GENETIC DISSECTION OF ESSENTIAL HYPERTENSION AND FAMILIAL INTRACRANIAL ANEURYSM

by

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who inspired my interest in science.

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LIST OF ABBREVIATIONS AND SYMBOLS:

AAA	Abdominal aortic aneurysm
ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotrophic hormone
AD	Autosomal dominant
ADPKD	Autosomal dominant polycystic kidney disease
ADRB2	β_2-adrenergic receptor
AFDS	Amish Family Diabetes Study
AGT	Angiotensinogen
AME	Apparent mineralocorticoid excess
Ang I	Angiotensin I
Ang II	Angiotensin II
APM	Affected pedigree member
AR	Autosomal recessive
ARB	Angiotensin receptor blockers
ASP	Affected sib pair
AT1R	Angiotensin receptor type I
ATR2	Angiotensin receptor type II
BB	BioBreeding rats, model of type I diabetes
BB/OK	Substrain of the BB rat strain originating from Canada.
BMI	Body mass index
BN	Brown Norway rat
BP	Blood pressure
BRIGHT	British Genetics of Hypertension Study
CCT	Cortical collecting tubule
CHD	Coronary heart disease
cM	CentiMorgan
COL3A1	Collagen type III
Dahl R	Dahl salt resistant rat
Dahl S	Dahl salt sensitive rat
DBP	Diastolic blood pressure
DCT	Distal convoluted tubule
ddNTP	Dideoxynucleotide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DOC	Deoxycortisone
DOCA	Deoxycorticosterone acetate
ECM	Extracellular matrix
EDS IV	Ehlers-Danlos syndrome type IV
ECSP	Extreme concordant sibpair
EDSP	Extreme discordant sibpair
ENaC	Epithelial sodium channel
EV-FBAT	Empirical variance FBAT
F1	First filial progeny
F2	Second filial progeny
FBAT	Family-based association test
FBPP	Family Blood Pressure Program
FBN1	Fibrillin I
FBPP	Family Blood Pressure Program

FGF1	Fibroblast growth factor 1
FHH	Fawn-hooded hypertensive rat
FIA	Familial intracranial aneurysm
g	Grams
GH	Genetically hypertensive rat
G-protein	Guanylate cyclase-activating protein
GPCR	G-protein coupled receptor
GRA	Glucocorticoid remediable aldosteronism
GRAF	GTPase regulator associated with focal adhesion kinase
GWS	Genome-wide scan
H^+	Hydrogen (cation)
HACP	Harvard-Anhui Collaborative Project
HDL	High density lipoprotein
HTN	Hypertension
ICSHB	International Collaborative Study on Hypertension in Blacks
IBD	Identity by descent
IBS	Identity by state
IA	Intracranial aneurysm
K^+	Potassium (cation)
kb	Kilobases
l	Litre
LD	Linkage disequilibrium
LE	Long Evans rat
LEW	Lewis rat
LH	Lyon hypertensive rat
LN	Lyon normotensive rat
LOD	Logarithm of odds
M	Molar
MAP	Mean arterial pressure
Mex Am	Mexican Americans
µg	Micrograms
mg	Milligrams
Mg^{2+}	Magnesium (cation)
MHS	Milan hypertensive rat
µl	Microlitres
mm	Millimetres
mM	Millimolar
MMP	Matrix metalloproteinase
MNS	Milan normotensive rat
MR	Mineralocorticoid receptor
MRFIT	Multiple Risk Factor Intervention Study
mRNA	Messenger RNA
Na^+	Sodium (cation)
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NHANES III	Third National Health and Nutrition Examination Survey
NO	Nitric oxide
NR3C1	Glucocorticoid receptor
OCP	Oral contraceptive pill

OOA	Old Order Amish
PCR	Polymerase chain reaction
PDE4D	Phosphodiesterase 4D
PHA I	Pseudohypoaldosteronism type I
PHA II	Pseudohypoaldosteronism type II
PI	Protease inhibitor (also known as SERPINA1, see below)
PKD1	Polycystin 1
PKD2	Polycystin 2
PP	Pulse pressure
PRA	Plasma renin activity
QTL	Quantitative trait locus or loci
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
s	Seconds
Sabra H	Sabra hypertensive rat
Sabra N	Sabra normotensive rat
SAFHS	San Antonio Family Heart Study
SAH	Subarachnoid haemorrhage
SBP	Systolic blood pressure
SD	Standard deviation
SERPINA1	Serine protease inhibitor A1
SHR	Spontaneously hypertensive rat
SHRSP	Spontaneously hypertensive rat, stroke-prone
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SP	Sibpair
SR	Salt-resistant Dahl rat strain
SS	Salt-sensitive Dahl rat strain
S-TDT	Sibling TDT
TAL	Thick ascending limb
TDT	Transmission disequilibrium test
TIMP	Tissue inhibitor of metalloproteinase
VSMC	Vascular smooth muscle cell
WHO	World Health Organisation
WKY	Wistar Kyoto rat
WNK1	Lysine-deficient protein kinase 1
WNK4	Lysine-deficient protein kinase 4

SUMMARY:

Essential hypertension and familial intracranial aneurysm (FIA) are complex disorders which may have, in common, features such as gene-gene and gene-environment interactions, small individual gene effects, genetic heterogeneity, incomplete penetrance and pleiotropy. Such features hamper efforts to discover disease genes and have resulted in continuous evolution of complex genetic strategies. Appropriate strategies and current thinking were applied to genetic dissection of essential hypertension and familial intracranial aneurysms.

Essential hypertension is a complex oligogenic or polygenic disorder with 30% to 60% of inter-individual differences determined by genes. Much is known from studies in Mendelian disorders, rat and mouse models, and human populations. The genetic basis of Mendelian disorders has been dissected, but susceptibility genes have yet to be confirmed for essential hypertension.

The studies of hypertension, described herein, combined complementary strategies to examine the relevance of human chromosome 5q31.1-qter to blood pressure as a quantitative trait, and hypertension as a qualitative trait. This distal region of chromosome 5 was focused upon due to its implication by other human and animal model studies and the cluster of cardiovascular candidate genes located there.

Eleven microsatellites across 55 cM of 5q31.1 to qter were genotyped in 212 hypertensive nuclear families of the Silesian Hypertension Study. Two-point and

multipoint analyses showed linkage of a 7 cM region to both hypertensive status and blood pressure phenotypes. A maximal multipoint Z-score of 2.2 for systolic blood pressure (SBP) was obtained proximal to the marker D5s1480. In light of the debate regarding significance in linkage studies, this quantitative trait locus (QTL) was confirmed in a second sample derived from the Scottish population. Genotyping of the same markers was done for 1,469 individuals from sibships of the MIDSPAN Family Study. Two-point and multipoint analyses confirmed a reproducible 7 cM QTL with a maximum multipoint logarithm of odds (LOD) score of 1.8 obtained for mean arterial pressure.

Several putative candidate genes were located within the QTL including the β_2-adrenergic receptor gene, ADRB2, which is known to have a major role in cardiovascular physiology. Three functional loci, Arg16Gly, Gln27Glu and Thr164Ile, were genotyped within the ADRB2 gene using the Silesian sample. Single locus and haplotype analyses using transmission disequilibrium tests and family-based association methods showed no association to hypertension status ($P>0.05$), effectively excluding this gene in the Silesian sample.

The fibroblast growth factor 1 gene, FGF1, is located at the centre of the QTL. This gene had previously been cited as a putative blood pressure candidate gene in the 5q region but not studied in humans. With roles in endothelial cell proliferation, possible oxidative stress defence and proven direct effects on blood pressure in rodent models, we considered it a possible novel candidate. Coding regions and 3' UTR were sequenced and family-based association analyses performed for three polymorphisms which were discovered in the 3' UTR.

Association of SBP (P=0.045), pulse pressure (P=0.019) and hypertension status (P=0.038) was demonstrated to the most proximal locus. Association of hypertension to a three-locus haplotype was also shown (P=0.020).

The work on essential hypertension demonstrated the merit of confirming linkage in independent populations, implicating a 7 cM region of 5q31.1-q33 linked to blood pressure. Subsequent candidate gene studies utilised the added value of haplotype analyses, excluding the ADRB2 locus but showing interesting data to implicate the novel FGF1 locus as a putative positional candidate. Further fine mapping strategies are now underway to define, more clearly, the haplotype tag across the FGF1 locus. Combination of genetic approaches with expression and functional profiling will be required to determine the importance of FGF1.

