

3.2 Genetic Epidemiology and Family Studies of Stroke

Before attempts at disease gene identification it is important to establish that a common disease has evidence of heritability. Most studies of stroke have generally involved unrelated individuals with little information on family history. Relatively few studies have investigated the role of genetics in the occurrence of stroke. Most of the evidence has been circumstantial, the results have often been conflicting, and the exact importance of genetic factors in the pathogenesis of stroke in the population is unknown. Nevertheless most studies have identified clear evidence of heritability.

3.2.1 Types of Genetic Epidemiological Studies

Twin, ecological, cohort and case control methodology have been used. Ecological and twin studies make up the minority of the published data but have provided valuable insights into disease susceptibility. Only a limited number of studies have addressed the familial aspects of stroke (see table 3.2) and comparisons have been difficult. Problems have included population variability, suboptimal disease classification, retrospective data collection often using unvalidated questionnaires, and no corrections for age or other risk factors in the analysis. Despite this there are a number of common trends.

The next section will discuss the basic principles and the evidence obtained from the different types of epidemiological study.

3.2.1.1 Twin Studies

These compare trait concordance rates between monozygotic (MZ i.e., identical) and dizygotic (DZ i.e., non-identical) twins and are useful in estimating the heritability of the trait. Their basis is the principle that environmental stimuli are shared by each twin within both MZ and DZ twin pairs. Allowing for this controlling influence, differences in the concordance rates of disease between MZ and DZ twins are thus likely to be due to their different genotypes (Hrubec and Robinette, 1984). If genes influence the risk of the trait, the concordance rates in the MZ will be higher than in the DZ pairs.

Twins can also be used to study effects of the environment. Comparison of the rates of disease in MZ twins who are discordant for a candidate environmental risk factor can provide information about the role of environmental triggers in disease aetiology.

3.2.1.2 Ecological Studies

These studies, focus on the comparison of disease frequency amongst specific geographic, racial, or ethnic groups and are useful in providing 'clues' for familial aggregation or genetic associations. Unfortunately such studies are rarely definitive. Ideal ecological studies are community-based with standardized definitions so as to ensure that the environmental contributions to risk are as homogeneous as possible between the two groups.

3.2.1.3 Cohort Studies

These studies follow groups of healthy individuals over time to identify the development of disease. The two advantages of this method are: (1) characterization of potential confounding factors, and (2) screening for endpoints can be performed in a systematic and well-controlled fashion. This can be especially important for stroke where the reliability of self-reporting varies widely (O'Mahony et al., 1995). Cohort studies do have significant disadvantages; large numbers of patient years of follow-up are often required to collect enough cases, detailed phenotyping is difficult and costs are often substantial.

3.2.1.4 Case-Control Studies

Case control studies compare individuals with a trait (cases) and those without (controls). These studies are valuable ways of investigating genotype-phenotype association in common disease. The nature of stroke, however, produces a number of potential problems for studies of this type. Stroke patients are more likely to have vascular risk factors which require careful investigation and thorough documentation and caution must be used in estimating the risk associated with an individual factor. Particular attention must be paid to ensure that the selected cases are representative and the controls are comparable so that potential biases are minimized. This methodology is often applied to candidate gene studies.

3.3 Evidence for the role of genetic factors in ischaemic stroke

3.3.1 Twin Studies

A small number of twin studies have been conducted on the heritability of ischaemic stroke. These are summarised in Table 3.1.

Table 3.1 Twin studies investigating the role of genetic factors in common ischaemic stroke

Study	Details	Results	Comments
(de Faire *et al 1975*)	10,900 same sex Swedish twins between 1886-1925.	Concordance rates did not significantly differ for fatal cerebrovascular disease between MZ and DZ pairs.	Assessed fatality rates and not morbidity rates so effects may have been underreported.
(Brass et al., 1992) US National Academy of Sciences Veteran Twin Registry	2722 twins 7 MZ twin pairs concordant, 65 discordant 1 DZ twin pair concordant, 53 discordant	Positive	4.3-fold increase risk in MZ twins
(Brass et al., 1996) US National Academy of Sciences Veteran Twin Registry	21 MZ twin pairs concordant, 205 discordant 23 DZ twin pairs concordant 204 discordant	Negative	Genetic influences comparatively less in an older twin population
(Carmelli et al., 1998)	MRI white matter lesions in healthy twins	Positive	MZ 61% concordance DZ 38% concordance

de Faire et al (1975) published the first twin study on stroke mortality from the Swedish twin registry which enrolled twins between 1886 and 1925. They identified 259 male and 222 female pairs who had both died before 1975. 85 male and 75 female pairs were concordant for cause of death however this concordance rate did not differ between the MZ and DZ pairs for cerebrovascular deaths. This study collected data by a postal questionnaire and only recorded fatality. Phenotyping of stroke aetiology is likely to have been sub-optimal as all diagnoses were self reported by non medical personnel and no cerebral imaging was performed. Additionally non fatal stroke was not included thereby potentially underestimating the true incidence of ischaemic stroke.

Brass *et al* (1992), using a postal health questionnaire found a 3.1% (292/9475) prevalence of stroke in the 58-68 year old twin cohort (Brass et al., 1992). Stroke rates were similar for MZ (131/4200) and DZ twins (143/4585) and approximated expected population rates. However, although numbers were small (7 MZ pairs and 1 DZ pair) concordance rates suggested that MZ twins were 4.3 times more likely to both suffer a stroke compared with DZ twins (17.3% vs 3.6% $p<0.05$). This study does provide a strong suggestion of a genetic component in stroke pathophysiology although its magnitude is smaller than that seen in other twin studies in IHD (MZ = 48-90% vs DZ 25-28% (Berg, 1987; Evans et al., 2003; Reed et al., 1991).

Brass et al followed up their twin cohort a decade later and found that genetic factors appeared to have less influence on the risk of stroke in the older population in whom much of the variance was accounted for by environmental exposure (Brass et al., 1996) suggesting that genetic stroke risk factors may exert much of their influence in younger adults. Carmelli *et al* in 1998, (Carmelli et al., 1998) used the volumes of white matter hyperintensities seen on MRI in healthy elderly individuals as a surrogate marker and found concordance rates of 61% in MZ twins compared with 38% in DZ twins.

Using twins to study the effects of environmental factors in stroke disease, Haapanen and colleagues compared the degree of carotid atherosclerosis between smoking and nonsmoking MZ twins. They found that the smokers had greater degrees of carotid stenosis. This association remained after adjusting for common risk factors (age, cholesterol, blood pressure, and body mass index) in a regression analysis implicating smoking as a strong risk factor for atherosclerotic diseases (Haapanen et al., 1989).

3.3.2 Ecological studies

Racial comparisons have suggested a genetic contribution to stroke. African-Americans have a higher burden of stroke risk factors and higher rates of ischemic and hemorrhagic stroke (Kittner et al., 1990). African subjects have more intracranial and less extracranial atherosclerosis than caucasians (Alter, 1994). Japanese populations are seen to have higher rates of intra-cranial haemorrhage (Alter, 1994; Suzuki et al., 1987) in addition to a preponderance of anterior circulation vascular disease in comparison to caucasians (Inzitari et al., 1990) and the recognition of a high frequency of lacunar infarction in China and Hong Kong has led to the idea that small vessel disease may be more prominent in Asian subjects (Davis et al., 1990; Huang et al., 1990).

Migration studies also provide interesting insight into the interaction of genes and environment. The Honolulu Heart study examined male Japanese immigrants to Hawaii. Results showed incidence rates reducing to reflect their new geographic location (Kagan et al., 1980) suggesting an environmental effect on modifying gene expression, possibly by retarding the rate of atherosclerosis (Knekt et al., 1991).

3.3.3 Cohort Studies

Positive family history as an independent risk factor in stroke has been well recognized in a number of cohort studies (Table 3.2). Whilst this is consistent with the role of genetic factors, alternative explanations, such as shared environmental influences, could also explain these results. Overall although these studies have included different measures of stroke risk, it appears that the RR of stroke associated with a positive family history lies between 1.8 - 3.3.

A minority of studies have found less convincing evidence that family history is a predictor of stroke (Boysen et al., 1988; Herman et al., 1983). The largest study of its kind, the Framingham heart study (Kiely et al., 1993) did identify a parental history of Stroke, TIA or coronary heart disease as a predictor of stroke in the offspring (RR= 3.3 (CI 1.27-8.72) however more specific measures of paternal , maternal and sibling history of stroke were not significantly predictive of stroke in the patients under study as confidence intervals overlapped 1.0 (RR=2.4, CI 0.96-6.03; RR=1.4, CI 0.6-3.25; RR=1.8, CI 0.68-4.94 respectively). Other studies have identified that family history of stroke is not independent of risk factors (Barnett et al., 1981; Diaz et al., 1986; Havlik et al., 1979). It is vital therefore that assessment of risk factors is incorporated into stroke genetic studies.

Table 3.2 Epidemiological studies investigating the role of genetic factors in common ischaemic stroke

Reference	Method	Details	Result	Comments
Rancho Bernardo Study (Khaw and Barrett-Connor, 1986)	Cohort	1491 men, 1491 women	Positive	First degree FH of stroke independent predictor of :- Stroke mortality in women aged 50-79, RR=2.3 ($p<0.05$) Coronary heart disease in males aged 50-64 (RR=3.3, $p<0.05$)
(Brass and Shaker, 1991; Welin et al., 1987)	Cohort	789 men born in 1913	Positive	Maternal history of stroke mortality associated with adjusted RR=3
(Howard et al., 1990)	Cross-sectional	55 probands selected from a stroke registry with 243 offspring	Positive	Association between young onset of stroke in probands and fatal coronary or vascular events in the offspring
(Brass and Shaker, 1991)	Cross-sectional	117 TIA patients	Positive	There was an association between FH and stroke in older patients >70 years
London Ontario Study (Graffagnino et al., 1994)	Case Control	85 patients, 86 controls	Positive	First degree FH of stroke predictor of : Stroke OR=2.33 (Cases 47% controls 24%). Ischaemic Heart Disease OR=2.14 But Not independent of other vascular risk factors.
(Duggirala et al., 1996)	Case control	Internal carotid artery intima-media thickness in siblings	Positive	Heritability of 92% independent of other risk factors
Family Heart Study (Liao et al., 1997)	Cross-sectional	3168 probands, 29325 first degree relatives	Positive	Significant independent association of stroke with:- Paternal history adjusted OR=2.0 (1.13-3.54) Maternal history adjusted OR=1.41(0.80-2.50)
(Jousilahti et al., 1997)	Cohort	14371 middle-aged men and women	Positive	Significant independent association of parental stroke. Adjusted OR in : Males = 1.5 Females = 1.8 Association strongest in 25-49 age group Positive parental history of CHD associated with stroke in women
Framingham Study (Kiely et al., 1993)	Cohort	4933 cohort individuals, comprising 604 sibships and 2317 offspring cohort individuals	Negative	Independent association of TIA or CVA with:- Paternal stroke history RR=2.4 (0.96-6.03) Maternal stroke history RR=1.4 (0.6-3.25) Sibling stroke history RR=1.8 (0.68-4.94) CHD Hx in both parents RR=3.33
(Herman et al., 1983)	Case control	132 stroke patients, 239 controls	Negative	
(Diaz et al., 1986)	Case control	Cases = 76 siblings of stroke patients Controls = 55 siblings of patients' spouses	Negative	Clustering of 2 or 3 vascular risk factors amongst siblings of stroke patients but no significance concordance of stroke.
(Boysen et al., 1988)	Cohort	19327 individuals	Negative	

3.3.4 Animal Models

An animal model for hypertension, the spontaneously hypertensive rat (SHR), (Okamoto and Aoki, 1963) has been studied for many years. Despite the close association of stroke and hypertension in humans, the SHR was not prone to stroke. In 1974, Yamori *et al* (Yamori, 1974) fed the SHR a stroke permissive diet and identified early fatal strokes in some whilst others were stroke resistant. This effect was independent of BP.

Experimental crosses of the stroke prone SHR (SPSHR) with the stroke resistant SHR generated F2 hybrids with marked variation in stroke susceptibility despite concordance for hypertension. Quantitative trait mapping experiments were performed on large numbers of progeny from these experimental crosses which identified three quantitative trait loci (QTL) which co-segregated with stroke (STR1 (rat chromosome 1), STR2 and STR3 (chromosome 4)(Rubattu et al., 1996). These accounted for 28% of the overall variance in the risk of stroke. STR2 seemed to protect against stroke. Comparison with human and mouse genetic maps revealed no obvious homologous genes to STRl and STR3 but STR2 mapped closely to the gene for atrial natriuretic peptide (*ANP*), located on rat chromosome 5.

Jeffs *et al* 1997 crossed stroke prone SHR rats with Wistar Kyoto rats and estimated infarct volume after a focal ischaemic insult (Jeffs et al., 1997). They found a highly significant QTL on the rat chromosome 5 near the *ANP* and brain natriuretic peptide (*BNP*) genes accounting for 67% of the phenotypic variance. This QTL was however not protective.

ANP's candidature is supported by biochemical data. Heidelberg colonies of stroke-prone SHR rats have impaired endothelium-dependent vasodilatation in response to *ANP* (Russo et al., 1998). In humans *ANP* has a role in controlling salt and water balance in hypertension and heart failure by increasing renal sodium excretion (Levin et al., 1998). *ANP* levels also increase in acute ischaemic stroke (Estrada et al., 1994)

In follow up experiments with the stroke prone F2 hybrids, Rubattu *et al* 1998 revealed functional DNA mutations which alter ANP levels. Subsequently a human *ANP* gene G-A polymorphism was identified at a highly conserved position of the gene (G664A) which confers an amino acid substitution in *ANP*. This polymorphism was found in significantly higher frequencies in human stroke cases compared to controls (Rubattu et al., 1999). These exciting studies have been the first to

identify a putative candidate gene in an animal model and then confirm a statistically significant association to human stroke. The relative risk conferred by the G664A is small and further replication is required to confirm these early findings especially in the light of other rat studies that exclude the *ANP* and *BNP* genes as candidates for sensitivity to cerebral ischaemia (Brosnan et al., 1999).

3.3.5 Human single-gene disorders associated with ischaemic stroke

Several rare Mendelian familial stroke syndromes exist (Table 3.3). They often manifest in a systemic manner with stroke merely part of the syndrome. They make up a more significant proportion of young stroke presentations especially in the absence of vascular risk factors. Underlying pathologies include cardioembolism, large and small vessel disease including arterial dissection, haematological, mitochondrial, and ion channel disorders and so they provide insights into the mechanisms contributing to or underlying the polygenic disorders as well as providing candidate genes for non familial stroke.

Table 3.3 Mendelian disorders associated with stroke.

Mechanism	Example
Cardioembolism	Cardiomyopathies: primary/secondary Familial atrial myxoma Familial dysrhythmias
Thromboembolism	Hereditary haemorrhagic telangectasia
Large Vessel Disease	Dyslipidaemias Ehler's Danlos syndrome Marfan's syndrome Pseudoxanthoma elasticum Neurofibromatosis type I Moya-Moya disease
Small Vessel disease	CADASIL
Small & Large Vessel Disease	Fabry's disease Homocysteinuria Sickle cell disease
Prothombotic states	Protein S deficiency Protein C deficiency Antithrombin III deficiency
Mitochondrial disorders	MELAS
Channelopathy	Familial hemiplegic migraine

Reviewed by (Natowicz and Kelley, 1987), Hassan and Markus 2000

3.3.6 Linkage Analysis In Common Ischaemic Stroke

Stroke research has often lagged behind cardiac research. In families with IHD recent linkage analysis using genome wide screens have yielded several linked loci (Broeckel et al., 2002; Francke et al., 2001; Harrap et al., 2002; Pajukanta et al., 2000; Wang et al., 2003) although no susceptibility genes have been identified to date.

Recent genome screens in families with stroke susceptibility in Iceland by the deCODE study group have also identified two linked loci (STRK-1) on chromosome 5q12 (Gretarsdottir et al., 2002) and another on 13q12-13 (Helgadottir et al., 2004). The gene encoding phosphodiesterase 4D (PDE4D) has been identified at 5q12 with evidence of dysregulation of multiple *PDE4D* isoforms in stroke patients (Gretarsdottir et al., 2003). At 13q12-13 haplotypes within the *5-lipoxygenase activating protein* gene (*ALOX5AP*) have been identified which confer almost a 2 fold increased risk of stroke and MI. A *ALOX5AP* haplotypic association with stroke has been replicated in a Scottish sample (Helgadottir et al., 2005).

The *PDE4D* gene is known to have at least eight different isoforms that encode functional proteins. Gretarsdottir et al identified several *PDE4D* isoforms in cell lines from individuals with stroke with significantly lower mRNA expression when compared to their controls (Gretarsdottir et al., 2003). The *PDE4D* proteins are known to degrade cANP which is a key signal transducer in multiple cell types including endothelium. Low levels of cANP enhance cell migration and proliferation and lead to atheroma (Indolfi et al., 1997). The role of these variant isoforms in stroke patients is therefore somewhat difficult to link with stroke disease. The authors propose that the identified *PDE4D* variants might up regulate cANP and thus affect either the expression of cANP regulated *PDE4D* isoforms or have effects on their intracellular compartmentalization (Houslay and Adams, 2003).

Gretarsdottir and colleagues (Gretarsdottir et al., 2002) identified *PDE4D* haplotypes associated with a combined subgroup of carotid atheroma related stroke and cardiogenic stroke which may have similar final common pathways but have rather different initial pathologies. There was no association between their ischaemic stroke group as a whole. Additionally the combined group of carotid and cardiogenic stroke represented 40 - 45% of all their ischaemic strokes which meant they excluded 55 - 60% of their stroke victims from this analysis. This would be a valid approach if their stroke phenotype had been refined however this was not the case as their phenotype was diluted. One possible mechanism for the association of the *PDE4D* gene with this rather heterogeneous group of

stroke patients is due to possible effects on the final common pathway of ischaemic stroke. Phosphodiesterase 4 is known to be involved in susceptibility to ischaemic brain damage in animal models (Kato et al., 1995; Nagakura et al., 2002; Nagasawa et al., 1992) and so individuals with the PDE4D mutations may suffer greater degrees of brain damage once cerebral vessels have been occluded (Funalot et al., 2004) . This mechanism however would not explain why similar risk ratios were seen in the deCODE study for TIAs as for completed cerebral infarcts as TIA patients would be expected not to carry less genetic susceptibility for higher degrees of cerebral ischaemia (Gulcher et al., 2004) unless those individuals also have protective alleles within other as yet unidentified genes which reduce the effects of ischaemia..

Despite the potential drawbacks in these studies they have provided promising data which has allowed stroke to join the few other complex traits such as type 1 and 2 diabetes, asthma, Crohn's and Alzheimer's disease witth at least one identified susceptibility gene (Glazier et al., 2002).

3.3.7 The Candidate Gene Approach to Common Ischaemic Stroke

Ischaemic stroke is a syndrome and not a single disease state and as such is a paradigm for late onset complex polygenic diseases. Recent advances in imaging allow the underlying pathogenic stroke mechanism to be determined in many cases. Although there is limited epidemiological work comparing risk factor profiles of different sub types of stroke, it is likely that they will differ significantly along with their genetic correlates. Stroke sub-classification plays a vital role however even with modern technology this may not be possible in as many as 30% of cases. For each of these stroke sub types, genetic factors may act either by predisposing to conventional risk factors, by modulating the effects of the risk factors on the end organs, or by a direct independent effect on stroke risk.

As identified by the INTERHEART study there are nine main risk factors which are important to contributors to myocardial infarction and probably stroke disease worldwide. These include hyperlipidaemia, hypertension, diabetes, abdominal obesity, consumption of fruits and vegetables, alcohol and smoking consumption and degree of regular exercise (Yusuf et al., 2004). Although not all are likely to be under genetic control there is evidence to suggest that hyperlipidaemia, hypertension, diabetes are controlled to a degree by genetic effects. Hypertension alone may have a genetic influence in the range of 20-60%. (Barnett et al., 1981; Havlik et al., 1979; Hunt et al., 1989; Kiely et al., 1993) It has however been estimated that up to 69% of the population attributable risk of

stroke may be unaccounted for by identifiable risk factors (Jamrozik et al., 1994).

Harrap (1994) calculated that within a population at least six genes may contribute to stroke aetiology (Harrap, 1994) and so even when stroke is defined in its purest sense our knowledge suggests that it is a complex disease resulting from numerous gene-gene and gene-environment interactions with each individual gene conferring a small relative risk. In a population the total spectrum of risk genes might be much larger however to develop the disease a single individual may only require a proportion of these genes in association with an environmental trigger or interaction with another susceptibility gene (i.e. an epistatic interaction) (Brown, 1994). The presence of several genes may increase the risk of disease in an additive manner (i.e., a gene dose effect).

Taking into account this complexity the emphasis of this review will be on independent stroke-causing genes rather than those acting through risk factors.

3.3.7.1 Some putative candidate genes

One possible method of classifying the putative candidate genes is outlined in Table 3.4. These categories may not be mutually exclusive as certain genes may both predispose to specific stroke subtypes and play a role in the after effects of all types of stroke by worsening neuronal injury.

Labelling these genes as candidates for stroke is based upon good scientific knowledge obtained from animal models, in-vitro and human studies. Much of the detail however is unclear and the candidacy of these genes is speculative. Many genes have diverse roles which would be difficult to predict from current knowledge. Now the human genome has been fully mapped enabling the recognition and investigation of the roles of numerous genes many more potential candidates are likely to emerge.

Table 3.4 Putative Candidate Genes For Ischaemic Stroke

Renin-Angiotensin System ACE Angiotensinogen Renin Angiotensin 2 receptor Aldosterone synthetase
Haemostasis Factor V Leiden Factor VII Factor XIII Prothrombin Fibrinogen PAI 1 GpIIb / IIIa GpIb / IX GpIa / IIa
Lipid Metabolism Apo E
Homocysteine Metabolism MTHFR
Vascular risk factor genes with stroke as a secondary phenomenon a) Hypertension Angiotensinogen Renin Angiotensin 2 receptor Aldosterone synthetase b) Diabetes MODY
Genes affecting the eventual infarct size a) Free Radicals and Anti-oxidants eNOS b) PDE4D

PAI1 = plasminogen activator inhibitor; GpIIb/IIIa, GpIb/IX, GpIa/IIa = Platelet glycoprotein receptors; ApoE=apolipoprotein E; MTHFR = Methylenetetrahydrofolate reductase; MODY = Maturity onset diabetes of the young; eNOS = endothelial Nitric Oxide synthase, PDE4E = Phosphodiesterase 4D

3.3.7.2 Candidate Gene Studies

Many genes have been labeled candidates for stroke following the demonstration of an association with IHD however this extrapolation may not be accurate. Many studies are based upon small numbers and are not reproduced in different populations. Tables 3.5-3.7 show details of the studies performed on the most widely studied candidates which have produced consistently positive associations. The *ACE* gene has received most attention as positive associations are regularly identified between ischaemic stroke and the deletion allele of an Alu insertion / deletion polymorphism (*ACE I/D*) in intron 16. A recent meta-analysis of 1918 patients with all causes of ischaemic stroke from 5 case control studies, which was calculated to have a power of 99.99% to detect a RR of 2, concluded that there was a moderate increased risk associated with the D allele acting recessively (OR = 1.31 (95% CI 1.06 - 1.62, p=0.01) (Sharma, 1998). This finding was similar to a meta analysis study of the ACE ID polymorphism in MI patients published by Samani in 1996

who found a DD vs II OR=1.36 (95% CI, 1.19 - 1 .55), and DD vs ID OR = 1.24 (95% CI, 1.11-1.38). (Samani et al., 1996). This risk appeared to increase in Japanese populations (OR=2.55: 95% CI, 1.75 - 3.70).

More recently Casas et al have performed a further meta-analysis of 11 case control studies in ischaemic stroke which included 2990 white adult patients. They identified an odds ratio of DD genotype versus II of 1.21 (95%CI, 1.08 - 1.35).(Casas et al., 2004).

Notwithstanding the problems of meta-analysis for example the comparison of a wide range of populations, the problem of publication bias (increasing the chances of missing negative studies) and the difficulties with multiple phenotypes, the studies in stroke and MI both provide some evidence that the *ACE* gene should be considered a risk factor for both these vascular diseases.

Other genes showing positive associations include *Fibrinogen, ApoE and the platelet glycoprotein receptor genes. Factor V and XIII, Prothrombin, PAI1, MTHFR, eNOS* and other lipoprotein genes have often produced negative results (Table 9.6 (Appendix).

The RR of between 1.21 – 2.55 would suggest that the role of *ACE* in the two complex vascular diseases is at best relatively modest however within the field of vascular disease the renin-angiotensin system (RAS) has been the focus of much interest over recent years. Genes involved in the RAS notably the *ACE* gene are known to play a vital role in many physiological and disease processes (see section 3.4). The evidence suggests that genetic variation of *ACE* may have pathological effects which makes it an excellent genetic candidate for further study in ischaemic stroke disease. Many researchers have also explored the links between *ACE* and other human vascular and non-vascular diseases. The following section will review the current understanding of these associations.

Table 3.5 Association studies Of Ischaemic Stroke and the *ACE* gene

Reference	Polymorphism	Methodology	Phenotype	Result	Association
(Markus et al., 1995)	*I/D*	Case-control: 100 cases, 137 controls	TOAST subtype	Positive	Lacunar stroke (no association with carotid atheroma)
(Castellano et al., 1995)	*I/D*	Cross-sectiona1: 199 cases	IMT patients aged 50-64 years	Positive	
(Hosoi et al., 1996)	*I/D*	Cross-sectiona1: 288 cases	IMT NIDDM patients	Positive	
(Kario et al., 1996)	*I/D*	Case-control: 228 cases, 90/104 controls	Symptomatic stroke or MRI silent lacunae in hypertensives. Hypertensive vs. normotensive controls	Positive	Association of D allele with clinical stroke or silent lacunae
(Margaglione et al., 1996)	*I/D*	Case-control: 101 cases, 109 controls	Ischaemic stroke	Positive	DD genotype
(Nakata et al., 1997)	*I/D*	Case-control: 55 cases, 61 controls	Ischaemic stroke	Positive	DD genotype
(Watanabe et al., 1997)	*I/D*	Cross-sectional: 169 cases	Carotid atherosclerosis/ asymptomatic lacunar stroke	Positive	Carotid atherosclerosis only
(Doi et al., 1997)	*I/D*	Case-control 181 cases, 271 controls	Ischaemic stroke (atheroembolic/ lacunar)	Positive	Young strokes (possibly post stroke mortality also)
(Elbaz et al., 1998)	*CT 2/3*	Case-control: 510 cases, 510 controls	Ischaemic stroke and subtype lacunar stroke	Positive	2/2 genotype associated with
(Sharma, 1998)	*I/D*	Meta-analysis: 1918 cases, 722 controls	Ischaemic stroke	Positive	Moderate increase in risk associated with D allele under a recessive model (OR= 1.31 (95% CI 1.06-- 1.62, p=0.01) of inheritance
(Casas et al., 2004)	*I/D*	Meta-analysis : 2990 cases, 11305 controls	Ischaemic stroke	Positive	Moderate increase in risk associated with D allele under a recessive model of inheritance (OR=1.21 (95%CI, 1.08 -- 1.35)
(Szolnoki et al., 2001)	*I/D*	Case-control ; 664 Cases, 199 controls	Ischaemic stroke, 3 subtypes (large vessel, small vessel, mixed)	Positive	ACE DD more common in small vessel stroke (OR=2.31, 95% CI, 1.49-3.57)
(Sharma et al., 1994)	*I/D*	Case-control: 100 cases, 73 controls	Ischaemic stroke	Negative	Trend towards DD genotype associated with young stroke
(Ueda et al., 1995b)	*I/D*	Case-control: 488 cases, 188 controls	Ischaemic stroke and OCSP sub type	Negative	DD genotype in hypertensive versus hypertensive controls
(Catto et al., 1996)	*I/D*	Case-control: 418 cases, 2 the 31 controls	Ischaemic stroke, OCSP subtype and mortality	Negative	No association with stroke subtype. D allele associated with early mortality
(Pullicino et al., 1996)	*I/D*	60 cases, No published controls	Lacunar stroke	Negative	
(Zee et al., 1999)	*I/D*	Nested case-control: 348 cases, 348 controls	Ischaemic stroke/PICH	Negative	No association following stratification for low risk
(Aalto-Setala et al., 1998)	*I/D*	Cross-sectional: 234 cases	Carotid atherosclerosis ischaemic stroke <60 years	Negative	

Adapted from (Hassan and Markus, 2000).

Table 3.6 Haemostatic Genes Positively Associated With Ischaemic Stroke

Reference	Polymorphism	Methodology	Phenotype	Result	Association
Fibrinogen					
(Carter et al., 1997)	*448(1/2)*	Case-control: 305 cases, 197 controls	Ischaemic stroke / OCSP subtype	Positive	Genotype distribution different amongst females only
(Kessler et al., 1997)	*G455A*	Case-control: 227 cases, 225 controls	Ischaemic stroke/TOAST subtype	Positive	Homozygotes only in large vessel stroke
(Nishiuma et al., 1998)	*G455A*	Case-control: 85 cases, 85 Hypertensive / 84 normotensive controls	Hypertensive strokes	Positive	
(Schmidt et al., 1998)	*148(C/T)*	Cross-sectional: 399 cases	Carotid atherosclerosis	Positive	Homozygotes *(T/T)* genotype
GpIlb/IIIa					
(Carter et al., 1998)	*P1A2*	Case-control: 505 cases, 402 controls	Ischaemic stroke/OCSP subtype and mortality	Positive	Association with atherothrombotic stroke in non smokers and <50 years
(Ridker et al., 1997)	*P lA2*	Nested case-control: 209 cases, 209 controls	Ischaemic stroke / PICH	Negative	
(Carlsson et al., 1997)	*HPA1/HPA3*	Case-control: 218 cases, 165 / 321 controls	Ischaemic stroke	Negative	
GpIb/IX					
(Gonzalez-Conejero et al., 1998)	*HPA2/VNTR*	Case-control: 104 cases, 104 controls	Ischaemic stroke	Positive	
GpIa/IIa					
(Carlsson et al., 1997)	*C807T*	Case-control: 227 cases,170 controls	Ischaemic stroke in patients aged <50 years	Positive	
(Carlsson et al., 1999)	*HPA5*	Case-control: 218 cases, 165/321 controls	Ischaemic stroke	Negative	
Factor V Leiden					
(Szolnoki et al., 2001)		Case-control ; 664 Cases, 199 controls	Ischaemic stroke, 3 subtypes (large vessel, small vessel, mixed)	Positive	V Leiden mutation more common in large vessel stroke. (OR=2.25, 95% CI, 1.16-4.34)
(Casas et al., 2004)		Meta-analysis; 4588 cases, 13798 controls	Ischaemic stroke	Positive	OR=1.33 (95% CI, 1.12-1.58) p=0.03

Adapted from (Hassan and Markus, 2000).

Table 3.7 Association studies Of Ischaemic Stroke and the *apoE* gene

Reference	Polymorphism	Methodology	Phenotype	Result	Association
(Pedro-Botet et al., 1992)	*apoE2/E3/E4*	Case-control: l00 cases, l00 controls	Ischaemic stroke	Positive	E4 a risk factor
(Couderc et al., 1993)	*apoE2/E3/E4*	Case-control: 69 cases, 68 controls	Ischaemic stroke or TIA	Positive	E3/E3 protective, E3/E2 risk factor
(de Andrade et al., 1995)	*apoE2/E3/E4*	Case-control: 145 cases, 224 controls	Carotid atherosclerosis age 45-64 yrs	Positive	E2/E3 genotype risk factor
(Terry et al., 1996)	*apoE2/E3/E4*	Cross-sectiona1: 260 cases	IMT in patients with and without CHD	Positive	E2 protective
(Schmidt et al., 1997)	*apoE2/E3/E4*	Cross-sectiona1: 280 cases	Silent white matter disease	Positive	E2/E3 genotype risk factor
(Kessler et al., 1997)	*apoE2/E3/E4*	Case-control: 227 cases, 225 controls	Ischaemic stroke/ TOAST subtype	Positive	E4 association with large vessel disease
(Ferrucci et al., 1997)	*apoE2/E3/E4*	Cohort study: 1664 subjects	Ischaemic stroke >71 years	Positive	E2 protective in patients 70-79 years
(McCarron et al., 1998)	*apoE2/E3/E4*	Cohort study: 714 subjects	Ischaemic stroke survivors	Positive	E4 associated with a favourable outcome
(Margaglione et al., 1998)	*apoE2/E3/E4*	Case-control 100 cases, 108 controls	Ischaemic stroke	Positive	E4 allele a risk factor, E3/E3 homozygotes protected
(Kuusisto et al., 1995)	*apoE2/E3/E4*	Cohort study: 1067 subjects	Ischaemic and haemorrhagic stroke 65-74 yrs at baseline	Negative	
(Basun et al., 1996)	*apoE2/E3/E4*	Cohort study: 1077 subjects	Ischaemic stroke in those >75yrs at baseline	Negative	No association with apo E genotype Reduced E3/E4 frequency in patients with previous stroke at baseline
(Aalto-Setala et al., 1998)	*apoE3/E3/E4*	Cross-sectional. 234 cases	Carotid atherosclerosis in patients with ischaemic stroke <60 years	Negative	
(Casas et al., 2004)	*apoE2/E3/E4*	Meta-analysis, 1805 cases, 10921 controls	Ischaemic stroke	Negative	OR=0.96 (95% CI, 0.84-1.11)
(McCarron et al., 1999)	*apoE2/E3/E4*	Meta-analysis, 1805 cases, 10921 controls	Ischaemic Stroke	Positive	Significantly higher frequencies of E4 allele in cases (OR=1.68, 95% CI, 1.36-2.09, p<0.001)

Table 3. 8 :Association Studies of Other Genes and Ischaemic Stroke

Reference	Polymorphism	Methodology	Phenotype	Result	Association
(Casas et al., 2004)	*MTHFR, C677T*	Meta-analysis, 3387 cases, 4597 controls	Ischaemic Stroke	Positive	OR=1.24 (95% CI 1.08-1.42)
(Cronin et al., 2005)	*MTHFR C677T*	Meta analysis, 6110 cases, 8760 controls	Ischaemic Stroke	Positive	OR=1.37, for TT (recessive model) (95% CI 1.15-1.64 p<0.001)
(Kelly et al., 2002).	*MTHFR C677T*	Meta analysis, 2788 cases, 3962 controls	Ischaemic Stroke	Negative	OR=1.23, for TT (recessive model) (95% CI 0.96-1.58 p=0.1)
(Casas et al., 2004)	*Prothrombin G20210A*	Meta-analysis, 3028 cases, 7131 controls	Ischaemic Stroke	Positive	OR=1.44 (95% CI 1.11-1.86)
(Casas et al., 2004)	*Factor XIII, Val-Leu*	Meta-analysis, 2166 cases, 1950 controls	Ischaemic stroke	Negative	OR = 0.97 (95% CI, (0.75-1.25)
(Casas et al., 2004)	*Leu 33 Pro*	Meta-analysis, 1467 cases, 2537 controls	Ischaemic Stroke	Negative	OR=1.11 (95% CI , 0.95-1.28)

ACE = Angiotensin Converting Enzyme. I/D = insertion / deletion polymorphism. PAI 1 = Plasminogen Activator Inhibitor. Gp = Platelet glycoprotein receptors. MTHFR = methylene tetrahydrofolate reductase. eNOS = endothelial Nitric Oxide synthase. apoE = apolipoprotein E. PICH = primary intracerebral haemorrhage; APC= activated protein C resistance; NIDDM = non-insulin-dependent diabetes mellitus; IMT = carotid intima media tlhickness; CHD = coronary heart disease; DVT = deep vein thrombosis; PE = pulmonary embolism;
Adapted from (Hassan and Markus, 2000).

3.3.8 *Angiotensin Converting Enzyme (ACE)* Gene Polymorphisms and Susceptibility to Human Disease.

3.3.8.1 Left Ventricular Size

Schunkert et al (1994) found an association between electrocardiographically defined left ventricular hypertrophy and the *ACE* DD genotype. This association was stronger in males and more prominent in normotensive individuals (Schunkert et al., 1994). Celentano et al 1999 however significantly associated LVH with the DD genotype in hypertensives independently of the other vascular risk factors (Celentano et al., 1999). In contrast Lindpaintner et al 1996 did not see an association between left ventricular mass, left ventricular hypertrophy and *ACE* DD genotype in a much larger sample of 2439 subjects from the Framingham heart study (Lindpaintner et al., 1996). Individuals undergoing a 10 week physical training course have an 18% greater increase of left ventricular mass if they carry the D allele (Montgomery et al., 1997).

3.3.8.2 Physical Performance

Although heart size may increase with physical training this may have a deleterious effect causing DD individuals to have lower levels of endurance performance. In a comparison with 1906 healthy

British males Montgomery et al (1998) identified an excess of the homozygous II genotype in 25 elite male British mountaineers who had been able to climb above 7000m without using supplementary oxygen. 15 of these climbers had previously ascended above 8000m and none were DD homozygotes (Montgomery et al., 1998). A second study of 123 Caucasian males recruited to the British Army undertook a period of elbow flexion training with a 15 kg barbel. An 11 fold higher level of improvement was identified in those with an II compared with a DD genotype. Similar effects of training on human skeletal muscle have been identified by Williams et al (2000). They proposed that the lower ACE activity seen in patients with an insertion allele was the explanation of the increased efficiency of training and this was likened to the reduction of ACE activity produced by ACE inhibitors which is beneficial for the survival of myocardial cells during ischaemia in patients with heart failure (Williams et al., 2000).

Zhang et al 2002 studied the effects of 10 weeks of mild exercise in 64 Japanese subjects with mild to moderate essential hypertension. They identified a significant reduction in blood pressure in those subjects with an I allele. No significant changes were seen in homozygous DD subjects (Zhang et al., 2002). Furthermore Gayagay et al. (1998) found similar results in 64 Australian national rowers who had a significantly higher frequency of the I allele compared with a control population. They proposed that the underlying mechanism related to a healthier cardiovascular system (Gayagay et al., 1998).

In 47 patients with the skeletal muscle disease, myophosphorylase deficiency Martinuzzi et al (2003) found a higher frequency of D allele carriers in those patients with a more severe clinical phenotype, suggesting a modulating role for the *ACE* gene in this type of muscle disease (Martinuzzi et al., 2003). The deleterious effects of the D allele has been supported by a study by Winnicki et al 2004 who found DD homozygous patients to be more likely to have a sedentary lifestyle (Winnicki et al., 2004)

3.3.8.3 Hypertension

Studies demonstrating linkage between the homologous rat *ACE* locus and elevated BP (Jacob et al., 1991) suggest that *ACE* is a good candidate gene in human hypertension. To date there has been no evidence of linkage of human hypertension to the *ACE* locus (Berge and Berg, 1994; Cambien et al., 1992; Jeunemaitre et al., 1992a; Kreutz et al., 1995; Zee et al., 1992). Julier *et al*. (1997) however

have found significant linkage of familial essential hypertension to two closely linked microsatellite markers on 17q which lie 18 cM proximal to the *ACE* locus (Julier et al., 1997).

3.3.8.4 Myocardial Infarction (MI) and Coronary Heart Disease (CHD)

Significantly higher frequencies of the *ACE* D allele have been found in fatal MI (Ettinger et al., 1978) as well as low-risk MI survivors (Cambien et al., 1992) and NIDDM patients with early-onset CHD (Ruiz et al., 1994). Ruiz et al (1994) attributed 24% of the risk of MI to the *ACE* D allele alone. *ACE* DD patients have more extensive coronary atherosclerosis (Arbustini et al., 1995; Ohishi et al., 1993) and experience twice as much restenosis following coronary artery stenting (Amant et al., 1997). The *ACE* I/D polymorphism may also be an important genetic marker for the risk of familial premature MI. Bohn *et al* 1993 found the frequency of premature parental MI to be 14% in DD homozygotes, 10.6% in heterozygotes and 6.1% in II individuals (Bohn et al., 1993b).

In a post-mortem study of definite and possible MI victims in Belfast, Northern Ireland, compared with non-MI related deaths in a control population, Evans et al (1994) identified an overall odds ratio of 2.2 for DD individuals versus II individuals (Evans et al., 1994).

Oike *et al* (1995) studied patients with coronary artery spasm and identified a greater risk for MI in those who are homozygous for the D genotype. The relative risk for MI conferred by the D allele was greatest in those who had coronary artery spasm but no fixed stenosis (Oike et al., 1995). In a large study in 1998 Gardemann *et al* investigated 2267 male caucasian patients and found an association of the D allele with coronary artery disease in those less than 62 years of age who had not suffered MI. When other cardiovascular risk factors were excluded and even stronger association of the D allele was seen (Gardemann et al., 1998).

As with many common disease genetic conditions not all published studies agree with these findings (Bohn et al., 1993a; Lindpaintner et al., 1996; Lindpaintner et al., 1995; Winkelmann et al., 1996). Lindpaintner *et al* 1995 in the largest study of its time which enrolled 1250 American physicians who had suffered either angina, coronary revascularisation or MI and 2340 age matched controls. They failed to identify any association with D allele. Winkelmann *et al* 1996 identified the D allele to cause higher levels of plasma ACE activity but were unable to find an association between the allele and coronary artery disease. There are many possible reasons for these differences including chance, undetected selection biases, different gene-environment interactions between the populations studied,

or to loss of DD individuals in the 'high-risk' groups due to mortality from other causes (Bohn *et al*. 1993).

More recently in the largest investigation of the role of the *ACE* in MI to date, Keavney and colleagues (2000) in the International Study of Infarct Survival (ISIS) studied 4629 MI patients and 5934 controls aged between 30 and 64 years. The control population were made up of siblings or spouses of the MI survivors who had no history of cardiovascular disease. The study identified a higher proportion of *ACE* DD genotypes in the MI cases (29.4%) compared with the controls (27.6%) giving a risk ratio of 1.10. Confidence intervals however included 1.0 (CI 1.00-1.21) and so the group concluded that their results did not confirm the existence of any substantial association. In a separate meta-analysis of their results and previously published studies Keavney et al (2000) calculated the RR for MI with the DD genotype to lie in the range of 1.0-1.1. Furthermore risks were not especially strong in the subgroups previously singled out (ie those with lower risk for cardiovascular disease) (Keavney et al., 2000).

A further Dutch large scale study by Sayed-Tabatabaei et al. (2005) genotyped the ACE I/D polymorphism in 6,714 individuals and recorded their smoking status and history of MI. There was no association between *ACE* genotype and MI however they did identify an increased risk of cardiovascular mortality for younger smokers below the age of 68.2 years who carried the D allele ($p = 0.03$) (Sayed-Tabatabaei et al., 2005).

3.3.8.5 Diabetes

Huang et al. (2001) engineered mice to have diabetes and 3 copies of the *ACE* gene. Compared to normal mice, those with a third copy of the *ACE* gene had higher enzyme levels, higher BP and greater degrees of overt proteinuria indicative of nephropathy suggesting that a modest genetic increase in ACE levels is sufficient to cause nephropathy in diabetic mice. (Huang et al., 2001).

In humans the *ACE* D allele has been associated with early onset MI in NIDDM patients (Ruiz et al., 1994). There is also evidence of an association between the D allele and nephropathy in diabetic individuals. Marre et al 1997 performed a large multicentre study on IDDM subjects with diabetic retinopathy. Those patients who possessed the D allele were considered to be more at risk of developing renal failure. Those with the combination of the *ACE* D allele and the angiotensinogen allele M235T were found to have an even greater risk of renal disease (Marre et al., 1997). Vleming et al. (1999) studied the contribution of the I/D polymorphism in 79 patients with end-stage renal failure

due to diabetic nephropathy and in 82 age-matched controls with 15 years of IDDM but without microalbuminuria. There was significant overrepresentation of the DD genotype with a significant increase of the D-allele frequency in the cases compared to controls. The presence of the DD genotype increased the risk of end-stage renal failure compared to other genotypes (OR, 2.1; 95% CI, 1.1-4.0) (Vleming et al., 1999).

These associations have not been universally identified with two studies both failing to identify the *ACE* gene as a risk factor in diabetic nephropathy (Schmidt et al., 1995; Tarnow et al., 1995).

3.3.8.6 Renal Disease

In non-diabetic patients there is an association of the *ACE* gene with renal diseases. Yoshida et al (1995) found the DD genotype to be a risk factor for progression to chronic renal failure in Immunoglobulin A associated nephropathy (IgA nephropathy). They found 43% of patients who showed decline of renal function had the DD homozygous genotype, whereas it was present in only 7% of age-matched individuals without a history of the proteinuria and in only 16% of a group of patients with IgA nephropathy and stable renal function. Once ACE inhibitors had been administered for 48 weeks, patients with the DD genotype gained more therapeutic benefit with lower levels of proteinuria (Yoshida et al., 1995).

In contrast Yoon et al. (2002) failed to find any association of the *ACE* gene with IgA nephropathy in 38 patients who showed progression of renal disease over a 3 year period. However there was a significant link between progressive renal disease and the combination of the *ACE* D allele and ala379-to-val polymorphism in exon 11 of *PLA2G7* , which encodes a functional agonist of platelet-activating factor (PAF) (Yoon et al., 2002). Similar results were seen in a study by Pei et al. (1997) who found that the presence of the *ACE* DD polymorphism adversely affected disease progression in IgA nephropathy only in patients with the met235/met235 (MM) genotype of the AGT gene (Pei et al., 1997). These results suggest that the interdependent effects of *ACE* and *PLA2G7* and *ATG* polymorphisms on the progression of IgA nephropathy may be more important than the effect of the individual polymorphisms alone and this has wider implications on the future design of common disease genetic studies.

In a study of the renal developmental anomally, renal tubular dysgenesis, Gribouval et al. (2005) identified a tyr266-to-ter (Y266X) mutation in the *ACE* gene arising from a 798C-G transversion in exon 5 in 2 affected siblings. It was the only mutation found and had been inherited from the father. This is the first published study to imply Mendelian inheritance of renal tubular dysgenesis due to a defect in the ACE gene. This highlights its crucial role in human kidney development. The authors

proposed that renal lesions and early anuria result from chronic low perfusion pressure of the fetal kidney, a consequence of renin-angiotensin system inactivity (Gribouval et al., 2005).

3.3.8.7 Alzheimer's Disease (AD)

In contrast to cardiovascular disease the *ACE* I allele has been associated with the development of Alzheimer's disease. In 133 Japanese sporadic AD patients and 257 controls Hu et al. (1999) found the *ACE* II genotype was 1.4 times higher in AD than controls (Hu et al., 1999). More recently Elkins et al. (2004) have investigated 23 independent published studies of the association between the ID polymorphism and Alzheimer's disease in a meta-analysis. They found the OR for AD in individuals with the I allele (II or ID genotype) was 1.27 compared to those with the DD genotype. The risk of AD was higher among Asians (OR of 2.44) and in patients younger than 75 years of age (OR of 1.54) although this risk is very small compared to the effects of other alleles, especially *APOE4* (Elkins et al., 2004).

3.3.8.8 Critical illness

The *I/D* polymorphism of the *ACE* gene has also been studied in patients with critical illness. A study of 110 consecutive caucasian paediatric patients with meningococcal disease by Harding et al. (2002) found the children with an I allele, to have a lower risk of mortality ($p = 0.01$), worse Glasgow Meningococcal Septicemia Prognostic Scores ($p = 0.014$), greater need for inotropes ($p = 0.034$) and ventilation ($p = 0.044$), and longer stays in the pediatric intensive care unit ($p = 0.021$) compared with DD individuals. DD genotype was 6% for the children not requiring intensive care (ITU), 33% for those ITU care but survived and 45% for those who died ($p = 0.013$) (Harding et al., 2002). These data suggesting adverse outcomes in the DD individuals were further supported by work from the same group in a prospective study on 148 preterm infants requiring ITU care. Infants with the DD genotype had more acid base deficits ($p = 0.020$), required more oxygen ($p = 0.028$) and inotropic support ($p = 0.039$) than those with the ID or II genotype (Harding et al., 2003).

Itoyama et al. (2004) obtained similar results in a small study of 44 Vietnamese severe acute respiratory syndrome (SARS) cases and proposed that *ACE* D allele may influence the progression to pneumonia in SARS. The group separated their stars cases into 22 hypoxaemic patients and 22 non-hypoxaemic patients. When compared with 103 healthy exposed and 50 unexposed controls the frequency of the D allele was significantly higher in the hypoxemic cases compared with the

nonhypoxemic cases (20 of 44 alleles vs 9 of 44 alleles), whereas there was no significant difference between the SARS cases and controls, regardless of contact history (Itoyama et al., 2004).

3.3.8.9 Effects of ACE Inhibitors

Angiotensin converting enzyme (ACE) inhibitors are now widely used in the field of heart failure and hypertension. One recognized and severe side-effect of ACE inhibitors is angioedema and the risk of its development appears to be associated with race. Brown et al (1996) found an adjusted RR of angioedema of 4.5 in African American's compared to whites. Seven of the eight patients admitted to the intensive care unit were black (Brown et al., 1996). It also seems that race affects the success of ACE inhibitors when used to treat heart failure. Exner et al 2001 found that whereas enalapril was associated with significant reduction in the risk of hospitalisation for heart failure among white patients with left ventricular dysfunction it had no such effects in similar black patients (Exner et al., 2001).

3.3.9 Other RAS Polymorphisms

3.3.9.1 Hypertension

Renin Gene

Initial studies of the renin gene have yielded conflicting results (Samani, 1991). Rat studies have identified a *Renin* allele associated with hypertension (Kurtz et al., 1990; Rapp et al., 1989) but these results are not universally reproduced and suggest different roles for the renin gene in different rat strains (Lindpaintner et al., 1990).

Analysis of polymorphisms within the human *Renin* gene has not demonstrated any linkage or association with hypertension (Jeunemaitre et al., 1992b; Naftilan et al., 1989; Soubrier et al., 1990).

Angiotensinogen Gene

The results of *Angiotensinogen* studies have been more successful. Unlike ACE, there is a correlation between plasma angiotensinogen levels and BP. Transgenic studies show that over expression of the *Angiotensinogen* gene is associated with higher BP (Kim et al., 1995). Human studies have revealed positive linkage between hypertension and the *Angiotensinogen* locus (Corvol et al., 1999) and a

recent meta-analysis of the M235T polymorphism in exon 2 has shown a weak but significant association with hypertension in white subjects (pooled OR = 1.2, 95% CI 1.1-1.3).

The RAS, in particular the *ACE* gene, has received much attention from common disease geneticists interested in vascular diseases. The candidacy of *ACE* is supported by a number of molecular biological roles which have been identified over the last few decades. These will be reviewed in the next section.

3.4 Molecular Biology of the Renin-Angiotensin System (RAS)

The RAS is the major regulator of BP and fluid homeostasis ensuring the constant perfusion of vital organs. Its primary components are Angiotensinogen, Renin, ACE, Angiotensin I (ANG I) and II (ANG II) and the ANG II receptor (Figure 3.1). Over recent years much attention has been given to the pathophysiological implications of the system. Inhibitors of components of the RAS such as the ACE inhibitors, ANG II receptor blockers have become important clinical tools in the treatment of cardiovascular and renal diseases. Angiotensinogen, predominantly synthesised in the liver, serves as the substrate for Renin which catalyses the conversion of Angiotensinogen to ANG I. This is the rate limiting step for the RAS cascade (Quinones MJ et al., 1997). The majority of ANG I is subsequently converted, predominantly on the pulmonary vascular endothelium, to the potent vasoconstrictor, ANG II, by *ACE* (Ng and Vane, 1968). Systemically ANG II also causes Aldosterone and catecholamine release as well as drinking, glycogenolysis and the secretion of Prolactin and Adrenocorticotrophic hormones (Peach, 1977).

Local and independent tissue based RAS also exist (Dzau et al., 2002) and have important roles within the vasculature (Andre et al., 1990; Samani and Swales, 1991), brain (Lenz and Sealy, 1990), heart (Lindpaintner et al., 1987), and kidney (Schunkert et al., 1991) and may affect vascular development and repair.

ACE inhibitors are widely used in the clinical setting. Pharmacological studies have shown that the antihypertensive, cardioprotective and anti-vascular smooth muscle proliferative effects of ACE inhibitors are independent of circulating somatic ACE inhibition (Lee et al., 1993; Weishaar et al., 1991). Indeed ACE inhibitors produce significant blood pressure reductions even in low systemic Renin states. Chronic hypertension and atherosclerosis are known to induce structural changes on the vascular wall leading to vasoconstriction. It has been speculated that tissue ANG II may be important roles in these chronic processes (Lee et al., 1993).

Rat studies have identified Renin, Angiotensinogen and *ACE* gene expression in the aorta, left ventricle and myocardium in experimental hypertension, cardiac hypertrophy and myocardial infarction respectively with no identified increase in the plasma levels. (Drexler H et al., 1989; Dzau et al., 1987; Samani et al., 1989; Schunkert et al., 1990; Shiota et al., 1992). Tissue levels of RAS components are also higher in the SHR than in the normotensive WKY rat but the plasma levels of ACE are lower in the SHR compared with its healthy counterpart. This suggests a greater pathological importance of tissue than circulating levels of RAS components (Nakamura and Nakamura, 1987).

Figure 3.1 The Renin Angiotensin System

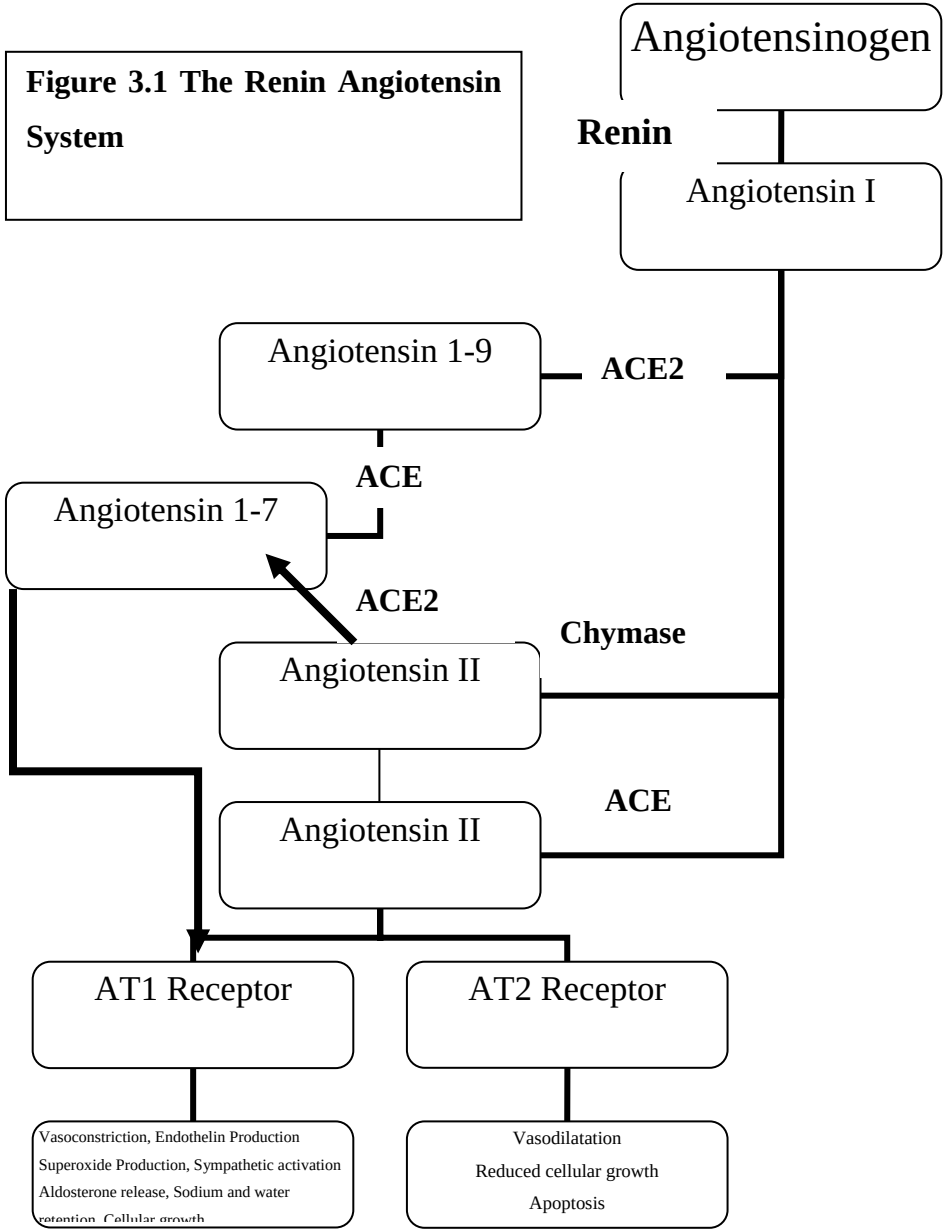
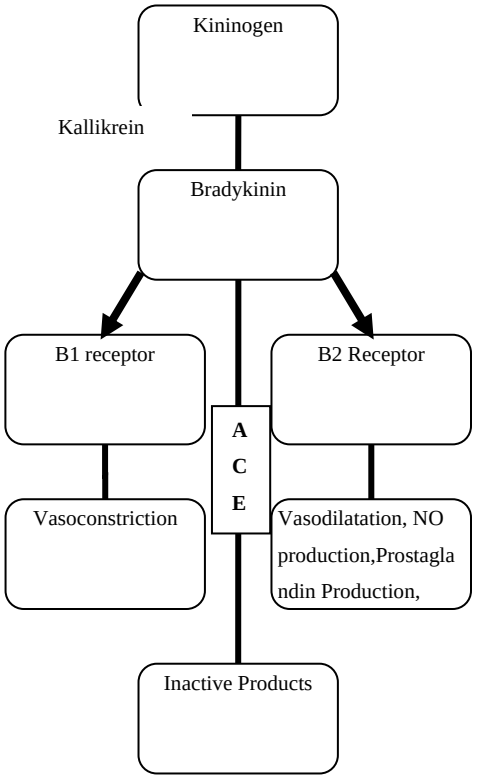


Figure 3.2 The role of ACE in Bradykinin Breakdown



These studies support the autocrine-paracrine effects of ANG II in normal and abnormal cardiovascular physiology. There have been some conflicting studies showing low levels of Renin in the vasculature suggesting that the Renin-like activity associated with the vascular wall is predominantly derived from uptake of circulating Renin of renal origin rather than in-situ production (Dzau, 1988; Fordis et al., 1983; Samani et al., 1988; Thurston et al., 1979). However despite these findings the existence and functional relevance of tissue based RAS is now generally accepted (Griendling et al., 1993).

During human development, random mutations can lead to dysfunctions of complex biological systems. Variability in the function of the important RAS components is evident and contributes to endothelial dysfunction, increased vascular resistance, myocardial hypertrophy and fibrosis, arteriosclerotic plaque rupture and reduced fibrinolysis. This variability may be under a degree of genetic control. The importance of the RAS in vascular physiology and pathology is shown by the effects of ACE inhibitors which block the ACE mediated conversion of ANG I to ANG II, and can reduce the risk of stroke (Ramipril group 3.4% vs placebo 4.9% RR = 0.68, $p<0.001$) and MI (Yusuf et al., 2000).

3.4.1 The Role of ANG II

ACE is ubiquitous and so ANG I conversion occurs in the entire vascular tree as well as the lungs (Campbell, 1985; Coghlan et al., 1982; Oliver and Sciacca, 1984). ANG II has a short half life, it is broken down in peripheral vascular beds by angiotensinogenases to ANG III which retains 30% of ANG II activity but has only a minor role in man. Many roles have been identified for ANG II. Its actions are mainly mediated through the second messengers Inositol triphosphate (IP3) and Diacylglycerol (DG). IP3 mobilises free calcium leading to cellular contraction via a Calmodulin pathway (Brock et al., 1985). DG sustains smooth muscle hypertonia by stimulating protein kinase C (Griendling et al., 1986). As a consequence, ANG II can induce vascular smooth muscle cells to undergo contraction, hyperplasia (Paquet et al., 1990) or hypertrophy (Geisterfer et al., 1988). Locally generated ANG II is also able to increase chatecholamine release from neuronal synapses (Kawasaki et al., 1982; Kawasaki et al., 1984) enhancing the vasoconstrictive effects.

ANG II also leads to the production of superoxide anions and hydrogen peroxide which are able to inactivate Nitric Oxide (Rajagopalan et al., 1996). This oxidative stress leads to expression of genes controlling cytokine formation and adhesion to the vessel wall (Marui et al., 1993). In addition, ACE

degrades and inactivates Bradykinin whose role is to promote the generation of Nitric Oxide (Drexler and Hornig, 1999). Nitric Oxide also inhibits vascular smooth muscle cell growth and migration and the expression of pro inflammatory molecules such as Vascular Cell Adhesion Molecule-1 and Monocyte Chemoattractant Protein-1, it also blocks platelet aggregation. Reductions in Nitric Oxide therefore produce vasoconstriction and also inflammation. The important role of Nitric Oxide can be seen in the more severe response to vascular injury in the endothelial Nitric Oxide synthase knockout mice (Moroi et al., 1998). ANG II can also promote thrombogenesis by activating Plasminogen Activator Inhibitor–1 (Vaughan et al., 1995) and by inhibiting Bradykinin it reduces the concentrations of Plasminogen Activator (Vaughan et al., 1997).

The effects of ANG II are greater in the presence of hypertension. In response to ANG II extracellular matrix growth within the vascular smooth muscle layer is greater in the spontaneously hypertensive rat (SHR) compared with normotensive rats (Scott-Burden et al., 1991). ACE inhibition in SHR is more effective in inhibiting vascular smooth muscle hypertrophy compared with other antihypertensives despite similar BP reduction (Owens, 1987). Furthermore treatment of young SHR can produce permanent reduction in blood pressure, an effect not seen in older animals (Harrap et al., 1990).

In vivo studies looking at the role of ANG II in the structural changes occurring during the disruption of normal vessel physiology have demonstrated vessel wall hypertrophy which was inhibited by ACE inhibitors but not by Beta blockers (Powell et al., 1989). Angiogenic properties of ANG II have also been identified within chick embryos (Le Noble et al., 1991).

Within the heart ANG II can have direct pathological effects including inotropic and chronotropic effects on myocardial cells, (Lindpaintner and Ganten, 1991) stimulation of DNA turnover (Khairallah PA et al., 1972) and protein synthesis (Tan et al., 1992) as well as increasing connective tissue deposition. ANG II infusions into mice can also promote aortic aneurysm (AA) formation (Nishijo et al., 1998).

3.4.1.1 Non ACE conversion of ANG I to ANG II

In rats ACE is thought to be the only converter of ANG I to ANG II (Tsunemi et al., 2002). In humans, although ACE is the main catalyst of ANG I to ANG II, other mechanisms have been identified in isolated arteries (Bund et al., 1989), heart preparations (Hirakata et al., 1990; Urata et al., 1990a; Urata et al., 1990b), the atherosclerotic aorta (Takai et al., 1997) and perfused vascular beds (Ideishi et al., 1990). A Chymase mediated conversion has been identified in the heart with a high substrate specificity for ANG I. In aortae Chymase activity rises in the presence of ACE inhibitors. These findings have lead to speculation that ANG II receptor antagonists may be more effective in vascular disease prevention than ACE inhibitors (Tsunemi et al., 2002).

3.4.1.2 Angiotensin Converting Enzyme 2 (ACE2)

This enzyme was discovered, cloned and mapped in 2000 (Tipnis et al., 2000). The *ACE* 2 gene has 18 exons and maps to Xp22. It encodes a zinc metalloproteinase which is made up of 805 amino acids and shares approximately 40% homology with *ACE*. ACE 2 has been detected in all 72 human tissues and cells except red blood cells with the highest concentrations found in the testis, kidney, gastrointestinal tract and cardiovascular tissues (Harmer et al., 2002). ACE2 is able to cleave angiotensin I and ANG II but not Bradykinin and is not inhibited by ACE inhibitors such as lisinopril (Tipnis et al., 2000). In contrast to ACE which converts angiotensin I to ANG II which consists of eight amino acids, ACE2 converts ANG I to ANG I-9 which has one extra amino acid. ANG I- 9 has no effect on blood vessels but can be converted by ACE to a shorter, vasodilatory peptide ANG I-7 (Boehm and Nabel, 2002; Crackower et al., 2002).

No human common disease studies of ACE2 has been published to date however (Gurley et al., 2006) generated ACE2-deficient mice and found that they were fertile, and had normal cardiac anatomy and function. After acute ANG II infusion, plasma concentrations of ANG II increased almost three fold higher in *ACE2*-deficient mice than in controls. In a model of ANG II -dependent hypertension, BPs were substantially higher in the *ACE2*-deficient mice than in wildtype mice, and severe hypertension in *ACE2*-deficient mice was associated with exaggerated accumulation of ANG II in the kidney. These studies suggest that *ACE2* is a functional component of the RAS, with effects on ANG II and contributing to the regulation of BP.

3.4.2 Angiotensin Converting Enzyme (ACE)

3.4.2.1 Protein Analysis

ACE, first characterised in 1956 (Lentz et al., 1956; Skeggs et al., 1956a; Skeggs et al., 1956b; Skeggs et al., 1956c) is a dipeptidyl carboxypeptidase. ACE has 2 isoforms, somatic and germinal (testicular). Somatic ACE is both endothelium bound and circulating. Its activity is higher in arteries compared with veins. Germinal ACE is smaller and expressed only in sperm (Ehlers MRW and JF, 1990).

Somatic ACE is ubiquitous (Lieberman and Sastre, 1983) with 90% being tissue bound (Cushman and Cheung, 1971). In situ synthesis also occurs as evidenced by the identification of ACE mRNA in vascular endothelial, smooth muscle cells and adventitial vascular layers (Battle et al., 1994) the choroid plexus (Arregui and Iversen, 1978), striatonigral neurones (Barnes et al., 1988) and blood cells (Costerousse et al., 1993).

ACE consists of 2 nearly identical lobes each of which has an active site. Each binding site has different substrate (Perich et al., 1994). Tissue binding occurs via an intracellular carboxy terminal tail. Zinc and chloride are both necessary for substrate binding, low zinc levels can lead to reduced ACE activity. In vivo endothelial ACE is variably glycosylated depending on the tissue of origin. Plasma ACE is similar to the pulmonary endothelial ACE from which it derives containing large numbers of sialic acid residues which protect it from hepatic breakdown. The enzyme is activated by chloride ions which cause a conformational change near the active binding site (Soubrier and Corvol, 1990). Although the structure of testicular ACE has recently been elucidated the structure of somatic ACE remains unknown (Brew, 2003).

3.4.2.2 Regulation of ACE Expression and Secretion

In normal rats in physiological conditions there is relatively low ACE expression (Cushman and Cheung, 1971; Schunkert et al., 1990). Tissue ACE deficient mice develop normal hearts but abnormal kidneys resulting in an inability to concentrate urine and hypotension (Bernstein, 1998).

In vitro, the secretion of ACE is induced by cAMP analogues, (Krulewitz and Fanburg, 1986) methylxanthines, the calcium ionophore, A231S7, the sodium ionophore, monensin (Dasarathy and Fanburg, 1991), glucocorticoid hormone (Fishel et al., 1995a; Friedland et al., 1977; Friedland et al.,

1978; Krulewitz et al., 1984) and fibroblast growth factor (Fishel et al., 1995b). In vivo, many vascular disease states lead to ACE induction (Table 3.8). In the failing heart most ANG II is locally synthesised mainly as a consequence of a 2-5 fold increase in ACE activity (Dzau et al., 2002).

Table 3.9 Disease States Leading to ACE Induction

Disease	Comments	Reference
Heart Failure	Pressure overload Volume overload	(Schunkert et al., 1990) (Ruzicka et al., 1995) (Fabris et al., 1990)
MI	Induction of brain ACE	(Tan et al., 2004) (Hokimoto et al., 1996)
Aging		(Heymes et al., 1994)

Paradoxically ACE levels are seen to increase in serum and tissues following ACE inhibitor therapy in both rats and humans (Fyhrquist et al., 1980; King and Oparil, 1992; Kokubu et al., 1980). ACE induction by ANG II has been observed in rat renovascular hypertension. (Arnal et al., 1994)

Overall little is known about in-vivo functions of the RAS, however the evidence suggests an important role in a complex physiological system regulating vascular tone, tissue perfusion, and inflammation.

3.5 Genomic Analysis of *ACE*

ACE mRNA was isolated in bovine lungs in 1986 (St Clair et al., 1986). The gene was subsequently mapped to 17q23 by in situ hybridization in 1989 (Mattei et al., 1989). The gene contains 26 exons which can be transcribed into either the 5kb somatic *ACE* mRNA or the 2.6kb testicular *ACE* mRNA (Thekkumkara et al., 1992). The somatic promoter is located 5' of the first exon and leads to transcription of all exons, exon 13 is subsequently spliced out. The testicular *ACE* promoter lies in exon 12 (Langford et al., 1991) and the mRNA contains exon 13-26 of the gene (Hubert et al., 1991).

3.5.1 Evolution of the *ACE* Gene

The structure or the human *ACE* gene suggests it has arisen from duplication of an ancestral gene which occurred over 300 million years ago (Villard and Soubrier, 1996). Exons 4-11 and 17-24 are similar and encode the two homologous domains of the ACE molecule (Hubert et al., 1991). An unduplicated Drosophila gene with similar enzymatic properties as ACE has been identified with 65% homology in the region of the zinc binding motif. Duplication has occurred in all other mammalian *ACE* genes identified to date suggesting that the Drosophila gene resembles the ancestral form.

ACE is likely to have played a vital during evolution as it is conserved between species with 80% homology in the upstream (5') regions of the human, rabbit and mouse genes (Kumar et al., 1991; Shai et al., 1990) as well as containing glucocorticoid and cAMP response elements within the distal 5' regions. (Shai et al., 1990).

3.5.2 The Genetic Control of ACE Levels

Apart from a temporary increase in adolescence, an individual's plasma ACE levels show little variation throughout life. A 5-fold variation can be seen between individuals due to a major gene effect accounting for 29% and 75% of the variance in parents and offspring, respectively (Cambien et al., 1988). The *ACE* gene was cloned in 1988 (Soubrier et al., 1988) and a functional 287 base pair insertion / deletion polymorphism in intron 16 (*ACE I/D*) was identified 2 years later which was thought to account for 47% of the plasma ACE variance. The D/D genotype conferred approximately double the amount of plasma ACE compared with I/I subjects, with I/D subjects having intermediate levels suggesting a co-dominant effect (Rigat et al., 1990). This was later confirmed by Tiret *et al* 1992 in a different caucasian population (Tiret et al., 1992) and by Kreutz *et al* in 1995 in the rat (Kreutz et al., 1995). Jeffrey et al (1999) has since disputed this co-dominance theory following results from a study of 97 Ghanaian individuals. They found significantly lower ACE levels in those

with the II genotype than in those with the ID or DD genotype, but no difference between the ID or DD groups and concluded that the D allele shows dominance relative to the I allele (Jeffery et al., 1999)

A proportion of the remaining variance may be accounted for a second QTL identified by McKenzie *et al* 1995. They identified two QTLs in a series of Afro-Carribean families, one within or close to the *ACE* locus on chromosome 17q accounting for 27% and a unlinked and as yet unmapped locus which accounted for 52% of ACE variability (McKenzie et al., 1995). They concluded that the ID polymorphism was unlikely to directly cause serum ACE variability itself although it does provide an ideal marker for a putative functionaly linked locus nearby.

Villard *et al* (1996) performed a quantitative trait segregation linkage analysis and proposed that two variants influence circulating ACE levels, one in complete linkage disequilibrium with the ID polymorphism (or identical to it) and the other mapping upstream of the translation start point (Villard et al., 1996). Follow up studies have suggested that there are independent 5' and 3' ACE – linked QTLs with effects on ACE concentrations (Soubrier F *et al* 2002; Zhu X *et al* 2001; McKenzie *et al* 2005). A study by Keavney *et al* 1998, using 10 of the polymorphisms identified by Villard *et al*, created intragenic haplotypes which segregated into 3 clades (A,B & C) (a clade is made up of haplotypes sharing DNA polymorphisms and thus are presumed to have undergone similar evolution from the ancestral haplotype). Clade A conferred low ACE levels, clade C had higher levels than A but B had the highest. They concluded that the clades had been generated by an ancestral recombination breakpoint but they felt it lay outside of the 5' region proposed by Villard *et al* (Keavney et al., 1998).

Rieder *et al* 1999 identified 78 ACE gene polymorphisms (Figure 3.3) which made up 13 distinct haplotypes (Table 4.2) segregating into two independent and distantly related clades (Figure 3.4). The two clades were distinct with respect to the ID polymorphism (i.e. an Insertion clade and a Deletion clade) (Rieder et al., 1999) (NCBI sequence reference AF118569).

Farrall *et al* (1999) used Rieder's data to refine the ancestral breakpoint, excluding a further 3.9kb of the gene (Farrall et al., 1999). They concluded that it lies 3' of exon 5 (approx 6435 bp Rieder location). Further studies by Cox *et al* (2002) in West African families have found more haplotype diversity (Cox et al., 2002). They propose the presence of clade D which is similar to clade B and

confers higher ACE levels than clades A and C. They report that clades B and D differ from clade C in the region 5’ to 8968 (Reider’s location) (T17944C Genecanvas marker).

These studies now make it possible to perform a more detailed association analysis of the ACE gene based upon haplotypes rather than single isolated polymorphsims (see Chapter 4).

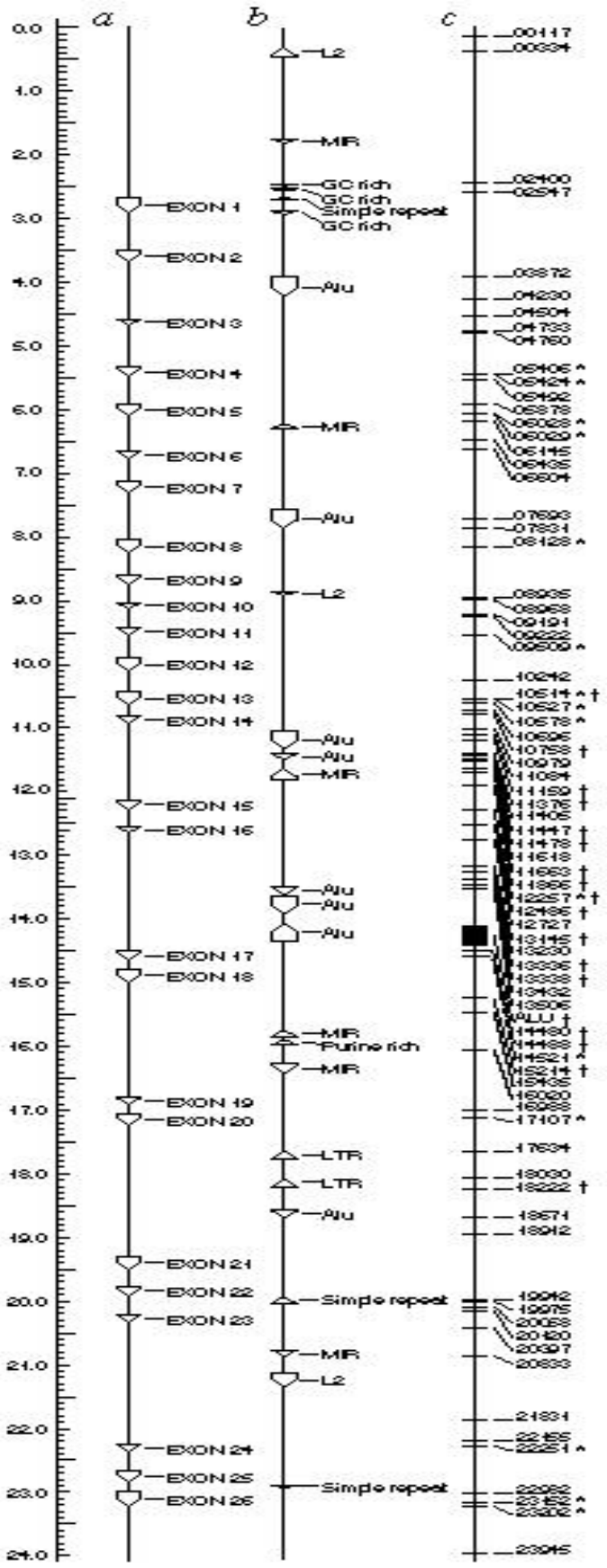


Figure 3.3

Location of ACE Gene SNPs identified by (Rieder et al., 1999)

a, Genomic structure of *ACE* consisting of 26 exons over 24 kb (~16% coding sequence) ACE is a duplicated gene with two homologous domains (exons 1-12, 14-26). Tissue-specific expression is found in testes, with a promoter in intron 12 driving expression of the testes-specific exon 13 and the second half of *ACE* (exons 14-26; ref.).

b, Repeat structure found in genomic *ACE* sequence. Genomic sequence was analysed for known repetitive elements .The *Alu* repeat element in intron 16 has been used as a common marker in association studies.

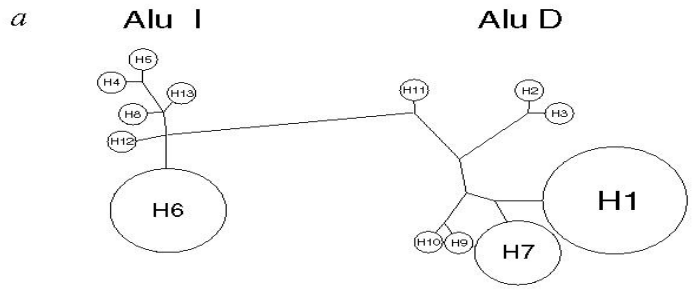
c, Variant distribution in *ACE showing* 74 SNPs (59 non-coding, 15 coding) and 4 insertion/deletion polymorphisms from 11 individuals (5 African-Americans, 6 European-Americans).

*Sites found in coding regions (5/15 coding sites resulted in nonsynonymous changes, 06029 (G/A; Ser→Ala), 09509 (C/T; Arg→Trp), 10527 (T/C; Ser→Ala), 10578 (A/G; Ser→Gly) and 23152 (C/A; Ser→ Arg).

t= Variant sites in absolute linkage disequilibrium with the *Alu* insertion/deletion.

Figure 3.4

***ACE* gene cladistic haplotype tree . (Rieder et al., 1999)**



Gene tree constructed from the 52 non-unique variant sites. The *Alu I/D* occurs along the long branch separating the two major clades. The relative frequency of each haplotype is indicated by the area of the circle

3.6 Haplotypes

Haplotypes are generated by the sexual reproduction over many generations of a species through chromosomal recombination during meiosis (Figure 3.5). Some ancestral chromosomal segments are shared by multiple individuals due to low levels of recombination (cold spots). These segments are the haplotypes that can be studied in common disease genetic studies.

Fossil and genetic evidence indicate that all humans today are descended from anatomically modern African *Homo sapiens* who lived about 150,000 years ago. Due to the relatively young age of the species, most of the modern genetic variation has originated from the ancestral human population. This variation is still more widespread in modern day Africans as recombination has had more time to break up the haplotypes. In contrast as only a fraction of the genetic variation has been carried away from Africa due to migration, these haplotypes tend to be subsets of the haplotypes seen inside Africa. Thus migrant populations have stronger LD and so haplotypes tend to be longer than their African ancestors.