The work on candidate genes of familial intracranial aneurysm (FIA) considers a complex disorder which has not benefited from the detailed study directed at essential hypertension. Genetic and physiological studies of this disorder are in their infancy. However, rupture of these lesions is often fatal, leading to subarachnoid haemorrhage, and contribution to all-stroke mortality and morbidity is disproportionate.

Four candidate genes were investigated in a small pilot study of 80 individuals from 21 Scottish families with a history of FIA. These genes were collagen type III (COL3A1), fibrillin 1 (FBN1), polycystin 1 (PKD1) and serine protease inhibitor 1 (SERPINA1). They were selected based on their importance to arterial wall integrity and remodelling; knowledge from Mendelian connective

tissue disorders; and studies in humans and animal models. A single intragenic or proximal microsatellite marker was genotyped for each gene and analysed with intracranial aneurysm status by linkage and association methods. A very small case-control paradigm comprising 18 cases and carefully matched controls suggested association of the marker proximal to SERPINA1 (P=0.028). Using a family-based association test (FBAT), a P value of 0.062 was obtained for the same marker. There was no suggestion that markers at the COL3A1, FBN1 or PKD1 loci were linked or associated (P>>0.05).

To increase sample size, the SERPINA1 marker was genotyped in 12 FIA families from the south of England. The combined dataset was analysed. Multiallelic FBAT and empirical variance-FBAT bordered on point-wise significance with P=0.054 and 0.071, respectively. This was supported by results from biallelic TDT analyses which implicated a 157 bp allele. Biallelic FBAT analyses implicated the same allele.

This pilot study is very small and a much larger study will be required to determine whether these preliminary data are robust. However, the results are interesting, being the first known study of this locus in FIA families. The SERPINA1 gene fits neatly with pathophysiological knowledge of FIA and further study of this gene is considered worthwhile to clarify its relevance to FIA aetiology. However, to fully take advantage of developing complex genetic strategies, considerable work is required on FIA to clarify important pathophysiological mechanisms.

CHAPTER 1

INTRODUCTION

1.1: HUMAN ESSENTIAL HYPERTENSION:

Blood pressure is a classic example of a complex polygenic or oligogenic trait under the multifactorial control of genes and environment (Dominiczak *et al*, 2000) (Fig. 1.1.1). The primary determinants of blood pressure (BP) in humans are cardiac output and peripheral resistance (Brown & Haydock, 2000). However, output and resistance are affected by many complex systems including the central nervous system; vasoconstrictor or vasodilator factors; vascular anatomy; body fluid volume and renal function (Carretero & Oparil, 2000b).

1.1.1: DEFINITION AND EPIDEMIOLOGY OF HYPERTENSION:

Debate raged in the 1960's regarding the population distribution of blood pressure and the mode of inheritance of hypertension (Pickering, 1967; Platt, 1967). Large population studies have since confirmed the opinion of Pickering, showing that blood pressure is normally distributed with a slight skew to the right. The continuous unimodal distribution implicates an oligogenic or polygenic regulation (Carretero & Oparil, 2000b).

At the individual level, blood pressure has a characteristic profile through life. Systolic blood pressure (SBP) increases progressively with age as the arterial tree loses its elasticity. Women's SBP increases more steeply. After 70 years women have as high or higher SBP than men (Burt *et al*, 1995). Diastolic blood pressure (DBP) increases until age 55 – 60 years (Carretero & Oparil, 2000b).

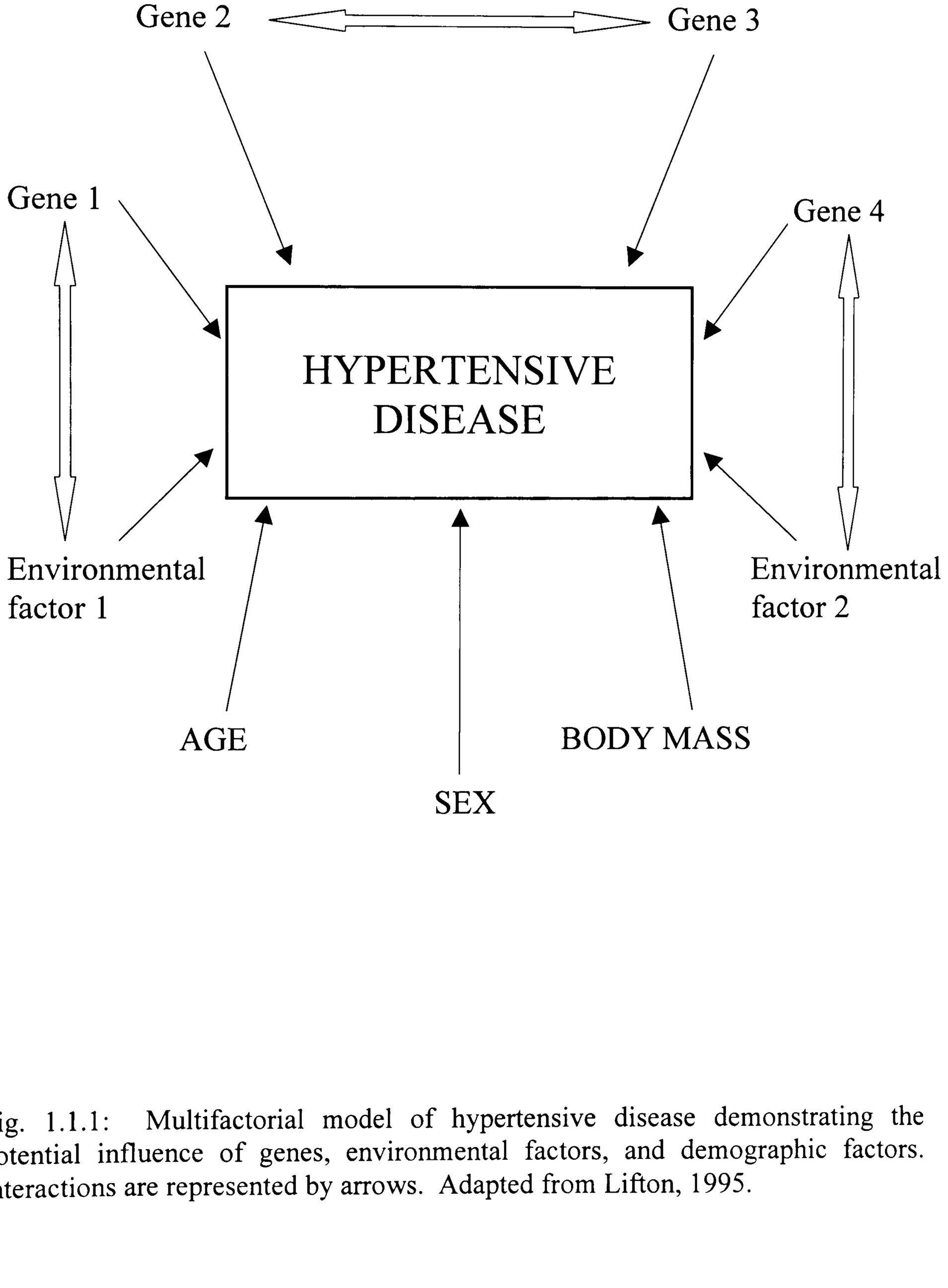


Fig. 1.1.1: Multifactorial model of hypertensive disease demonstrating the potential influence of genes, environmental factors, and demographic factors. Interactions are represented by arrows. Adapted from Lifton, 1995.