As modern humans have continued to spread throughout the world different regions have developed their own variations through random chance, natural selection, and other genetic mechanisms and so haplotype freqencies vary widely. Geographically isolated populations show the smallest number of haplotypes as they are unlikely to exchange much DNA through mating. Although specific disease causing variations are more likely to be identified in genetically diverse populations with the largest numbers of haplotypes available to show up co-segregation of diseases with specific haplotypes this leads to the requirement of huge study populations as each haplotype will have a low frequency. In order to increase the chance of identifying disease haplotypes researchers have often studied

geographically isolated populations with a smaller number of haplotypes each with a higher frequency therbey reducing the numbers of patients needed to generate sufficient statistical power.

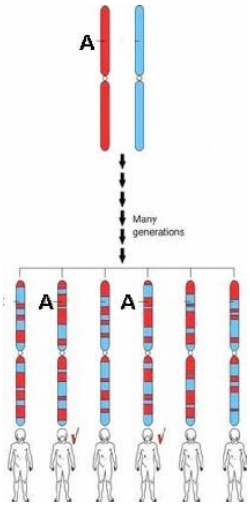


Figure 3.5 : **The generation of haplotypes**

Chromosomal recombination over many generations to yield different descendant chromosomes. If a genetic variant marked by the A on the ancestral chromosome increases the risk of a particular disease, the two individuals in the current generation who inherit that part of the ancestral chromosome will be at increased risk. Adjacent to the variant marked by the A are many SNPs that can be used to identify the location of the variant. (http://www.hapmap.org/originhaplotype.html)

3.7 The Case for ACE Having an Important Role in Vascular Pathology

Although the rate of ANG II production by the RAS is generally limited by the action of Renin, there is evidence, at least in some tissues, that ACE is capable of exerting some control over its production as well (Danser et al., 1992). Morishita *et al* (1994) discovered that following the injection of *ACE* cDNA into rat carotid artery, DNA synthesis was stimulated by the production of ANG II (Morishita et al., 1994). This suggests that an increase in ACE can stimulate ANG II production and in view of its control over the ACE levels the *ACE ID* polymorphism may have an important role in this context. Ueda *et al* 1995 investigated the differential effects on human ANG II production following the infusion of angiotensin I in *ACE* DD and *ACE* II subjects. They observed a greater elevation in blood pressure and a higher venous ANG II levels in DD subjects compared with other genotypes (Ueda et al., 1995a). Furthermore survival of human endothelial cells (ECs) in vivo is enhanced by low levels of ACE. Hamdi and Castellon (2004) (Hamdi and Castellon, 2004) found that after slow starvation human ECs with the II genotype showed enhanced growth and reduced ANG II levels compared with ECs with the DD genotype. This effect was reversed by the ACE inhibitor Captopril which improved the viability of DD cells, but it had little effect on II cells.

In 1996, Villard and Soubrier proposed a 'Vascular or Endothelial Hypothesis' as a possible explanation of the pathological effects of the *ACE* ID polymorphism (Villard and Soubrier, 1996). This hypothesis implies that the induction of *ACE* gene expression during neointimal proliferation may vary with respect to *ACE* ID genotype. The D allele being associated with an increase in the ACE level may increase the local ANG II concentration and reduce the level of the vasodilator Bradykinin. This would reduce Nitric Oxide levels and may lead to increased neo-intimal proliferation and subsequent arteriosclerosis. At times of stress, ANG II stimulation of thrombogenesis may lead to vessel occlusion and subsequent cerebral infarction.

3.8 Conclusions

Ischaemic stroke is a major cause of death and disability throughout the world, but attempts to define the genetic basis of this condition have lagged behind studies in other polygenic disorders. Strong evidence from epidemiological and animal studies has implicated genetic influences, but the identification of individual causative mutations in human polygenic stroke remains problematic. The recent biological revolution catalysed by the human genome project promises the advent of novel technologies supported by bioinformatics resources that will transform the study of disorders such as stroke. The ultimate goal of these endeavors will be not only to provide new avenues for prevention for such a devastating human disease which touches the lives of most of us but also to provide insights into factors that influence the outcome of stroke, and new therapeutic targets when preventative strategies have failed.

There are many putative candidate genes for ischaemic stroke. The RAS and more specifically ACE is unrivaled in the amount of molecular biology, genetic and pharmacological research performed to date. The evidence for a widespread role in the development of chronic vascular disease processes as well as acute thrombogenesis is strong. Recent genetic advances have identified new avenues of research into *ACE* using haplotype based genotyping and consequently it provides an excellent candidate for this study.

CHAPTER 4

PATIENTS AND METHODS

Chapter 4: List of Tables

Chapter 4: List of Figures

4.1 Ethics Approval

Ethics approval was obtained from the Leicestershire Area Ethics Committee in 1998 to conduct this project in each of the three Leicester NHS hospitals and Leicester General Practice settings. A period of 18 months was allowed for each of the clinical and laboratory phases of the research.

4.2 Subject Ascertainment & Clinical Data Collection

4.2.1 Ischaemic Stroke Patients

The clinical phase of the study simultaneously identified adult patients (>18yrs) both retrospectively and prospectively from hospital stroke databases as well as through attendance at stroke assessment clinics and acute stroke ward rounds. The clinical assessment is detailed in Table 4.1. Entry criteria included the recorded presence of acute symptoms and signs of cerebral dysfunction lasting more than 6 hours diagnosed as cerebral ischaemia/infarction by an experienced consultant physician. Patients who had experienced a historic event with good hospital notes were also included. Supporting evidence of an ischaemic lesion on cerebral imaging was preferred although patients with no imaging or normal imaging were entered if the clinical syndrome was clearly compatible with ischaemia. The patient or a close relative were required to give written consent to recording of clinical data and collecting DNA samples. Information of vascular risk factors was obtained from hospital and general practice notes, laboratory, radiology and cardiology departments.

4.2.2 Control Population

Our aim was to identify an elderly control population at risk of stroke and other vascular diseases but naive of clinical cerebrovascular events. This would control for vascular risk factors and suggest that any differences between the groups may be accounted for by independent genetic susceptibility to ischaemic stroke. Advertisments were posted in the local press asking for caucasian volunteers born in the East Midlands area of England. Hospital hypertension and care of the elderly outpatient clinics were also targeted. Additionally hypertensive subjects who had enrolled as controls in another study of stroke disease at the Glenfield General Hospital were approached. Where possible a full medical assessment was performed on the responders and a review of hospital and GP records was made to exclude individuals with previously confirmed or possible cerebrovascular events. Methods of data collection mirrored those used for the stroke patients. All the data obtained was stored electronically in a Microsoft Excel spreadsheet.

Table 4.1 The clinical assessment.

A	A request for informed written consent to participate from the patient or next of kin or close relative
B	Full clinical history obtaining information on :- Stroke Myocardial Infarction (MI) Angina / Coronary Heart Disease (CHD) Hypertension Valvular Heart Disease Cardiac Arrhythmias Diabetes (DM) Hyperlipdaemia Thrombophilia Neoplasia Current Medications Smoking history Body Mass Index (BMI) Kg/m^2
C	Clinical examination
D	Review of medical case notes
E	Assessment of available blood tests, imaging, vascular studies, cardiology studies, and any other relevant investigations.
F	Extraction of 20 ml of whole venous blood for DNA extraction
G	Communication with the patient's GP to inform them of entry into the study.

4.3 Molecular Methodology

4.3.1 DNA Extraction

Leucocyte DNA was extracted from whole venous blood samples using a guanidine hydrochloride salt precipitation method (Table 9.7 Appendix).

4.3.2 Molecular Study of the Angiotensin Converting Enzyme Gene

In 1999, 78 ACE gene polymorphisms were identified in a group of 11 healthy volunteers by DNA sequencing (Rieder et al 1999). Using ancestral haplotype trees the polymorphisms were segregated into 13 haplotypes (Table 4.2). It is hypothesised that the ACE produced from these haplotypes may be structurally and functionally different, thereby playing different roles in vascular biology and pathology. The discovery of this haplotypic variation has allowed for a more detailed analysis of the gene by comparing haplotype frequencies of stroke patients and control subjects. Due to time and financial reasons it was not possible to genotype all study subjects for all 78 polymorphisms. It was therefore necessary to generate the haplotypes from a smaller but highly discriminative subgroup of polymorphisms.

Table 4.2 *ACE* Gene Haplotypes (Rieder *et al* 1999)

Polymorphism

Haplotype

Polymorphism	**2**	**3**	**4**	**5**	**7**	**10**	**12**	**13**	**17**	**20**	**21**	**23**	**24**	**25**	**28**	**29**	**30**	**31**	**32**	**33**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**52**	**53**	**54**	**55**	**56**	**57**	**61**	**62**	**63**	**65**	**69**	**71**	**73**	**74**	**75**	**76**	**78**
Haplotype																																																					
H1	C	T	C	G	C	C	C	T	**A**	A	C	T	**A**	C	C	T	A	(G-)	G	T	C	A	C	G	C	**T**	A	A	A	T	T	A	**G**	T	G	**D**	C	A	G	G	A	**C**	G	G	A	**C**	T	A	T	G	**C**	(CTCT
H2	C	A	T	G	G	C	C	C	**A**	G	C	C	**A**	C	C	C	G	(G-)	G	T	C	A	C	G	C	**T**	A	A	A	T	T	A	**A**	T	G	**D**	C	A	A	G	G	**T**	A	G	A	**C**	C	G	C	A	**C**	(CTCT
H3	C	A	T	G	G	C	C	C	**A**	G	C	C	**A**	C	C	C	G	(G-)	G	T	C	A	C	G	C	**T**	A	A	A	T	T	A	**A**	T	G	**D**	C	A	A	G	G	**C**	A	G	A	**C**	C	G	C	A	**C**	(CTCT
H4	T	T	T	G	C	C	T	T	**A**	G	C	C	**A**	C	T	T	A	(GG)	A	C	T	G	C	A	G	**T**	G	C	G	C	C	C	**A**	A	T	**I**	G	C	A	A	A	**T**	G	A	G	**T**	C	A	C	A	**A**	(CTCT
H5	T	T	T	G	C	C	T	T	**A**	G	C	C	**A**	C	T	T	A	(GG)	A	C	T	G	C	A	G	**C**	G	C	G	C	C	C	**A**	A	T	**I**	G	C	A	A	A	**T**	G	A	G	**T**	C	A	C	A	**C**	(CTCT
H6	C	A	T	A	G	C	C	T	**G**	G	T	C	**A**	C	T	T	A	(GG)	A	C	T	G	A	A	G	**T**	G	C	G	C	C	C	**A**	A	T	**I**	G	C	A	A	A	**C**	G	A	G	**C**	C	A	C	A	**C**	(CTCT
H7	C	A	T	A	G	C	C	T	**G**	G	C	T	**A**	C	C	T	A	(G-)	G	T	C	A	C	G	C	**T**	A	A	A	T	T	A	**G**	T	G	**D**	C	A	G	G	A	**C**	G	G	A	**C**	T	A	T	G	**C**	(CTCT
H8	C	A	T	G	G	T	C	T	**A**	G	C	C	**A**	C	T	T	A	(GG)	A	C	T	G	C	A	G	**T**	G	C	G	C	C	C	**A**	A	T	**I**	G	C	A	A	A	**T**	G	A	G	**T**	C	A	C	A	**C**	(CTCT
H9	C	A	T	G	G	T	C	T	**A**	G	C	C	**T**	T	C	T	A	(G-)	G	T	C	A	C	G	C	**T**	A	A	A	T	T	A	**G**	T	G	**D**	C	A	G	G	A	**C**	G	G	A	**C**	T	A	T	G	**C**	(CTCT
H10	C	A	T	G	G	C	C	T	**A**	G	C	C	**A**	C	C	T	A	GG)	G	C	C	A	C	G	C	**T**	A	A	A	T	C	A	**A**	T	G	**D**	C	A	A	G	A	**C**	G	G	G	**C**	C	A	C	A	**C**	(CTCT
H11	C	A	T	G	G	T	C	T	**A**	G	C	C	**T**	T	C	C	G	(G-)	G	T	C	A	C	G	C	**T**	A	A	A	T	T	A	**A**	T	G	**D**	C	A	A	G	A	**C**	G	G	A	**C**	T	A	T	G	**C**	(CTCT
H12	C	A	T	G	C	C	C	T	**A**	G	C	C	**A**	C	T	T	A	(GG)	A	C	T	G	C	A	G	**C**	G	C	G	C	C	C	**A**	A	T	**I**	G	C	A	A	A	**T**	G	A	G	**T**	C	A	C	A	**A**	(CTCT
H13	C	A	T	G	C	C	C	T	**A**	G	C	C	**A**	C	T	T	A	(GG)	A	C	T	G	C	A	G	**T**	G	C	G	C	C	C	**A**	A	T	**I**	G	C	A	A	A	**C**	G	A	G	**C**	C	A	C	G	**C**	(CTCT
	2	**3**	**4**	**5**	**7**	**10**	**12**	**13**	**17**	**20**	**21**	**23**	**24**	**25**	**28**	**29**	**30**	**31**	**32**	**33**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**52**	**53**	**54**	**55**	**56**	**57**	**61**	**62**	**63**	**65**	**69**	**71**	**73**	**74**	**75**	**76**	**78**

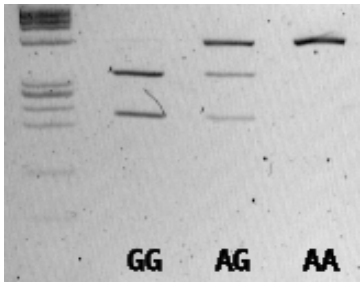
4.3.2.1 SNP Genotyping

There are many ways of genotyping single nucleotide polymorphisms (SNPs). Direct DNA sequencing is the 'gold standard' but is expensive when used in large population based studies. Restriction Fragment Length Polymorphism (RFLP) Analysis, using bacterial restriction enzymes, by comparision is relatively simple and cost efficient and was the genotyping method chosen at the outset of this study.

4.3.2.1.1 RFLP Analysis

In order to protect the host bacterium, each restriction enzyme recognizes short foreign DNA sequences and cuts at those specific sites. SNPs alter DNA sequences and can either disrupt a cleavage site preventing the enzyme from binding or can produce new restriction sites. This SNP induced variability of enzyme action can be harnessed to allow SNP identification. See Figure 4.1. Assigning genotypes using RFLP analysis is open to errors due to incomplete enzymic digestion of the DNA in sub-optimal conditions. Positive control samples for each of the 3 possible genotypes were used in each plate of experiments and any plates in which the positive control failed to digest the DNA was repeated in its entirety. Any apparently homogygous sample that appeared to have not been cut by the enzyme (ie the AA sample in figure 4.1) was then repeated with a higher concentration of enzyme to ensure that the failure to cut the DNAs was due to the lack of a restriction site and not failure of enzymic action. Differences in the sizes of the DNA bands was often easy to see with the naked eye (see Figure 4.1) however where there was doubt about apparently heterozygous samples the digestion assay was again repeated.

Figure 4.1 Example of RFLP analysis



	G	A
Phi X	337	No cut
	209	site

The wild type (WT) sequence (GG) produces an enzyme cleavage site resulting in 2 RFLP fragments. The SNP (A) disrupts this site resulting in no cleavage ie a single band. These differentially sized RFLPs can often be easily visualised on agarose gels.

4.3.2.1.1.1 Development of a subset of SNPs

Primer sequences and PCR conditions for each of the primer pairs designed for DNA sequencing of the ACE gene were available on the internet (http://droog.mbt.washington.edu). To choose the best subset of SNPs from which to generate haplotypes, restriction enzyme cleavage sites were identified in the wild type (WT) and polymorphic sequences by the computer program *GENEJOCKEY*. The specific ACE DNA sequences (amplicons) generated by each of the ACE gene primer pairs were entered into *GENEJOCKEY* and RFLPs were identified (Table 4.3). Sites which produced small size differences were excluded as these would be difficult to visualise on ethidium bromide-stained agarose gels.

Using probability based assessments it was calculated that genotype data from 8-10 SNPs could accurately differentiate the 13 ACE haplotypes. Eleven of the most discriminative SNPs (ie those with high heterozygosity rates), which produced restriction enzyme sites easily identifiable on agarose gels, as well as the ID polymorphism were chosen for genotyping. A computer program ('Hmatch', Ambler, S. University of Leicester, Personal Communication, Section 9.7, Appendix) was written to assign haplotypic genotypes (ie H1 / H6) from the genotype data. Hmatch merely excluded haplotypes identified by Rieder et al 1999 if the genotype data that was entered was not compatible. Once all but 2 haplotypes had been excluded a patient was labelled with the genotypic haplotype of the 2 haplotypes that remained.

Polymerase chain reaction (PCR) methodology (Section 9.6.2) was used to amplify ACE amplicons and restriction digests were performed according to the protocols in Table 8.10-11, 9.13 (Appendix). The RFLP products were visualised on agarose gels (Table 4.4) and sequencing reactions were performed on random samples for each of the SNPs to establish the accuracy of the RFLP method (Table 4.4).

4.3.2.1.2 Genotyping Errors

All PCR products were run on agarose gels prior to restriction enzyme digestion to ensure that the PCR amplicons had been produced successfully. All PRC reactions which failed to produce sufficient product were repeated until adequate quantities of product was obtained. Genomic DNA was discarded if no product could be obtained. All digestion plates included a gold standard positive and negative control sample. Plates were discarded if these controls failed to digest adequately. Following enzyme digestion on plates where control sample had been adequately digested samples that failed to digest or could not be adequately assigned a genotype because of sub-optimal visualisation of the DNA bands were then repeated. Three

repeat attempts were allowed for each RFLP digestion experiment. Following three failures no further attempts were made.

4.3.2.1.3 Genotyping the ACE I/D Polymorphism

The *ACE* I/D Alu repeat polymorphism is 287 bp long. In the amplicon generated by primers *ACE* IDF & ACE IDR the insertion and deletion alleles are easily separated on 2% agarose gels stained with ethidium bromide (Table 4.4). In 10.5% of cases however, heterozygous (ID) individuals may appear as DD homozygotes due to preferential amplification of the smaller (190 bp) D allele. This results in the absence of the 490 bp I allele (Ueda et al (1996). Ueda et al (1996) designed a PCR reaction using 3 primers (ACE 1, 2 & 3) to counteract this phenomenon. The extra primer (ACE 2) is complementary to sequence within the insertion and so an additional band is produced in heterozygous individuals (Table 4.4). This extra PCR step for all apparent DD genotypes was incorporated into the study design.

Table 4.3 SNPs Producing Restriction Enzyme Cleavage Sites.

SNP Number (Rieder *et al*. 1999)	**NCBI SNP Database ID**	**Base Number AF118569**	**Restriction Enzyme**	**Visualised On Agarose Gel (Y/N)**
2	rs4290	00334	*Taq* I	Y
13	rs4301	5878	*Alu* I	N
17	rs4305	6435	*Hha* I	Y
20	rs4424958	7831	*Pst* I	Y
21 *	rs4309	8128	*Bsm*BI	Y
23	rs4311	8968	*Alu* I	Y
24	rs4312	9191	D*pN* II	Y
28 * +	rs4316	10514	*Bfa* I	Y
29 *	rs4317	10527	*Ban* I	Y
30 *	rs4318	10578	*Hae* III	Y
32 +	rs4320	10758	*Eco* NI	Y
35 +	rs4323	11159	*Nla* III	Y
37	rs4325	11405	*Mbo* II	N
39 +	rs4327	11478	*Bst* NI, *Scr*FI	N
40	rs4328	11618	*Eco* NI	Y
41 +	rs4329	11663	*Tth* III	N
43 * +	rs4331	12257	*Hae* II	Y
44 +	rs4332	12486	*Mwo* I	Y
45	rs4333	12727	*Ban* II	Y
47	rs4335	13230	*Hae* III	Y
48 +	rs4336	13336	*Nla* III	N
49 +	rs4337	13338	*Mbo* II	Y
53 +	rs4341	14480	*Bsm* I	Y
55 *	rs4343	14521	*Fok* I	N
56 +	rs4344	15214	*Fok* I	Y
57	rs4345	15435	*Ava* II	N
61	rs4349	17634	*Dde* I	Y
62	rs4350	18030	*Nla* III	Y
69	rs4357	20120	*Ban* I, *Dde* I	Y
75	rs4363	22982	*Mwo* I	Y
76 *	rs4364	23152	*Pvu* II	Y

* Coding SNPs

SNPs chosen for genotyping

+ = absolute LD with the I/D polymorphism (ie D’ = 1)

Table 4.4 Restriction Enzyme Cleavage Sites, Agarose Gel Appearances and Sequencing Histograms For The Chosen SNPs

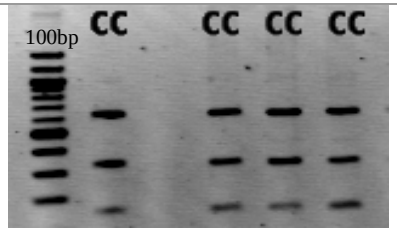
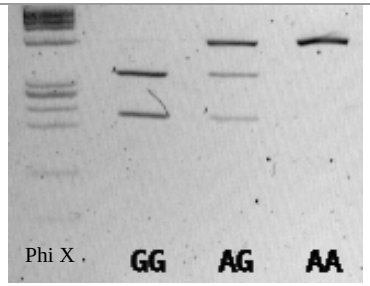
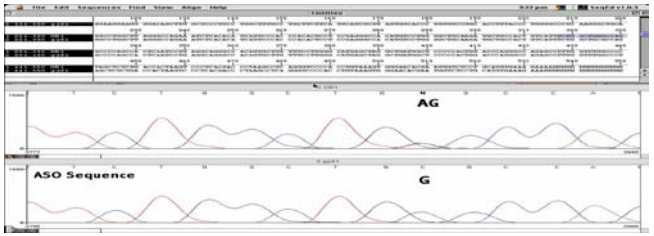
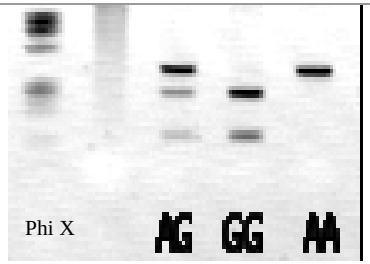
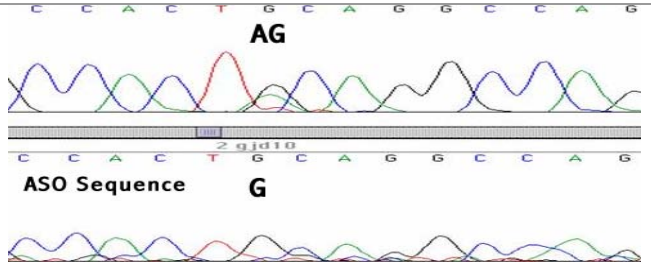
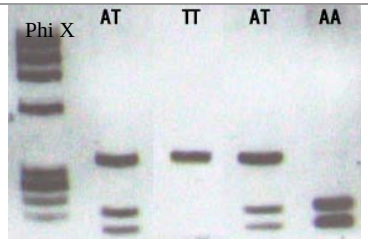
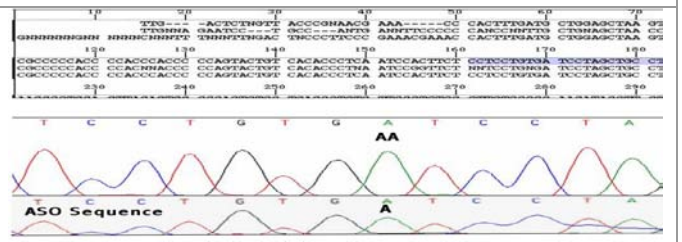
SNP	ACE Gene Location (BP) (NCBI Sequence AF118569)	Enzyme	Wild Type RFLP Sizes (BP)	Polymorphic RFLP Sizes (BP)	Gel Appearance	Sequencing Histogram
2 (rs4290)	0334	*Taq* I	C 617 333	T No Site		
17 (rs4305)	6435	*Hha* I	A No Site	G 337 209		Complementary sequence 
20 (rs4424958)	7831	*Pst* I	G 278 127	A No Site		
24 (rs4312)	9191	DpN II	A 193 149 44 17	T 324 44 17		

Table 4.4 Restriction Enzyme Cleavage Sites, Agarose Gel Appearances and Sequencing Histograms For The Chosen SNPs (Cont)

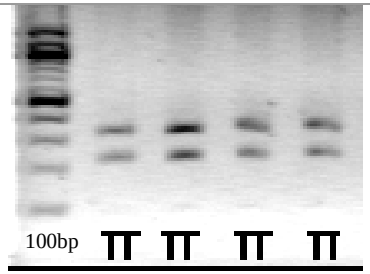
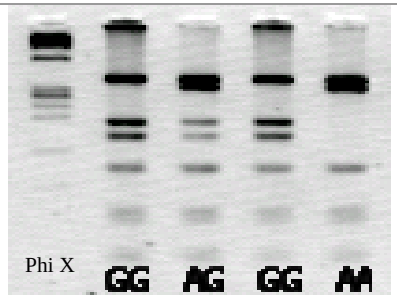
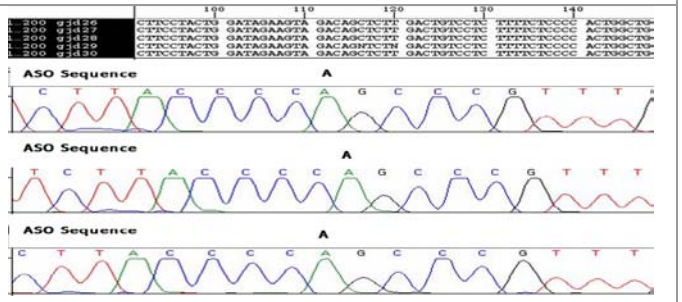
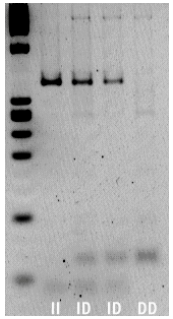
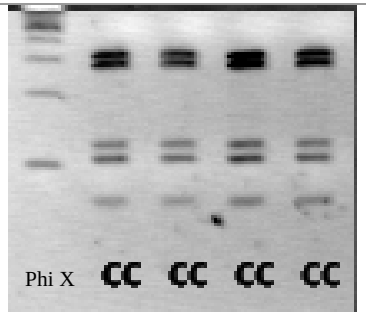
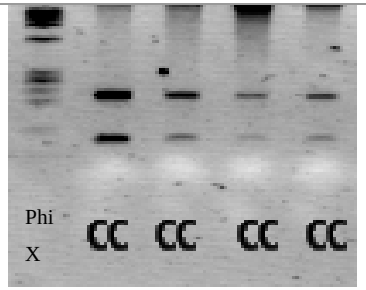
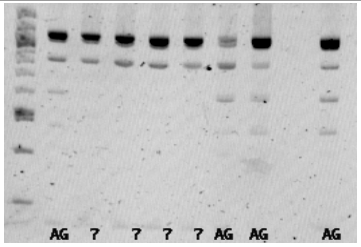
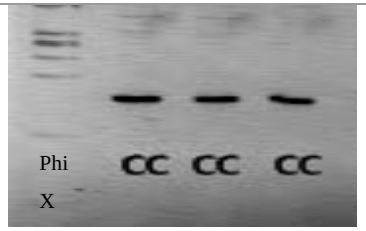
29 * (rs4317)	10527	*Fok* I	**T** 331 233 227 79	**C** 564 228		
47 (rs4335)	13230	*Hae* III	**A** 379 327 92 50 44	**G** 379 180 147 92 50 44		
52 (Alu I/D) (rs1799752)	14094	No enzyme required	**I**	**D**	Standard 2 Primer I/D PCR Phi X  ID Triple Primer PCR Phi X 	

Table 4.4 Restriction Enzyme Cleavage Sites, Agarose Gel Appearances and Sequencing Histograms For The Chosen SNPs (Cont)						
61 (rs4349)	17634	*Dde* I	**C** 308 270 116 102 101 69	**T** 339 308 116 102 101		
69 (rs4357)	17634	*Ban* I	**C** 206 86	**T** No sites		
75 (rs4363)	22982	*Fau* I	**A** 559 212 133	**G** 559 212 81 52		
76 * (rs4364)	23152	*Pvu* II	**C** No sites	**A** 144 24		

* Coding SNPs

4.3.3 Statistical Methods

To assess differences between two variables, where possible, each variable was converted into a binary format (present or not present) and the null hypothesis was assumed (i.e., the observed frequencies did not differ from the frequencies that we would expect by chance alone). Differences between groups were analysed in contingency tables with Pearsons chi-squared tests. A probability (p value) of 0.05 or less was used as justification for rejecting the null hypothesis. Comparisons with groups of less than 50 was analysed with the Fisher's exact test due to inaccuracies in the Pearson's chi-square statistic below this figure. For continual variables the Student's t-test was used to test the difference of the means.

In complex situations where multiple interacting aetiological variables are involved more sophisticated statistical tests are required. For example, we wish to assess the role of variables x and y on outcome z. It is suspected that x has an effect on z and it is known that y independently leads to z. It is also suspected that x interacts with y. It is therefore important that we 'control' for the effects of y in order to assess the independent effects of x.

A regression analysis is commonly used to test for the association between the dependent (genotype / haplotype) and multiple independent variables. Logistic regression is a variation of ordinary regression, useful when the observed outcome is restricted to two values, which usually represent the occurrence or non-occurrence of some outcome event, (usually coded as 1 or 0, respectively). It produces a formula that predicts the probability of the occurrence as a function of the independent variables and generates Odds Ratios (O.R.) associated with each predictor value. The odds of an event is defined as the probability of the outcome event occurring divided by the probability of the event not occurring. The odds ratio for a predictor tells the relative amount by which the odds of the outcome increase (O.R. greater than 1.0) or decrease (O.R. less than 1.0) when the value of the predictor value is increased by 1.0 units.

Continuous and non continuous clinical data was available for each patient (ie Blood pressure = continuous (190/90) and smoking status = non – continuous (present / not present). In order to analyse all this data simultaneously all data was converted to a non continuous (binary) format and tested for association against the dependent variable in a logistic regression analysis using the statistical package *STATA.*

4.3.3.1 Statistical Power

The power of a study will determine the sample size that is required to identify any genetic locus which has a functional role in disease aetiology. The greater the power the smaller the sample size required. The determination of the power of a genetic study which exploits LD is based upon many assumptions including the strength of LD among trait influencing and marker loci (D'), the frequencies of the marker locus and trait-influencing alleles, and the density of the markers which are genotyped. Many genetic studies are unfortunately underpowered {Ambrosius, 2004 #986} despite researchers undertaking power and sample-size calculations prior to data collection. As much of the information required to enter into such calculations is assumed, the resulting calculations are often unrealistic. When researchers study common genes or well characterised areas of the genome, frequency and LD data may have been established a priori, such as the frequencies of alleles at the *ACE* ID locus (I = 0.45, D = 0.55 in caucasian populations). However recently characterised alleles such as the *ACE* gene haplotypes require a more assumptive approach as they have only been identified in 11 individuals to date (Rieder *et al*. 1999) .

Calculating power requires a few initial decision to be made. Firstly it is important to establish the required level of power. Most studies set this to be at least 80% which gives an 80% chance of identifying a real difference if one exists. Secondly the desired significance level needs to be set. This level is conventionally 0.05 (ie a 95% probability that a result has not been identified by chance alone) however more stringent levels of 0.01 would be ideal to reduce chance findings, especially in studies where multiple testing has occurred (see Section 2.3.3.2.2.4). Thirdly the size of the effect that is being sought should also be calculated ie by how much does the given genetic risk factor increase the odds of disease development. The larger the size of the effect, the smaller the sample size required.

Table 4.5 shows a variety of power calculations (80-95% power and significance levels of 0.05 – 0.001) for the proposed study. Two high risk allele frequencies have been modelled, the first 0.55 is equivalent to the ACE D allele frequency in a European population and the second a much lower frequency of 0.10 to cover the possible scenario of a low frequency haplotype being associated with ischaemic stroke. Higher frequency disease causing alleles were not modelled as these are unlikely to have escaped identification due to the great interest in this area if the genome. The prevalence of ischaemic stroke of 0.0018 was calculated from UK data of age standardised prevalence rates between 1994 and 1998 from the Office of National Statistics (Office of National Statistics (ONS) 1998). As the RR of each of the ACE haplotypes was unknown genotypic RR figures between 1.0 and 2.5 were used. These

incorporated figures identified for the ACE D allele acting in a dominant ((RR for ID = 1.14 {Sharma, 1998 #803} and recessive manner (RR for DD = 1.21 {Casas, 2004 #975} & 1.31 {Sharma, 1998 #803}. A higher RR of a disease causing allele of 2.5 was also modelled as it is possible that other disease causing alleles are present at the *ACE* locus however it was considered unlikely that such an allele would have an effect on common ischaemic stroke disease of greater than this due to multifactorial nature of stroke aetiology. Two levels of LD were used in the model (0.8 & 1.0) to reflect the high degrees of LD seen in this region.

The results of the various power calculations show that at the least stringent level assuming the identification of a high frequency (0.55) stroke associated allele with a genotypic relative risk of 2.5, absolute LD between risk allele and marker allele and 80% power at the 0.05 significance level 98 cases and controls would be required to identify a difference between the two groups. However the application of more stringent assumptions of having a 95% power to find a low frequency stroke causing allele (0.10) even with a high genotypic RR of 2.5 but with incomplete LD between marker and disease alleles (D'= 0.8) would require 11440 cases and controls. This would clearly be unmanageable in the timeframe of this project.

Given less stringent criteria however equal number of cases and controls between 285 and 1364 would give an 80% power at the p = 0.05 level of identifying a common disease causing allele (frequency 0.55) with a genotypic RR of stroke between 1.31 and 2.5 with a D' of between 0.80 and 1.0 between it and the marker allele. At the time of conception of this study no data was available to suggest that these favourable conditions were unlikely to be achieved and so it was considered that this number of individuals could feasibly be ascertained. At the outset of the data collection phase of the study the goal was to identify at least 300 cases and controls and if possible as close a number of subjects to 1364 as possible.

Table 4.5 Genetic Power calculations (using the Genetic Power Calculator at http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html)

Power Calculations To Identify Necessary Sample Sizes With Variable High Risk Allele Frequency (0.10 & 0.55), Genotype Relative Risk (1.0 – 2.5), D' (0.8-1.0) and Degrees of Power (80-95%)										
High Risk Allele Frequency (D)	**Prevalence of Stroke**	**Genotypic Relative Risk ID**	**Genotypic Relative Risk DD**	**Marker Allele Frequency**	**LD (D')**	**Alpha**	**Power**	**N cases for 80% Power**	**N cases for 90% Power**	**N cases for 95% Power**
Modelling for a D' of 1.0										
0.55	0.0018	1.0	1.21	0.55	1	0.05	0.26	2820	3775	4668
0.55	0.0018	1.0	1.21	0.55	1	0.01	0.058	4196	5345	6400
0.55	0.0018	1.0	1.21	0.55	1	0.001	0.011	6134	7510	8751
0.55	0.0018	1.14	1.21	0.55	1	0.05	0.13	4148	5553	6867
0.55	0.0018	1.14	1.21	0.55	1	0.01	0.04	6172	7863	9414
0.55	0.0018	1.14	1.21	0.55	1	0.001	0.007	9023	11050	12870
0.55	0.0018	1.0	1.31	0.55	1	0.05	0.30	1364	1826	2258
0.55	0.0018	1.0	1.31	0.55	1	0.01	0.13	2030	2586	3096
0.55	0.0018	1.0	1.31	0.55	1	0.001	0.03	2967	3633	4233
0.55	0.0018	1.0	2.0	0.55	1	0.05	0.98	181	243	300
0.55	0.0018	1.0	2.0	0.55	1	0.01	0.91	269	343	411
0.55	0.0018	1.0	2.0	0.55	1	0.001	0.74	394	482	562
0.55	0.0018	1.0	2.5	0.55	1	0.05	0.99	98	131	163
0.55	0.0018	1.0	2.5	0.55	1	0.01	0.99	146	186	223
0.55	0.0018	1.0	2.5	0.55	1	0.001	0.98	214	262	305
0.10	0.0018	1.0	2.5	0.10	1	0.05	0.12	4475	5991	7409
0.10	0.0018	1.0	2.5	0.10	1	0.01	0.04	6659	8483	10160
0.10	0.0018	1.0	2.5	0.10	1	0.001	0.006	9735	11920	13890

Modelling for LD of 0.8

0.55	0.0018	1.0	1.21	0.55	0.8	0.05	0.13	4412	5907	7305
0.55	0.0018	1.0	1.21	0.55	0.8	0.01	0.04	6565	8364	10000
0.55	0.0018	1.0	1.21	0.55	0.8	0.001	0.006	9598	11750	13690
0.55	0.0018	1.14	1.21	0.55	0.8	0.05	0.10	6488	8686	10740
0.55	0.0018	1.14	1.21	0.55	0.8	0.01	0.03	9654	12300	14730
0.55	0.0018	1.14	1.21	0.55	0.8	0.001	0.004	14110	17280	20140
0.55	0.0018	1.0	1.31	0.55	0.8	0.05	0.21	2136	2860	3536
0.55	0.0018	1.0	1.31	0.55	0.8	0.01	0.08	3178	40490	4848
0.55	0.0018	1.0	1.31	0.55	0.8	0.001	0.02	4647	5689	6629
0.55	0.0018	1.0	2.0	0.55	0.8	0.05	0.88	285	382	473
0.55	0.0018	1.0	2.0	0.55	0.8	0.01	0.71	425	541	648
0.55	0.0018	1.0	2.0	0.55	0.8	0.001	0.44	621	761	887
0.55	0.0018	1.0	2.5	0.55	0.8	0.05	0.99	156	209	258
0.55	0.0018	1.0	2.5	0.55	0.8	0.01	0.95	232	296	354
0.55	0.0018	1.0	2.5	0.55	0.8	0.001	0.83	339	416	484
0.10	0.0018	1.0	2.5	0.10	0.8	0.05	0.09	6911	9252	11440
0.10	0.0018	1.0	2.5	0.10	0.8	0.01	0.02	10280	13100	15690
0.10	0.0018	1.0	2.5	0.10	0.8	0.001	0.004	15040	18410	21450

4.3.3.2 Hardy Weinberg Equilibrium Calculations

Genetic equilibrium is a basic principle of population genetics. This equilibrium assumes that the relative frequency of genotypes and the frequency of alleles stay constant unless matings are non-random, or mutations accumulate. The Hardy–Weinberg principle of population genetics is a relationship between the frequencies of alleles and the genotype of a population and can be likened to a punnett square for populations, instead of individuals, thereby testing the uniformity and suitability of the population under study. If the Hardy Weinberg equilibrium is established in a study population then it is a valuable guide to the quality of genotyping methodology.

The Hardy Weinberg principle is based upon a number of assumptions, the organism under study is diploid, sexually reproducing and the trait under consideration is not likely to be sex linked (ie autosomaly inherited). The population needs to have discrete generations, undergo Random mating within a single population and be sufficiently large so as to minimize the effect of genetic drift. Finally the population should participate in random mating with no selection and there should be no mutation and no migration (gene flow). These principles ensure that the alleles for the next generation for any individual undergo random selection and are independent of each other.

4.3.3.2.1 The Derivation of the Hardy Weinberg Principle

The HW principle is derived as follows using the example of two alleles, A and a, with frequencies *p* and *q*, respectively, in the population. The potential genotypes can be derived using the following punnett square, where the fraction in each cell is equal to the product of the row and column probabilities.