There is a graded and continuous increase in cardiovascular disease mortality and morbidity as blood pressure increases (Stamler *et al*, 1993). Hypertension is thus defined with regard to disease risk. The World Health Organisation (WHO) currently describes hypertension as "*SBP of 140 mmHg or greater and/or DBP of 90 mmHg or greater in subjects who are not taking antihypertensive medication*" (Chalmers *et al*, 1999). By these criteria, approximately one quarter to one third of adults in the Western economies are hypertensive (Burt *et al*, 1995; Mancia *et al*, 1997). The third National Health and Nutrition Examination Study (NHANES III) in the US showed that significant differences in prevalence exist between racial groups (Burt *et al*, 1995). However, population blood pressures and prevalence of hypertension are decreasing as treatment and awareness improve (Burt *et al*, 1995). There is also a concomitant reduction in stroke and coronary heart disease (CHD). Despite the improvements it is generally the case, in Western economies, that about half of hypertensives are treated and a quarter adequately controlled (Burt *et al*, 1995; Mancia *et al*, 1997). Some suggest that this may be an overestimate. Treatment of hypertension therefore remains a major public health challenge (Brown, 1997).

A minority of hypertensive cases are due to secondary causes, for example renovascular disorders, phaeochromocytoma or aldosteronism or rare single gene disorders. The vast majority of hypertensive disease is due to primary or essential hypertension in the absence of secondary causes and simple genetic disorders (Brown & Haydock, 2000; Carretero & Oparil, 2000b).

1.1.2: BLOOD PRESSURE AND DISEASE RISK:

Studies as early as the 1930s demonstrated that blood pressures greater than 140/90 mmHg were associated with excess mortality and a blood pressure of 110/70 mmHg was optimal for longevity (Lew, 1973). This was confirmed by large studies, for example the Multiple Risk Factor Intervention (MRFIT) study which included 300,000 US middle-aged men. Table 1.1.1 shows the increasing risk of CHD as SBP and/or DBP increase in the MRFIT cohort. Table 1.1.2 shows the striking relationship between adjusted stroke risk and SBP (Stamler *et al*, 1993). Current evidence suggests disease risk increases above 115/75 mmHg, doubling for every 20/10 mmHg increment thereafter (Chobanian *et al*, 2003).

In the 1970s the Framingham study showed that SBP rather than DBP was the primary risk factor for both CHD and stroke (Kannel *et al*, 1970; Kannel, 2000). These results were later upheld in the 5,000-strong Framingham offspring cohort (Wilson *et al*, 1991) and studies such as MRFIT (Stamler *et al*, 1993) and the Chicago Heart Association Detection in Industry (CHA) study (Miura *et al*, 2001). Such studies confirmed that this is true of all age/ sex classes and ethnicities (Stamler *et al*, 1993). Other studies showed that the risk was reversible and that lowering of blood pressure was beneficial even in the elderly. Studies in patients over 60 years of age recorded reductions of one third for stroke (Probstfield, 1991), and one quarter for myocardial infarction (Staessen *et al*, 1997) as a result of anti-hypertensive treatment.

TABLE 1.1.1: Effect of baseline SBP and DBP on adjusted coronary heart disease death in the MRFIT study.

Systolic Blood Pressure, mmHg	Diastolic Blood Pressure, mmHg				
	<80	80-84	85-89	90-99	≥100
<120	1.00	1.35†	1.36‡	0.98	3.23
120-129	1.19§	1.30†	1.49†	1.49†	1.84
130-139	1.67†	1.61†	1.67†	1.91†	2.64†
140-159	2.52†	2.22†	2.67†	2.56†	2.99†
≥160	4.19†	3.20†	3.41†	3.41†	4.57†

Adjusted for age, race, serum cholesterol, cigarettes per day, medication and income. †P<0.001 §P<0.01 ‡P<0.05. Taken from Stamler *et al*, 1993.

TABLE 1.1.2: Deciles of SBP and adjusted stroke mortality in the MRFIT study.

Decile of SBP	SBP, mmHg	No. of deaths	Rate*	Relative risk
1	<112	19	0.59	1.00
2	112-117	28	0.77	1.33
3	118-120	30	0.89	1.56
4	121-124	59	1.44	2.62‡
5	125-128	39	0.97	1.67
6	129-131	44	1.30	2.25‡
7	132-136	78	1.74	3.06§
8	137-141	82	2.04	3.60§
9	142-150	116	2.53	4.44§
10	≥151	238	4.76	8.21§

*Rate per 10,000 person-years adjusted for age, sex, race, serum cholesterol, cigarettes per day, diabetes medication, and income. Average follow-up of 11.6 yrs. ‡P<0.01; §P<0.001. Taken from Stamler *et al*, 1993.

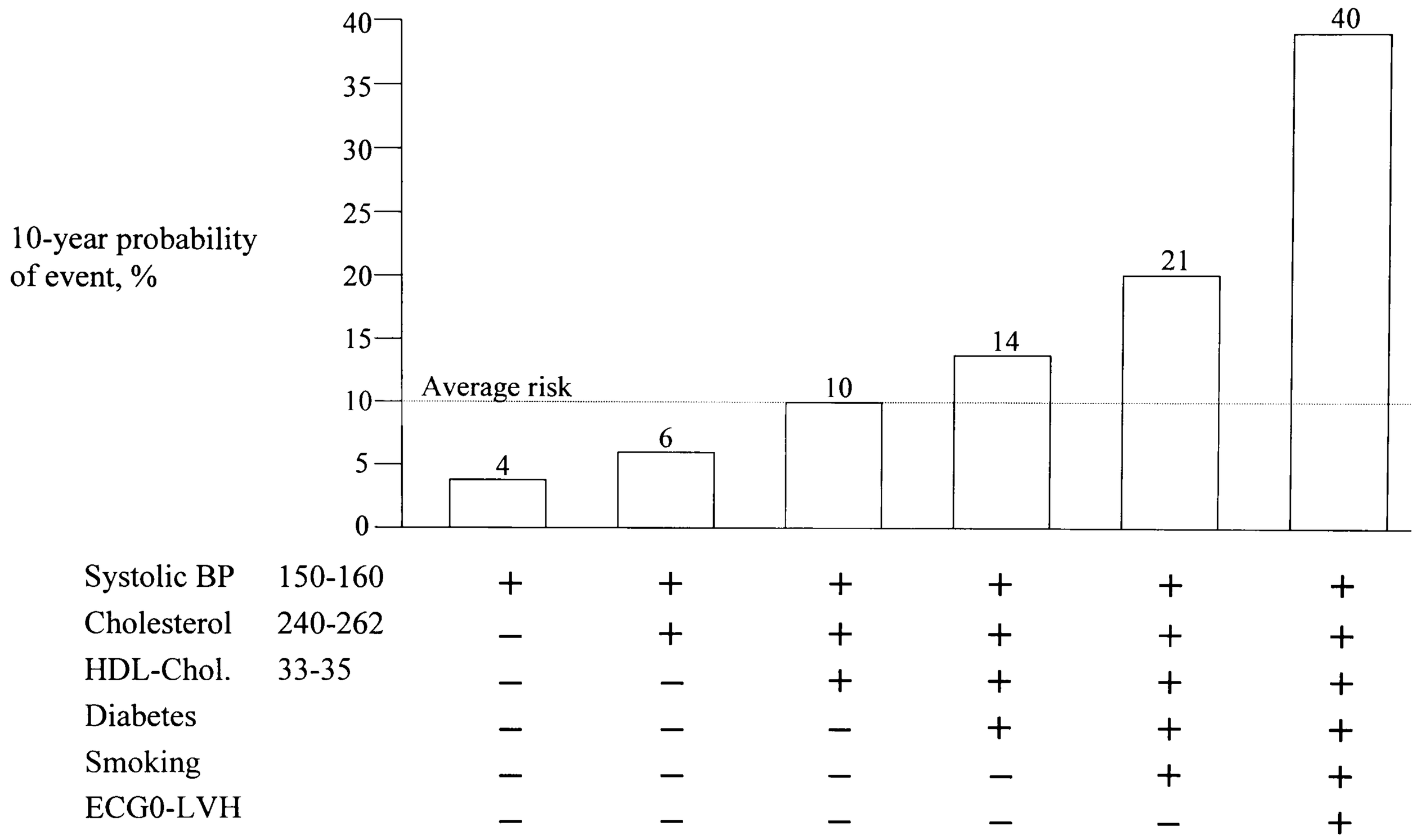


Fig 1.1.2: Cumulative effect of multiple risk factors on coronary heart disease risk in Framingham Heart Study men aged 45 years. BP = blood pressure; Chol. = Cholesterol; ECG = electrocardiography; HDL = high-density lipoprotein; LVH = left ventricular hypertrophy. Taken from Kannel, 2000.