Punnett square for Hardy–Weinberg equilibrium

		Females	
		A (*p*)	**a (*q*)**
Males	**A (*p*)**	AA (p^2)	Aa (pq)
	a (*q*)	Aa (pq)	aa (q^2)

The possible genotypic frequencies (Hardy Weinberg frequencies) in the offspring are

$f(AA) = p^2n$

$f(Aa) = 2pqn$

$f(aa) = q^2n$

Where n = population size

A chi square value is then obtained by comparing observed and expected genotype frequencies using the Pearson's chi-square test.

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

Using one degree of freedom the 5% significance level (p) is 3.84, and so a χ^2 value of less than this will establish the genotype frequencies of the study population to be in Hardy–Weinberg equilibrium.

4.3.4 Verification of Haplotypes by Allele Specific PCR and DNA Sequencing

The classification of the 13 *ACE* gene haplotypes by Rieder *et al* 1999, was derived from sequencing bi-allelic PCR amplicons generated from only 11 Caucasian and African American individuals from the USA. The haplotypes were then generated by calculating the frequency with which SNPs were associating with each other in the 22 chromosomes sequenced. Direct confirmation of these haplotypes by single stranded DNA sequencing has not previously been performed and so the validity of the haplotypes is not completely clear. We studied a different population to Rieder *et al* 1999. Thus, it was necessary to validate the haplotypes in our population. In order to do this directly, we planned to isolate the single stranded haplotype structure of the ACE gene by performing allele specific PCR experiments followed by DNA sequencing. At each polymorphic locus an allele specific oligonucleotide primer (ASO) will anneal to one allele but not the other. This directly isolates haplotypes within allele specific amplicons.

4.3.4.1 The Choice of Patients for Verification of *ACE* Gene Haplotypes

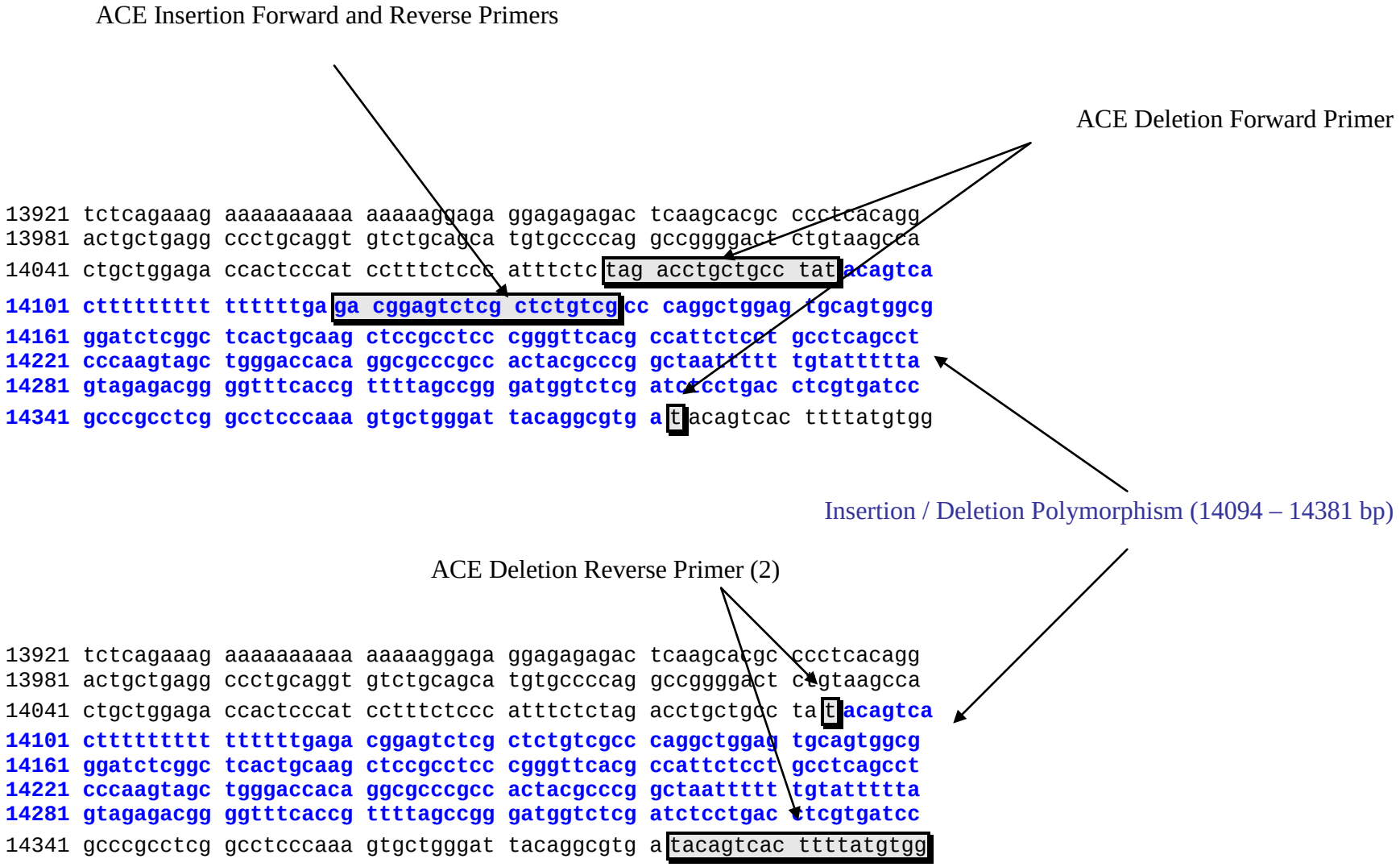
Initial analysis of the haplotype data obtained from RFLP genotyping (see results section) suggested that the most frequent haplotypes were H1, H6 and H7 and so individuals were randomly selected from these groups to preferentially validate these haplotypes. Two patients heterozygous for the chosen SNP on which the (ASO) would anneal were chosen from each of the H1/H6, H6/H7 and H1/H13 genotypic subgroups. It was envisaged that long range ASO PCR would be difficult to perfect and so as well as using ASOs for each of the 2 alleles, non-ASO PCRs were performed so that ASO sequence could theoretically be inferred by subtracting one ASO sequence from genomic sequence for the same region

4.3.4.2 ASO PCR complimentary to the I/D polymorphism

The ACE gene is 24 kb in size with SNPs distributed throughout the gene. The aim was to obtain large allele specific PCR amplicons incorporating as many polymorphisms as possible especially the ID polymorphism as it is a major determinant of the cladistic haplotype tree. The central position of the ID polymorphism (14094 bp from the 5’ end of the gene) makes it ideal to use as the 'anchor' for complimentary forward and reverse ASOs to produce large allele specific PCR amplicons in both 5’ and 3' directions. Sequencing of these amplicons would provide direct evidence for the haplotype structure of each of the two alleles present. ASOs were designed to the insertion and deletion alleles respectively (Figure 4.2).

DNA from patients heterozygous for the I/D polymorphism was then used in PCR experiments with each of the forward and reverse primer pairs in an attempt to amplify DNA from the insertion (primers ACE Insertion Forward with ACE 3 and ACE 241 ; ACE Insertion Reverse with ACE 182) and deletion (ACE Deletion Forward with ACE 3, ACE 241 and ACE 271; ACE Deletion Reverse with ACE 210, ACE 182 and ACE 170) alleles respectively (Table 9.10, Appendix).

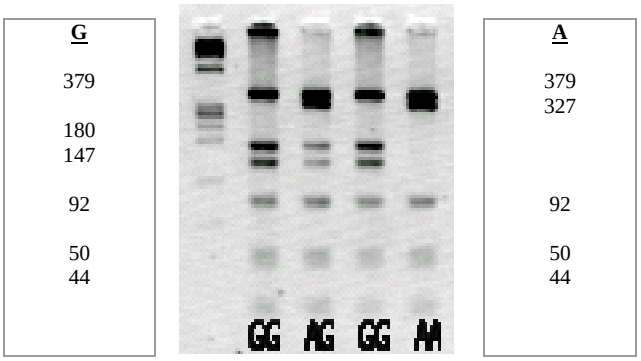
Figure 4.2 ASO Primer Location in the ACE I/D Polymorphism Region



4.3.4.3 ASO PCR using Primers Complimentary to SNP 47 (rs4335)

To provide verification of the ASO PCR experiments with the ID polymorphism similar ASO PCR experiments were designed using random heterozygous subjects for the nearby polymorphism SNP 47 (rs4335 Figure 4.3).

Figure 4.3 RFLP analysis of Snp 47 (rs4335)

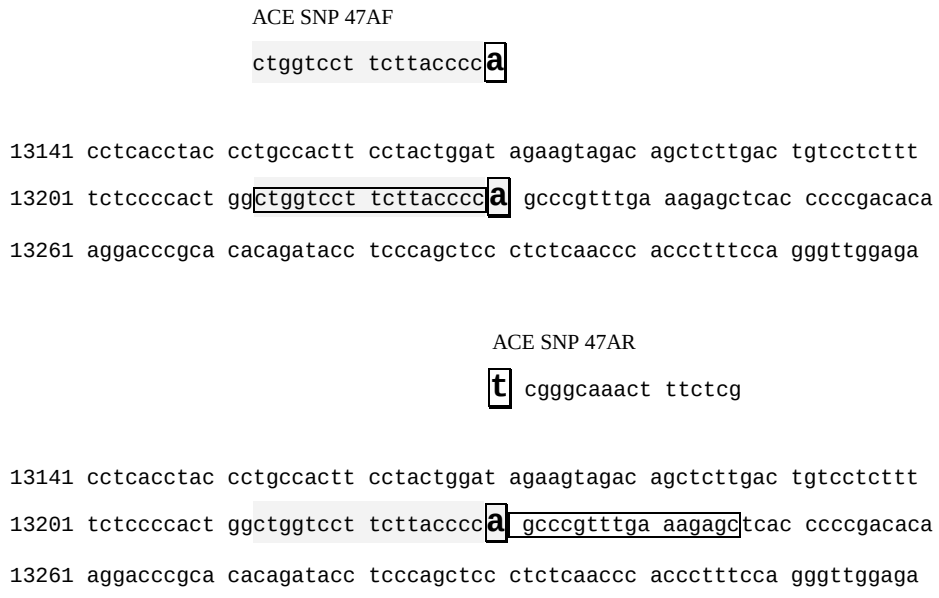


ASOs were designed for the A and G alleles respectively (ACE SNP 47 A Forward and Reverse; ACE SNP 47 G Forward and Reverse). In the 5' direction a high concentration of SNPs were found in the region between 5 and 15 kb of the gene and so further primers were designed to amplify this region (Figure 4.4).

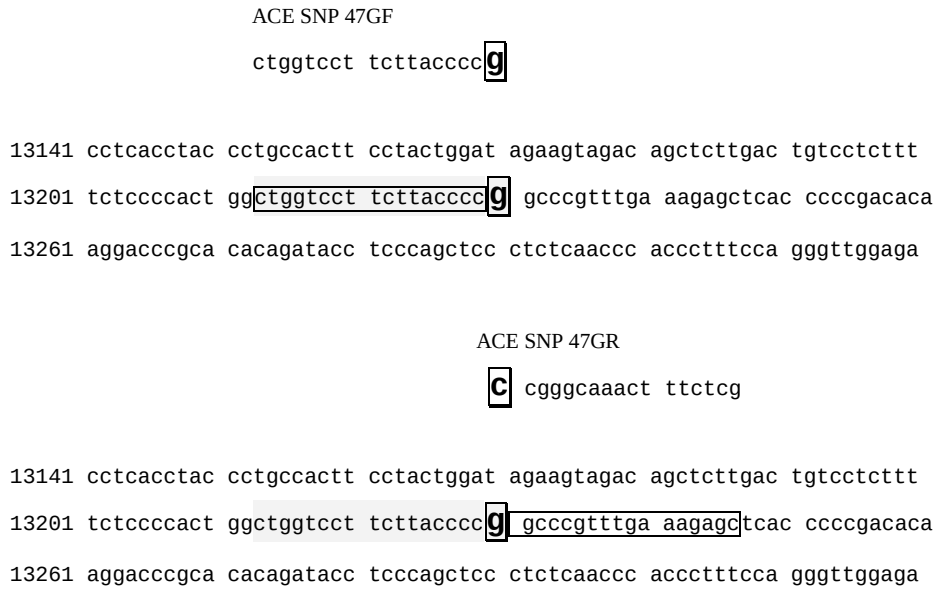
In the 3' direction there were fewer SNPs in the range of single allele PCR experiments anchored to SNP 47 and so 3' experiments concentrated upon identifying the I/D polymorphism.

Figure 4.4 Design and location of ASOs to SNP 47 (rs4335) (13230 bp) (A/G)

a) A allele primers



b) G allele primers



CHAPTER 5

RESULTS OF EXPERIMENTS TO VERIFY *ACE* HAPLOTYPES IN A UK CAUCASIAN POPULATION

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5.1 ASO PCR Experiments

This chapter will show the results of long range ASO PCR experiments. The aim of these was to amplify large allele specific amplicons to enable sequencing and direct visualisation of as much of the gene as possible in an attempt to validate the published *ACE* haplotypes.

- **Primers Specific to the I/D Polymorphism Insertion and Deletion Alleles**

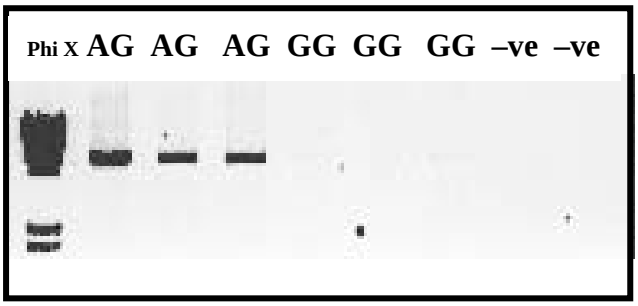
All attempts at amplifying the respective PCR amplicons in these experiments were unsuccessful despite repeated attempts in differing PCR buffers (ABT 10X, 11.1X (Table 8.12, Appendix)), annealing (45-65°C) and extension temperatures (72-64°C) and extension times (30s–7 min).

- **Primers Specific to SNP 47 (rs4335)**

5.1.1.1 A Allele PCR in the 5' region

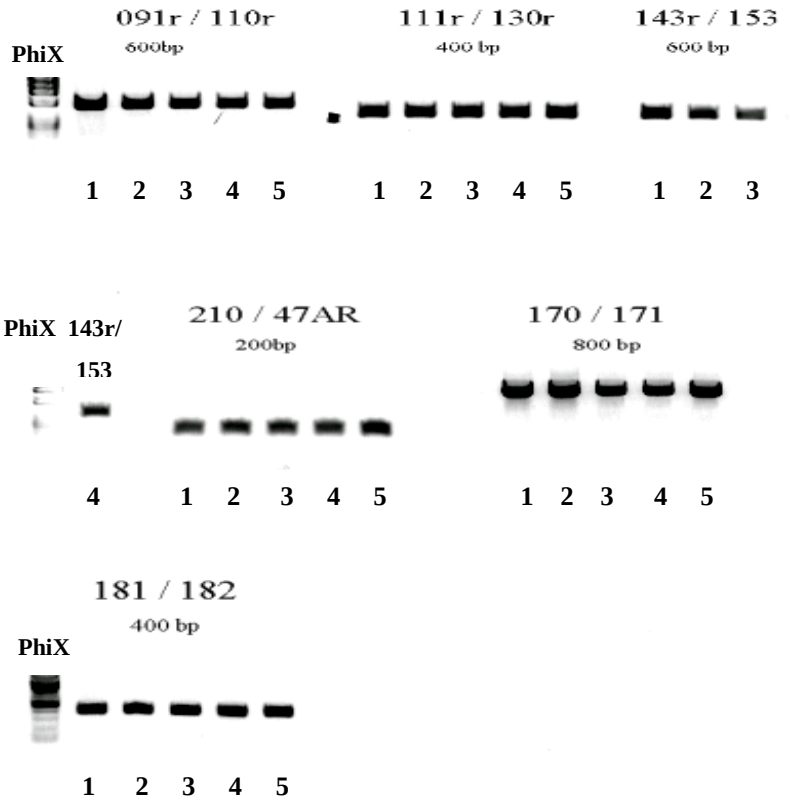
Using heterozygote DNA at the SNP 47 locus a 7.0 kb amplicon was successfully obtained from A allele specific primers, 091R/47AR, and no product was obtained from the GG homozygote DNA (Figure 5.1) (PCR conditions are detailed in the Table 9.14 (Appendix). Initial DNA concentrations were inadequate to use in sequencing reactions and so the DNA was extracted from the agarose gel, purified (using a QIAquick Gel Extraction Kit, Qiagen) and used as a template in multiple PCR experiments using primers designed for DNA sequences within the 091R/47AR amplicon. The ASO amplicons generated from this second stage of PCRs (Figure 5.2) were designed to house all of the SNPs chosen to differentiate among the 13 haplotypes (17, 20, 24, 29, 40 and 47) and as many as possible of the other SNPs identified by Rieder et al 1999 (Figure 5.5). ASO amplicons were successfully obtained from these PCR experiments and were then used as templates in sequencing reactions (Example are shown in Figure 5.3).

Figure 5.1 5' ASO PCR using primers 091R/47AR (7003 bp allele A specific amplicon for SNP 47 (rs4335)



Comparison of 3 randomly selected heterozygous and homozygous GG DNA samples showing the ACE47AR ASO primer producing a PCR product from the A allele but not the G allele.

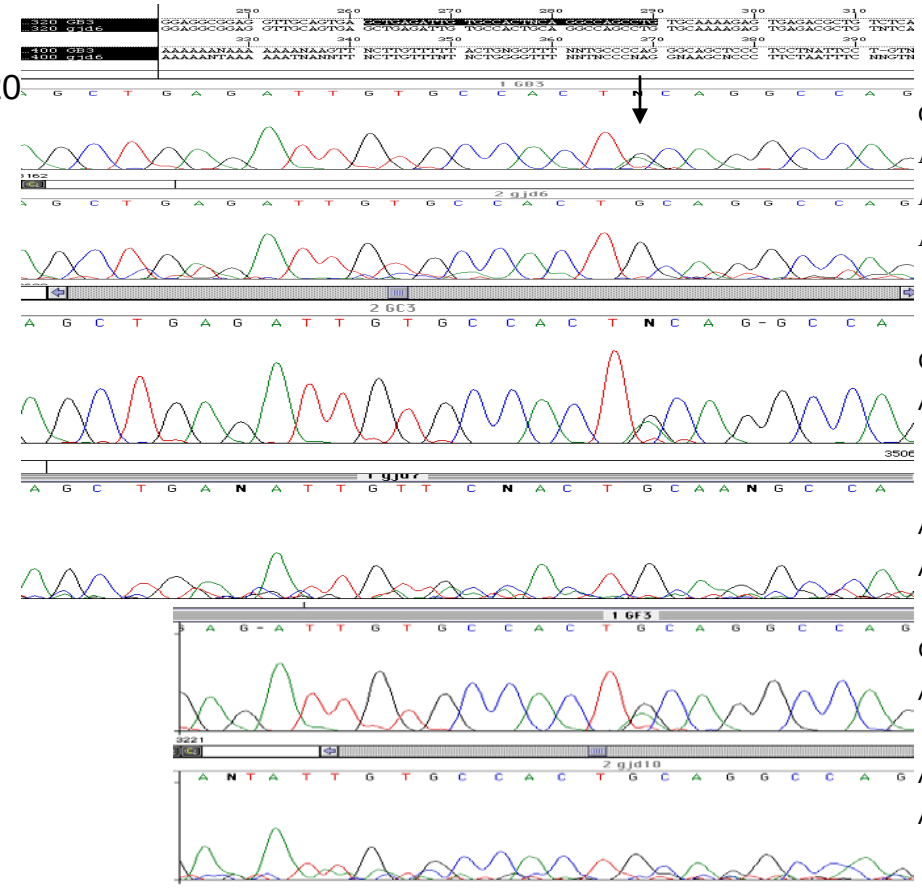
Figure 5.2 PCR products used in sequencing reactions.



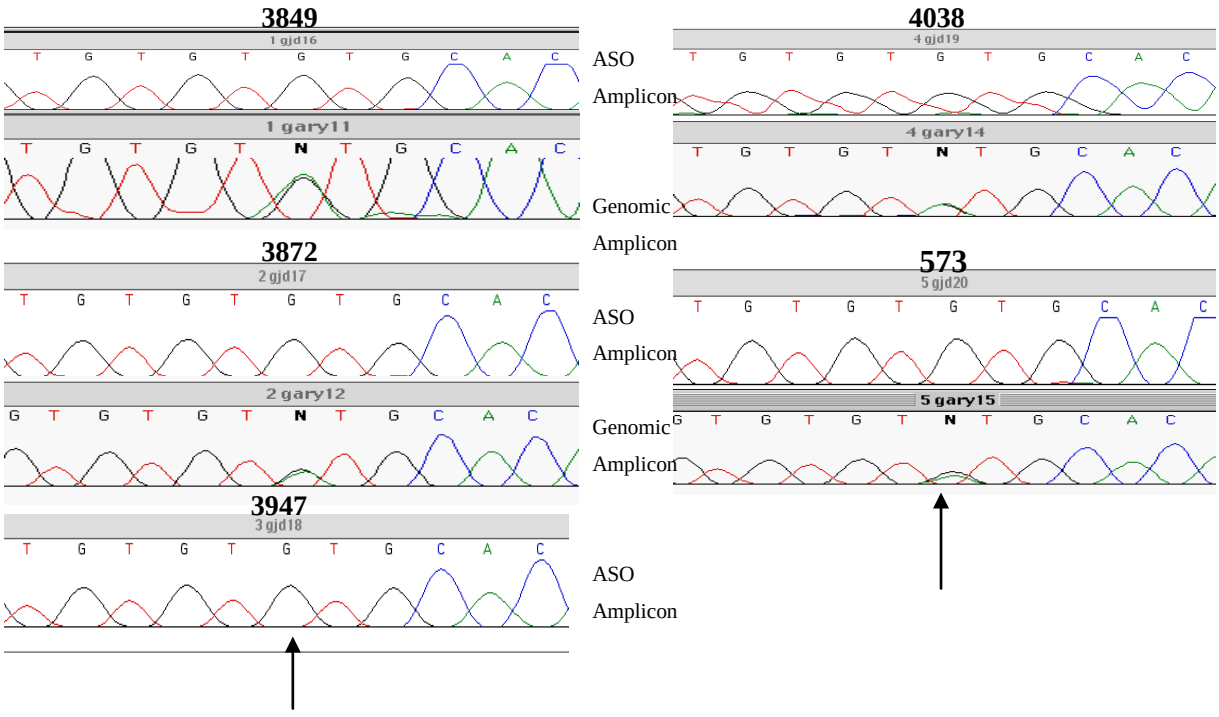
Products shown were obtained by PCR with ACE gene primers using a SNP 47 A allele specific DNA template generated from primers 091R / 47AR .using DNA from genotypes H1/6 (lanes 1-2), H6/7 (lanes 3-4) and H1/13 (lane 5) .

Figure 5.3 Selected Sequencing Histograms for 5 patients (H1/6; 3849, 3872), (H6/7; 3947, 4038) and (H1/13 5739) comparing genomic DNA sequence with ASO amplicon (091R/47AR) sequence in the region of a) SNP 20, b) SNP 33

a) SNP 20



b) SNP 33



5.1.1.2 G Allele PCR in the 5' region

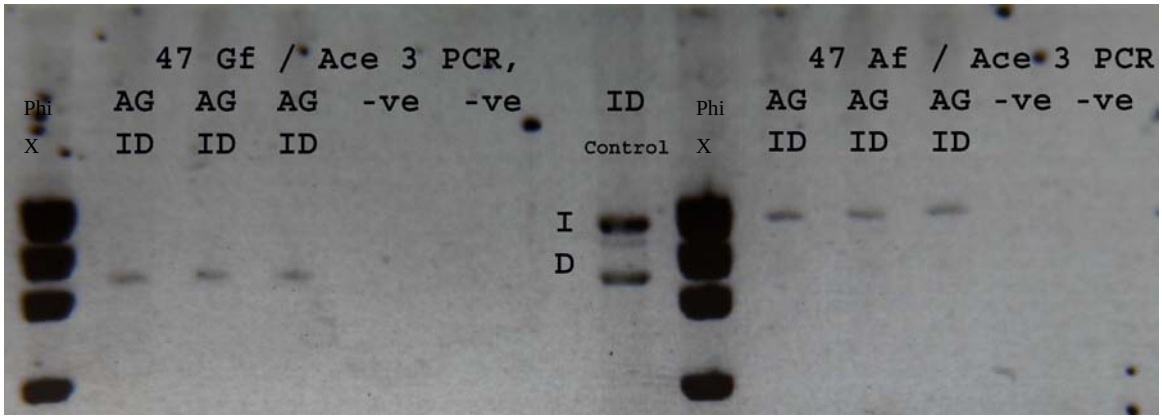
The ASO PCR experiment using primers 091R/47GR failed to generate an allele specific amplicon. However, genomic amplicons were obtained for the region in question and so G allele sequence could be inferred by subtracting the A allele sequence in the 091R/47AR amplicon from the bi-allelic sequence.

5.1.1.3 A & G Allele PCRs in the 3' region

3' ASO amplicons were obtained using primers 47AF/Ace3 and 47GF/Ace 3. These were run on agarose gels to identify the ID polymorphism. Figure 5.4 confirms the haplotype structure of SNP 47 (rs4335) and the ID polymorphism (rs1799752) to be GD and AI respectively.

Successful sequencing reactions were then performed for each of the chosen subjects using the primers outlined in the Figure 5.2. The direct haplotype data generated from these experiments was then compared with the published data of Rieder et al 1999.

Figure 5.4 ASO PCR of the ID region.



ASO PCR with 3 random heterozygous DNA samples using primers 47AF/Ace3 47GF/Ace3 in the region of the I/D polymorphism. Only the D allele amplifies with the G primer and the I allele with the A primer.

Figure 5.5

GeneSNPs Display of ACE: angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
Chromosomal Location: 17q23

***Image courtesy of GeneSNPs**

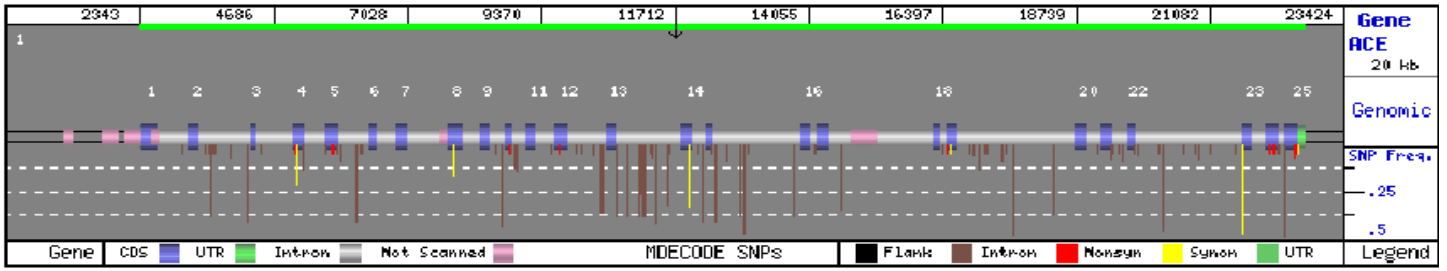
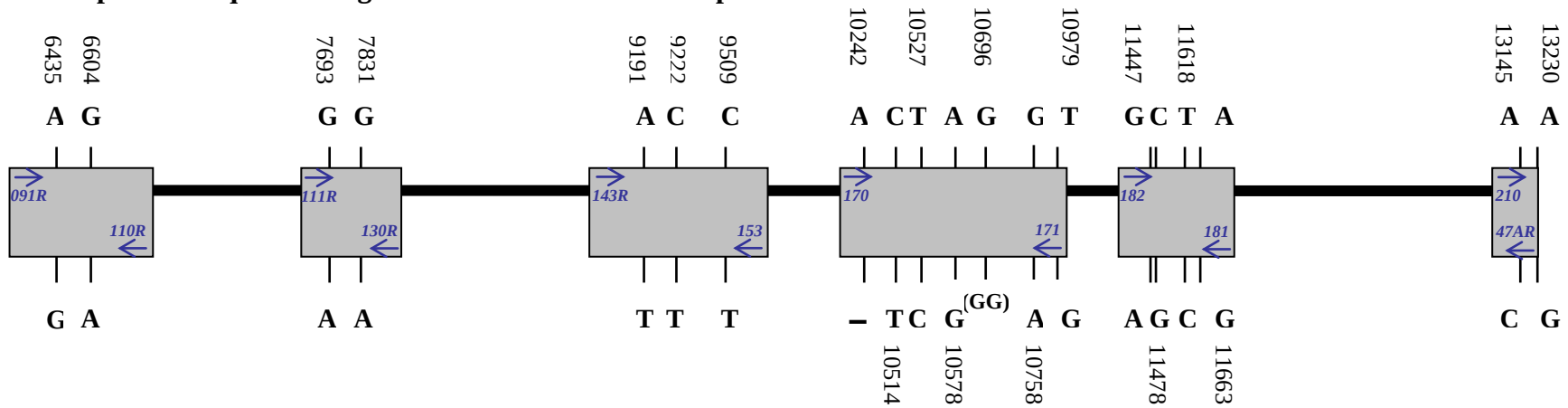


Figure 5.6 Map of the sequenced regions of the 091R / 47AR Amplicon



Exon	Begins(BP)…Ends (BP)	Exon	Begins(BP)…Ends (BP)	Exon	Begins(BP)…Ends (BP)	Exon	Begins (BP)…Ends (BP)	Exon	Begins (BP)…Ends (BP)
1	2662……...2910	6	6658……6755	11	9415……...9537	16	14499……14642	21	19773……19917
2	3498……3665	7	7133……7305	12	9896……...10107	17	14792……14983	22	20223……20321
3	4574……4667	8	8032……8255	13	10802……10938	18	16805……16902	23	22245……22367
4	5336……5479	9	8595……8739	14	12123……12281	19	17060……17232	24	22649……22836
5	5904……6095	10	9026……9124	15	12552……12639	20	19287……19510	25	22988……>23217

5.1.1.3.1 Results of Sequencing Reactions using the 091R/47AR amplicon template

Table 5.1 shows the comparison between Reider *et al's* (1999) indirect haplotype calculations and those obtained in the present study by direct sequencing of the single stranded 091R/47AR amplicon. DNA from 2 patients in each of the H1/H6 and H6/H7 haplotypes and one in the H1/H13 was successfully sequenced for the majority of the SNPs. Genomic DNA sequence was obtained for the majority of the region and genotype data was available for the I/D polymorphism.

The data obtained is consistent between patients assigned to the individual haplotypes. Additionally, the haplotype structure obtained from direct allele specific sequencing is consistent with that inferred by Rieder *et al* for each of the common haplotypes, H1, H6, H7 and H13, in Caucasian subjects.

Due to limitations with long range PCR methods data has only been obtained for a proportion of the ACE gene and has been validated on a small number of individuals, however this result provides good evidence for the validity in a UK caucasian population of the commoner of the 13 *ACE* gene haplotypes identified by Rieder et al. It is now possible to assign haplotypes and produce valid comparisons of *ACE* haplotype frequencies in a large UK case control study of ischaemic stroke using RFLP analysis and the computer program Hmatch.

Table 5.1 Haplotypes H1, H6, H7 & H13 Identified in the ASO 091R / 47AR Amplicon Compared With Haplotypes Published by Rieder et al 1999.

Sample		3849	3849			3872	3872			3947	3947		4038	4038			5739	5739	
	Rieder et al 1999	Genomic DNA	ASO DNA	Rieder et al 1999	Rieder et al 1999	Genomic DNA	ASO DNA	Rieder et al 1999	Rieder et al 1999	Genomic DNA	ASO DNA	Rieder et al 1999	ASO DNA	Genomic DNA	Rieder et al 1999	Rieder et al 1999	Genomic DNA	ASO DNA	Rieder et al 1999
HAP	**H1**	**H1/H6**	**H6**	**H6**	**H1**	**H1/H6**	**H6**	**H6**	**H7**	**H6/H7**	**H6**	**H6**	**H6**	**H6/7**	**H7**	**H1**	**H1/H13**	**H13**	**H13**
SNP																			
17	A	AG	G	G	A	AG		G	G	GG	G	G	G	GG	G	A	AA	A	A
18		GG	G			GG				GG	G		G	GG				A	
19		GG	G			GG	G			GG	G			GG			GG	G	
20	A	AG	G	G	A	AG	G	G	G	GG	G	G		GG	G	A	AG	G	G
24	A	AA	A	A	A	AA		A	A	AA	A	A		AA	A	A	AA	A	A
25	C	CC	C	C	C	CC		C	C	CC		C		CC	C	C	CC	C	C
28	C		T	T	C		T	T	C		T	T			C	C		T	T
29	T		T	T	T		T	T	T		T	T			T	T		T	T
30	A			A	A			A	A			A			A	A			A
31	(G-)	GGG	GG	(GG)	(G-)	GGG	GG	(GG)	(G-)	GGG	GG	(GG)	GG	GGG	(G-)	(G-)	GGG	GG	(GG)
32	G	AG	A	A	G	AG	A	A	G	AG	A	A	A	AG	G	G	AG	A	A
33	T	CT	C	C	T	CT	C	C	T	CT	C	C	C	CT	T	T	CT	C	C
38	G	AG	A	A	G	AG	A	A	G		A	A	A	AG	G	G		A	A
39	C	CG	G	G	C	CG	G	G	C	CG		G	G	CG	C	C		G	G
40	T	TT	T	T	T	TT	T	T	T	TT	T	T	T	TT	T	T	TT	T	T
41	A	AG	G	G	A	AG	G	G	A	AG	G	G	G	AG	A	A	AG	G	G
46	A	AC	C	C	A	AC	C	C	A	AC	C	C	C	AC	A	A		C	C
47	G	AG	A	A	G	AG	A	A	G	GA	A	A	A	AG	G	G	AG	A	A
52	D	DI	I	I	D	DI	I	I	D	DI	I	I	I	ID	D	D	DI	I	I

CHAPTER 6

RESULTS OF A CASE CONTROL ASSOCIATION STUDY OF *ACE* HAPLOTYPE FREQUENCIES IN ISCHAEMIC STROKE

Chapter 6: List of Tables

6.1 Demographic and Clinical Characteristics

359 ischaemic stroke patients and 328 unrelated controls were enrolled into the study. Despite most of the data being collected retrospectively, clinical and laboratory data was available for a high proportion of the subjects (Table 6.1). 90% of the stroke patients had cranial imaging with cerebral infarction confirmed in 80%. Carotid and cardiac imaging was performed less frequently, (36% & 26% respectively) often because they were not clinically indicated. None of the controls had cerebral, cardiac or carotid imaging. Data on hypertension was available for 94% of patients and 90% of controls. Serum glucose was recorded in 79.9% of patients and 91.4% of controls, and serum cholesterol in 71.6% patients and 84.0% controls.

Table 6.1 Clinical and Imaging Data Obtained

Data		Patients	Controls
Hypertension		94.4% (339/359)	90.2% (294/328)
Diabetes Mellitus		79.9% (287/359)	91.4% (298/328)
Hypercholesterolaemia		71.6% (257/359)	84.0% (274/328)
BMI		86.4% (310/359)	90.2% (294/328)
Cranial Imaging Studies		90.0% (322/359)	0% (0/328)
CT Only		88.0% (312/359)	
MRI Only		2.0% (7/359)	
Results	Cerebral Infarction	80.0% (287/359)	
	Normal	9.0% (32/359)	
Cardiac Imaging	Echocardiography	26.5% (95/359)	0% (0/328)
Carotid Imaging	Doppler Studies	36.2% (130/359)	0% (0/328)
Results	> 75% ipsilateral stenosis	3.6% (13/359)	

The demographic and clinical characteristics of the study sample are summarized in Table 6.2. The strongest clinical associations of stroke were DM, hypercholesterolaemia, hypertension, family history of stroke in a first degree relative, age over 65 years, smoking, valvular heart disease, cardiac arrythmias (mainly AF), IHD, CCF and PVD (Table 6.3). A separate analysis of gender (Table 6.4) revealed significantly higher proportions of males who were current or ex smokers or had BMI>25, and significantly more females who had BP>160/90 and reported a FH a first degree relative with stroke, though both sexes had an equal risk of stroke. The mean BMI was equivalent in both groups. There was a significantly higher proportion of stroke victims less than 65 years old than controls (25.6% vs

Table 6.2. Characteristics of the Study Population

Demographic Characteristics	Patients (n=359)	Controls (n=328)	P
Mean Age	71.9 (+/- 12.1)	73.4 (+/- 8.0)	ns
Under 65 yrs	25.6% (92/359)	13.7% (45/328)	<0.0001
Male	53.0% (190/359)	53.7% (176/328)	ns
Age at First Stroke	70.2 (+/- 13.1)		
Known Family History of Stroke	50.2% (128/255)	28.3% (69/244)	<0.0001
Current Smoker	18% (66/359)	10% (33/328)	0.002
Ex Smoker	45% (159/359)	45% (146/328)	ns
Smoking > 10 pack years	41% (146/359)	42% (136/328)	ns
History of Hypertension	58.1%(207/359)	23.3% (76/328)	<0.0001
Measured BP >160/90 mmHg	77% (258/336)	51.6% (165/320)	<0.0001
Mean SBP	153.8 (+/-23.7)	146.4 (+/-19.5)	<0.0001
Mean DBP	83.3 (+/- 13.1)	82.5 (+/- 12.5)	ns
History Of Diabetes	16.6% (59/359)	6.1% (20/328)	<0.0001
Measured random blood glucose > 7.0	32% (92/284)	7% (22/298)	<0.0001
History of Hypercholesterolaemia or measured total cholesterol >6.0	52% (131/254)	13% (36/274)	<0.0001
BMI >25 Kg/m^2	51% (157/308)	50.7%(150/296)	ns
BMI >30 Kg/m^2	12.5% (38/308)	12.3%(37/296)	ns
Mean BMI	25.3 (+/-5.0)	25.6 (+/-5.7)	ns
Heart Failure	7.3% (26/359)	2.8% (9/328)	0.009
Angina	14.6% (52/359)	11.0%(36/328)	ns
Valvular Heart Disease	6.5% (23/359)	0.61% (2/328)	<0.0001
Cardiac Arrythmias	23.0% (82/359)	5.2% (17/328)	<0.0001
Atrial Fibrillation	14.0 % (50/359)	5.2% (17/328)	<0.0001
Peripheral Vascular Disease	11.8% (42/359)	0% (0/328)	<0.0001
Myocardial Infarction	10.4% (37/359)	8.0% (26/328)	ns
CABG	3.9% (14/359)	4.6% (15/328)	ns
Respiratory Disease	15.4%(55/359)	12.6% (41/328)	ns
Cancer	7.3% (26/359)	5.8% (19/328)	ns

Table 6.3 Odds Ratios of Clinical Risk Factors for Ischaemic Stroke

Clincal Parameters	OR	95% CI	p
BMI (>25 vs <25)	1.0	0.73-1.40	0.95
Never Smoked	0.76	0.55-1.05	0.09
Ever Smoked	1.31	0.95-1.81	0.09
Current Smoker	1.96	1.24-3.10	0.004
Age >65 yrs	2.11	1.43-3.12	<0.0001
Family History	2.37	1.63-3.42	<0.0001
Hypertension (>160/90)	2.98	2.13-4.15	<0.0001
Hypercholesterolaemia	4.13	2.78-6.14	<0.0001
Diabetes	5.11	3.09-8.44	<0.0001

13.7% respectively) although the mean age of the groups was equivalent (71.9 (+/-12.1) vs 73.4 (+/-8.0). Severe hypertension (>160/90) was significantly more

prevalent in the stroke group (77%) but there was still a high proportion of severely hypertensive controls (51%). DM on the other hand affected 32% of the strokes and a significantly smaller proportion of controls (7%).