The Framingham study noted that hypertension must be considered in the context of other cardiovascular disease risk factors since the effects were cumulative as shown in Fig. 1.1.2. This has been confirmed by others who show that low risk groups have only 25% the mortality of high-risk groups (Stamler *et al*, 1999). The Framingham study also noted that such additional risk factors tended to occur in hypertensives more than twice as often as expected (Kannel, 2000).

1.1.3: PATHOPHYSIOLOGY OF HYPERTENSION:

The variety of physiological, environmental and genetic factors affecting regulation of blood pressure and, potentially, development of hypertension is immense (Fig. 1.1.3). Pathophysiology, acquired factors and the evidence for genetic factors will now be discussed.

1.1.3.1: SYMPATHETIC NERVOUS SYSTEM:

Increased sympathetic nervous system activation increases blood pressure and maintains hypertension via stimulation of heart, peripheral vasculature and kidneys. Population-based studies show positive correlation between increased heart rate and development of hypertension (Oparil *et al*, 2003) and studies demonstrate that sympathetic cardiac stimulation is greater in young hypertensives than controls (Esler, 2000). Renal stimulation, on infusion of the α-adrenergic antagonist phentolamine, is also increased in hypertensives (Hollenberg *et al*, 1975; Esler *et al*, 1990).

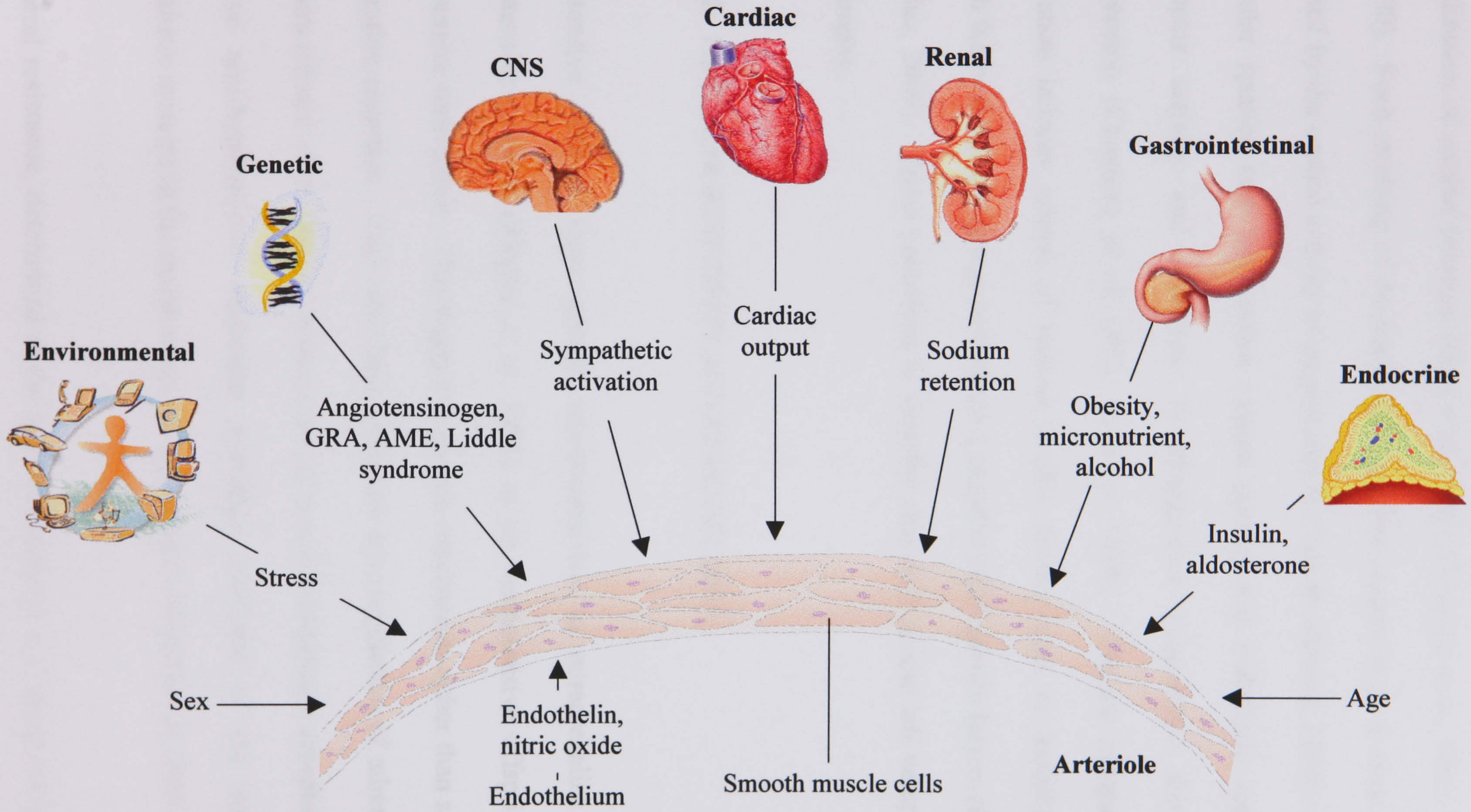


Fig. 1.1.3: Pathophysiological mechanisms of hypertension. AME = apparent mineralocorticoid excess; CNS = central nervous system; GRA = glucocorticoid-remediable aldosteronism. Taken from Oparil *et al*, 2003.

Arterial baroceptors, which feed back to decrease sympathetic outflow, are reset at a higher pressure in hypertensive patients and this offset can be reversed by normalisation of arterial pressure (Guo *et al*, 1983; Chapleau *et al*, 1988; Xie *et al*, 1990). Such resetting of the baroreflex function appears to be at least partly mediated by the central activity of angiotensin II (Guo & Abboud, 1984). Small molecular species such as reactive oxygen species and endothelin suppress baroceptor activity and contribute to exaggerated sympathetic drive of hypertension (Chapleau *et al*, 1992; Li *et al*, 1996). Chronic sympathetic stimulation induces release of various trophic factors such as transforming growth factor β, insulin-like growth factor 1 and fibroblast growth factors (Brook & Julius, 2000). These contribute to vascular remodelling and left ventricular hypertrophy.

1.1.3.2: VASCULAR REACTIVITY AND REMODELLING:

Hypertensive patients show greater vasoconstriction to noradrenaline than normotensive controls (Ziegler *et al*, 1991) as do normotensive offspring of hypertensive individuals. This suggests a genetic mechanism rather than simply an adaptive response. They also have impaired downregulation of adrenergic receptors (Oparil *et al*, 2003). Antagonists of α and β-adrenergic receptors are effective anti-hypertensive treatments providing evidence of the role of sympathetic systems in the maintenance of hypertension (Oparil *et al*, 2003).

Peripheral resistance, determined at the level of arterioles and small arteries, is generally elevated in hypertension due to structural and mechanical changes in

the vessels (Oparil *et al*, 2003). Vascular remodelling contributes to high blood pressure and the associated target organ damage (Folkow, 1982; Mulvany & Aalkjaer, 1990). Gluteal skin biopsies from untreated hypertensives show increased media-lumen ratios due to decreased lumen area (Oparil *et al*, 2003). Several classes of antihypertensive treatment normalise vessel structure, including ACE inhibitors, angiotensin-receptor blockers (ARB) and calcium-channel blockers (Schiffrin, 2001). β-blocker therapy lowers blood pressure but does not improve vessel remodelling. Also, hypertension in animal models can be reversed by acute manipulation (for example, by unclipping the renal artery in the Goldblatt model), again without affecting vascular hypertrophy. This suggests that resistance vessel structure may not be strictly secondary to hypertension (Oparil *et al*, 2003).

1.1.3.3: ARTERIAL STIFFNESS:

Collagen deposition, muscle cell hypertrophy and decreased integrity of elastic fibres are observed in aging arteries. These contribute to arteriosclerosis. In conjunction with these structural abnormalities, endothelial dysfunction develops due to aging and hypertension in both rodent models and humans (Hamilton *et al*, 2001). Thus arteries become stiffer through life (Berry *et al*, 2001). Reduced nitric oxide (NO) synthesis or release, in this setting, contributes to increased wall thickness of conduit vessels. Such deficiency appears to be important. NO donors, such as nitrates, increase arterial compliance and distensibility, reducing systolic blood pressure without affecting diastolic pressures (Oparil *et al*, 2003). Stiffness of arteries results in increased velocity of reflected pulse waves. This,

in turn, increases SBP because reflected pulse waves reach the aortic valve before closure, and may result in decreased DBP and coronary perfusion pressure. This phenomenon explains the predictive value of the SBP-DBP derivative, pulse pressure, on cardiovascular disease risk (Oparil *et al*, 2003).

1.1.3.4: RENIN-ANGIOTENSIN SYSTEM:

Angiotensin II (Ang II) increases blood pressure by mechanisms including vasoconstriction, aldosterone synthesis, renal salt handling, stimulation of thirst and sympathetic outflow from the brain (Oparil *et al*, 2003). Constriction to Ang II is increased in hypertensive individuals. It signals through two angiotensin receptors, type I (AT1R) and type II (AT2R). Via the AT1R, Ang II induces cardiac and vascular hypertrophy and hyperplasia. It also stimulates release of several growth factors and cytokines (McConnaughey *et al*, 1999). AT2R inactivates the signalling pathway downstream of AT1R, opposing the biological effects and leading to vasodilatation, growth inhibition and cell differentiation. This is thought to be important under stress conditions (Oparil *et al*, 2003). Local renin-angiotensin systems are now recognised. These may make an important contribution to remodelling of resistance vessels and the development of target organ damage in hypertensives (McConnaughey *et al*, 1999).

1.1.3.5: ANGIOTENSIN II AND OXIDATIVE STRESS:

Hypertension associated with long-term infusion of Ang II is linked to upregulation of vascular p22phox mRNA, a component of the oxidative enzyme

NAD(P)H oxidase (Fukui *et al*, 1997). This results in increased production of superoxide ($\cdot O_2^-$) which oxidises nitric oxide to peroxynitrite ($ONOO^-$). Blockade of AT1R has beneficial effects on superoxide levels in rat models of hypertension and stroke (Brosnan *et al*, 2002). This reduced NO bioavailability may be part of the reason for the increased vasoconstriction to Ang II in hypertensive individuals. NAD(P)H oxidase may also mediate the hypertrophic response to Ang II since antisense p22phox inhibition in cultured vascular smooth muscle cells (VSMCs) inhibits Ang II-induced protein synthesis (Zafari *et al*, 1999).

1.1.3.6: ENDOTHELIAL DYSFUNCTION:

Nitric oxide is a potent vasodilator, inhibitor of platelet adhesion and aggregation, and suppressor of VSMC migration and proliferation (Oparil *et al*, 2003). The cardiovascular system in healthy individuals is exposed to continuous NO-dependent vasodilator tone, but NO-related vasodilatation is reduced in hypertensive individuals (Berry *et al*, 2001; Oparil *et al*, 2003). Superoxide dismutase (SOD) reduces superoxide and *in vivo* delivery of SODs have been shown to reduce blood pressure and increase NO bioavailability in hypertensive models (Nakazono *et al*, 1991; Laursen *et al*, 1997; Fennell *et al*, 2002). This provides further evidence that oxidative stress contributes to the reduction of NO bioavailability, and development of endothelial dysfunction in hypertensive models.

1.1.4: ACQUIRED RISK FACTORS FOR ESSENTIAL HYPERTENSION:

The most important modifiable environmental risk factors are salt intake, alcohol consumption and obesity (Kornitzer *et al*, 1999).

1.1.4.1: SALT INTAKE:

In the 1960's, Dahl showed a heterogeneous blood pressure response to salt in rats. The trait was captured by inbreeding, developing salt-sensitive and resistant strains (Dahl *et al*, 1962a; Dahl *et al*, 1962b). This confirmed the influence of both salt and genes on blood pressure. Effect of salt has also been demonstrated in chimpanzees (Denton *et al*, 1995) and humans.

The Intersalt study showed correlation of SBP with salt excretion and blood pressure increase over life with salt intake in more than 10,000 individuals from 32 countries (Stamler *et al*, 1989). Meta-analyses have confirmed this between populations (Law *et al*, 1991) and within populations (Frost *et al*, 1991). There is also an ethnic component as shown by 73% prevalence of salt-sensitivity in black hypertensives versus 56% in white hypertensives (Kornitzer *et al*, 1999). Moderate salt reduction decreases blood pressure in both hypertensive and, to a lesser extent, normotensive individuals and the effect is dose dependent (Law *et al*, 1991; Cutler *et al*, 1997). This applies to all ages from new born (Geleijnse *et al*, 1997) to elderly (Whelton *et al*, 1998). Thus, many advocate a population-wide reduction in salt intake (Kornitzer *et al*, 1999).

Salt's effect on blood pressure could involve several mechanisms. Salt intake has been observed to increase sympathetic activity (Koolen & Vanbrummelen, 1984) and hyperinsulinaemia has been noted in salt-sensitive individuals (Bigazzi *et al*, 1996). Interaction with factors such as stress has been proposed (Staessen *et al*, 1994). Reduced nitric oxide bioavailability, as observed in animal models, may enhance the pressor effect of sodium (Weinberger, 1996).

1.1.4.2: POTASSIUM INTAKE:

Data from NHANES I showed that increased blood pressure in the black population was partly attributable to a relatively low potassium intake (Frisancho *et al*, 1984). The MRFIT study showed an inverse relationship of potassium intake and SBP, and to a lesser extent DBP, in men (Stamler *et al*, 1997). This correlation was not observed in women of the Nurses Health Study (Kornitzer *et al*, 1999) but a substudy did observe a significant 2 mmHg decrease in SBP with potassium supplementation for 16 weeks (Sacks *et al*, 1998). High potassium diets were reported to reduce SBP and DBP by 5.5 mmHg and 3.0 mmHg in US adults (Appel *et al*, 1997). Meta-analysis of 33 potassium supplementation trials indicated a significant reduction in pressures, again of the order of 3 mmHg (Whelton *et al*, 1997).

1.1.4.3: ALCOHOL CONSUMPTION:

Studies in US, European and Asian populations show a J-shaped correlation between alcohol intake and blood pressure (MacMahon *et al*, 1990). The

correlation of alcohol use and BP is also dependent on age and sex (Fortmann *et al*, 1983). The Intersalt study showed that high alcohol consumption was independently related to the prevalence of hypertension (Stamler *et al*, 1989) and reduction of alcohol in hypertensives has been associated with decreases in SBP and DBP (Kornitzer *et al*, 1999). Complete withdrawal of alcohol in normotensive persons also causes a reduction in blood pressures (MacMahon *et al*, 1990). 7% to 11% of male hypertension prevalence may be due to alcohol (MacMahon *et al*, 1990). Thus, a population-wide limit of alcohol is advocated. Current recommendations suggest daily limits of 30 ml of alcohol for men and 15 ml for women (Chobanian *et al*, 2003). Mechanisms for this relationship may involve several pathways, not least increased catecholamine secretion, disruption of normal Na^+-Li^+ countertransport in erythrocytes or a decrease in insulin sensitivity (Kornitzer *et al*, 1999).

1.1.4.4: METABOLIC SYNDROME:

The metabolic syndrome is a clustering of disease risk factors, including abdominal obesity, glucose intolerance, dyslipidaemia and often hypertension (Isomaa, 2003). The link between these features is not clear, but it is clear that patients with hypertension are at greater risk of having other features of the metabolic syndrome (Reaven, 2003). For example, insulin resistance is found in 45% of hypertensives versus 10% of normotensives (Reaven *et al*, 1996).

Features of the metabolic syndrome are, themselves, risk factors for the development of hypertension. Body mass is related to blood pressure and the

Framingham study showed that changes in weight were accompanied by changes in blood pressure (Kannel *et al*, 1967; Stamler *et al*, 1975). Weight loss in middle-aged, borderline hypertensives has been shown to reduce BP by 3.7/2.7 mmHg (Whelton *et al*, 1997). Intervention in the elderly also indicates a beneficial effect from weight loss (Whelton *et al*, 1998). Distribution of fat is important. Abdominal, visceral fat is metabolically more active and insulin resistant than subcutaneous fat (Arner, 1995) and correlates with hypertension (Bjorntorp, 1990).