Table 6.4 Gender Analysis of Stroke Risk Factors

Risk Factor	Male	Female	P
Ever smoked	75.3% (263/349)	47.1% (140/297)	<0.00001
BMI > 25	54.5% (175/320)	46.5% (132/284)	0.04
BP > 160/90	17.6% (58/329)	24.9% (73/293)	0.03
FH	34.6% (93/269)	43.3% (104/240)	0.04

6.2 Sub-Classification of Stroke

35% of the strokes were classified as cardiogenic, 17% small vessel, 12% large vessel in origin. The majority were localized to the anterior circulation using OCSP classification, 44% PACS and 29% TACS. 9% fell into each of the POCS and LACS groups and the minority were TIAs (3%) (Table 6.5). Insufficient clinical and investigation data was available to assign a TOAST aetiological classification in 34% of patients

Table 6.5 Sub-Classification of Stroke

TOAST CLASSIFICATION	Numbers (n=359)	%
1 - Large artery disease	44	12
2 - Cardioembolic	124	35
3 - Small Vessel	62	17
5 – Undetermined	123	34
OCSP CLASSIFICATION		
PACS	157	44
TACS	104	29
POCS	33	9
LACS	32	9
TIA	14	3
UNKNOWN	16	4

6.3 Genotyping Experiments

6.3.1 ACE ID polymorphism

The frequency of the D allele was not significantly different between patients (55%) and controls (53%). The genotype of the I/D polymorphism showed no association with any other risk factor.

6.3.2 Selected ACE Gene SNPs

Genotype data was obtained for 9 of the 10 chosen SNPs (see Table 6.6). Enzymatic cleavage of the amplicon incorporating SNP 75 (rs4363) by Fau I was inefficient. SNPs 2 (rs4290), 29 (rs4317), 61 (rs4349), 69 (rs4357) and 76 (rs4364) were not informative in our caucasian population due to 100% homozygosity. Table 6.6 shows a comparison of the SNP allele frequencies in this sample and the European caucasian sample studied by Rieder et al 1999. Frequencies vary considerably for SNPs 17 (rs4290), 20 (rs4424958), 24 (rs4312), and 47 (rs4335) though these do not reach significance as Rieder et al only studied 6 subjects.

Table 6.6 Comparison of Allele Frequencies between the Leicester Sample and European Caucasians (Rieder et al 1999)

SNP	NCBI SNP Database ID	Bp	Alleles	Leicester Sample of Caucasian Europeans SNP Frequency n=697	European Americans SNP Frequency n=11	P
2	rs4290	00334	C/T	1.00/0.00	1.00/0.00	ns
17	rs4305	06435	A/G	0.48/0.52	0.68/0.32	ns
20	rs4424958	07831	G/A	0.39/0.61	0.77/0.23	ns
24	rs4312	09191	A/T	0.56/0.44	0.91/0.09	ns
29	rs4317	10527	T/C	1.00/0.00	1.00/0.00	ns
47	rs4335	13230	A/G	0.47/0.53	0.59/0.41	ns
52(I/D)	rs1799752	14094	(ALU)/-	0.45/0.55	0.41/0.59	ns
61	rs4349	17634	C/T	1.00/0.00	1.00/0.00	ns
69	rs4357	20120	C/T	1.00/0.00	1.00/0.00	ns
76	rs4364	23152	C/A	1.00/0.00	1.00/0.00	ns

Hardy Weinberg Calculations

Figure 6.1 shows an example of the HW calculation using the genotyped data from SNP 47 (rs4335). Table 6.7 shows the HW calculations for all of the genotyped SNPs. The HW equilibrium was upheld for all SNPs where the entire study population was tested. SNP 24 (rs4312) was not in HW equilibrium however this was an invalid analysis as the SNP was used to differentiate between H6/H9 and H7/H13 in a selected subset of subjects.

Figure 6.1 : Example : Hardy–Weinberg calculation for SNP 47 (rs4335)

Genotype	**AA**	**AG**	GG	Total (n)
Number	72	175	98	345

Allele frequencies:

$$p = \frac{2\text{x observed (AA)} + \text{observed (AG)}}{2\text{x }\{\text{observed (AA)} + \text{observed (AG)} + \text{observed (GG)}\}}$$

$$p = \frac{(2\text{x}72) + 175}{2\text{x }\{72 + 175 + 98\}}$$

$p = 319 / 690$

$p = 0.46$

$q = 1\text{-}p = 0.54$

The Hardy–Weinberg expectation is:

Expected AA = $p^2n = (0.46)^2 \text{ x } 345 = 73.0$

Expected AG = $2pqn = 2 \text{ x } (0.46 \text{ x } 0.54 \text{ x } 345) = 171.4$

Expected GG = $q^2n = (0.54)^2 \text{ x } 345 = 100.6$

Pearson's chi-square test states:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

$\chi^2 = (72 - 73.0)^2 / 73.0 + (175 - 171.4)^2 / 171.4 + (98 - 100.6)^2 / 100.6$

$\chi^2 = 0.014 + 0.076 + 0.067 = 0.157$

No significant difference exists between observed and expected genotype frequencies and so the HW equilibrium is upheld in this example.

Table 6.7 Genotype Frequencies

Population	SNP	Unknown	Genotype1	Genotype2	Genotype3	Total	p	sig vs ns
	47 rs4335		**AA**	**AG**	**GG**			
Cases		14	72	175	98	345	0.93	ns
Controls		44	62	141	81	284	0.99	ns
	52 rs1799752		**DD**	**ID**	**II**			
Cases		6	107	172	74	353	0.95	ns
Controls		1	86	173	68	327	0.54	ns
	17 rs4305		**AA**	**AG**	**GG**			
Cases		1	81	186	91	358	0.75	ns
Controls		10	62	176	78	317	0.12	ns
	20 rs4424958		**AA**	**AG**	**GG**			
Cases		4	46	177	132	355	0.53	ns
Controls		13	52	157	105	314	0.87	ns
	24 rs4312		**AA**	**AT**	**TT**			
Cases		331 (untyped)	6	22	0	28	0.003	sig
Controls		314 (untyped)	5	9	0	14	0.21	ns

6.3.3 Haplotype Identification

Haplotypes were successfully identified by 'Hmatch' for the majority of subjects using genotype data from SNPs 17 (rs4305), 20 (rs4424958), 47 (rs4335) and the I/D polymorphism (rs1799752). Discrimination between genotypes H7/H13 and H6/H9 could not be achieved in 42 subjects by genotyping these 4 polymorphisms alone. Analysis of SNP 24 (rs4312) was required in these individuals. From the available data Hmatch could not assign 8.1% of the study sample to any of the haplotypes published by Rieder et al 1999 suggesting that there are more than the 13 ACE haplotypes identified in Rieder *et al's* small sample of 11 individuals.

6.4 Comparisons of Ischaemic Stroke Patients vs Controls

6.4.1 SNP Genotype Frequencies

The genotype frequencies at each of the chosen polymorphic loci (Table 6.8) did not differ significantly between stroke patients and controls. No significant associations were found between any of the genotyped loci and any of the vascular risk factors regardless of whether an individual had been labeled as having a stroke or not. Analysis of the subclasses of ischaemic stroke (large vessel, cardiogenic, small vessel or undetermined) also failed to identify any significant phenotype / genotype correlations.

Table 6.8 Case control frequency comparison of `chosen *ACE* SNP Genotypes

Genotyped SNP	NCBI SNP Database ID	Allele	Stroke Patients	Controls	p
17	rs4305	A	48% (348/716)	47% (299/636)	ns
17		G	52% (368/716)	53% (334/636)	ns
		Failed	0.3% (1/359)	3.0% (10/328)	p=0.004
20	rs4424958	A	38% (269/710)	41% (258630)	ns
20		G	62% (441/710)	59% (372/630)	ns
		Failed	1.1% (4/359)	3.9% (13/328)	p=0.02
47	rs4335	A	46% (319/690)	47% (267568)	ns
47		G	54% (371/690)	53% (301/568)	ns
		Failed	3.9% (14/359)	13.4% (44/328)	p<0.0001
52	rs1799752	I	45% (320/706)	47% (308/654)	ns
52		D	55% (386/706)	53% (346/654)	Ns
		Failed	1.7% (6/359)	0.3% (1/328)	Ns

6.4.2 *ACE* Haplotype Frequencies

The distribution and frequencies of the haplotypes were not significantly different from Rieder *et al* 1999. The commonest haplotypes were H6 (39.3%), H1 (35.3%), and H7 (8.6%). H2, 3, 4, 5, 8, 11 and 12 were not identified in this sample.

In case control comparisons there was a significant association of H9 with ischaemic stroke (OR = 2.11, 95% CI = 1.33-3.80, p=0.004). Multivariate regression analysis showed this association to be independent of other vascular risk factors. H9 also showed an apparent gene dose effect with homozygotes having greater OR (3.93) of ischaemic stroke than homozygotes, however this result was afflicted by wide confidence intervals reflecting the low frequency of the H9 haplotype (Table 6.9).

Table 6.9 Case Control Frequencies Comparisons Of *ACE* Haplotypes

Haplotype	Patients	Controls	OR	95% CI	P
H1	35.2 % (253/718)	36.0 % (236/656)	0.99	0.80-1.24	Ns
H6	40.1% (288/718)	38.9 % (255/656)	1.07	0.90-1.32	Ns
H7	8.7 % (63/718)	8.3 % (55/656)	1.05	0.72-1.52	Ns
H9	7.2% (52/718)	3.5% (20/656)	2.05	1.25-3.35	0.004
H9/H9	1.1 % (4/359)	0.3% (1/328)	3.93	0.43-35.5	0.222
H10	0.97% (7/718)	1.2% (8/656)	0.79	0.28-2.22	Ns
H13	2.1% (15/718)	2.1% (14/656)	0.98	0.46-2.10	Ns
Unknown	5.6% (40/718)	10.1% (66/656)	2.10	1.18-3.70	<0.001

Table 6.10 Details of the Unknown Haplotypes

Haplotype	Patients	Controls	p
Total Unknown	5.6% (20/359)	10.1% (33/328)	<0.001
Unable to assign haplotypes from Reider et al's data despite adequate genotyping	17	26	0.11 (ns)
Undifferentiated haplotypes due to genotyping failures	3	7	0.21 (ns)

Due to PCR and restriction enzyme failures, despite repeated attempts, we were unable to assign haplotypes to 53 subjects (Table 6.10). A significantly higher proportion of the failures occurred in the control subjects than the patients. (10.1% vs 5.6%). This is potentially a serious confounding factor. In 3 patients and 7 controls the failures affected the differentiation of genotype H6/H9 from H7/H13. These subjects were coded as 'unknown', however in view of the small numbers involved

in the H9 analyses it is possible that these failures are the reason for the positive association of H9. In order to assess this further ,2 scenarios were explored ;

1) all failed controls were H9 heterozygotes

2) all failed subjects (cases and controls) were H9 heterozygotes.

A sensitivity analysis was performed by recoding subjects to comply with both these 2 scenarios. The results are shown in Table 6.11. Both scenarios maintained the association of H9 with ischaemic stroke but with reduced levels of significance.

Table 6.11 Results of sensitivity analyses following recoding of potential H9 subjects

Scenario	OR	Lower 95% CI	Upper 95% CI	P
1) All failed controls who were potentially H9 recoded as H9	1.65	1.01	2.70	0.04
2) All failed subjects (cases and controls) who were potentially H9 recoded as H9	1.64	1.07	2.53	0.02

6.4.3 Genotypic Haplotype Frequencies (Table 6.12)

The commonest ACE genotypes were H1/H6, H6/H6, H1/H1, and H1/H7. H6/H9 was significantly more prevalent in the stroke group although the confidence interval was wide reflecting the small numbers (OR 2.31, 95% CI 1.04-5.10, p=0.04). The sensitivity analysis described above to correct for possible confounding by failed genotypes found no significant association between H6/H9 and ischaemic stroke (Scenario 1; OR = 1.19, 95%, CI = 0.62 - 2.29, p=0.60 : Scenario 2; OR = 1.31, 95%, CI = 0.69 - 2.48, p=0.41)

Table 6.12 *ACE* Genotypic Haplotype Frequencies

Haplotype	Patients (n=359)	Controls (n=326)	OR	Lower 95% CI	Upper 95% CI	P
H1/H1	11.7% (42/359)	13.1% (43/326)	1.18	0.88	1.57	ns
H1/H10	0.83% (3/359)	0.3% (1/326)	2.07	0.34	12.5	ns
H1/H13	1.7% (6/359)	1.5% (5/326)	1.18	0.37	3.73	ns
H1/H6	31.2% (114/359)	32.8% (107/326)	1.02	0.65	1.58	ns
H1/H7	8.6% (31/359)	6.1% (20/326)	1.38	0.74	2.56	ns
H1/H9	4.7% (17/359)	2.5% (8/326)	2.13	0.94	4.85	ns
H6/H10	0.77% (2/359)	2.0% (6/326)	0.45	0.09	2.40	ns
H6/H13	1.4% (5/359)	1.8% (4/326)	1.38	0.40	5.02	ns
H6/H6	17.8% (64/359)	16.3% (53/326)	1.09	0.68	1.75	ns
H6/H7	5.0% (18/359)	5.8% (19/326)	0.94	0.46	1.88	ns
H6/H9	9.6% (25/359)	4.0% (13/326)	2.31	1.04	5.10	0.04
H7/H13	0.83% (3/359)	1.5% (5/326)	0.69	0.15	2.40	ns
H7/H7	0.83% (3/359)	1.2% (4/326)	0.59	0.15	3.11	ns
H9/H10	0.28% (1/359)	0.3% (1/326)	1.10	0.28	4.32	ns
H9/H13	0.28% (1/359)	0.3% (1/326)	2.76	0.24	31.2	ns
H9/H9	1.1% (4/359)	0.3% (1/326)	5.52	0.59	51.0	ns
Unknown	5.6% (20/359)	11.0% (36/326)	2.10	1.18	3.70	0.01

6.4.3.1 Subgroup Analysis of The Most Accurately Phenotyped Patients

The above analysis included a small number of patients whose cranial imaging was normal or no imaging was performed at all. This could potentially introduce inaccuracies in the data. To address this potential problem, further analysis was performed with these patients excluded. The results did not differ significantly from those of the entire patient sample. There was a slight strengthening of the association of H9 with ischaemic stroke ((OR= 2.12, 95% CI 1.3 - 3.3, p=0.001) vs (OR= 2.00, 95% CI 1.3 - 3.1, p=0.002)). This suggests that the clinical phenotyping was only marginally less accurate when cranial imaging was not included and this has not had a significant effect on the final results.

6.4.4 SNP / Polymorphism Analysis

Although only a selection of the *ACE* SNP loci have been genotyped in this study, the successful validation of Rieder *et al's ACE* haplotypes in our subjects has allowed for case control comparisons of each of the *ACE* SNP loci (Table 6.13).

Table 6.13 Significant Case Control Comparisons of Calculated Genotypes at SNP loci

SNP	NCBI SNP Database ID	Genotype	OR	95% CI	P
10	rs4298		1.94	1.18-3.18	0.009
10		C/T	1.94	1.12-3.35	0.017
10		T/T	3.71	0.41-33.5	0.243
24	rs4312		1.94	1.18-3.18	0.009
24		A/T	1.94	1.12-3.35	0.017
24		T/T	3.71	0.41-33.5	0.243
25	rs4313		1.94	1.18-3.18	0.009
25		C/T	1.94	1.12-3.35	0.017
25		T/T	3.71	0.41-33.5	0.243
52	rs1799752		1.07	0.87-1.33	0.519
52		I/D	0.89	0.61-1.31	0.575
52		D/D	1.12	0.73-1.73	0.598

Only three SNPs, (10 (rs4298), 24 (rs4312) & 25 (rs4313)) have significant associations with ischaemic stroke. SNP 10 (rs4298) differentiates H8, H9 & H11 from the other haplotypes and SNPs 24 (rs4312) and 25 (rs4313) differentiate H9 and H11 from all of the others. H8 & H11 were not identified in our or Rieder *et al's* caucasian samples and so there was no requirement to differentiate between H8, H9 and H11. This analysis of SNPs 10, 24 and 25 is therefore not valid in this dataset as it is essentially giving us the same result as the H9 haplotype analysis.

A study of the independent effects of the I/D polymorphism is a valid analysis in this sample however it was not found to be significantly associated with ischaemic stroke.

CHAPTER 7

DISCUSSION

Chapter 7: List of Tables

The first part of this study directly established the validity of a selection of the previously inferred *ACE* haplotypes in a caucasian UK population by direct allele specific PCR and sequencing of 7 kb of the gene. The validity of the remaining un-sequenced haplotypes was inferred due to the accuracy of the selected sequencing experiments. Haplotypes were then assigned to the majority of individuals by genotyping five polymorphisms. This allowed for a case control association study of the *ACE* haplotypes in a large sample of ischaemic stroke victims (n=359) and unrelated controls (n=328) which discovered a significant association of the low frequency H9 haplotype with ischaemic stroke.

7.1 Haplotype Validation

Using allele specific oligonucleotides annealed to the A allele of SNP 47 (rs4335) we have successfully amplified a single allele of the *ACE* gene in the region between 6227-14429 bp (Rieder location (Rieder et al., 1999)). Sequencing of 43.7% of this region, which includes 21 polymorphisms (27% of total number of polymorphisms), using DNA from patients in the most common haplotypes 1,6,7, & 13 has confirmed the exact structure of these haplotypes in this region. We have not performed experiments on the less common haplotypes nor have we confirmed the haplotype structure in the remaining 66% of the gene. Nevertheless for a substantial proportion of the gene all 4 haplotypes that were studied exactly matched the previously published data (Rieder et al., 1999) ; Genecanvas) so we feel justified in inferring a reasonable degree of validity of the haplotypes identified in the Rieder et al 1999 study which were not analysed in this study. This inference does presume that no new mutations have occurred in our sample to generate new haplotypes and as we have not sequenced the entire region for all of our subjects this possibility cannot be excluded. The numbers of unknown haplotypes (53/687 , 7.7%) in the total sample is more than we would have liked and this does limit the confidence with which the results can be presented.

7.2 Patient Ascertainment

The heritability of stroke is relatively low, suggesting that genetic risk factors are present but each is likely to have a small role to play in stroke development. In order to

identify these small effects it is essential to study large populations. With relatively few resources and a finite time in which to conduct this study it was not possible to ascertain sufficient numbers whilst also conducting detailed prospective phenotyping. We chose to obtain as many subjects as possible but as a consequence the phenotyping was based upon incomplete retrospective data which we accept is less than ideal. At the time that this study was conducted, however, many other similar genetic stroke studies suffered the same phenotyping difficulties.

Ascertainment of patients in the acute hospital setting proved difficult. Patients were often elderly and had suffered significant morbidity as a consequence of their stroke. This resulted in problems with comprehension and subsequent difficulties with the consent procedure. More patients were willing to consent in the setting of the out-patient clinic which resulted in the collection of more retrospective data and also biased the patient group in favour of survivors who may be genetically different than patients who experience fatal strokes.

These difficulties of patient ascertainment resulted in only 359 cases and 328 controls being enrolled in to the study. Earlier power calculations had suggested that these numbers would have roughly given an 80% power of identifying a common disease causing allele (frequency of 0.55), in absolute LD with the marker allele, producing a RR of ischaemic stroke of 2.0 at the 0.001 significance level. In view of the identification of an associated haplotype (H9) with a much lower population frequency (0.05), post hoc power calculations using the same power and significance levels (Table 7.1) would suggest that at least 10 fold greater numbers of subjects would be required to have more confidence in the accuracy of this association.

Table 7.1

Post Hoc Power Calculation To Identify The Necessary Sample Sizes In This Study Using The Identified H9 Allele Frequency

High Risk Allele Freq(H9)	Prevalence of Stroke	Genotypic Relative Risk H9/Hx	Genotypic Relative Risk H9/H9	High Risk Marker Allele Freq	LD (D')	Alpha	Power	N cases for 80% Power	N cases for 90% Power	N cases for 95% Power
0.0052	0.0018	2.05	3.93	0.0052	1	0.05	0.20	2253	3016	3730
0.0052	0.0018	2.05	3.93	0.0052	1	0.01	0.073	3352	4271	5113
0.0052	0.0018	2.05	3.93	0.0052	1	0.001	0.015	4901	6000	6991
0.0052	0.0018	2.05	3.93	0.0052	0.8	0.05	0.15	3274	4383	5421
0.0052	0.0018	2.05	3.93	0.0052	0.8	0.01	0.05	4872	6207	7431
0.0052	0.0018	2.05	3.93	0.0052	0.8	0.001	0.01	7123	8720	10002

7.3 Population Demographics

Although the mean age of the cases and controls was equivalent, there were significantly more controls over the age of 65 years. As common ischaemic stroke is usually a late onset disease the development of stroke after entry into the study would invalidate the case control comparisons. Thus, having an older control group strengthens their validity as they have had more time in which to have developed symptomatic cerebrovascular ischaemia if they were prone to it. Individuals with a genetic predisposition are likely to have younger age of onset and the younger patient group would also enhance the chances of identifying those genetically prone. The average age of first stroke of 70.2 years in the patient group cannot be considered victims of 'young' stroke and so it is likely that they reflect common forms of multi-factorial ischaemic stroke.

Our aim was to identify an elderly control population who were at risk of stroke but naive of clinical events. The frequencies of ex-smokers and mean cigarette dose (pack years) did not differ significantly between the groups however the prevalence rates of hypertension, FH, DM, hyperlipidaemia, current smoking, cardiac arrhythmias, and other vascular diseases were significantly higher in the cases than controls. Nevertheless, risk factor frequencies in the control group were higher than would be predicted from population studies for hypertension (51% vs. 30-38%), IHD (11.0% vs. 4.5-7%) and DM (4% vs. 1.5-3%) (Table 7.1). The control population should therefore

be considered a reasonably valid control population for this type of study however it must be emphasised that the results require further assessment using a variety of control groups of differing ages and risk factor profiles. Additionally, the true status of a 'control' should ideally be kept under review in case a clinical stroke occurs in a control subject as they age.

Table 7.1 Comparisons of Risk Factor Prevalence In This Study and Published UK Studies

Demographic Characteristics	Patients (n=359)	Controls (n=328)	p value	Leicester Study OR	Population Based Studies OR or RR	UK Prevalence Of Risk Factor
Mean Age	71.9 (+/- 12.1)	73.4 (+/- 8.0)	0.03			
Under 65 yrs	26.4%(94/356)	9.5% (31/326)	<0.001			
Known FH of Stroke	35%(126/356)	9% (31/326)	<0.001	2.37	RR 1.4-3.3 (See table of epidemiological studies in Ischaemic Stroke	
Current Smoker	18% (66/356)	10% (33/326)	0.015	2.24	1.5 (Shinton and Beevers, 1989)	Men = 28% Women= 27% (Erens and Primatesta, 1999)
Ex Smoker	45%(159/356)	45% (146/326)	ns	1.62		
Hypertension (>160/90 mmHg)	77% (258/336)	51% (149/294)	0.003	OR=3.86	RR=7, 160/95 vs. 120/80 (Markus, 2003)	BP>140/90 mmHg Men = 38% Women = 30% (Erens and Primatesta, 1999)
Diabetes Mellitus (glucose > 7.0)	32% (92/284)	7% (22/298)	<0.001	OR= 5.11	2-3 (Ebrahim and Harwood, 1999)	Men = 2-3% Women = 1.5-2.5% (Erens and Primatesta, 1999; Gatling et al., 1998) Men = 1% Women = 1% (Bennett et al., 1995)
Hypercholesterolaemia (total cholesterol > 6.6)	52% (131/254)	13% (36/274)	<0.0001	OR=4.13	1.4 between lowest & highest quartiles of total cholesterol (Benafante et al 1994)	
Angina	14.6% (52/356)	11.0%(36/326)	0.02		2.5 (Ebrahim and Harwood, 1999)	Men = 7% Women = 4.5% (Erens and Primatesta, 1999)
Cardiac Arrhythmias	23.0% (82/356)	5.2% (17/326)	<0.001		4-5 (Brand et al., 1985; Kopecky et al., 1999)	5% of 65+ yrs (Sudlow et al., 1998; Wheeldon et al., 1998)

7.4 Phenotyping

Unfortunately the accuracy of clinical phenotyping was variable and is a significant limitation of this study. Nevertheless, an exhaustive review of the medical case notes

and whenever possible personal assessments of controls was achieved. All cases were examined promptly by experienced consultant physicians and cranial imaging was performed in the majority of the sample usually allowing sufficient time for a cerebral infarct to be visualised (i.e. >6 hours post symptom onset).

Cardiac and carotid imaging was performed in the minority of cases (36% and 26% respectively) often because the cause of cerebral infarction was evident or the individual's prognosis was felt to be poor. Data on hypertension was only missing in 5.6% of patients and 9.8% of controls. The deficits in this data ascertainment resulted in an inability to determine the pathological subtype of stroke in 34% of patients. Despite this our results compare favourably with the 39% of unclassifiable stroke seen in the deCODE genetics genome screen (Gretarsdottir et al., 2002). Nevertheless, this has limited the confidence with which any conclusions can be made regarding the role of H9 in the pathophysiology of the different subtypes of stroke.

In order to evaluate whether phenotyping errors have affected the results, we reanalysed the data after excluding patients who had normal scans or had not undergone cranial imaging. Results showed only slight strengthening of the association of ischaemic stroke with H9 (OR=2.12, 95% CI 1.3-3.3, p=0.001 vs. OR=2.00, 95% CI 1.3-3.1, p=0.002). Additionally the lack of genetic associations of H9 with hypertension, hypercholesterolaemia and diabetes, independent of the presence or absence of stroke, would also suggest that the deficits in phenotyping have not significantly affected the results.

Of the patients who were given a TOAST classification (66% of the total sample), 17% were considered to have small vessel disease, 12% large vessel disease as the primary cause of the stroke and 34% were classified as having a cardiogenic aetiology. Cardiogenic stroke is often related to cardiac arrhythmia and although there are associations between this and atheromatous disease it is clearly a very different pathological process than large or small vessel cerebrovascular disease. Analysis did not identify any specific differences between these stroke subgroups and so the overall analysis was performed on the total sample made up of multifactorial causes of stroke. A similar approach of combining cases of cardiogenic and cerebral atherothrombotic large vessel stroke was also taken by researchers in the Icelandic deCODE study who argued that there were common mechanisms between the development of coronary

artery disease and cerebrovascular disease (Gulcher et al., 2004). Although this approach is less than ideal it should be considered to have some validity and whilst accepting the limitations of this method it would not preclude identification of a role of genetic mechanisms in the final common pathway of stroke, irrespective of the cause of the initial insult.

7.5 Genotyping

7.5.1 Genotyping Methods

PCR and RFLP analysis proved to be a successful combination for the majority of the samples analysed. All of the genotypes at the SNPs loci where data was available for the entire population were not significantly different than expected by the Hardy Weinberg equilibrium in both the case and control groups. This therefore suggests that the genotyping methods produced no significant genotyping inaccuracies for the study population as a whole.

Genotype frequencies at the SNP 24 (rsr312) locus were significantly different from Hardy Weinberg expectations however this is a highly selected group as SNP 24 was only used to differentiate between the haplotypic genotypes H6/H9 and H7/H13. Under these circumstances it is not surprising that the genotype frequencies differ from Hardy Weinberg expectations as none of the genotyped patients were likely to have a TT genotype at this SNP locus as they had been selected out by the previous genotyping experiments at the other loci.

None of the chosen SNPs were within the coding region of ACE and although this would have reduced the likelihood of identifying any functionally relevant SNPs it is unlikely to have had any effect on the outcome of the haplotype association analysis as the chosen SNPs were merely being used as markers for the generation of the haplotypes.

Haplotypes could not be ascribed to 66 individuals partly due to PCR and restriction enzyme failures but also due to the likely presence of more than 13 haplotypes in our population. Since the commencement of this study large quantities of SNP and

haplotype data has been published on the dbSNP (www.ncbi.nlm.nih.gov/sites/entrez?db=snp) and Hapmap (www.hapmap.org/) databases which identify many more than the 13 haplotypes studied in this project. These developments will be covered in the additional chapter on 'Advances in Common Disease Genetics' as they have clear relevance for interpretation of the results of this study.

Unfortunatley our methods did produce genotyping errors despite repeated testing of failed experiments. This has introduced a small element of doubt into the study findings particularly as a significantly larger number of PCR and RFLP failures occurred in the control group which could have produced a spurious association of H9 with ischaemic stroke. However the sensitivity analyses, which assumed the worst scenario of all the failed samples in the control group having the H9 haplotype and none of the failed patient samples being H9, was marginally significant (p=0.04, 95% CI = 1.01-2.70) thereby suggesting that this may still be a real phenomenon in this sample. Nevertheless these genotyping errors are clearly less than ideal and need further evaluation with alternative genotyping methodologies. Since this study was conducted, high throughput SNP genotyping techniques have been developed and this may provide a better solution for any future attempts at validating the findings of this study in larger study populations (see Chapter 8).

7.5.2 The Choice Of SNPs

SNPs 2 (rs4290), 29 (rs4317), 61 (rs4349), 69 (rs4357) and 76 (rs4364) were all monomorphic in our caucasian subjects. This finding is in line with Rieder et al's data as each of these SNPs were only identified in their Afro-Carribean patients. Although in our population of caucasian European subject we could have reasonably assumed that the genotype frequencies of these SNPs would have been identical to those found in Rieder's caucasian population we chose not to make this assumption and proceeded to genotype these SNPs in our sample. The finding that all of these SNPs are monomorphic has made them uninformative markers in our caucasian individuals however it has provided us with useful confirmation that our chosen subjects are derived from a stable and homogeneous population.

The monomorphic nature of these SNPs served to reduce the potential number of Rieder haplotypes that could be identified to 6 by excluding H2, H3, H4, H5, H8, H11 & H12. Genotyping only 4 SNPs and the I/D polymorphism was required to successfully identify haplotypes for the majority of the sample. A further SNP (rs4312) was genotyped in 41 subjects to differentiate between haplotypes 7/13 and 6/9 (this explains why genotype frequencies cannot be generated and Hardy Weinberg calculations cannot be performed for this SNP a.s not all subjects were analysed).

SNP frequencies did not differ significantly from Rieder's European and American sample. The HapMap data of the SNPs typed in this study is limited to rs4305 and rs4357. This suggests that the SNPs genotyped in this study which were chosen on the grounds that they could be analysed on agarose gels were sub-optimal and could have been improved. Future studies should ideally chose tagging SNPs within exonic regions identified from the HapMap database with high heterozygosity rates to optimally differentiate between haplotypes. Newer SNP genotyping methods which use easily recognisable non-gel based techniques are clearly better than those used in this study.

7.6 Haplotype 9 as a Genetic Risk Factor For Ischaemic Stroke

Accepting the limitations of this study outlined above, for the first time we have advanced the genetic assessment of the ACE gene in population based vascular disease by analysing haplotypes rather than single polymorphisms. We have found H9 twice as frequently in stroke patients in this study group as in the elderly controls (7.6% vs. 3.5%, OR=2.00, 95%CI=1.30-3.08, p=0.002) and the association is independent of the other vascular risk factors studied.

The validation of the ACE haplotypes in our population allowed for a search of all the recognised polymorphisms in H9 as well as the other polymorphisms, identified by Rieder et al 1999, in different haplotypes, in an attempt to locate any possible functional variants which are associated with ischaemic stroke. H9 incorporates the D allele of the I/D polymorphism however no independent association of the D allele was identified in our population. This is at odds with a number of studies (Castellano et al., 1995; Doi et al., 1997; Elbaz et al., 1998; Hosoi et al., 1996; Kario et al., 1996; Margaglione et al.,

1996; Markus et al., 1995; Nakata et al., 1997; Sharma, 1998; Watanabe et al., 1997) but in keeping with a large number of negative outcomes (Aalto-Setala et al., 1998; Catto et al., 1996; Pullicino et al., 1996; Sharma et al., 1994; Ueda et al., 1995; Zee et al., 1999). These studies were performed in widely differing populations. In the present study this finding supports the view that the I/D polymorphism does not lead to *ACE* dysfunction itself but is in LD with a genetic variant(s) which affects the function of the gene. The variability of LD within different populations may account for the variability in the results of the association studies looking at the I/D polymorphism in ischaemic stroke and other vascular diseases.

Three SNPs were found to have a significant association with ischaemic stroke in our population. One exonic, SNP 10 (rs4298T; p=0.004), and two intronic SNPs, 24 (rs4312T; p=0.004) & 25 (rs4313T; p=0.004), all are present in the H9 as well as the H11 haplotype and differentiate these haplotypes from the other D allele linked haplotypes in caucasian populations. H11 was not identified in our population. These SNPs are therefore likely to be the reason for the positive association with H9. SNP 10 (rs4298T) is intuitively more likely to have some functional relevance although it is synonymous and so would not affect the amino acid composition of the protein. Nevertheless synonymous SNPs have been found to have functional affects as identified by Duan et al 2003 in the human *DRD2* gene through affects on mRNA stability and translation (Duan et al., 2003).

Despite being intronic the other 2 SNPs may also have functional roles as seen in studies on Drosophila where two non-coding polymorphisms have been found to influence alcohol dehydrogenase expression (Stam and Laurie, 1996) and a further 2 intronic nucleotides have been strongly associated with variation in bristle number (Long et al., 1998). In humans, non-coding SNPs are also associated with measurable differences in mRNA levels in 6 out of 13 loci (Yan et al., 2002) and the intronic *ACE* I/D polymorphism has long been thought to have a directly functional role in regulating ACE levels (see Chapter 3).

SNP 10 (rs4298T) lies within the 5' region of the gene, falling within the QTL proposed by Villard et al 1996 and within the region 5' of 8968 bp (Rieder's location) (T17944C http://ecgene.net/genecanvas/modules/icontent/index.php?page=70) which may differentiate high ACE level clades B & D from lower ACE level clade C as proposed

by Cox et al 2002. SNPs 24 (rs4312T : 9191 bp) and 25 (rs4313T : 9222 bp) both lie 3' of exon 5 (approx 6435 bp Rieder location) and may lie close to the ancestral breakpoint of *ACE* giving them some possible functional significance. Both of these SNPs are located very close to each other and are likely to be in strong LD.

The functional relevance of these polymorphisms remains speculative as further analysis has not been performed. The positive result may merely be due to chance as a consequence of the multiple testing required to generate the results although the highly significant p values at the level of 0.004 are stringent enough to suggest these results are real and clearly warrant future analysis of these polymorphisms in association and functional studies.

The frequency of H9 is low in this study and is clearly not related to the pathogenesis of ischaemic stroke in all cases. One can speculate that interactions with other risk factors are required before it exerts its effects. Additional interactions not assessed by this study could also be relevant in the same way that epistatic phenomena have been identified between *ACE* and *angiotensinogen* (Nakata et al., 1997) and the Factor V Leiden mutation and *MTHFR* (Lalouschek et al., 1999). Such interactions warrant further assessment in future studies and chip based micro-array genotyping techniques (see Chapter 8) which allow for the study of multiple polymorphisms in large numbers of genes would make this possible.

The relevance of these findings is unclear especially when considered in the context of the many complex biological interactions leading to stroke. ACE is not the only converter of ANGI to ANGII and it is possible that pathological enhancement of other pathways, for example the Chymase system, may override the ACE system in patients with genetically low ACE levels. Similarly, deficiencies in the pathways which down regulate or degrade ACE activity may lead to relative enhancement of the enzyme in the absence of any genetic predisposition to ACE over activity. Both these mechanisms may serve to produce an ischaemic stroke phenotype thereby reducing the chances of identifying other strong independent allelic or haplotypic associations in population studies.

Additionally as small vessel disease manifesting as lacunar stroke (Elbaz et al., 1998; Markus et al., 1995) or silent white matter ischaemia on MRI, (Kario et al., 1996)

appears to have a greater association with the D allele, further study of H9 associations in this stroke subset are now indicated. Unfortunately, this study did not find any associations with the different stroke subtypes but this may have been due to relatively small numbers in each subgroup or sub-optimal phenotyping

CHAPTER 8

ADVANCES IN COMMON DISEASE GENETICS SINCE THE COMMENCEMENT OF THIS STUDY

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8.1 Introduction

Since the development of this project in 1998 many advances have been made in the field of common disease genetics. As a consequence the techniques used in the study have become outdated. Modern studies are generally multi-centered using higher throughput automated genotyping technologies and integrating the huge amount of genetic data held in public databases such as dbSNP and HapMap, available on the internet. The following chapter will summarise the advances in the field over the last decade. It will cover the topics of high throughput genotyping methods, haplotype identification techniques and optimal SNP identification using these internet resources. There will be an update of recent *ACE* gene haplotype studies before concluding with a section on the potential for future research based upon the findings in this study.

8.1.1 Updates on SNP Genotyping

Many different methods of high throughput SNP genotyping are now available which would have undoubtedly improved the quality of the genotyping data in this study. In general the methods use either hybridisation, enzyme cleavage, other post amplification techniques based upon DNA structure or a combination thereof.

8.1.1.1 Hybridization-based methods

These include dynamic allele-specific hybridization, molecular beacons and SNP microarrays. Cross hybridisation can complicate the process but can usually be overcome by increasing the stringency of the conditions to improve specificity of the hybridisation.

8.1.1.1.1 Dynamic allele specific hybridisation (DASH)

This easily automated method makes use of the differences in melting temperature of DNA which results from the instability of mismatched base pairs produced by SNPs. Initially the amplicon under study is amplified and attached to a bead through a PCR reaction with a biotinylated primer. The amplicon is attached to a streptavidin column and the unbiotinylated strand is removed by washing with sodium hydroxide. An allele specific fluorescently labelled oligonucleotide is then added. The intensity of fluorescence is then measured as the temperature is increased until the melting temperature (™) can be determined. Any polymorphism will result in a lower than expected Tm {Howell, 1999 #1083}.

8.1.1.1.2 Homogeneous solution hybridisation with fluorescence resonance energy transfer detection.

Fluorescence resonance energy transfer (FRET) occurs when two fluorescent dyes (fluorophores) are placed in close proximity. The energy is transferred when the emission spectrum of one dye overlaps the excitation spectrum of the other (Clegg, 1992). There are now three FRET-based technologies , TaqMan assays, molecular beacons and the Invader assay.

8.1.1.1.1.1 TaqMan genotyping

This assay uses the 5' nuclease activity of *Taq* polymerase to detect a fluorescent reporter signal generated during or after PCR reactions (Ranade et al., 2001)(Livak et al., 1995). The reaction requires one pair of PCR primers and one pair of allele specific TaqMan probes differing only at the polymorphic SNP site. One of the allele specific primers has a 5’ reporter dye and a 3’ quencher dye linked to it. During the annealing step of the PCR reaction the TaqMan probes hybridise to the targeted polymorphic site. During extension the 5’ reporter dye is cleaved by *Taq* polymerase generating fluorescence of the reporter dye which allows detection of the allelic SNP.

8.1.1.1.1.2 Molecular beacons.

These are oligonucleotide probes that have 2 complimentary DNA sequences flanking the target DNA sequence as well as fluorescently labelled fluorophores at opposite ends of each probe. No fluorescence occurs unless the probes hybridise to the target sequence (Tyagi and Kramer, 1996)(Tyagi et al., 1998). Two allele specific molecular beacons labelled with different fluorescent dyes are used in each PCR reaction to enable detection of all three possible allelic combinations.

8.1.1.1.1.3 Invader assay

This FRET-based technique does not require PCR amplification. Two allele specific primers plus an upstream invader probe are required. Specific hybridisation of these probes to genomic DNA occurs when they anneal to the same region with overlapping of the probe by at least one base pair. The structure is then cleaved by a cleavase endonuclease (Lyamichev et al., 1999). Each cleavage product becomes an invader oligonucleotide in a secondary reaction directing the cleavage of a FRET probe template construct labelled with a donor and acceptor fluorophore. Cleavage of this template separates the fluorophores, quenching is abolished and fluorescence generated. The fluorescently labelled allele specific products are then read on a plate reader able to recognize this fluorescence.

8.1.1.1.3 DNA micro-array genotyping

This hybridisation method allows simultaneous identification of multiple polymorphisms. The micro-arrays are created by attaching many allele specific oligonucleotides to a solid silicon surface. Fluorescently labelled nucleotides are incorporated into a PCR generated amplicon which is then hybridised to the array. The degree of fluorescence directly corresponds to the strength of the hybridisation and signals can then be quantified by high-resolution fluorescence scanning techniques and analysed by computer software. (Chee et al., 1996; Lipshutz et al., 1999) . Once a comprehensive high-density SNP map is generated, micro-array technology will allow the rapid assessment of tens of thousands of genetic variations in large patient groups. At present heterozygous variation is less easily detected than

homozygous variation however with improvements in detection of degrees of fluorescence this potential error should be minimised.

8.1.1.2 Enzyme Based Methods

Along with restriction enzymes used in RFLP analysis (described in Chapter 4) DNA ligases, polymerases and nucleases have been used to identify SNPs.

8.1.1.3 PCR-based methods

Tetra-primer ARMS-PCR employs two pairs of primers to amplify two alleles in one PCR reaction. The primers are designed such that the two primer pairs overlap at a SNP location but each match perfectly to only one of the possible SNPs. Thus if a given allele is present in the PCR reaction, the primer pair specific to that allele will produce a product but not from the alternative allele. The two primer pairs should be designed to produce PCR products of significantly different lengths allowing for easily distinguishable bands by gel electrophoresis.

Primer extension

Primer extension is a two step process that first involves the hybridization of a probe to the bases immediately upstream of the SNP followed by a 'mini-sequencing' reaction in which DNA polymerase extends the hybridized primer by adding a base that is complementary to the SNP. This incorporated base is detected and determines the SNP allele {Syvanen, 2001 #1084}. Because, primer extension is based on the highly accurate DNA polymerase enzyme, the method is generally very reliable. Primer extension is able to genotype most SNPs under very similar reaction conditions making it also highly flexible. The primer extension method is used in a number of assay formats.

Primer extension techniques generally use two main approaches which incorporate either fluorescently labelled dideoxynucleotides (ddNTP) or deoxynucleotides (dNTP). ddNTPs, probes hybridize to the target DNA immediately upstream of SNP

nucleotide, and a single, ddNTP complementary to the SNP allele is added to the 3' end of the probe (the missing 3'-hydroxyl in dedioxynucleotide prevents further nucleotides from being added). Each ddNTP is labelled with a different fluorescent signal allowing for the detection of all four alleles in the same reaction. dNTPs, allele-specific probes have 3' bases which are complementary to each of the SNP alleles being interrogated. If the target DNA contains an allele complementary to the probe's 3' base, the target DNA will completely hybridize to the probe, allowing DNA polymerase to extend from the 3' end of the probe. Fluorescent labelling of the dNTPs allows detection of the probe. If the target DNA does not contain an allele complementary to the probe's 3' base, the target DNA will produce a mismatch and DNA polymerase will not be able to extend from this end. The benefit this approach is that several labelled dNTPs may get incorporated into the growing strand which increases the strength of the signal. In rare cases DNA polymerase can extend from mismatched 3' probes giving a false positive result.

The flexibility and specificity of primer extension make it amenable to high throughput analysis. Two commercially available methods of primer extension include arrayed primer extension (APEX) and the Infinium assay (Illumina). The Infinium assay uses hapten-labelled nucleotides which can be recognized by anti-bodies, which in turn are coupled to a detectable signal. This technique allows over 100,000 SNP to be detected at the same time{Gunderson, 2006 #1085}.

Oligonucleotide ligation assay genotyping

The oligonucleotide ligation assay (OLA) relies on hybridisation with specific oligonucleotide probes which discriminates between the polymorphic and wild type sequences. Three oligonucleotide primers are required, one each for the wild type and mutant SNP and one fluorescently labelled common primer. The 3' ends of the allele specific probes are immediately adjacent to the 5' end of the common probe. Following amplification of the desired region by PCR techniques the amplicon is incubated with all 3 probes. In the presence of a thermally stable DNA ligase, ligation of the allele specific probe to the common probe occurs when there is a perfect match between the wild type or variant probe and the PCR amplicon. Electrophoresis is then

used to separate and recognise the products (Ross et al., 1998). Multiple reactions can be conducted at the same time by using different fluorescent markers (Baron et al., 1996).

8.1.1.4 Post-Amplification SNP Detection Methods Based Upon the Physical Properties of DNA

Conformation based mutation scanning

Single-stranded conformation polymorphism analysis is a widely used method of mutation detection. The underlying principle of these methods is that the melting characteristics of double stranded DNA are largely defined by its sequence. Following genomic amplification using PCR single-stranded regions of DNA are generated by denaturation of the amplicon. These are then separated on a non-denaturing polyacrylamide gel. Single base modifications produced different conformations of the amplicon which migrate differently. The sensitivity can be increased to nearly 100% in combination with restriction enzyme fingerprinting (Liu and Sommer, 1995) or dideoxy-sequencing fingerprinting (Sarkar et al., 1992) Other methods include conformation sensitive gel electrophoresis (Ganguly and Prockop, 1995), chemical or enzymatic mismatch cleavage detection (Youil et al., 1995) denaturing gradient gel electrophoresis (Myers et al., 1987) and denaturing high-performance liquid chromatography (dHPLC) (O'Donovan et al., 1998).

Temperature gradient gel electrophoresis (TGGE)

The temperature gradient gel electrophoresis (TGGE) or temperature gradient capillary electrophoresis (TGCE) method is based on the principle that partially denatured DNA is more restricted and travels slower in a porous material such as a gel. This property allows for the separation of DNA by melting temperature. To adapt these methods for SNP detection, two fragments are used; the target DNA which contain the SNP polymorphic site being interrogated and an allele-specific DNA sequence, referred to the normal DNA fragment. The normal fragment is identical to the target DNA except potentially at the SNP polymorphic site, which is unknown in

the target DNA. The fragments are denatured and then re-annealed. If the target DNA has the same allele as the normal fragment, homoduplexes will form that will have the same melting temperature. When run on the gel with a temperature gradient, only one band will appear. If the target DNA has a distinct allele, four products will form following the re-annealing step; homoduplexes consisting of target DNA, homoduplexes consisting of normal DNA and two heteroduplexes of each strand of target DNA hybridized with the normal DNA strand. These four products will have distinct melting temperatures and will appear as four bands in the denaturing gel

8.1.1.5 Other SNP Genotyping Methods

In silico SNP mapping

This method uses the large amount of sequence data published in public databases generated from the human genome project. Expressed sequence tags (ESTs) and genomic DNA sequences provide a rich source for in silico identification of SNPs. Multiple alignment of the available sequences from redundant ESTs and bacterial artificial chromosome clones is required however this does not require sequencing of multiple individuals and is considered a fast and cost-effective way to identify new SNPs. A computer-based SNP screening algorithm (POLBAYES) is available for this purpose (Marth et al., 1999)

Allele specific ligation

This technique is a combination of FRET and PCR. Three labelled ligation probes for each SNP with low melting temperatures along with PCR primers designed with high melting temperatures are used. The 5' labelled common probe is designed to terminate one base upstream of the polymorphic site. Two allele specific acceptor dye labelled probes have polymorphic nucleotides at the 5' end. A thermostable DNA ligase and DNA polymerase with no 5' nuclease activity are used in the experiment. Ligation probes prevented from annealing in the first stage of PCR by keeping the temperature high. In the second stage of the reaction after sufficient PCR products are generated a lower annealing temperature allows ligation to occur. The

fluorescents signals of all the dyes enables genotypes to be determined (Chen et al., 1998)

Rolling Circle amplification (RCA)

This analysis is sensitive enough to work from genomic DNA (Lizardi et al., 1998) Two allele specific probes are designed for each SNP, each probe consisting of between 80 and 90 base pairs. The 5' end of the probe is phosphorylated with a sequence of approximately 20 nucleotides which is complimentary to the region immediately 5' of the SNP. The 3' end of the probe contains between 10 and 20 nucleotides complimentary to the region immediately 3' of the SNP. Each probe differs in one base complimentary to the SNP designed at the 3' end. In the remaining 40 bases the two probes contain two different generic backbone sequences that encode binding sites for two rolling circle amplification primers.

The first stage of this assay involves ligation of each of the probes to their respective target DNA sequences. After denaturation both allele specific probes are then added to the denatured DNA and if a perfect match between the 3' base of the probe and the polymorphic nucleotide is present in a stable hybrid is formed. Complete hybridisation aligns the two ends of the probe on the target DNA and circularises the probe using a thermostable ligase. Amplification of a circularised probe by rolling circle amplification requires the first primer to hybridise to its complimentary region on the probe backbone. A strand displacing DNA polymerase is then required to extend the primer which eventually displaces it at its 5' end once one complete revolution of the circularised probe is made. Continued polymerisation and displacement generates single-stranded DNA copies of the original probe.

Allele specific nucleotide incorporation

Pyrosequencing

This detects de novo incorporation of nucleotides based on the specific template. During the process of incorporation a pyrophosphate is released which is converted to ATP in the presence of adenosine 5 phosphosulphate. In turn a luciferase enzyme is stimulated which is able to produce light which can be detected by a charge coupled

device camera. The amount of light produced correlates with the number of nucleotides incorporated. Current pyrosequencing technology allows the detection of 500 SNPs per hour in a 96 well plate. (Alderborn et al., 2000).

Single base extension with fluorescence detection

This is a popular SNP genotyping method. It is a template directed dye terminator incorporation assay with fluorescence polarisation (FP) detection. FP is based on the principle that a small molecule tumbles rapidly in solution. When plane polarised light is shone on fluorescent dyes the molecules tumble rapidly and the omission is polarised. The fluorescence polarisation is directly proportional to molecular volume and molecular weight (Chen et al., 1999). The sequencing primer is designed immediately upstream of the polymorphic site. Fluorescent dideoxynucleotide triphosphate target DNA and the allele specific probes are then incubated and specific sequence generated. The genotype of the target DNA molecule can then be determined by exciting the fluorescent dye in the reaction in determining the change in fluorescent polarisation. A commercial high throughput version of this method is now available.

Mass spectrometry (MS)

This technique provides precise information on the molecular mass of DNA fragments. Each nucleotide base differs slightly in their mass which allows detection with this technique (Ross et al., 1998). Unlike fluorescent genotyping methods MS offers specificity and accuracy without needing to label the probes. MS does however require samples to be free of impurities. This technique can be incorporated into a chip based genotyping assay which provides high throughput at relatively low cost (Tang et al., 1999).

In conclusion, I have outlined the many different techniques that are now available for SNP genotyping. Many of these are user-friendly, cost efficient and allow automation which improves efficiency and increases their throughput. Of the options available FRET and micro-array chip based technologies are likely to be the most popular in the near future for large scale genetic studies.

8.2 Publicly Available Internet Based Genetic Databases

The last 10 years has seen the expansion and wide spread use of the internet. The field of genetics has grasped this opportunity and has posted vast amounts of easily accessible genetic information in public databases for the benefit of the world's geneticists. The two most relevant databases for common disease geneticists are the HapMap and dbSNP databases and these will now be covered in turn. An independednt SNP and haplotype database held at the Washington University website, the Seattle SNP database, will also be referred to later in the chapter.

8.1.2 International HapMap Project

The International HapMap Project (International HapMap Consortium (2003) has been organized as an extension to the human genome project. Its aim was to develop a haplotype map of the human genome (the HapMap), which will describe the common patterns of human genetic variation and assist in the discovery of genes affecting health, disease and responses to drugs and environmental factors.

The project began after the commencement of this study in 2002, and was expected to take about three years. It comprises two phases; the complete data obtained in Phase I were published on October 27, 2005 (International HapMap Consortium (2005). The Phase II data has been published very recently {Frazer, 2007 #1087}.

Background

A haplotype is a series of consecutive alleles on a particular chromosome. In many parts of chromosomes nearly a handful of haplotypes may be identified. Meiotic recomination should potentially ensure a constant variation in haplotype structure however preferential recombination (recombination hotspots) and reduced levels of recombination (recombination cold spots) occur in specific regions. This has lead to the development of the concept of haplotype blocks. The generally stable structure of a haplotype block through the generations allows researchers to identify and genotype the most important and stable SNPs (tagging SNPs) within a specific haplotype block and infer other alleles without genotyping all of the SNPs (Figure 8.1). Within the

human genome it is estimated that 300,000 to 600,000 tagging SNPs are present which is far fewer than the 11 million common SNPs currently deposited in the dbSNP database.

Figure 8.1 Haplotypes. The construction of the HapMap occurs in three steps. (a) SNPs are identified in DNA samples from multiple indivduals. (b) Adjacent SNPs that are inherited together are compiled into "haplotypes." (c)"Tag" SNPs within haplotypes are identified that uniquely identify those haplotypes. By genotyping the three tag SNPs shown in this figure, researchers can identify which of the four haplotypes shown here are present in each individual. (http://www.hapmap.org/whatishapmap.html.en)

Samples used to generate Hapmap

Haplotypes are generally shared between populations, but their frequency can differ widely. Four populations were selected for inclusion in the HapMap: 30 Nigerian offspring and parents trios (YRI), 30 trios of U.S. residents of northern and western European ancestry (CEU), 44 unrelated Japanese individuals from Tokyo (JPT) and 45 unrelated Han Chinese individuals from Beijing (CHB). Parallel studies are currently examining the usefulness of including additional populations in the project.

Scientific strategy

At the start of the project, less than 3 million SNPs had been identified and published. In phase I of HapMap 1 million polymorphic SNPs spaced approximately 5000 bases

apart were genotyped in 10 centres using 5 different genotyping technologies. By August 2006, there were more than 10 million SNPs in the public domain, 40% of which were polymorphic and therefore informative for haplotype generation. Phase II of the project has genotyped another 4.6 million SNPs incorporating DNA samples from additional populations and it is hoped to make the phase I data even more robust.

The data generated from HapMap will hopefully increase the chances of association studies successfully identifying alleles conferring susceptibility or resistance to common disease. Additionally it may provide a deeper understanding of the architecture of disease, for example the number of genes involved, the extent of allelic interaction and the role of environmental factors. The HapMap consortium have highlighted the importance of heavily investing in the discovery and characterisation of relevant lifestyle factors, environmental exposures, detailed characterisation of clinical phenotypes and the ability to obtain such information in longitudinal studies of adequate size. They warn that unless we can learn to capture environmental variation with the same precision and completeness as genotypic variation, replication of association studies will be a difficult task (International HapMap Consortium (2005).

Along with HapMap other internet linked haplotype databases generated by independent researchers are also available and can provide unique information that is not available in the HapMap dataset. One such example is the Seattle SNP (Parc) database which can be viewed at http://pga.gs.washington.edu/gty_data/ace/

8.1.3 dbSNP

The dbSNP database was created in the late 1990s through a collaboration between the US National Centre for Biotechnology Information (NCBI) and the US National Human Genome Research Institute. It serves as a multi-species repository for SNPs, short deletion and insertion polymorphisms and haplotypes which have been generated from this information. The database is freely available on the World Wide Web (www.ncbi.nlm.nih.gov/). dbSNP entries record the sequence information around the polymorphism, its frequency, the specific experimental conditions needed to perform experiments and descriptions of the population studied. The dbSNP database also provides links to other external databases. This will therefore facilitate searches across the major axes of information linking in sequence location, function, cross species homology and degree of heterozygosity.

8.1.4 Methods of Generating Haplotypes

Haplotypes have gained increasing attention in the mapping of complex disease genes because of the limited power of conventional single locus analyses and the abundance of recognised SNPs within the genome. The haplotype approach which combines the information of adjacent SNPs into composite multilocus haplotypes is more desirable. Not only does it reduce the need for Bonferroni corrections which would be required when performing multiple testing of multiple SNP loci it is also more informative as it catches the regional LD information which is arguably more robust and powerful (Akey et al., 2001; Daly et al., 2001; Pritchard, 2001).

Generating haplotypes for autosomal loci when only the multilocus genotype (i.e. the unphased genotype configurations) for each individual is available is inherently ambiguous. Diploid genotype information for multiple sites allows an individual haplotype phase to be resolved with certainty when no more than one heterozygous site is present. Phase information is difficult to identify when a large amount of heterozygous data has been generated.

Two direct solutions to this difficulty are genotype studies of close biological relatives or molecular haplotyping. Genotyping of close relatives increases the size of population studies and is usually difficult to perform in genetic studies of late-onset adult diseases. A number of molecular haplotyping methods have been identified, these include single molecule dilution (Ruano et al., 1990), allele specific long-range PCR (Michalatos-Beloin et al., 1996) isothermal rolling circle amplification (Lizardi et al., 1998), long insert cloning (Ruano et al., 1990) and carbon nano tube probing (Woolley et al., 2000). All of these techniques are difficult to automate, they have low throughput, are prone to experimental errors and are therefore costly and often impractical. The novel diploid to haploid conversion method identified by Douglas et al in 2001 (Douglas et al., 2001) has been shown to have some promise however it is also technically difficult and expensive and has not been widely adopted at present.

More recently indirect algorithm-based approaches based upon explicit statistical inference models have been devised which provide a robust economical and accurate method of generating haplotypes. There are generally four algorithms for the inference of haplotype phases of individual genotype data; Clark's parsimony algorithm (Clark, 1990), the expectation-maximization (EM) algorithm, (Chiano and Clayton, 1998; Excoffier and Slatkin, 1995; Hawley and Kidd, 1995; Long et al., 1995) the pseudo-Gibbs sampler (PGS) (Stephens et al., 2001b) and a pseudo-Bayesian algorithm method (Stephens et al., 2001b). The following section will study these methods in turn.

Haplotype Inference Methods

Each of the following methods have advantages and disadvantages when applied to haplotype inference in large genetic studies. Clark's parsimony approach begins by using data from individuals whose phase is known and then assigns the smallest number of haplotypes for the observed genotype data. This popular algorithm has produced successful results in the delineation of the gene-based haplotype variations (Stephens et al., 2001a) as well as genomewide LD studies in populations with different histories (Reich et al., 2001). However the handling of a very large number of linked SNPs has not been satisfactorily addressed by this method.

The EM algorithm initially guesses haplotype frequencies and then updates them to maximize the log-likelihood function. This approach has been shown to be reasonably accurate under a wide range of conditions (Fallin and Schork, 2000) but it does suffer in the same way as Clark's algorithm when handling very large numbers of linked SNPs.

Another method was described by Stephens et al (2000b) who used a stochastic-sampling strategy—the pseudo-Gibbs sampler (PGS) to assign haplotype phases. By coalescence-based simulations, they showed that this method performs better than both Clark's and the EM algorithm (Stephens et al., 2001b). However although these conclusions hold for stable populations that have evolved over long periods of time Niu et al 2002 have shown that deviations from this assumption which often occur in real populations can adversely affect the performance of the PSG technique (Niu et al., 2002). Such deviations include past gene flow, stratifications, or bottlenecks. Furthermore all of these algorithms have difficulties with the commonly occurring technical problem of missing data.

More recently Niu et al (2002) have developed a robust Bayesian procedure that builds on the EM algorithm and makes no assumptions on the population evolutionary history. In this model, each individual's haplotype pair is treated as two random draws from a pool of haplotypes with unknown population frequencies. Two novel techniques are then applied (partition ligation (PL) and prior annealing) which are considered to improve both the accuracy and capacity of the technique. This method has been shown to deliver improved performance, in comparison to existing methods, in a wide variety of simulated and real data sets (Niu et al., 2002).

Once haplotypes are constructed, various statistical methods can be applied to detect associations between haplotypes and the disease under study including the chi-squared (χ^2) test, the likelihood-ratio test (Fallin et al., 2001), logistic regression analyses (Wallenstein et al., 1998), cladistic analysis (Heng and Low, 2000; Templeton, 1995), and the haplotype pattern-mining method (Toivonen et al., 2000).

8.3 Current Understanding of Genetic Variation and Haplotype Structure at the *ACE* locus

The following section will study the current data held in the HapMap and dbSNP databases pertaining to the *ACE* gene and apply this to the results that have been identified in the current study.

8.1.5 LD and Genotype Frequency Data

Out of the four subsets used to generate HapMap data those with European ancestry are the most similar to our native Leicester subjects. The most comparable HapMap data has been obtained from 88 individuals from CEPH trios (U.S. residents with northern and western European ancestry by the Centre d'Etude du Polymorphisme Humain). In this dataset comparable LD information is only available for SNP 61 (rs4357). Frequency data is however available for SNPs 2 (rs4290), 17 (rs4305), 29 (rs4317), 61 (rs4349), 69 (rs4364) and 76 (rs4364) and is shown in Table 8.1. The frequencies of all the SNPs genotyped in all of our subjects are not significantly different to those generated from caucasian subjects in the HapMap database. This provides strong evidence of the validity of our Leicestershire caucasian population as well as the accuracy of the genotyping methodology that we used.

Table 8.1 Comparison of Allele Frequencies between the Leicester Sample, HapMap and Seattle SNP (Parc) data

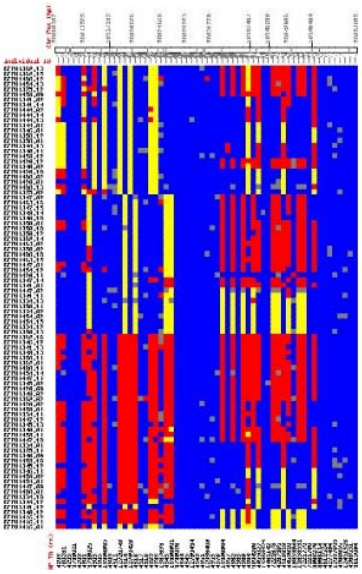
SNP	NCBI SNP Database ID	Bp	Alleles	Leicester Sample of Caucasian Europeans SNP Frequency	HapMap Caucasian American Population (CEU) SNP Frequency	Seattle SNP (Parc) European Caucasian Population SNP Frequency
2	rs4290	00334	C/T	1.00/0.00	1.00/0.00	no data
17	rs4305	06435	A/G	0.48/0.52	038/0.62	no data
20	rs4424958	07831	G/A	0.39/0.61	no data	no data
24	rs4312	09191	A/T	0.56/0.44*	no data	0.91/0.09
29	rs4317	10527	T/C	1.00/0.00	1.00/0.00	no data
47	rs4335	13230	A/G	0.47/0.53	no data	0.34/0.66
52(I/D)	rs1799752	14094	(ALU)/-	0.45/0.55	no data	no data
61	rs4349	17634	C/T	1.00/0.00	1.00/0.00	no data
69	rs4357	20120	C/T	1.00/0.00	0.90/0.10	no data
76	rs4364	23152	C/A	1.00/0.00	1.00/0.00	no data

* Selected data generated from only 42 individuals as SNP 24 was only genotyped to differentiate haplotypes in individuals H6/9 or H7/13

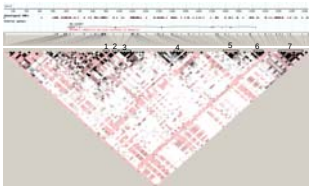
Figure 8.2 shows the LD map of the *ACE* gene from the HapMap Database. Each horizontal line in figure 8.2 represents genotypes from one individual for more than 50 SNPs across the *ACE* gene. Many more than 13 haplotypes have now been identified from HapMap and these have been segregated into 7 haplotype blocks.

a) Haplotypes generated by genotyping CEPH trios (U.S. residents with northern and western European ancestry by the Centre d'Etude du Polymorphisme Humain). Each horizontal line represents genotypes from one individual for multiple SNPs across the ACE gene

a)



b)



b) Linkage disequilibrium plot. Each square represents the LD between 2 SNPs in the genotyping panel. The darker the colour the greater the degree of LD.

Figure 8.2

Haplotype Structure of *ACE* (HapMap Database)

http://www.hapmap.org

Genotype data is also available on the Seattle SNP (Parc) database for 69 *ACE* SNPs from a sample of 61 individuals (Table 8.1 & Figure 8.3). Unfortunately there are only 2 common SNPs in our study and the Parc database, SNP 24 (rs4312) and 47 (rs4335). No significant differences in frequency are found between the 2 studies for SNP 47. Frequencies of SNP 24 genotypes do significantly differ from our dataset however as we only genotyped a selected group of 42 individuals in order to differentiate between haplotypes H6/H9 and H7/H13 this is not a valid comparison.

Figure 8.3 shows the haplotype data for the Parc European caucasian dataset. 11 haplotypes have been identified from this data however comparisons between them and those published by Rieder et al 1999 that were analysed in our study are difficult to make due to the different SNPs used to generate the haplotypes. Table 8.2 shows

the structure of the Parc haplotypes and Table 8.3 attempts to show the similarities between these and the haplotypes published by Rieder et al 1999. Only Rieder et al's haplotype 7 matches with Parc haplotype 9. Rieder et al's haplotypes 1, 2, 3, 6, 9, 10, & 11 match with at least more than one other Parc haplotype. Rieder et al's haplotypes 4, 5, 11 & 12 were not seen in their European subjects which is likely to explain why there were no comparable haplotypes seen in the Parc European sample.

Figure 8.3
ACE Haplotype Data published on the Seattle SNP website (Parc database)
http://pga.gs.washington.edu/gty_data/ace/

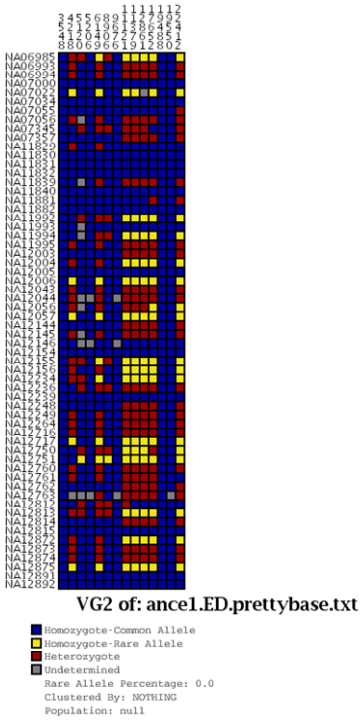


Table 8.2: The Structure of the Seattle SNP (Parc) ACE gene Haplotypes in a European Caucasian Sample													
PARC Database SNP Location	**NCBI BP Number** AF118569	**SNP Number Used in This Study**	**PARC Haplotype 1**	**PARC Haplotype 2**	**PARC Haplotype 3**	**PARC Haplotype 4**	**PARC Haplotype 5**	**PARC Haplotype 6**	**PARC Haplotype 6**	**PARC Haplotype 8**	**PARC Haplotype 9**	**PARC Haplotype 10**	**PARC Haplotype 11**
3548	3834		T	T	T	T	T	T	T	T	T	T	T
3586	3872		G	A	A	A	G	G	G	G	A	A	A
3944	4230		A	A	A	A	A	A	A	A	A	A	A
4218	4504		C	G	G	G	C	G	G	C	G	G	G
5120	5406	10	C	C	C	C	C	T	T	C	C	C	C
5206	5492	12	C	C	C	C	C	C	C	C	C	C	C
5693	5979												
5743	6029		G	G	G	G	G	G	G	G	G	G	G
5821	6107		G	G	G	G	G	G	G	G	G	G	G
5859	6145		C	C	C	C	C	C	C	C	C	C	C
6149	6435	17	A	G	G	G	A	A	A	A	G	G	G
6240	6526		C	C	C	C	C	C	C	C	C	C	C
7843	8129		C	C	T	T	C	C	C	C	C	C	T
8581	8867		C	C	C	C	C	C	C	C	C	C	C
8683	8969		T	C	T	C	T	C	C	T	T	C	C
8824													
8858	9144		C	C	C	C	C	C	C	C	C	C	C
8906	9191	24	A	A	A	A	A	T	T	A	A	A	A
8937	9223		C	C	C	C	C	T	T	C	C	C	C
9676	9962		A	A	A	A	A	A	A	A	A	A	A
10091	10377		G	G	G	G	G	G	G	G	G	G	G
10293	10579		A	A	A	A	A	A	A	A	A	A	A
10414	10700		D	D	G	G	D	D	D	D	D	G	G

Table 8.2 (continued): The Structure of the Seattle SNP (Parc) ACE gene Haplotypes in a European Caucasian Sample													
PARC Database SNP Location	**NCBI BP Number** AF118569	**SNP Number Used in This Study**	**PARC Haplotype 1**	**PARC Haplotype 2**	**PARC Haplotype 3**	**PARC Haplotype 4**	**PARC Haplotype 5**	**PARC Haplotype 6**	**PARC Haplotype 6**	**PARC Haplotype 8**	**PARC Haplotype 9**	**PARC Haplotype 10**	**PARC Haplotype 11**
10474	10760		G	G	A	A	G	G	G	G	G	A	A
10695	10981		T	T	C	C	T	T	T	T	T	C	C
10876	11162		C	C	T	T	C	C	C	C	C	T	T
11092	11378		A	A	G	G	A	A	A	A	A	G	G
11121	11405	37	C	A	C	A	C	C	C	C	C	A	A
11163	11449		G	G	A	A	G	G	G	G	G	A	A
11194	11480		C	G	C	G	C	C	C	C	C	G	G
11334	11620		T	T	T	T	T	T	T	T	T	T	T
11379	11663	41	A	A	G	G	A	A	A	A	A	G	G
11582	11868		A	A	A	C	A	A	A	A	A	C	A
11973	12259		A	G	A	G	A	A	A	A	A	G	G
12202	12488		T	T	C	C	T	T	T	T	T	C	C
12443	12729		T	C	T	C	T	T	T	C	T	C	C
12619	12905		A	A	A	A	A	A	A	A	A	A	A
12626	12912		C	C	C	C	C	C	C	C	C	C	C
12861	13147	46	A	C	A	C	A	A	A	A	A	C	C
12946	13232		G	G	A	A	G	G	G	G	G	A	A
12990	13276		A	A	A	A	A	A	A	A	A	A	A
13813	14099		D	+	D	+	D	D	D	D	D	+	+
14644	14930		G	A	G	A	G	G	G	G	G	A	A
16419	16705												
16497	16783												
16896	17182												
16940	17226		G	G	G	G	G	G	G	G	G	G	G
17064	17350		C	C	C	C	C	C	C	C	C	C	C

Table 8.2 (continued): The Structure of the Seattle SNP (Parc) ACE gene Haplotypes in a European Caucasian Sample													
PARC Database SNP Location	**NCBI BP Number** AF118569	**SNP Number Used in This Study**	**PARC Haplotype 1**	**PARC Haplotype 2**	**PARC Haplotype 3**	**PARC Haplotype 4**	**PARC Haplotype 5**	**PARC Haplotype 6**	**PARC Haplotype 6**	**PARC Haplotype 8**	**PARC Haplotype 9**	**PARC Haplotype 10**	**PARC Haplotype 11**
17448	17734		C	C	C	C	C	C	C	C	C	C	C
17460	17746		G	G	G	G	G	G	G	G	G	G	G
17652	17938		G	A	G	A	G	A	G	G	G	A	A
18342	18628		A	A	G	G	A	A	A	A	A	G	G
19130	19416												
19372	19658		C	C	C	C	T	C	C	C	C	C	C
19488	19774		T	T	T	T	T	T	T	T	T	T	T
19550	19836		C	C	C	C	C	C	C	C	C	C	C
19756	20042		C	C	C	C	C	C	C	C	C	C	C
19827	20113		G	G	G	G	A	G	G	G	G	G	G
20263	20549		T	C	T	C	T	T	T	T	T	C	C
20616													
20898	21184		G	G	G	G	G	G	G	G	G	G	G
21095													
21681	21967		T	C	T	C	T	T	T	T	T	C	C
22287	22573		T	T	T	T	T	T	T	T	T	T	T
22412	22698		G	A	G	A	G	G	G	G	G	A	A
22582	22868		C	C	C	C	C	C	C	C	C	C	C
22632	22918												
Numbers in each Haplotype			15	2	2	13	1	1	3	1	6	1	1

Table 8.3 : Similarities Between the Parc and Rieder et al (1999)Haplotypes Generated from the Common Data on SNPs 10, 12, 17, 24, 37, 41, & 46.

		Parc	Parc	Parc	Parc	Parc	Parc	Parc	Parc	Parc	Parc	Parc
		1	2	3	4	5	6	7	8	9	10	11
Rieder	1	✓	X	X	X	✓	X	X	✓	X	X	X
Rieder	2	✓	X	X	X	✓	X	X	✓	X	X	X
Rieder	3	✓	X	X	X	✓	X	X	✓	X	X	X
Rieder	4	X	X	X	X	X	X	X	X	X	X	X
Rieder	5	X	X	X	X	X	X	X	X	X	X	X
Rieder	6	X	X	X	✓	X	X	X	X	X	✓	✓
Rieder	7	X	X	X	X	X	X	X	X	✓	X	X
Rieder	8	X	X	X	X	X	X	X	X	X	X	X
Rieder	9	X	X	X	X	X	✓	✓	X	X	X	X
Rieder	10	✓	X	X	X	✓	X	X	✓	X	X	X
Rieder	11	X	X	X	X	X	✓	✓	X	X	X	X
Rieder	12	X	X	X	X	X	X	X	X	X	X	X
Rieder	13	X	X	X	X	X	X	X	X	X	X	X

8.1.1.6 Tagging SNPs

Tagging-SNPs capture most of the genetic variation in a region. Small numbers of these highly informative markers can be used in association studies to reduce the number of SNPs needed to detect LD-based association between a trait of interest and a genomic region. Both the HapMap and Parc datasets have identified tagging SNPs within the *ACE* region and have placed them in a number of 'bins'. Within each 'bin' only one tagging SNP needs to be genotyped as the extent of LD within the 'bin' ensures that information is also obtained for other SNPs within the same bin. Unfortunately the nomenclature differs between the 2 databases and so the data is not directly comparable. Additionally the location of tagging SNPs also differs. Table 8.4 details the most informative tagging SNPs published on the HapMap website showing that genotyping merely 5 SNPs would give genotypic information about 22 other linked SNPs. Table 8.5 shows a comparison of the tagging SNP 'bins' published on the HapMap and Parc databases. These bins are made up of highly informative SNPs and also other SNPs (*) which give slightly less information but are nevertheless important markers. It can be seen that there are 14 'bins' identified from the Parc dataset and 4 from the HapMap study. Some bins only contain a single tagging SNP.

Unfortunately out of the SNPs that were genotyped in our study only SNPs 17 (rs4305) and 24 (rs4312) have been highlighted as tagging SNPs. SNP 24 and SNP 10 make up the Parc 'bin 2' which confirms that they are in tight LD and supports our conclusions that they play a similar role in differentiating H9 individuals from those with other haplotypes. Despite being an intronic SNP, SNP 17 has been identified by both databases as a tagging SNP. This SNP is linked with 2 other SNPs close by and collectively these make up Parc 'bin 3' and supports its use as one of the chosen SNPs in our study.

Table 8.4: *ACE* gene Tagging SNPs identified the HapMap Project

Tagging SNPs Suggested by HapMap Consortium (http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B35)	SNPs Linked with the Tagging Snp (NCBI dbSNP No.)	Rieder sequence position (Bp) AF118569	SNP No. in this study
rs4344	Rs4344	15214	56
	Rs4316	10514	28
	Rs4329	11663	41
	Rs4333	12727	45
	Rs4344	15214	56
	Rs4351	18222	63
	Rs4353	18912	65
	Rs4362	22251	74
	Rs4363	22982	75
rs7221780	Rs7221780	?	na
	Rs4316818	?	na
	Rs4459610	?	na
	Rs11658531	?	na
	Rs10853044	?	na
	Rs4267385	?	na
	Rs4611524	?	na
	Rs7221779	?	na
rs8066114	Rs8066114	?	na
	Rs12451328	?	na
	Rs4968591	?	na
rs4461142	Rs4461142	?	na
rs8066276	Rs8066276	?	na

Table 8.5 Comparison of *ACE* Tagging SNPs identified in HapMap (http://pga.gs.washington.edu/gty_data/ace/) and Seattle SNP (Parc) (http://droog.gs.washington.edu/parc/data/ace/) Databases

PARC Database SNP Location	NCBI BP Number AF118569	SNP 'rs' Number	SNP Number Used in This Study	Parc Dataset Tagging SNP Bin Number	HapMap Dataset Tagging SNP Bin Number
3604				11	
3586	3872			3	2
4218	4504			3	2
5120	5406	s4298	10	2	3
5693	5979			8	
6149	6435	rs4305	17	3	2
7843	8129			1*	
8683	8969			1*	1*
8824				14	
8906	9191	rs4312	24	2	3
8937	9223			2	3
10414	10700			1	1
10474	10760			1	1
10695	10981			1	1
10876	11162			1	1
11092	11378			1	1
11121	11405	rs4325	37	1	1
11163	11449			1	1
11194	11480			1	1
11379	11663	rs4329	41	1	
11582	11868			1*	1*
11973	12259			1	1*
12202	12488			1	1
12381	12667			6	
12443	12727	rs4333		1*	1*
12861	13147	rs4334	46	1	1
12946	13232			1	1
13813	14099			1	1
14644	14930			1	1*
16419	16705			10	
16497	16783			4	
16896	17182			4	
17652	17938			1*	1*
18342	18628			1	1
19130	19416			9	
19372	19658			5	
19827	20113			5	
20263	20549			1	1
20616				13	
21095				12	
21681	21967			1	1
22412	22698			1	1
22632	22918			7	

8.1.6 *ACE* Gene Haplotype Studies

Over recent years haplotype studies of the *ACE* gene have begun to appear in the literature. To date no *ACE* gene haplotype studies on ischaemic stroke patients have been published. The following section will summarise the result on studies conducted on the *ACE* gene in the fields of hypertension, MI, diabetes and Alzheimer's disease.

Zhu et al (2000) genotyped 7 polymorphisms in separate samples of Afro-Caribbean and European individuals spanning a 13 kb region within the 3' end of *ACE*. They identified 3 distinct haplotype segments. The greatest linkage disequilibrium was identified in the middle region and using cladistic analysis they concluded that this was most likely to house the *ACE* linked quantitive trait locus (Zhu et al., 2000). Fewer haplotypes were identified in the sample of European patients showing that the greater genetic diversity of Afro-Caribbean subjects is more informative for fine scale mapping. In a follow-on study of 332 Nigerian families in 2001, the same group genotyped 13 *ACE* polymorphisms and found all to be significantly associated with serum ACE levels. A240T and A2350G were found to explain the greatest variation and these were the most significantly associated with high blood pressure (Zhu et al., 2001). This finding had not been identified in previous studies of ACE levels and hypertension.

Further cladistic analyses have been conducted in 4000 Swedish individuals by Katzov et al (2004). In males they demonstrated significant associations between *ACE* SNPs and the metabolic traits of fasting plasma glucose levels, insulin levels and obesity. When analysing patients with MI and Alzheimer's disease using cladistic haplotype analyses they noted that this analysis consistently outperformed analyses based upon single markers. They identified significant associations with a greater effect size than those previously seen in analyses of the *ACE* I/D polymorphism. Frequencies of *ACE* genotypes varied with age suggesting that certain genotypes may be protective and lead to greater longevity (Katzov et al., 2004).