Insulin sensitivity has been reported to be inversely correlated with blood pressure in both sexes (Ferrannini *et al*, 1997). Baseline fasting plasma insulin levels were shown to predict hypertension status a decade later in 2,322 middle-aged men (Skarfors *et al*, 1991). Similar results have been reported in women with 12 years follow-up with a threefold prevalence of hypertension in the top quartile compared with the bottom (Lissner *et al*, 1992).

Insulin resistance is not increased in patients with secondary hypertension, but there is a higher prevalence in normotensive relatives of hypertensive individuals (Reaven, 2003). This suggests that hypertension does not cause the metabolic anomalies. Some suggest that insulin resistance and hyperinsulinaemia are causally linked to essential hypertension (Reaven, 2003). There is evidence that endothelial damage may occur in conditions of insulin resistance and hyperglycaemia (Tooke & Hannemann, 2000). Sympathetic nervous system stimulation by high plasma levels of insulin (Julius & Jamerson, 1994) or

insulin's action on sodium retention by the kidney may also be potential mechanisms (Sechi & Bartoli, 1997).

1.1.4.5: OTHER FACTORS:

Many studies show an inverse relationship between exercise and both blood pressure and hypertension risk (Kornitzer *et al*, 1999). The benefit of exercise and improved fitness in lowering blood pressure has been demonstrated in African American men (Kokkinos *et al*, 1995), and Caucasian men (Paffenbarger *et al*, 1983) and women (Kornitzer *et al*, 1999). Psychosocial stress has also been shown to contribute to risk. Work-related stress correlates with ambulatory blood pressures in men and women (Van Egeren, 1992; Theorell *et al*, 1993).

1.1.5: EVIDENCE FOR A GENETIC COMPONENT IN ESSENTIAL HYPERTENSION:

Evidence from human population studies, rat model studies and Mendelian blood pressure disorders demonstrate a genetic component in essential hypertension (Rapp & Deng, 1995).

1.1.5.1: EVIDENCE FROM HUMAN POPULATION STUDIES:

An early study used longitudinal blood pressures over 9 years to assess aggregation of blood pressure in families in Wales (Miall *et al*, 1967). It showed a correlation of BP in probands and first-degree relatives which was absent in

spouses, despite their shared environment. Similarly, the Framingham study showed correlation between the BPs of parents and offspring but no correlation between spouses. Like Miall *et al*, above, the conclusion was that genetic factors could explain this (Havlik *et al*, 1979). Studies in Evans County in the US showed that blood pressure aggregation was present in both black and white populations and an individual's BP correlated with the number of hypertensive parents (Hayes *et al*, 1971). Examination of related and unrelated individuals living together or apart in Michigan was used to quantify the genetic effect. Heritability of SBP was 0.42 and DBP was 0.30. Genetic factors accounted for 25% of SBP variance (Longini *et al*, 1984). Comparable results have been published in populations lacking some of the Western risk factors, such as urban Nigerian families with low BMI and hypertension prevalence. Heritability estimates of 0.34 and 0.29 were obtained for SBP and DBP (Adeyemo *et al*, 2002).

Twin studies support population studies. A significant genetic effect on BP was demonstrated in 500 monozygotic (MZ) and dizygotic (DZ) middle-aged twin pairs by the National Heart, Lung and Blood Institute (NHLBI) (Feinleib *et al*, 1977). Resting blood pressures and response to stress, tested by the cold pressor test, demonstrated that a genetic component was involved in both cases in young to middle-aged German twins (Busjahn *et al*, 1996). Adoption studies support the findings of other study designs. One of the most informative studies in French-Canadian families compared BP correlation between adoptees, or natural children, and parents. It concluded that most of BP correlation was explained by heredity (Biron *et al*, 1976). Subsequently the same group estimated that up to

61% and 58% of the SBP and DBP correlation could be explained by shared genes. About a third of the phenotypic variance in the population was attributed to genes.

Overall, different study designs have confirmed that blood pressure is highly heritable. This also applies, as one might expect, to hypertension (Williams *et al*, 1989). The precise contribution of inherited genes has been more difficult to define. At best one can say that between 30% and 60% of interindividual variation is attributable to genes (Ward, 1990).

1.1.5.2: EVIDENCE FROM RAT MODELS:

In rodent models, it has been shown very clearly that blood pressure must be influenced by genetic variation. The 20th century saw the development of a number of inbred rat strains. These were produced by selectively breeding rats that showed a propensity for high blood pressure. Some were developed from outbred rats, being brother-sister mated to fix the phenotype and the genomic complement (Smirk & Hall, 1958). Others were derived from normotensive inbred strains using animals which displayed unusually high blood pressures. Thus the widely used spontaneously hypertensive rat (SHR) was derived from atypical Wistar rats (Okamoto & Aoki, 1963). Dahl developed partially inbred salt sensitive and resistant strains (Dahl *et al*, 1962a). From these initial strains, fully inbred strains were created later (Rapp & Dene, 1985). Sabra H and Sabra N rats were developed by Ben-Ishay, having a marked BP response to DOCA-salt administration or negligible response, respectively (Ben-Ishay *et al*, 1972).

These rat strains develop spontaneous hypertension which is relatively consistent, within a strain, in terms of its severity and age of onset (Lee *et al*, 2000).

Mating of normotensive and hypertensive rats to produce an F2 generation results in continuously distributed blood pressures in the progeny. This suggests that, as in humans, the rats develop polygenic forms of hypertension (Dominiczak *et al*, 1998). This has been confirmed by the finding that multiple genetic loci contribute to the hypertension in each model (Rapp, 2000). Rodent models elegantly demonstrate that high blood pressure levels are inherited from generation to generation and this phenotype can be captured by inbreeding. Use of congenic strain production provides definitive proof for the effect of a chromosomal segment on blood pressure (Rapp & Deng, 1995; Dominiczak *et al*, 1998). As far as possible environment is a constant so inbred rat strains allow us to directly observe the effects of genes on blood pressure.

1.1.5.3: EVIDENCE FROM MENDELIAN HYPERTENSIVE DISORDERS:

Whilst contributing very little to the overall burden of hypertensive disease, Mendelian hypertensive disorders have confirmed not only the crucial role of genes in human blood pressure control, but also indicated mechanisms by which this control is achieved and disrupted.

1.1.5.3.1: Glucocorticoid-remediable aldosteronism:

Glucocorticoid-remediable aldosteronism (GRA) is characterised by early onset of hypertension with normal or elevated aldosterone levels with suppressed, or undetectable, plasma renin activity (PRA) (Sutherland *et al*, 1966). Hypokalaemia and metabolic alkalosis may also feature (Rich *et al*, 1992). Exogenous glucocorticoids relieve symptoms (Sutherland *et al*, 1966).

Linkage analysis in multiple GRA pedigrees showed the cause to be unequal crossing over between two highly homologous genes on chromosome 8q (Lifton *et al*, 1992a; Lifton *et al*, 1992b). CYP11B1, normally regulated by adrenocorticotrophic hormone (ACTH), expresses steroid 11β-hydroxylase, an enzyme involved in cortisol synthesis in the adrenal fasciculata. CYP11B2, normally regulated by angiotensin II (AngII), expresses aldosterone synthase, the rate-limiting enzyme in aldosterone synthesis in the adrenal glomerulosa. The resulting chimaeric gene expresses a protein with aldosterone synthase activity under the control of ACTH. Thus, efforts to maintain normal levels of cortisol result in ectopic and constitutive production of aldosterone from the adrenal fasciculata.

The hyperaldosteronism causes hyperactivation of the mineralocorticoid receptor leading to increased Na^+ reabsorption via the epithelial sodium channel (ENaC) (Fig. 1.1.4). Plasma volume expansion and hypertension result (Lifton *et al*, 2001). Increased secretion of K^+ and H^+ is driven by the lumen-negative potential from the low Na^{2+} concentration.