Haplotype studies of Alzheimer's disease (AD) involving 2861 individuals from three European populations have been conducted by Kehoe et al 2004. Two markers were

linked with earlier age at onset of the disease and were independent of the genotype at the other associated gene in AD, *APOE4,* in both male and female subjects. The 2 markers were rs4343 (SNP 55) in exon 17 (P<0.0001), and rs4291 (P=0.0095) located in the ACE promoter (Kehoe et al., 2004).

More recently Meng et al (2006) (Meng et al., 2006) has looked at 15 SNPs within the *ACE* gene (including the *I/D* polymorphism) in a sample of 92 AD patients compared with 166 controls in an Israeli Arab community. They also identified a significant association with SNPs rs4343 as well as an adjacent SNP rs4351 (p= 0.00001 and 0.01 respectively). The haplotype generated by these SNPs was seen in a frequency of 0.21 in cases and 0.01 in controls conferring a 45 fold increased risk of developing AD compared with any of the three other haplotypes identified.

Neither rs4343 or rs4291 are tagging SNPs for this region of the *ACE* gene in the HapMap (http://droog.gs.washington.edu/parc/data/ace/) or Seattle SNP (Parc) databases (http://pga.gs.washington.edu/gty_data/ace/) nor are they in tight linkage disequilibrium with any tagging SNPs and so this would enhance their candidacy as functional variants. Neither of these SNPs were genotyped in our study. In contrast, rs4351 is in tight linkage disequilibrium with the tagging rs4344 in the HapMap database. Genotyping this SNP also generates information from SNPs rs4316, rs4329, rs4333, rs4344, rs4351, rs4353, rs4362 & rs 4363 and so this suggests that this particular haplotype may be associated with a functional variant in the *ACE* gene in this location. These 2 studies have identified interesting results which will need further analysis of larger numbers of AD in different populations.

8.1.7 The Application of Contemporary Knowledge to This Study

The use of the dbSNP and the HapMap databases and haplotype inference methods would have negated the need to perform the time consuming allele specific PCR experiments detailed in Chapters 4 and 5. The use of tagging SNPs in place of the chosen SNPs would have been more informative and improved the depth and quality of information that was generated from the genotyping experiments. Many more than 13 haplotypes have now been identified at the *ACE* locus by the HapMap and Seattle SNP projects and this information would have provided a much more robust association analysis than was possible using the chosen methodology. Additionally it may have reduced the number of individuals who we were unable to assign haplotypes to.

Any one of the newer SNP genotyping techniques that have been described is likely to have improved the quality and quantity of genotype and haplotype data that was generated by RFLP analysis. The likely time savings of the newer higher throughput techniques would have released more time to be spent in the clinical phase of the study. This would have allowed for the enrolment of much larger numbers of individuals with subsequent improvements in the power of the study and thus more confidence in the study results.

8.1.8 Future Research

This study has generated a number of exciting avenues of future research. The finding of an association between an *ACE* haplotype and ischaemic stroke needs further evaluation in the current dataset in view of the genotyping difficulties that occurred. The use of one of the more contemporary genotyping methods outlined earlier would allow for a higher throughput of samples and thus be more cost effective and time efficient. If the association with H9 is maintained then larger scale long term prospective studies would then be indicated involving the collection of between 5000

– 10,000 cases and controls in order to give sufficient power to identify any significant associations. Ascertaining individuals from different geographical backgrounds would increase the numbers of *ACE* haplotypes and thus allow for a more robust study of the gene.

Phenotyping should ideally utilise information from MRI (including diffusion, perfusion and angiographic studies to give clearer information about the presence of large or small vessel disease), carotid dopplers (studying the extent of extra-cranial atheromatous large vessel disease), transoesophageal echocardiography (to identify routes of paradoxical emboli), clotting studies (fibrinogen), inflammatory markers (ESR, CRP) and serum ACE levels. Using surrogate markers such as cerebrovascular white matter hyperintensity {Carmelli, 1998 #621} and carotid intima / media thickness may improve the power to detect real differences.

Genotyping experiments should incorporate analyses of the tagging SNPs published on the HapMap and other SNP databases in order to harness the power of LD to produce a more complete study of the entire gene. As SNP 10 has been identified in this study as being a major discriminator of H9 from other haplotypes and is one of the tagging SNPs identified in the HapMap and Seattle SNP projects, it suggests either a functional role or tight linkage with another functional polymorphism, thus direct genotyping of this marker should be incorporated into future studies. Similarly the results of the studies on AD by Meng and Kehoe and their colleagues (Kehoe et al., 2004(Meng et al., 2006)) which has identified SNPs rs4343 and rs4291 as possibly having functional relevance in AD should make these important markers for any future studies on the *ACE* gene.

The findings of genetic associations of haplotypes spanning multiple genes ({Marre, 1997 #1054}{Pei, 1997 #1078}{Yoon, 2002 #1075}) suggesting functional interactions of genes within common physiological pathways should also be explored in ischaemic stroke by harnessing the power of micro-array chip technology to perform a wider ranging study of multiple SNPs within many genes.

Confirmation of haplotype associations with ischaemic stroke would then allow in-vitro and in-vivo analysis of *ACE* in order to study whether different haplotypes produce different functions of the gene under a variety of physiological conditions.
From the clinical view-point *ACE* haplotype assessment could potentially be incorporated into routine assessments of 'at risk' individuals to allow preventative strategies to be more focussed, initiated earlier and more aggressive than is currently possible.

The hugely rewarding development of the clinical and DNA resource in this project will allow for future case control analyses of other candidate genes for ischaemic stroke, and the other comorbid conditions that were identified in the study subjects, thereby allowing study of epistatic phenomena and gene-environment interaction. Collaboration with other national and international groups will also be possible as the interest in this fascinating, complex but vastly important area of medicine increases.

CHAPTER 9

APPENDICES

Chapter 8: List of Tables

9.1 Diagnosis of Stroke

A detailed history and bedside examination are usually all that is required to differentiate acute stroke from its mimics (tumours, systemic infection, post-ictal deficits, and metabolic disturbances) (Bamford 1992). However little directed research has been carried out to assess the process of stroke diagnosis (IWP 2000). US studies have shown that emergency personnel (ambulance and A+E staff) are extremely good at identifying the signs of acute stroke with levels of accuracy ranging from 76-95% (Kothari et al. 1995a; Kothari et al. 1995b; Libman et al. 1995). Experienced stroke physicians have a higher pick up rate with only 5 out of 325 false positive 'clinically definite first stroke' diagnoses made in the OCPS (2 sub-dural; 2 glioma; 1 metastasis) (Sandercock et al. 1985) and 3 out of 411 false negatives. However in the acute setting (within 6 hours of symptom onset) the diagnostic accuracy may be somewhat lower. In a series of 70 patients clinically diagnosed within 6 hours of symptom onset with an anterior circulation ischaemic stroke, 15 (21.4%) had another type of stroke (intracerebral haemorrhage; small vessel occlusion) and 6 (8.6%) had not had a stroke (3 metabolic upset; 1 migraine; 1 alcohol withdrawal; 1 hysteria) (Allder et al. 1999).

9.1.1 Imaging Stroke

X-Ray (Computerised Axial Tomography (CT) and magnetic (Magnetic Resonance (MRI) imaging are the commonest forms of cerebral imaging.

9.1.1.1 CT Imaging

CT is easily accessible in most clinical settings and is helpful in the identification of cerebral haemorrhage and tumour which may not be identified clinically. The importance of early radiological diagnosis is now being highlighted by the availability of immediate thrombolysis therapy for acute cerebral infarction which requires rapid exclusion of cerebral haemorrhage – a task that cannot be performed clinically. CT signs of cerebral infarction may be seen a few hours after the ictus however up to 50% of clinically definite strokes may not have a diagnostic lesion on a CT scan (Davenport and Dennis 2000) and after two weeks, the scan may be classified incorrectly (Dennis et al. 1987).

9.1.1.1.1 Acute Infarcts

The earliest suggestion of acute cerebral infarction may be a hyperdensity within a cerebral vessel. The first parenchymal changes visible on CT reflect the cytotoxic oedema affecting primarily the gray matter and can be seen within a few hours. As the effects become permanent the normal gray-white contrast is lost as the oedematous cortex becomes isodense to the underlying white matter. After 6-8 hours the accompanying vasogenic oedema highlights the areas of brain infarction. With large infarcts the fluid shifts increase local pressure which is responsible for sulcal and ventricular effacement and subsequent mass effect which becomes maximal at about five days.

9.1.1.1.2 Subacute Infarcts

The subacute stage begins during the second week with capillary proliferation in the area of infarcted brain tissue which has lost the protection afforded to it by the blood brain barrier (BBB). This allows radio-opaque contrast to freely diffuse into the interstitial spaces and give distinctive gyral enhancement which can last up to 8 weeks. As an infarct evolves, it becomes progressively lower in density on CT eventually approaching the density of CSF. As the mass effect resolves the infarcted tissue is resorbed allowing 'ex-vacuo' enlargement of the adjacent sulci and ventricle. The end result is a chronic infarct with focal areas of cystic encephalomalacia and some surrounding parenchymal change due to gliosis.

9.1.1.1.3 Arterial Infarction

In large cerebral vessel occlusion the CT abnormality often follows these vascular territories involving both the cortex as well as underlying white matter. This allows differentiation from inflammatory and neoplastic disease. If ischaemia results from systemic hypotension or hypoxia, infarction is commonly found in watershed areas between the major vascular territories.

Lacunar infarcts are smaller infarcts (less than 1 cm) and are found in the basal ganglia, thalamus and brainstem. MRI is far more sensitive than CT for detecting small lacunar infarcts, particularly in the brainstem where CT scans are often degraded by artifacts from the bone at the skull base (Brown et al. 1988).

9.1.1.1.4 Venous Infarction

Venous infarction occurs as a consequence of cerebral venous sinus occlusion leading to elevation in venous pressures and engorgement of the brain parenchyma. This may mimic arterial ischaemic stroke. Acute thrombus is hyperdense on CT and may be detected within one of the major sinuses or cortical veins. The "empty delta sign" may be seen due to nonfilling of the superior sagittal sinus on a contrasted scan. Venous infarction is often seen soon after the ictus (earlier than expected with arterial occlusion) and affects peripheral areas of the brain, sometimes bilaterally in a parasagital distribution. As arterial perfusion is maintained to the damaged tissue hemorrhagic infarction is not uncommon. MR imaging is far more superior to CT for diagnosing venous abnormalities.

9.1.1.2 MR Imaging Of Acute Stroke

Conventional CT and MR imaging are not sufficiently sensitive to evaluate acute stroke. MR scans can detect acute stroke changes by 6 to 12 hours, but the few available therapies are only successful within the first 3 hours after onset. The ultimate goal for imaging is to define the area of brain infarction and perfusion deficit, and to identify any ischemic tissue that can be salvaged by medical or surgical therapy. MR diffusion and perfusion -weighted imaging can provide valuable information in the early stages of cerebral infarction. Diffusion weighted imaging can detect acute brain infarction within 1 to 2 hours and perfusion imaging is positive immediately after arterial occlusion has occurred.

9.1.1.2.1 Diffusion-Weighted Imaging

By making use of the random movement ("Brownian motion") of water molecules in brain tissue diffusion imaging is very helpful in identifying acute ischemia and separating this from all other conditions which are not associated with cytotoxic oedema (old strokes, infection, neoplasia, contusions, and demyelinating diseases). In healthy tissues cell membranes, vascular structures, and axons limit the amount of water movement. Diffusion-weighted images are obtained by adding a pair (dephasing and rephasing) of strong gradient pulses to the MR sequence. If net movement of spins occurs between the gradient pulses, signal attenuation occurs.

The diffusion data can be presented as signal intensity or as an image map of the apparent diffusion coefficient (ADC). A low ADC corresponds to high signal intensity (restricted

diffusion), and a high ADC to low signal intensity. In acute cerebral ischemia, as the cell membrane ion pump fails, excess sodium and then water enters the cell causing cytotoxic oedema. Diffusion of the intracellular water molecules is restricted by the cell membranes resulting in a decreased ADC and increased signal intensity on density-weighted images. Severe ischemia can lower the ADC by as much as 56% of normal tissue at 6 hours.

9.1.1.2.2 Perfusion Imaging

Measurements of brain perfusion include vascular transit time, cerebral blood volume, and cerebral blood flow. Cerebral blood volume measurements can be obtained easily with conventional 1.5 Tesla MR systems using a gradient-echo technique. The most common technique is called dynamic contrast-enhanced susceptibility-weighted perfusion imaging. It measures the changes in intravascular susceptibility as a bolus of paramagnetic contrast material, (i.e. gadolinium), passes through the circulation. The amount of signal attenuation is proportional to the cerebral blood volume.

9.1.1.2.3 Comparison of Diffusion and Perfusion Weighted imaging.

The goal of these techniques is to detect the 'ischaemic penumbra'). The presence of the penumbra implies that local thrombolysis will allow reperfusion of theses areas and thereby salvage ischaemic tissue. The ischaemic penumbra would therefore appear as an area with reduced or absent perfusion but some evidence of water diffusion suggesting that the tissue may still be viable.

9.1.1.2.4 MR Angiography (MRA)

In the context of acute stroke MRA has an important role in the posterior circulation which is inaccessible to ultrasound. Imaging the neck vessels takes about 10 minutes and overall the accuracy is reasonable for estimating stenoses, although the method tends to overestimate the degree and length of stenosis. Very slow flow distal to a critical stenosis is difficult to detect and may be confused with a complete occlusion. MRA is also useful in cases of suspected arterial dissection with spin-echo images being very sensitive for detecting the intramural haemorrhage in the false lumen.

MRA can also evaluate the major intracranial arteries around the circle of Willis. The resolution is lower, and flow artifacts limit accuracy in arteriosclerotic disease. Estimates of

carotid artery stenosis are possible following anterior circulation stroke however catheter angiography remains the gold standard.

9.1.1.2.5 Catheter Angiography

Catheter angiography provides views of the aortic arch, carotids and the intracranial circulation. When contemplating carotid angioplasty and stenting it is important to identify arch disease which may produce distal emboli during the procedure. The techniques allow accurate detection of carotid artery plaques and stenoses which may be amenable to surgery or endovascular techniques.

In acute ischaemic stroke angiography provides a method of localizing cerebral arterial occlusion and direct infiltration of thrombolytic agents to improve perfusion.

9.2 The Treatment Of Stroke

The last 10 years has been an exciting time for researchers and clinicians working in the field of stroke medicine. We are increasing our understanding of the precise underlying pathological mechanisms of the various subtypes of stroke which is improving the scope of acute and chronic treatments.

Over the last few decades the rates of hospital admission for acute stroke has increased from 55% in the 1980s in the OCSP (Bamford et al. 1986) to 70-84% in the 1990s (Wolfe et al. 1993; Wolfe et al. 1995; Du et al. 1997; Stewart et al. 1999; Collaboration 2000). However patients continue to be cared for in a variety of different settings including home, residential and nursing homes, day centres, hospital at home schemes, outpatient clinics and hospital medical wards. Consensus exists that in order to comply with the acute stroke guidelines, patients should be urgently referred to specialist care services so that they can be monitored and treated for fever, hyperglycaemia and dehydration as well as signs of impaired airway control so as to reduce risks of aspiration (Wolfe et al. 2001).

9.2.1 Acute Treatments

Table 8.2 shows the conclusions from recent Cochrane Stroke group reviews of acute stroke treatments. Although there have been many exciting prospective interventions based upon animal studies and studies in similar human diseases, very few have shown beneficial effects in acute ischaemic human stroke. There are currently only two interventions for which there is some RCT evidence of beneficial effect, aspirin and thrombolysis.

9.2.1.1 Aspirin

The pooled results of the International Stroke Trial (IST) and the Chinese Stroke Trial comparing aspirin with placebo, concluded that medium dose aspirin (160-300 mg) started in the acute phase of an ischaemic stroke produces a small net benefit (6% risk reduction, 13 fewer patients per 1000 dead or disabled) (Chen et al. 2000). It is unclear whether this benefit is due to an effect on the stroke itself or simply through earlier initiation of secondary prevention of a further stroke or other thrombotic complications (Gubitz et al. 2000b).

9.2.1.2 Thrombolysis

A recent systematic review of 12 randomised controlled trials of thrombolysis suggests that although thrombolytic therapy (with recombinant tissue plasminogen activator (r-TPA), streptokinase, or urokinase) is associated with 70 symptomatic (50 fatal) intracranial bleeds per 1000 patients treated, its use is associated with 65 more patients surviving free of dependency 3 to 6 months after the stroke (Wardlaw et al. 1997). Results of treatment with r-TPA within 3 hours of symptoms demonstrate greater long term benefit with 130 extra patients alive and independent per 1000 treated as well as a lower risk of early intracranial haemorrhage. The current UK recommendations have taken a conservative approach and as a result 94.1% of UK stroke physicians have 'rarely or never used thrombolysis' (Ebrahim 1999) The Cochrane review concluded that further trial evidence was required before thrombolytic therapy is adopted on a wider scale (Wardlaw et al. 2000). The 3rd International Stroke Trial is currently studying the effects of thrombolysis within 6 hrs of acute ischaemic stroke (http://www.dcn.ed.ac.uk/ist3/).

9.2.1.3 Anticoagulants (Heparins)

Anticoagulants are thought to prevent progression of thrombosis and early recurrence of embolic stroke. The use of high dose (20,000 U/24 h) heparin followed by oral anticoagulant therapy is well accepted as early treatment for ischaemic stroke in patients with a cardiac source of embolism or disorders such as the antiphospholipid antibody syndrome. The ideal timing of intervention is often disputed because of the early risk of haemorrhagic transformation in the ischaemic area. Two other conditions are commonly associated with the early administration of full dose intravenous heparin, progressing ischaemic stroke, and crescendo TIA, however, there is no randomised evidence in support of this intervention.

A recent systematic review of 20,000 patients receiving immediate anticoagulant therapy for all sub-categories of ischaemic stroke concluded that the risk of DVT and pulmonary embolism were reduced but there were no short or long term benefits in terms of independent survival (Gubitz et al. 2000a).

9.2.1.4 Other Acute Stroke Therapies

These have been reviewed by the Cochrane collaboration and summarized in Table 8.2. Interventions that show some promise, but for which there is insufficient evidence of effectiveness to recommend them currently outside clinical trials, include cooling therapy and

fibrinogen depleting agents (Ancrod). Acute treatments that are in common use in several parts of the world but without good supportive evidence include anticoagulants, including low molecular weight heparins and deliberate lowering of BP.

Table 8.2 Summary of Cochrane Reviews of Acute Stroke Treatments

Date of Review	Review	Reviewer's Conclusion
Effective / promising treatments		
May 1999	Antiplatelet therapy for acute ischaemic stroke	Aspirin (PO or PR 160 - 300 mg od) within 48 hours of onset reduces the risk of early recurrent ischaemic stroke without a major risk of early haemorrhagic complications and improves long-term outcome.(Gubitz et al. 2000b)
July 1999	Thrombolysis for acute ischaemic stroke	Increases deaths within the first 7-10 days and at final follow-up as well as significantly increasing symptomatic and fatal intracranial haemorrhage. These risks are offset by a reduction in disability in survivors giving a significant net reduction in the proportion of patients dead or dependent in activities of daily living Recombinant tissue Plasminogen Activator (tPA) has less hazards and more benefit. Further trials are needed to identify the patients most likely to benefit from treatment (Wardlaw et al. 2000)
Ineffective Treatments		
Feb 1999	Anticoagulants	No Benefit (Gubitz et al. 2000a)
Jan 1999	Piracetam	Some suggestion of an unfavourable effect (Ricci et al. 2000)
July 2001	Tirilazad	Appears to worsen outcome (TISC 2000)
Unproven Treatments		
May 1999	Cooling therapy	Insufficient evidence but improvements seen for severe closed head injury, so trials in acute stroke are warranted (Correia et al. 2000)
Nov 1996	Fibrinogen-depleting agents	Ancrod appears to be promising but not possible to draw reliable conclusions from the available data (Liu et al. 2000)
March 1999	Interventions for dysphagia	Too few studies with too few patients. PEG feeding may be better vs NG feeding. Further research is required to assess type & timing of feeding and the effect of swallowing or drug therapy on dysphagia (Bath et al. 2000c)
Oct 2000	Mannitol	Insufficient evidence (Bereczki et al. 2000)
Aug 1999	Haemodilution	As used in RCTs, not proven to improve survival or functional outcome (Asplund et al. 2000)
May 2000	Glycerol	Insufficient evidence (Righetti et al. 2000)
Oct 1998	Corticosteroids	Insufficient evidence (Qizilbash et al. 2000)
Feb 2001	Interventions for deliberately altering blood pressure in acute stroke	Insufficient evidence((BASC) 2001a)
Oct 1999	Calcium antagonists	Insufficient evidence (Horn and Limburg 2000)
Aug 2001	Low molecular weight heparins or heparinoids versus standard unfractionated heparin	Insufficient evidence (Counsell and Sandercock 2000)
Aug 1997	Nitric oxide donors (nitrates), L-arginine, or nitric oxide synthase inhibitors	Insufficient evidence (Bath et al. 2000a)
June 1996	Pentoxifylline, propentofylline and pentifylline	Insufficient evidence (Bath et al. 2000b)
Jan 1998	Prostacyclin and analogues	Insufficient evidence (Bath and Bath 2000)
March 1999	Theophylline, aminophylline, caffeine and analogues	Insufficient evidence (Mohiuddin et al. 2000)
April 2000	Vasoactive drugs	Insufficient evidence ((BASC) 2001b)
July 1997	Vinpocetine	Insufficient evidence (Bereczki and Fekete 2000)
Aug 2000	Gangliosides	Insufficient evidence, may cause GBS (Candelise and Ciccone 2000)

The recommendations on acute ischaemic stroke management of the Intercollegiate Working Party for Stroke (IWP), Scottish Intercollegiate Guidelines Network (SIGN) and the Edinburgh Consensus Conferences on stroke (ECC) are summarised in Table 8.3

Table 8.3 Summary of the Guidelines of the Acute Treatment of Ischaemic Stroke

CT scanning/brain imaging	Should be undertaken within 48 h (ideally within 24 h (ECC)) (and no later than seven days.) (SIGN) unless there are good clinical reasons for not doing so.(IWP) Urgent imaging if the following are suspected: Sub-arachnoid haemorrhage, Hydrocephalus, Trauma. OR in the context of : Anticoagulant treatment, or with a known bleeding tendency. Clinical deterioration
Aspirin	300 mg should be given as soon as possible after the onset of stroke symptoms (if a diagnosis of haemorrhage is considered unlikely). (IWP) Early treatment with aspirin (150-300 mg daily) is recommended, starting as soon as intracranial haemorrhage is excluded by CT brain scanning. (SIGN) Should be commenced within 48 h, or as soon as the diagnosis of cerebral infarction has been made (dose: 150-300 mg). (ECC)
Thrombolytic therapy	Thrombolytic treatment with tPA should be given only in a specialist centre, within 3 h of stroke onset (when haemorrhage has been definitely excluded). (IWP) It is reasonable to use thrombolytic therapy (for example rtPA) in highly selected patients in a carefully monitored environment. (ECC)
Other drug therapies	No other drug treatment aimed at treatment of the stroke should be given unless a part of a randomised controlled trial. (IWP)
Compression stockings	Where stroke has caused weak or paralysed legs, full length compression stockings should be applied (unless contraindicated) to prevent venous thrombosis. (IWP) Physical methods of preventing DVT in stroke patients should be evaluated. (ECC)
Other	There should be local policies for the early management of hypertension, hyperglycaemia, hydration and pyrexia. (IWP)
Notes: IWP: Intercollegiate Working Party for Stroke; SIGN: Scottish Intercollegiate Guidelines Network; ECC: Edinburgh Consensus Conferences on stroke.	

9.2.2 Medium and Long Term Treatments

9.2.2.1 Stroke Rehabilitation

The evidence in favour of the process of stroke rehabilitation as a whole is good. (Cochrane Collaboration 2000) The evidence for the individual components of rehabilitation however is a lot weaker.

9.2.2.1.1 Stroke unit care

Among patients admitted to hospital, a major distinction is whether patients are treated on a stroke unit, or in a general medical ward. The term 'stroke unit' care has come to be synonymous with 'organised in-patient' care. The Stroke Association's 1998 survey of consultant physicians in the UK estimated that approximately 50% of stroke patients were cared for in an organised stroke service however only 12% of the responders admitted patients directly to a stroke unit (Ebrahim 1999). This is reflected in the National Sentinel Audit for stroke conducted in the same year which showed only 18% of patients spent more than half of their in-patient stay on a stroke unit, 15% on a rehabilitation unit, and 67% on general wards (Rudd et al. 1999).

The benefits of stroke units was highlighted by Evans et al who reported that there was more intensive monitoring, earlier use of assisted feeding with measures to reduce aspiration and greater use of oxygen and antipyretics (Evans et al. 2001)

9.2.2.1.2 Physiotherapy

A systematic review of 7 RCTs of post stroke physiotherapy involving 597 patients showed that more intensive physiotherapy resulted in lower rates of deterioration and death (OR 0.54; 95% CI: 0.34-0.85) (Langhorne et al. 1996). A separate meta-analysis reached similar conclusions (Kwakkel et al. 1997) and a more recent randomised trial (n=101) of physiotherapy comparing three patient groups (arm training, leg training, and controls) with severe disability following a middle cerebral artery stroke, demonstrated that a greater intensity of rehabilitation improved functional recovery and health-related functional status (Kwakkel et al. 1999).

Table 8.4 Summary of Cochrane Stroke Group Reviews of Rehabilitation Following Stroke

Date of Review	Review	Reviewers' conclusion
Effective OR Promising Treatments		
May 2000	Cognitive rehabilitation for attention deficits	'Improves alertness and sustained attention but no evidence to support or refute the use of cognitive rehabilitation for attention deficits to improve functional independence following stroke.'(Lincoln et al. 2000)
Ineffective & unproven treatments		
Feb 2000	Cognitive rehabilitation for memory deficits	'Insufficient evidence'(Majid et al. 2000)
April 1999	Electrical stimulation for preventing and treating post-stroke shoulder pain	'Benefits for passive humeral lateral rotation but no benefit on pain. Further studies are required.'(Price and Pandyan 2000)
July 2001	Pharmacological treatment for aphasia following stroke	Piracetam may be effective. Further research is needed with comparisons with SALT (Greener et al. 2000a)
July 1999	Speech and language therapy for aphasia	SALT has not been shown either to be clearly effective or clearly ineffective within a RCT. Further research is required (Greener et al. 2000b)
Sept 2000	Speech and language therapy for dysarthria due to non-progressive brain damage	'There is no evidence of the quality required by this review to support or refute the effectiveness of SALT. Urgent need for good quality research in this area.'(Sellars 2000)

9.2.2.1.3 Occupational Therapy (OT)

Domiciliary OT for stroke, both in patients who stay at home (Walker et al. 1999) and patients after discharge from hospital has been shown to be effective in recent RCTs (Gilbertson et al. 2000). A meta-analysis of controlled studies of OT for older people in general including stroke victims found some evidence in support of OT in nursing homes and psychogeriatric wards (Carlson et al. 1996).

9.2.2.1.4 Speech and Language Therapy (SALT)

There is too little evidence from RCTs about the effects of SALT after stroke to draw any conclusions (Greener et al. 2000b). However intensive targeted therapy is effective for some specific dysphasic syndromes (Enderby and Emerson 1996).

9.2.2.1.5 Feeding

Observational studies using historical controls suggest that identification and treatment of dysphagia reduces the risk of pneumonia ((AHCPR) 1999). However, a Cochrane review

concluded that there is a lack of evidence to guide feeding policies (Bath et al. 2000c). Percutaneous endoscopic gastrostomy (PEG) feeding may be better than naso-gastric tube (NG) feeding, but this is based on two small trials only (Norton et al. 1996; Bath et al. 2000c)

9.2.2.1.6 Depression

Trials have been performed of both pharmacological and psychological treatments of post-stroke depression. A recent systematic review concluded that patients with depressive symptoms respond to short-term antidepressant therapy, however psychological treatments suffer from a lack of evidence to make any firm conclusions (House 2001).

9.3 Association Studies Of Candidate Genes in Ischaemic Stroke With Predominantly Negative Results

Table 8.5 Association studies of Ischaemic Stroke for Genes with Predominantly Negative Results

Angiotensinogen					
(Barley *et al*. 1995)	M235T	Case-control: 100 cases, 45 spouse controls	Ischaemic stroke /Carotid IMT/ Carotid Atheroma	Negative	
(Nakata *et al*. 1997)	M235T	Case-control: 55 cases, 61 controls	Ischaemic stroke	Negative	TT-positive interaction with ACE DD genotype

Table 8.6 Haemostasis Gene Studies

Factor V					
(Catto *et al*. 1995)	Q506 Leiden	Case-control: 348 cases, 247 controls	Ischaemic stroke/stroke mortality	Negative	
(Kontula *et al*. 1995)	Q506 Leiden	Case-control: 236 cases, 137 controls	Ischaemic stroke	Negative	
(Ridker *et al*. 1995)	Q506 Leiden	Nested case-control: 209 cases, 209 controls	Ischaemic stroke/PICH	Negative	Association only with DVT and PE
(Markus *et al*. 1996)	Q506 Leiden	Case-control: 180 cases, 80 controls	Ischaemic stroke	Negative	
(van der Bom *et al*. 1996)	Q506 Leiden	Case-control: 112 cases, 222 controls	1schaemic stroke	Negative	Increased APC resistance independent of factor V
(Martinelli *et al*. 1997)	Q506 Leiden	Case-contro1: 155 cases, 155 controls	Ischaemic stroke	Negative	
(Halbmayer *et al*. 1997)	Q506 Leiden	Case-control: 229 cases, 71 controls	Ischaemic stroke	Negative	
Prothrombin					
(De Stefano *et al*. 1998)	G20210A	Case-control: 72 cases, 198 controls	Ischaemic stroke <50 years	Positive	
(Poort *et al*. 1996)	G20210A	Case-control: 104 cases, 104 controls	Ischaemic stroke	Negative	Association with DVT only
(Martinelli *et al*. 1997)	G20210A	Case-control: 155 cases, 155 controls	1schaemic stroke		
(Longstreth *et al*. 1998)	G20210A	Case-control: 106 cases, 391 controls	Ischaemic stroke young women age 18-44 yrs	Negative	
(Ridker *et al*. 1999)	G20210A	Nested case-control: 259 cases, 1744 controls	Ischaemic stroke/PICH	Negative	Weak association with DVT
Factor VII					
(Heywood *et al*. 1997)	R353Q	Case-control: 286 cases, 198 controls	Ischaemic stroke/OCSP subtype/stroke mortality	Negative	No association with stroke subtype or mortality
(Corral *et al*. 1998)	R353Q/323 A2	Case-control: 104 cases, 104 controls	Ischaemic stroke	Negative	
PAI1					
(Catto *et al*. 1997)	*4G/5G*	Case-control: 421 cases, 172 controls	Ischaemic stroke/OCSP sub type and mortality	Negative	No association with stroke sub type or mortality
Factor X III					
(Catto *et al*. 1998)	Va134Leu	Case-control: 529 cases, 427 controls	Ischaemic stroke/OCSP subtype	Negative	Weak association with PICH. No association with ischaemic stroke subtype

Table 8.6 (Continued) Association studies Of Ischaemic Stroke for Genes with Predominantly Negative Results

Reference	Polymorphism	Methodology	Measure	Results	Comments
MTHFR					
(Harmon *et al*. 1999)	C677T	Case-control: 174 cases, 183 controls	Ischaemic stroke CT proven >60 years	Inconclusive	OR 1.59 (Cl = 0.85-2.97)
(Markus *et al*. 1997)	C677T	Case-control: 345 cases, 161 controls	Ischaemic stroke/TOAST subtype	Negative	No association in folate deficient patients, young individuals or carotid atheroma
(Lalouschek *et al*. 1999)	C677T	Case-control: 81 cases, 81 controls	TIA or minor ischaemic stroke	Negative	Possible synergism between MTHFR and Q506 al1eles
(De Stefano *et al*. 1998)	C677T	Case-contro1: 72 cases, 198 controls	Ischaemic stroke <50 years	Negative	
(Reuner *et al*. 1998)	C677T	Case-contro1: 91cases, 182 controls	Ischaemic stroke	Negative	
eNOS					
(Yahashi *et al*. 1998)	eNOS 4a/b	Case-control: 127 cases, 91 controls	Atherothrombotic/lacunar/ silent stroke	Negative	
(Markus *et al*. 1998)	Glu298Asp	Case-control: 361 cases, 236 controls	Ischaemic stroke/TOAST subtype	Negative	No association with carotid atheroma
(MacLeod *et al*. 1999)	Glu298Asp	Case-control: 265 cases, 293 controls	Ischaemic stroke	Negative	
Apo A1/CIII					
(Patsch *et al*. 1994)	*XmnI*	Cross-sectional: 268 cases	IMT in groups with different lipid profiles	Positive	6.6 kb allele a risk factor in group with elevated cholesterol and triglyceride
(Aalto-Setala *et al*. 1998)	*SstI*	Cross-sectional: 234 cases	Carotid atherosclerosis in patients with ischaemic stroke <60 years	Negative	
Apo B					
(Aalto-Setala *et al*. 1998)	*XbaI*	Cross-sectional: 234 cases	Carotid atherosclerosis in patients with ischaemic stroke <60 years	Negative	
Lipoprotein lipase					
(Huang *et al*. 1997)	A291G	Case-control: 125/56 cases, 95 controls	Ischaemic stroke / carotid atherosclerosis	Negative	
Paraoxonase I					
(Schmidt et al. 1998a)	glul92arg	Cross-sectional: 316 cases	Carotid atherosclerosis 44-75 years	Positive	leu541eu genotype met541eu
(Cao *et al*. 1998)	glu192arg	Cross-sectional: 197 cases	IMT in NIDDM	Negative	

ACE = Angiotensin Converting Enzyme. I/D = insertion / deletion polymorphism. PAI 1 = Plasminogen Activator Inhibitor. Gp = Platelet glycoprotein receptors. MTHFR = methylene tetrahydrofolate reductase. eNOS = endothelial nitric oxide synthase. apoE = apolipoprotein E. OCSP = Oxford Community Stroke Project Classification; TOAST = trial of org10172 in acute stroke treatment; PICH = primary intracerebral haemorrhage; APC= activated protein C resistance; NIDDM = non-insulin-dependent diabetes mellitus; IMT = carotid intima media thickness; CHD = coronary heart disease; DVT = deep vein thrombosis; PE = pulmonary embolism; TIA = transient ischaemic attack.

Adapted from (Hassan and Markus 2000).

Molecular Methodology

9.3.1 DNA Extraction

Table 8.7 Preparation of DNA from 10 ml of Whole Blood (in EDTA Tubes using Guanidine Hydrochloride).

1)	Thaw blood samples at room temperature.
2)	Lyse red cells by adding 45 ml of ice cold distilled water to the 10 ml of blood.
3)	Centrifuge at 2500 revolutions per minute (rpm) for 20 min at 4°C to produce a pellet of cellular debris.
4)	Pour off supernatant and retain the pellet.
5)	Wash the pellet and dissolve any remaining lipid rich leucocyte membranes by adding 35 ml of ice cold 0.1% (v/v) Nonidet (NP40) detergent. Vortex until the pellet has dissolved.
6)	Centrifuge at 2500 rpm for 20 min at 40°C to re-precipitate the pellet.
7)	Pour off the supernatant.
8)	Preciptate the DNA by adding 7 ml filtered Guanidinium Hydrochloride (6 M) and 0.5 ml Ammonium Acetate (7.5 M). Vortex to disperse the pellet.
9)	Enzymatically remove unwanted intracellular proteins by adding 0.5 ml of Sodium Sarcosyl (20%, w/v) and 150 µl Proteinase K (10 mg/ml).
10)	Incubate at 60°C for at least 90 min.
11)	Precipitate the DNA by adding 1 ml 96% (v/v) ethanol and mix gently.
12)	Spool out the DNA and redissolve overnight in 1 ml TE buffer (10mM Tris-HCl buffer, 1mM EDTA, pH 8.0).
13)	Re-precipitate the samples by adding 100 µl Sodium Acetate (3 M, pH 5.5) and 2.5 ml ice cold 96% (v/v) ethanol.
14)	Spool out the DNA, allow to dry and re-dissolve overnight in 1 ml TE buffer
15)	Calculate the concentration of the stock DNA using an optical densitometer.
16)	Dilute the stock DNA to 50 ng/µl for use in polymerase chain reactions.

9.3.2 The Polymerase Chain Reaction (PCR)

PCR is a novel method to selectively amplify DNA fragments without cloning. PCR involves the annealing of flanking chemically manufactured oligonucleotide probes (primers) usually 15-20 bases in length, complementary to the DNA sequence of interest (amplicon). Specific amplification of the amplicon is achieved by repeated rounds of DNA synthesis between the flanking primers. One primer is complementary to the DNA sequence 5' of the target sequence, and the second is complementary to the antisense strand 3' to the target sequence.

After the DNA sequence is denatured the primers anneal to their target sequences. Annealing temperatures differ depending upon the primers used. The amplification process is catalysed by a DNA polymerase which is resistant to the heat required by the denaturation process. When DNA synthesis is completed, the old and newly synthesized DNA strands are separated by denaturation, and the cycle is repeated. Subsequent cycles produce greater quantities of newly amplified specific DNA sequences compared with the initial non specific template thereby making the annealing process more specific. Typically 35 cycles of PCR are performed per experiment giving exponential increases in the mass of the required sequence.

Automated PCR reactions are possible using purpose built PCR blocks. A typical PCR cycle is as follows:

Denaturation at 95° C for 30 seconds.
Annealing at 55° C for 1 minute
Primer extension at 72° C for 1 minute
Repeated for 35 cycles
Final extension 72° C for 5 minutes

The specificity of PCR is primarily determined by the annealing temperature. Lower annealing temperatures lead to reduced sequence specificity. Higher temperatures may prevent annealing.

9.3.2.1 Allele Specific PCR

This is identical to conventional PCR in every way except the final base in the primer sequence is designed to specifically anneal to a SNP but not the wild type base, or vice versa. A particular locus may either have an Adenine (A) or Cytosine (C) base, for example, and if PCR conditions are stringent enough then the primer complementary to the A will not anneal to the C and so pure A allele sequence will be obtained.

9.3.3 DNA SEQUENCING

The ability to decipher the DNA sequence of a fragment has provided the basis for elucidation of the genetic code and has generated the human genome project. There are several sequencing methods, (enzymatic chain termination, hybridization based approaches, and chemical sequencing), however only the most commonly used enzymatic chain termination method is described further. In this method, a DNA fragment is provided as a template for the synthesis of new DNA strands with a DNA polymerase. The reaction is primed by a sequencing primer usually 17 to 22 bp long that specifically binds to the region being sequenced. In addition to the regular deoxynucleotide triphosphates (dNTPs; N stands for any of the four bases, Adenine (A), Thymidine (T), Guanine (G) & Cytosine (C)), smaller amounts of dideoxynucleotide triphosphates (ddNTPs) are added to the reaction mix. ddNTPs can be incorporated into the growing newly synthesized DNA chain but cause abrupt termination because their lack of the 3' hydroxyl group does not permit formation of the phosphodiester bond.

For each nucleotide a separate sequencing reaction is prepared, each containing the four dNTPs but only one of the ddNTPs in a much smaller concentration. For example, one of the four reactions contains ddATP. Chain termination occurs randomly whenever a ddATP is incorporated into the growing chain and as the number of dATP molecules greatly outnumbers the ddATP molecules, many fragments of different lengths are generated. The length of the fragments can be determined by polyacrylamide gel electrophoresis indicating the presence of an A at this position of the DNA chain. Fragments for the remaining three DNA bases are generated in similar reactions. The fragments can be visualized by radioisotope labelling or with

Fluorophores incorporated into the primer or the dNTPs. The use of different coloured fluorophores for each DNA base permits the running of the four sequencing reactions in one lane of the polyacrylamide gel, thus greatly increasing throughput. The DNA sequence is represented as an intensity profile for each fluorescent dye as detected by a fluorescent detector at the bottom of the gel. Automated sequencing with fluorescent dyes provides generally longer sequences than manual sequences and is currently the preferred method for large scale sequencing.

9.4 The Haplotype Generator HMATCH

(Dr. Simon Ambler, Leicester University)

```
{-
     File: Hmatch.hs
   Author: Simon Ambler
Copyright: University of Leicester, 1999.
  Created: 7th December 1999.
Time-stamp: <99/12/08 12:28:53 sja4>

  Program to analyse the possible haplotypes of a DNA sample from the results of typing a selection of sites.

-}
module Main where
import Char
import List
import System

-------------------------
-- Haplotype sequences --
-------------------------
-- We use the following sites.

sites :: [Int]
sites = [2,13,17,20,21,23,24,28,29,30,32,35,37,39,40,43,47,49,52,53,55,56,57,61,62,69,76]

-- Translate back and forth between the site number and its position in the above list.

site :: Int -> Int
site p  | 0 <= p && p <= 51         = sites !! p
              | otherwise                           = error "Position out of range"

position :: Int -> Int
position s = case lookup s (zip sites [0..]) of
                            Just i  -> i
                            Nothing -> error "Site not listed"

-- The haplotype sequences restricted to the sites listed.
h :: Int -> [Char]

h 1  = "CTAACTACTAGCCCTAGGDCGGACGCC"
h 2  = "CCAGCCACCGGCCCTAAGDCAGGTACC"
h 3  = "CCAGCCACCGGCCCTAAGDCAGGCACC"
h 4  = "TTAGCCATTAATCGTGATIGAAATGTA"
h 5  = "TTAGCCATTAATCGCGATIGAAATGTC"
h 6  = "CTGGTCATTAATAGTGATIGAAACGCC"
h 7  = "CTGGCTACTAGCCCTAGGDCGGACGCC"
h 8  = "CTAGCCATTAATCGTGATIGAAATGTC"
h 9  = "CTAGCCTCTAGCCCTAGGDCGGACGCC"
h 10 = "CTAGCCACTAGCCCTAAGDCAGACGCC"
h 11 = "CTAGCCTCCGGCCCTAAGDCAGACGCC"
h 12 = "CTAGCCATTAATCGCGATIGAAATGTA"
h 13 = "CTAGCCATTAATCGTGATIGAAACGCC"

-- Picking suitable sites for the analysis.
group0, group1, group2, group3 :: [Int]

-- The following sites have already been typed.
group0 = [2, 29, 47, 52, 61, 76]

-- Ideal sites for further testing.
group1 = [17, 20, 24, 32, 40, 43, 55, 56, 69]

-- Okay sites (not too bad but preferably not).
group2 = [23, 28, 35, 37, 49, 53, 57, 62]

-- Poor sites (only if absolutely necessary).
group3 = [13, 21, 30, 39]

---------------
-- Key sites --
---------------
keySites :: [Int]
keySites = group0 ++ [17, 24, 20, 69, 40]

----------------------
```

```
-- Expected results --
----------------------
-- Mixed results are ordered alphabetically,
-- i.e. we write CT rather than TC.

orientate :: String -> String
orientate [c1,c2] = if c1 < c2 then [c1,c2] else [c2,c1]

-- The expected results from Haplotype type pair Hi/Hj.

expect :: Int -> Int -> Int -> String
expect i j s = orientate [h i !! position s, h j !! position s]

expectedResults :: Int -> Int -> [String]
expectedResults i j = [expect i j s | s <- keySites]

--------------------------------
-- Table of expected results --
--------------------------------
type Table = [([String],String)]

-- The expected results for each pair Hi/Hj should be distinct.

testTable :: Table -> Bool
testTable tab = [(nm1,nm2) | (rs1,nm1)<-tab, (rs2,nm2)<-tab,
                             nm1/=nm2, rs1==rs2] == []

table :: Table
table = if testTable tab
            then tab
            else error "Key sites do not discriminate all haplotype pairs ..."
            where
            tab = sort [(expectedResults i j, "H"++show i ++ "/H"++show j)
                          | i<-[1..13], j<-[i..13]]
--------------------------
-- Displaying the table --
--------------------------
layoutTable :: String
layoutTable = unlines [commaList rs ++ " " ++ nm | (rs,nm) <- table]
                  where
                  commaList = foldr1 (\s t -> s++","++t)

outputTable :: String -> IO()
outputTable outfile = writeFile outfile layoutTable

------------------------
-- Processing results --
------------------------
match :: [String] -> [String] -> Bool
match [] [] = True
match [] (y:ys) = False
match (x:xs) [] = False
match (x:xs) (y:ys) = (x==y || x=="??") && match xs ys

possibleMatches :: [String] -> [String]
possibleMatches xs
 = [name | (results, name) <- table, match xs results]

---------------------
-- Read input file --
---------------------
-- items are separated by commas

items :: String -> [String]
items "" =  []
items s  =  let (x, s') = break (== ',') (dropWhile isSpace s)
                          in  x : case s' of
                                      []      -> []
                                      (_:s") -> items s"

readResult :: String -> (String,[String])
readResult str = (label, map orientate results)
            where
            label:results = items str


readResults :: String -> [(String,[String])]
readResults str = map readResult (filter (/="") (lines str))
```

```
getResults :: String -> IO [(String,[String])]
getResults infile
 = do
                contents <- readFile infile
                return (readResults contents)

------------------------
-- Processing results --
------------------------
showMatches :: (String,[String]) -> String
showMatches (identifier, results)
 = identifier ++","++ concat (map (++",") results)
                ++ unwords (possibleMatches results)

------------------------
-- Write output file --
------------------------
putMatches :: String -> [(String,[String])] -> IO()
putMatches outfile results
 = writeFile outfile (unlines (map showMatches results))

------------------
-- Main program --
------------------
hmatch :: String -> String -> IO()
hmatch infile outfile
 = do
                results <- getResults infile
                putMatches outfile results

main :: IO()
main
 = do
                args <- getArgs
                case args of
                  [infile,outfile] -> hmatch infile outfile
                  _ -> putStr "\n\n\nusage: hmatch <infile> <outfile>\n\n\n"

---------
-- End --
---------
```

9.5 SNPs Identified in *ACE*

Table 8.8 Comparison of SNP notation used in this study and sequence position of 2 published sequences of the ACE gene ; Rieder et al 1999 (NCBI sequence no. AF118569) and Genecanvas
sequence published at http://ecgene.net/genecanvas/modules/icontent/index.php?page=70,

Rieder at al 1999 BP position Sequence AF118569	No. in this study	NCBI SNP Database No.	Markers in genecanvas sequence	Alleles	Rieder at al 1999 BP position Sequence AF118569	No. in this study	NCBI SNP Database No.	Markers in genecanvas sequence	Alleles
00117	1			C/T	13336	48 +	rs4336	T22313A	T/A
00334	2	rs4290	A11377T	C/T	13338	49 +	rs4337	G22315T	G/T
02400	3	rs4291		A/T	13432	50	rs4338		A/G
02547	4	rs4292		T/C	13506	51	rs4339		G/T
03872	5	rs4293		G/A	14094	52	rs1799752	D/I-23069	(ALU)/-
04230	6	rs4294		A/C	14480	53 +	rs4341	G23454C	G/C
04504	7	rs4295	G13479C	G/C	14488	54	rs4342	A23462C	A/C
04733	8	rs4296		G/A	14521	55 *	rs4343	A23495G	A/G
04760	9	rs4297	G13735C	G/C	15214	56 +	rs4344	G24188A	G/A
05406	10	rs4298	C14379T	C/T	15435	57	rs4345	A24409G	A/G
05424	11	rs4299		G/A	16020	58	rs4346		T/C
05492	12	rs4300	C14465T	C/T	16988	59	rs4347		C/G
05878	13	rs4301		T/C	17107	60	rs4348		C/T
06028	14	rs4302		C/T	17634	61	rs4349	C26608T	C/T
06029	15	rs4303		G/T	18030	62	rs4350		G/A
06145	16	rs4304		C/T	18222	63	rs4351	A27196G	G/A
06435	17	rs4305	G15408A	A/G	18671	64	rs4352		G/A
06604	18	rs4306		G/A	18912	65	rs4353	A27888G	A/G
07693	19	rs4307	G16664A	G/A	19942	66	rs4354	C28919T	C/T
07831	20	rs4424958	G16804A	G/A	16020	58	rs4346		T/C
08128	21 *	rs4309	C17105T	C/T	16988	59	rs4347		C/G
08935	22	rs4310		G/C	17107	60	rs4348		C/T
08968	23	rs4311	T17944C	C/T	17634	61	rs4349		C/T
09191	24	rs4312		A/T	18030	62	rs4350		G/A
09222	25	rs4313		C/T	18222	63	rs4351		G/A
09509	26	rs4314		C/T	18671	64	rs4352		G/A
10242	27	rs4315		A/-	18912	65	rs4353		A/G
10514	28 * +	rs4316	C19490T	C/T	19942	66	rs4354	C28919T	C/T
10527	29 *	rs4317		T/C	19975	67	rs4355	G28952C	G/C
10578	30 *	rs4318	A19554G	A/G	20058	68	rs4356	T29035C	T/C
10696	31	rs4319	(3G)19673(4G)	G/GG	20120	69	rs4357	C29097T	C/T
10758	32	rs4320	G19735A	G/A	20397	70	rs4358	G29373A	G/A
10979	33	rs4321	C19956T	T/G	20833	71	rs4359	C29809T	C/T
11084	34	rs4322		C/T	21831	72	rs4360		A/G
11159	35 +	rs4323	C20136T	C/T	22155	73	rs4361		A/G
11376	36	rs4324	G20354A	A/G	22251	74	rs4362		C/T
11405	37	rs4325	C20383A	C/A	22982	75	rs4363	A31958G	A/G
11447	38	rs4326	G20425A	G/A	23152	76 *	rs4364	C32128A	C/A
11478	39	rs4327	G20456C	C/G	23202	77	rs4365	G32178A	G/A
11618	40	rs4328	T20596C	T/C	23945	78	rs4366	$(CT)_{3/2}$-32915	(CT)2/3
11663	41 +	rs4329	G20641A	A/G	22155	73	rs4361		A/G
11866	42	rs4330	A20844C	A/C	22251	74	rs4362	C31226T	C/T
12257	43 * +	rs4331	A21235G	A/G	22982	75	rs4363	A31958G	A/G
12486	44 +	rs4332	C21464T	T/C	23152	76 *	rs4364	C32128A	C/A
12727	45	rs4333	C21705T	T/C	23202	77	rs4365	G32178A	G/A
13145	46	rs4334	A22122C	A/C	23945	78	rs4366	$(CT)_{3/2}$-32915	(CT)2/3
13230	47	rs4335	A22207G	A/G					

*=coding

+=LD with I/D polymorphism

.

Table 8.9 Primer Sequences

SNP	Rieder at al 1999 Location. Sequence AF118569	Forward Primer	Reverse Primer
2 (rs4290)	00334	ACE 010 TGCCCAGACAGCCTTATCTC	ACE 011 CTAACAGCTTGGCCTTCCAG
17 (rs4305)	6435	ACE 091 R TTGCTTCACATGCAGGAGAC	ACE 110R TTGAGCCCAGAGCTTACCTG
20 (rs4424958)	7831	ACE 111 R GGGCAGGGTAAAGAATTTGC	ACE 130 R ATTGGCACCTGAAAGACAGG
24 (rs4312)	9191	ACE 143 R AACCAAGTATCAGGGGAT	ACE 152 R AACTCACAAAGTACCTGCA
29 (rs4317)	10527	ACE 170 CTGAGCCCTGGTACCCTGT	ACE 171 CGGCCCGAAATTGAGTATTG
40 (rs4328)	11618	ACE 182 TCAGTCTCTGGGAGGCCTAG	ACE 181 GCTCTGTGGCCTTTCTGAAC
47 (rs4335)	13230	ACE 210 CTCTCCCAGCATGTGACTGA	ACE 211 GCAGTGGCTTACAGAGTCCC
52 (Alu I/D) (rs1799752)	14094	ACE I/D F CTGGAGACCACTCCCATCCTTTCT ACE 1 CATCCTTTCTCCCATTTCTC	ACE I/D R GATGTGGCCATCACATTCGTCAGAT ACE 2 TGGGATTACAGGCGTGATACAG ACE 3 ATTTCAGAGCTGGAATAAAATT
61 (rs4349)	17634	ACE 270 GTTTGGGCAGAACTCCCTCT	ACE 271 CTAGCCTGGAGAGATGCCTG
69 (rs4357)	20120	ACE 302 AATATATGTTGGAAGATGAG	ACE 3l2 AGCCTCAGGTTCTGGAACAC
76 (rs4364)	23152	ACE 353 AGACAGCGGCCGCGTGACCTTC	ACE 76 R AGTGCCGGTGGAGGCAGC

Table 8.10 PCR Reaction Conditions

SNP	Primers (2 µM)	Vol of Each Primer (µl)	Vol of DNA (50 ng/µI) (µl)	Vol of PCR Buffer (µl)	Vol of dNTPs (2 mM) (µl)	Vol of $MgCL_2$ (µl)	Vol of Taq Polymerase	Vol of dH_20 (µl)	Total Vol	Annealing Temp (°C)
2 (rs4290)	ACE 010 ACE 011	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (20 mM)	0.1	4.9	10	61
17 (rs4305)	ACE091R ACE 110R	0.5	1.0	1.0 (ABT10X)	1.0	1.0 (20 mM)	0.1	4.9	10	58
20 (rs4424958)	ACE111R ACE 130R	0.5	1.0	1.0 (ABT10X)	1.0	1.0 (20 mM)	0.1	4.9	10	54/58
24 (rs4312)	ACE 143R ACE 152R	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (20 mM)	0.1	4.9	10	58
29 (rs4317)	ACE 170 ACE 171	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (20 mM)	0.1	4.9	10	63
40 (rs4328)	ACE 182 ACE 181	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (15 mM)	0.1	4.9	10	54
47 (rs4335)	ACE 210 ACE 211	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (20 mM)	0.1	4.9	10	61/ 65
52 (I/D) (rs1799752)	ACE I/DF ACE I/DR	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (15 mM)	0.1	4.9	10	55
52 (I/D) (rs1799752)	ACE 1 , ACE 2 , ACE 3	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (15 mM)	0.1	4.4	10	55
61 (rs4349)	ACE 270 ACE 271	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (20 mM)	0.1	4.9	10	65
69 (rs4357)	ACE302 ACE 312	0.5	1.0	0.9 (11.1 X)	0.0	0.0	0.1	7.0	10	48
76 (rs4364)	ACE 353 ACE 76R	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (20 mM)	0.1	4.9	10	61

Table 8.11 PCR Protocols for all primer pairs included in Table 8.9

PCR Step	
Initial Denaturation Temperature (°C)	95.0
Initial Denaturation Time (min)	5.00
Annealing Temperature (°C)	Variable (see table)
Annealing Time (min)	0.30
Extension Temperature (°C)	72.0
Extension Time per cycle (min)	0.30
Number of cycles	35
Final Extension Time (min)	5.00

Table 8.12 Composition of 11.1X Buffer

Reagent	**Volume ul**	**Final Concentration**
Tris (2M, pH 8.8)	167.0	4.5mM
MgCl2 (1 M)	33.5	0.45 mM
(NH4)2 SO4 (1 M)	83.0	1.1 mM
EDTA (10 mM)	3.4	0.44 µM
dATP (100 mM)	75.0	20.0 µM
dCTP (100 mM)	75.0	20.0 µM
dGTP (100 mM)	75.0	20.0 µM
dTTP (100 mM)	75.0	20.0 µM
Bovine Serum Albumin	85.0	11.0 µl /µl
β-Mercaptoethanol	3.6	0.67 µM

Table 8.13 Restriction Enzyme Digest Conditions

SNP	**2 rs4290**	**17 rs4305**	**20 rs4424958**	**24 rs4312**	**29 rs4317**	**47 rs4335**	**61 rs4349**	**69 rs4357**	**76 rs4364**
Enzyme	***Taq*I**	***Hha*I**	***Pst*I**	**DpN II**	***Fok*I**	***Hae* III**	***Dde*I**	***Ban*l**	***Pvu* II**
Enzyme Units	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Volume of Enzyme (µl)	0.1	0.1	0.1	0.1	0.25	0.1	0.1	0.1	0.1
Restriction Digest Buffer	Gibco React 2	Gibco React 2	Gibco React 2	NE Biolabs 1	NE Biolabs 2 or 4	Gibco React 2	Gibco React 3	NE Biolabs 2 or 4	Gibco React 6
Volume of Buffer (µl)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Volume of PCR Products (µl)	10	10	10	10	10	10	10	10	10
dH20 (µl)	16.9	16.9	16.9	16.9	16.75	16.9	16.9	16.9	16.9
Total Volume (µl)	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Incubation Temperature °C	65	37	37	37	37	37	37	37	37
Incubation Period	12 Hours	12 Hours	12 Hours	12 Hours	12 Hours	12 Hours	12 Hours	12 Hours	12 Hours

Table 8.14 Successful ASO PCR Conditions

SNP	Primers (2 µM)	Vol of Each Primer (µl)	Vol of DNA (50 ng/µI) (µl)	Vol of PCR Buffer (µl)	Vol of Tris 2M) (µl)	Vol of Herring Sperm DNA_2 (µl)	Vol of Taq Poly –merase (µl)	Vol of PFU Taq (µl)	Vol of dH_20 (µl)	Total Vol	Annealing Temp (°C)
47 (rs4335)	ACE 091 R TTGCTTCACATGCAGGAGAC ACESNP47AR GCTCTTTCAAACGGGCT	0.5	1.0	11.1 X	0.062	0.17	0.1	0.03	6.7	10	53

Poly-morphism	Primers (2 µM)	Vol of Each Primer (µl)	Vol of DNA (50 ng/µI) (µl)	Vol of PCR Buffer (µl)	Vol of dNTPs (2 mM) (µl)	Vol of $MgCL_2$ (µl)	Vol of Taq Poly-merase	Vol of dH_20 (µl)	Total Vol	Annealing Temp (°C)
ID (rs1799752)	ACESNP47AF CTGGTCCTTACCCCA ACE3 ATTTCAGAGCTGGAATAAAATT	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (20 mM)	0.1	4.9	10	46
ID (rs1799752)	ACESNP47GF CTGGTCCTTACCCCG ACE3 ATTTCAGAGCTGGAATAAAATT	0.5	1.0	1.0 (ABT 10X)	1.0	1.0 (20 mM)	0.1	4.9	10	46

Table 8.15 Protocol for Successful Long Range ASO PCR Experiments

Initial Denaturation Temperature (°C)	96.0	Extension Temperature (°C)	64.0
Initial Denaturation Time (min)	5.00	Extension Time per cycle (min)	7.0
Denaturation Time per cycle	20 secs	Number of cycles	36
Annealing Temperature (°C)	Variable (see table)		
Annealing Time	30 secs		

9.6 Patient Consent Form

A Genetic Study of Stroke Disease

Principal Investigator *: Dr. Gary Dennis*
Supervisors : Prof Trembath (Professor of Medical Genetics),
: Dr. Abbott (Consultant Neurologist)

I have been given a full explanation of the purpose of this research project and I have read and understood the information leaflet provided. Yes * / No *
Would you like to ask any questions and discuss this study further ? Yes * / No *
If YES, have you received satisfactory answers to your questions ? Yes * / No *
Have you been provided with sufficient information about the study ? Yes * / No *
Who has spoken to you or provided information to you .....
I am aware that a researcher will seek information about my medical history. Yes * / No *
I am aware that I will be required to provide a blood sample for genetic studies. I am also aware that the sample will be stored and used for the purpose of this study alone, any further testing in other studies will require me to give further consent. Yes * / No *

I realise that any information I give and all results obtained will be treated **confidentially** and that I may at any time in the future obtain full details of information held or stored electronically arising from this study. Yes * / No *

I have been given the contact telephone number of Dr. G. Dennis should I require further information about any aspect of the research in the future. Yes * / No *

I am aware that I am free to decline and withdraw from participating in this research without giving a reason. I am aware that I can request that my DNA sample is destroyed at any stage. I am aware that this will **NOT** affect my own or my family's medical care in the future. Yes * / No *

I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

I am willing to participate in this research.

To be completed by the Patient
Signature of patient
.....Date.....
(Name in BLOCK LETTERS)
[This space has been left blank for any additional comments that you would like to make regarding the research.]

To be completed by the researcher
I confirm I have explained the nature of the Study, as detailed in the Patient Information Leaflet, in terms which in my judgement are suited to the understanding of the patient.
Signature of Investigator .....Date.....

(Name in BLOCK LETTERS) .....

9.7 Sequence variation in the human angiotensin converting enzyme (ACE)

Rieder,M.J., Taylor,S.L., Clark,A.G. and Nickerson,D.A.

Nat. Genet. 22, 59-62 (1999)

1 : AF118569 . Homo sapiens Protein, Related Sequences, angio...[gi:4732025] LinkOut

```
LOCUS       AF118569    24070 bp    DNA         PRI       28-OCT-1999
DEFINITION  Homo sapiens angiotensin I converting enzyme precursor (DCP1) gene,
            alternative splice products, complete cds.
ACCESSION   AF118569
VERSION     AF118569.1  GI:4732025
KEYWORDS    .
SOURCE      human.
ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;
            Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 24070)
AUTHORS     Rieder,M.J., Taylor,S.L., Clark,A.G. and Nickerson,D.A.
TITLE     Sequence variation in the human angiotensin converting enzyme (ACE)
JOURNAL   Nat. Genet. 22, 59-62 (1999)
REFERENCE   2  (bases 1 to 24070)
AUTHORS   Rieder,M.J.
TITLE     Direct Submission
JOURNAL   Submitted (07-JAN-1999) Molecular Biotechnology, University of
          Washington, UW Health Sciences Building, Rm. K316, 1705 NE Pacific,
          Seattle, WA 98195, USA
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:9606"
                     /chromosome="17"
                     /map="17q23 between D17S794 and D17S795"

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                     /frequency="0.09"
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                     /rpt_family="L2"
                     /rpt_type=dispersed
          repeat_region  <1785..>1841
                     /note="putative"
                     /rpt_family="MIR"
                     /rpt_type=dispersed
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                     /replace="t"
                     /frequency="0.32"
SNP 4     variation      2547
                     /replace="c"
                     /frequency="0.23"
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16805..16902,17060..17232,19287..19510,19773..19917,
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/product="angiotensin I converting enzyme precursor"
gene <2662..>23217
/gene="DCP1"
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9026..9124,9415..9537,9896..10107,10802..10938,
12123..12281,12552..12639,14499..14642,14792..14983,
16805..16902,17060..17232,19287..19510,19773..19917,
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/note="somatic form of ACE; dipeptidyl carboxypeptidase 1;
kininase II"
/codon_start=1
/product="angiotensin I converting enzyme precursor"
/protein_id="AAD28560.1"
/db_xref="GI:4732026"

/translation="MGAASGRRGPGLLLPLPLLLLLPPQPALALDPGLQPGNFSADEA

GAQLFAQSYNSSAEQVLFQSVAASWAHDTNITAENARRQEEAALLSQEFAEAWGQKAK

ELYEPIWQNFTDPQLRRIIGAVRTLGSANLPLAKRQQYNALLSNMSRIYSTAKVCLPN

KTATCWSLDPDLTNILASSRSYAMLLFAWEGWHNAAGIPLKPLYEDFTALSNEAYKQD

GFTDTGAYWRSWYNSPTFEDDLEHLYQQLEPLYLNLHAFVRRALHRRYGDRYINLRGP

IPAHLLGDMWAQSWENIYDMVVPFPDKPNLDVTSTMLQQGWNATHMFRVAEEFFTSLE

LSPMPPEFWEGSMLEKPADGREVVCHASAWDFYNRKDFRIKQCTRVTMDQLSTVHHEM

GHIQYYLQYKDLPVSLRRGANPGFHEAIGDVLALSVSTPEHLHKIGLLDRVTNDTESD

INYLLKMALEKIAFLPFGYLVDQWRWGVFSGRTPPSRYNFDWWYLRTKYQGICPPVTR

NETHFDAGAKFHVPNVTPYIRYFVSFVLQFQFHEALCKEAGYEGPLHQCDIYRSTKAG

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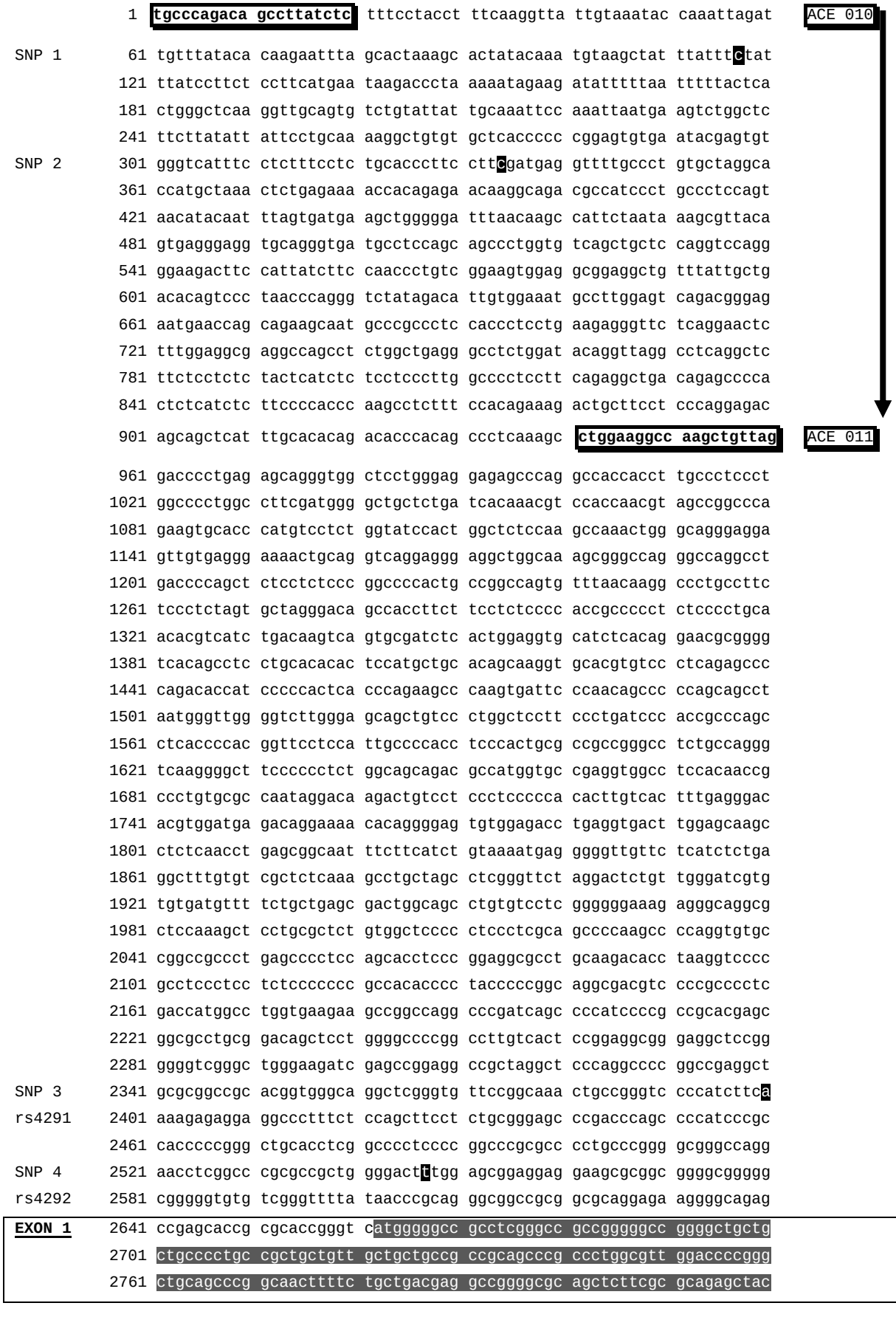

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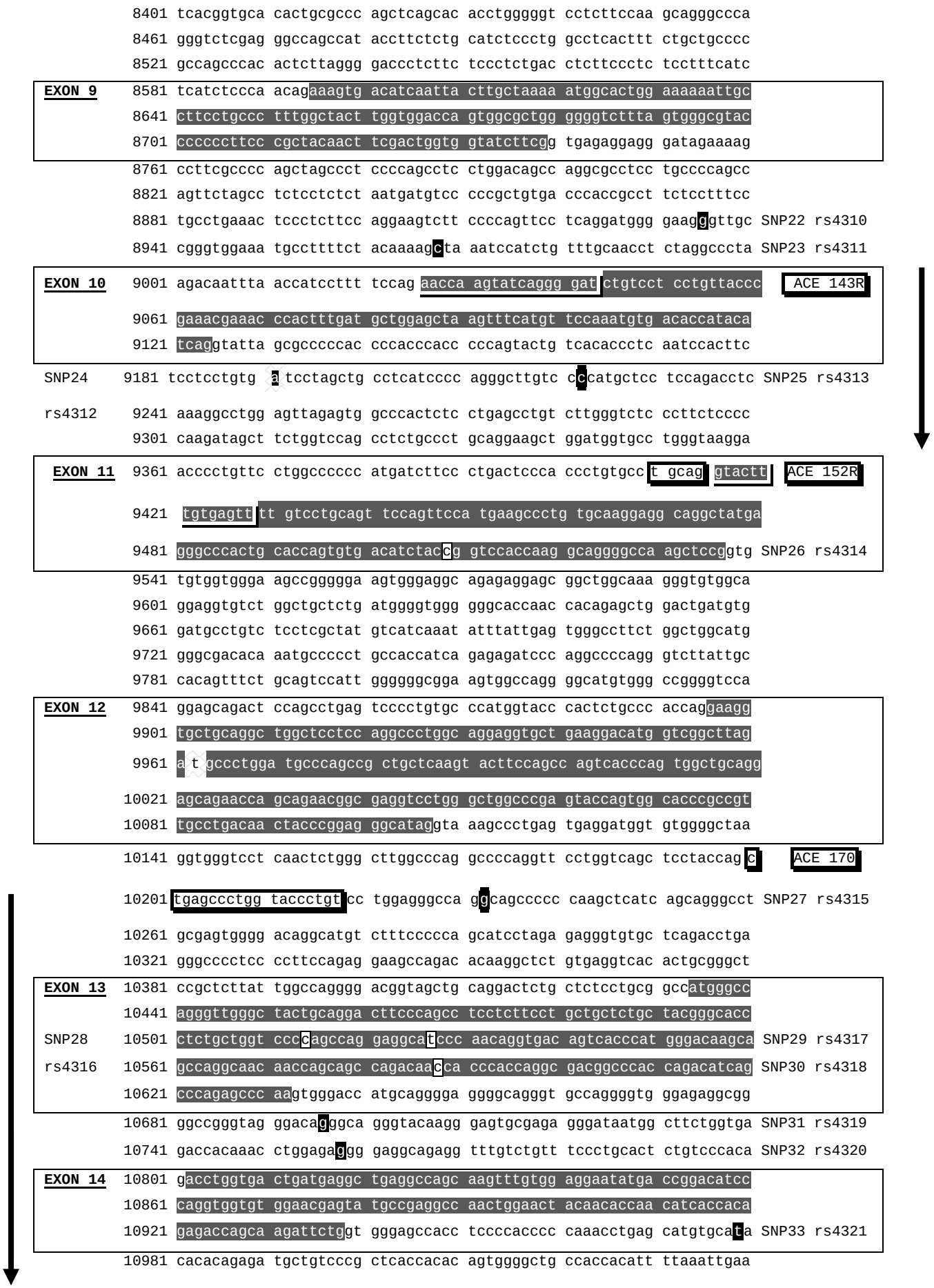
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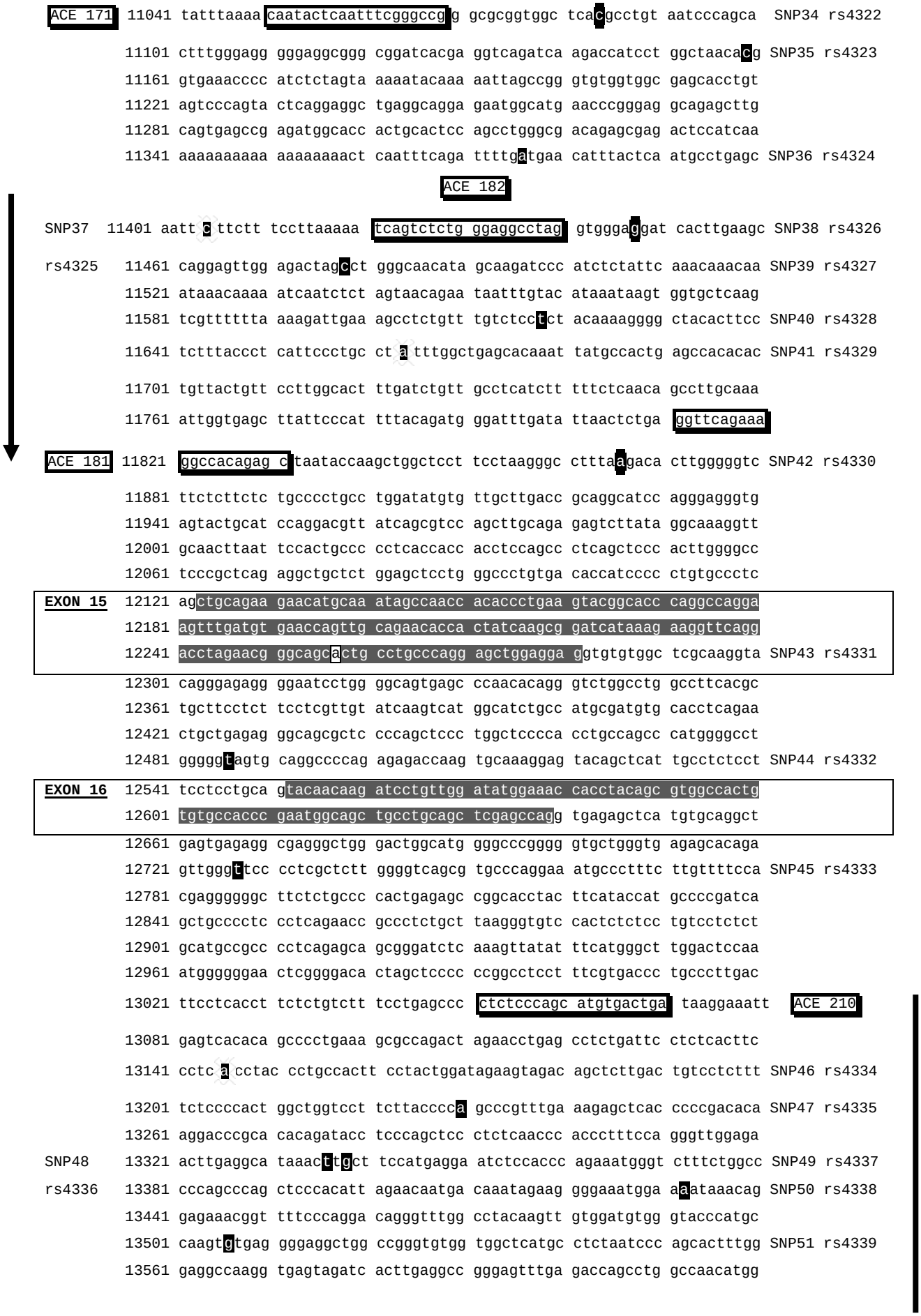
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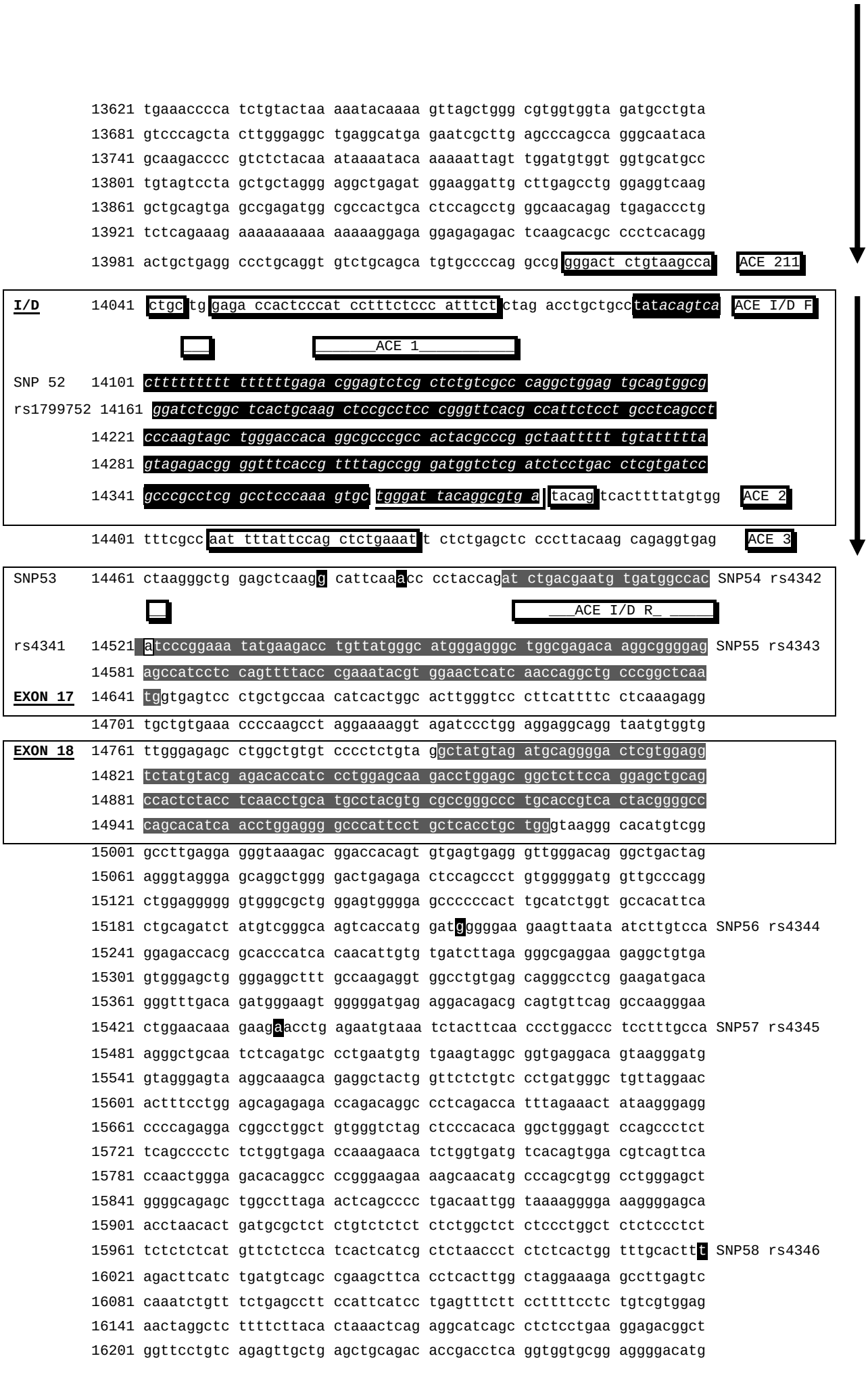
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CHAPTER 10

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