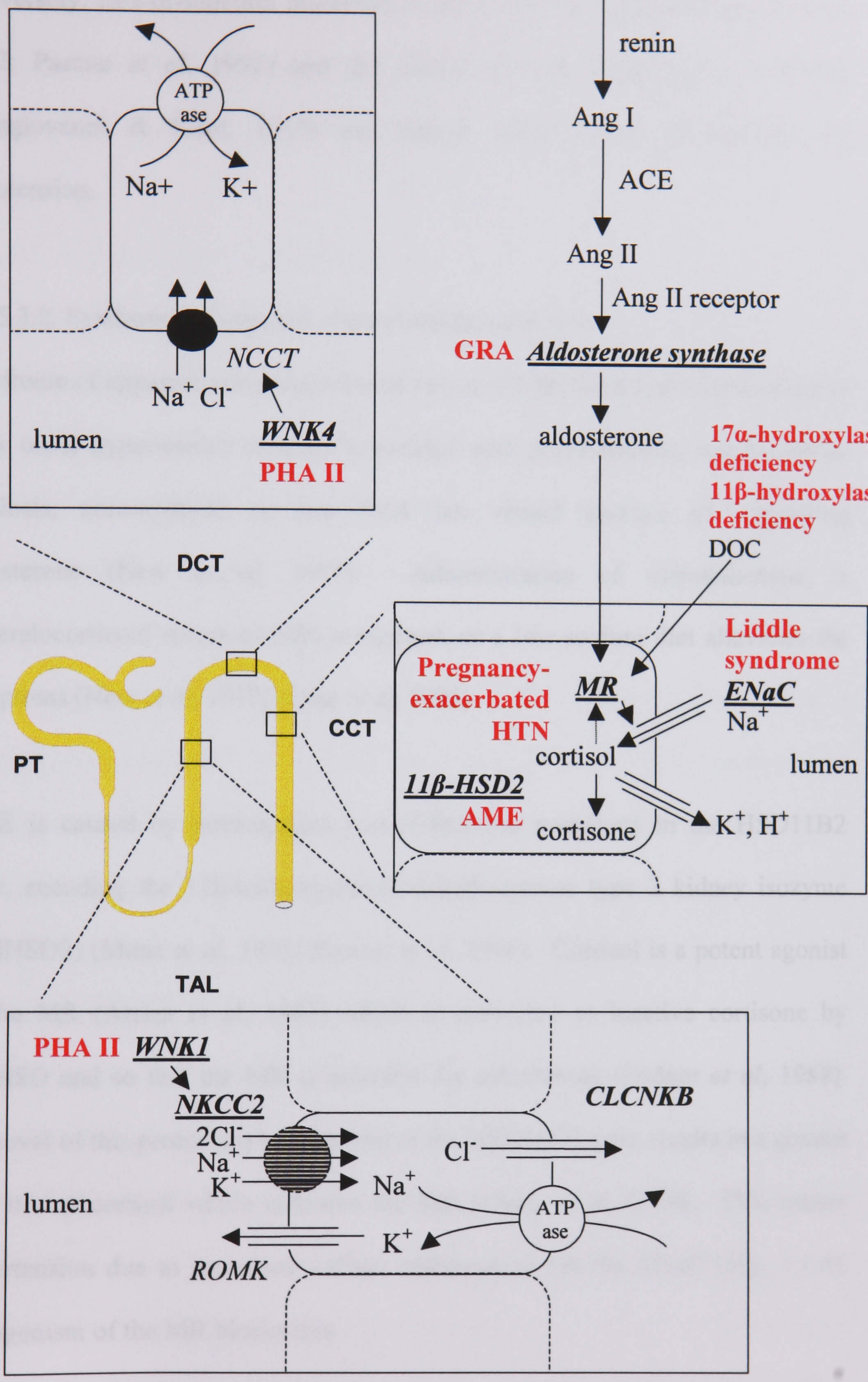


Fig 1.1.4: Mendelian hypertensive disorders in humans. A schematic kidney nephron is shown in yellow. NaCl reabsorption pathways are shown with hypertensive disorders in red. PT = proximal tubule; TAL = thick ascending limb; DCT = distal convoluted tubule; CCT = cortical collecting tubule. Ang I = angiotensinogen I; Ang II = angiotensinogen II. PHA II = pseudohypoaldosteronism type II. Adapted from Lifton *et al*, 2001.

Conversely, loss-of-function mutations in the CYP11B2 gene (Mitsuuchi *et al*, 1992; Pascoe *et al*, 1992) and the steroid 21-hydroxylase gene, CYP21B, (Bongiovanni & Root, 1963) are known which cause salt-wasting and hypotension.

1.1.5.3.2: Syndrome of apparent mineralocorticoid excess:

Syndrome of apparent mineralocorticoid excess (AME) is an autosomal recessive early onset hypertensive disorder associated with hypokalaemia and metabolic alkalosis, accompanied by low PRA but virtual absence of circulating aldosterone (New *et al*, 1977). Administration of spironolactone, a mineralocorticoid receptor (MR) antagonist, or a low sodium diet alleviates the symptoms (New *et al*, 1977; Mune *et al*, 1995).

AME is caused by homozygous loss-of-function mutations in the HSD11B2 gene, encoding the 11β-hydroxysteroid dehydrogenase type 2 kidney isozyme (11βHSD2) (Mune *et al*, 1995; Stewart *et al*, 1996). Cortisol is a potent agonist of the MR (Arriza *et al*, 1987) which is converted to inactive cortisone by 11βHSD and so that the MR is selective for aldosterone (Fudner *et al*, 1988). Removal of this protection by mutation of the HSD11B2 gene results in a greater half life of cortisol which activates the MR (Ulick *et al*, 1979). This causes hypertension due to increased sodium reabsorption via the ENaC (Fig. 1.1.4). Antagonism of the MR blocks this.

High cortisol levels associated with heritable mutations of the glucocorticoid receptor or pituitary or adrenal adenomas also result in hypertension (Hurley *et*

al, 1991). Deficiencies in steroid 11β-hydroxylase and 17α-hydroxylase can result in low renin hypertension due to the accumulation of MR-agonist precursors of cortisol synthesis (Kagimoto *et al*, 1988; White *et al*, 1991).

1.1.5.3.3: Pregnancy-accelerated hypertension:

Mutation of the mineralocorticoid receptor causes an autosomal dominant form of hypertension which worsens during pregnancy (Geller *et al*, 2000) (Fig. 1.1.4). The mutations disrupt the receptor's specificity such that progesterone will activate it. Progesterone increases 100-fold in pregnancy, and exacerbates the hypertension during this time (Lifton *et al*, 2001).

Conversely, loss-of-function mutations in the MR cause autosomal dominant pseudohypoaldosteronism type I (AD PHA I) which is a form of neonatal salt wasting associated with hypotension in the presence of elevated aldosterone levels and metabolic acidosis (Lifton *et al*, 2001).

1.1.5.3.4: Liddle syndrome:

Liddle syndrome is characterised by autosomal dominant transmission of early onset hypertension associated with hypokalaemic alkalosis, suppressed plasma renin activity and low plasma aldosterone levels (Lifton *et al*, 2001).

The disease is caused by mutations in either the β or γ subunit of the epithelial sodium channel (ENaC). ENaC is a heterotrimeric protein consisting of α, β and γ subunits (Fig. 1.1.4). Mutations result in loss of the C terminus of the affected subunit (Shimkets *et al*, 1994; Hansson *et al*, 1995). The effect of mutations in

either subunit is increased channel activity due to increased numbers of ENaC molecules at the cell surface (Schild *et al*, 1995; Snyder *et al*, 1995). All mutations described affect a highly conserved PPPXY motif in the C terminus (Hansson *et al*, 1995; Inoue *et al*, 1998). Studies showed that mutation of any conserved PPPXY residues resulted in increased amiloride-sensitive Na^+ conductance in *X. laevis* oocytes (Schild *et al*, 1996). The PPPXY motif was subsequently implicated in internalisation of channels or subsequent degradation hence the increased channel numbers at the membrane (Shimkets *et al*, 1997). Nedd4-1 (Staub *et al*, 1996), and Nedd4-2 (Kamynina *et al*, 2001) interact with the PPPXY motif, ubiquinating the channel and targeting it for degradation.

Loss-of-function mutations in ENaC cause recessive pseudohypoaldosteronism type I (Chang *et al*, 1996) which results in neonatal salt wasting with hypotension, elevated aldosterone and metabolic acidosis. It is more severe than the dominant form (Strautnieks *et al*, 1996).

1.1.5.3.5: Pseudohypoaldosteronism type II:

Pseudohypoaldosteronism type II (PHA II), or Gordon's syndrome, is an autosomal dominant form of hypertension with hyperkalaemia, possible metabolic acidosis and suppressed renin activity, despite otherwise normal renal function. It is particularly sensitive to thiazide diuretics (Lifton *et al*, 2001).

Genome wide scans identified linkage on chromosomes 1q31-42, 17p11-q21 and 12p13 (Mansfield *et al*, 1997; Disse-Nicodeme *et al*, 2000). Subsequently, two serine-threonine kinases were shown to underlie the linkage results on

chromosomes 12 and 17. Large deletions in the first intron of lysine-deficient protein kinase 1 (WNK1) caused increased expression whilst missense mutations caused loss-of-function of lysine-deficient protein kinase 4 (WNK4) in PHA II pedigrees. Both proteins co-localise to the distal convoluted tubule (DCT) and cortical collecting duct (CCD) of the distal nephron (Wilson *et al*, 2001). The renal downstream target of WNK1 is not known but may be the Na-K-2Cl channel, which is known to be regulated by a kinase dependent mechanism (Choate *et al*, 2003). WNK4 limits cell surface expression of the diazide-sensitive Na-Cl cotransporter expressed in the DCT (Wilson *et al*, 2003). Mutations in the Na-K-2Cl channel cause the hypotensive Bartter syndrome type 1 (Simon *et al*, 1996) whilst a mutant Na-Cl cotransporter causes Gitelman syndrome (Bettinelli *et al*, 1992). Both feature hypotension with hypokalaemia and alkalosis (Simon *et al*, 1996).

1.1.5.3.6 Hypertension with brachydactyly:

Hypertension with brachydactyly is an autosomal dominant disorder showing complete cosegregation of severe early-onset hypertension with abnormal skeletal development of the hand and wrist (Lifton *et al*, 2001). The syndrome was mapped to 12p12.2-11.2 in a Turkish pedigree (Schuster *et al*, 1996). Deletions in this same interval can result in brachydactyly in the absence of hypertension and so the possibility that contiguous genes are altered has been raised (Bahring *et al*, 1997).

1.1.6: THE ROLE OF GENETICS IN TREATMENT OF HYPERTENSION:

As discussed, despite advances in the understanding of pathophysiology of essential hypertension, health education and development of novel medicines, essential hypertension is still not adequately controlled in the majority of patients (Chobanian *et al*, 2003). Essential hypertension has a multifactorial aetiology, a combination of environmental and genetic influences (Lifton *et al*, 2001). Therefore, lifestyle modifications have been promoted as an important non-pharmacological approach to treatment. They effectively lower blood pressure and disease risk (Carretero & Oparil, 2000a). However, in reality lifestyle targets are difficult to achieve and harder to maintain. Alone they cannot solve the problem (Chobanian *et al*, 2003).

What, then, can genetic studies contribute to the situation and how would this benefit patients? Recent opinion dichotomises the role of genetics. Essentially, there are two distinct questions. The first concerns the genetic basis of essential hypertension – answered by disease genetics. The second concerns the role of genes in determining individual response to therapy – answered by pharmacogenetics (Roses, 2000a).

Disease genetics studies, the focus of this thesis, may lead to new insights into hypertensive disease and could potentially inform future drug development (Roses, 2000a). However, Roses notes that, in reality, disease genes and drug targets are not the same thing. Most susceptibility genes will not be tractable targets or amenable to high-throughput screening methods to identify active

compounds (Roses, 2000a). Despite this, understanding of individual aetiologies at the genetic level would allow mechanism-based drug discovery (McLeod & Evans, 2001). This should ultimately enable a more customised treatment regimen based on an individual's subtype of hypertensive disease. Another possibility, once disease genes are known, would be genetic-testing. However, whilst it might be useful to determine an individual's risk, in practice there is a minefield of social, ethical and legal issues (Roses, 2000b).

From the pharmacogenetics standpoint, an understanding of why a given patient fails to respond well to a drug may be of more immediate use in treating the individual. One of the greatest obstacles in treating patients is non-compliance with medication or change in medication. Between 50 and 70% of hypertensive patients will change their medication within 6 months of commencing treatment (Carretero & Oparil, 2000a). Unpleasant side effects may result in complete non-compliance, and at least will reduce the treatment options available. Even in patients tolerating their medication it is poorly understood why response to drugs is so variable. The standard deviation of response to medication is as great as the mean response. Ranges of response are greater than the mean response. Ten to twenty percent of patients have no response at all or a paradoxical increase in BP (Turner & Boerwinkle, 2003). Pharmacogenetics could be used to predict if an individual patient is likely to benefit from a medicine and be free of serious side-effects (Roses, 2000a).

1.2: FAMILIAL INTRACRANIAL ANEURYSM AND SUBARACHNOID HAEMORRHAGE:

Formation of intracranial saccular aneurysms (IA) is a complex process involving genetic and acquired risk factors. Focal dilatation of intracranial arterial walls results in the formation of an extruded pouch – the aneurysm - which compromises the integrity of the vessel and may lead to rupture (Zhang *et al*, 2003). Within the cranium, rupture of aneurysms on the surface of the brain results in bleeding into the subarachnoid space, between the pia mater and arachnoid membrane. This is termed 'subarachnoid haemorrhage' (SAH). Both IA and aneurysmal SAH exist as sporadic and familial disorders (Zhang *et al*, 2003).

1.2.1: DEFINITION & EPIDEMIOLOGY OF FAMILIAL INTRACRANIAL ANEURYSM & SUBARACHNOID HAEMORRHAGE:

The suggestion that IA formation may involve a heritable component was first raised by Chambers *et al* when they described IAs in a father and son (Chambers *et al*, 1954). Many families have since been described in the literature (Lozano & Leblanc, 1987; Elshunnar & Whittle, 1990; Schievink *et al*, 1991; Schievink *et al*, 1994; Bromberg *et al*, 1995) and the familial aggregation of IAs is now accepted. The term 'familial intracranial aneurysm' (FIA) describes the disorder in families having two or more affected first to third-degree relatives, this being distinct from 'sporadic' aneurysms which have no accompanying family history (Schievink *et al*, 1994; Bromberg *et al*, 1995).

The majority of aneurysms remain asymptomatic (Schievink *et al*, 1994) and, as such, calculation of the population prevalence of sporadic and familial intracranial aneurysms is very difficult. Autopsy studies have been used to address this question. A meta-analysis by Rinkel *et al* (Rinkel *et al*, 1998) of 19 studies suggested a total population prevalence of 4.3%. The proportion that are familial aneurysms is estimated to be from 4% in a Scottish population (Elshunnar & Whittle, 1990) and 6.7% in a Swedish study (Norrgard *et al*, 1987) to 29% in French-Canadians (Mathieu *et al*, 1997). However, the latter population may be atypical since it is a geographically isolated population and may include founder effects (Mathieu *et al*, 1997).

Whilst asymptomatic intracranial aneurysms are not detrimental in themselves, should they rupture the prognosis for the patient is very poor. About 80% to 90% of non-traumatic subarachnoid haemorrhage cases are attributable to a ruptured so-called saccular or 'berry' intracranial aneurysm (Alberts, 1999). Mortality is about 50% and more than 35% of those admitted to hospital die within one month. One third of survivors have serious neurological deficits (Gaist *et al*, 2000). Of SAH due to saccular aneurysmal rupture, about 5 to 10% are thought to be familial cases (Bromberg *et al*, 1995) although some authors report as high as 20% having a family history (Schievink *et al*, 1995).

1.2.2: EVIDENCE FOR A FAMILIAL DISORDER:

Most reported FIA families have only two affected members raising the possibility that the observed aggregation is purely fortuitous. This has been

addressed by several studies. US studies comparing SAH incidence in first-degree relatives of SAH cases with the population prevalence demonstrated between twofold (Wang *et al*, 1995) and fourfold risk (Schievink *et al*, 1995). Alberts *et al* (Alberts *et al*, 1994) showed fourfold risk of IA or SAH in siblings of cases from large sibships in the literature. Comparable recurrence risks of 4 for IA in Finnish first-degree relatives (Ronkainen *et al*, 1997) and 3 to 5 for SAH in Danish first-degree relatives (Gaist *et al*, 2000) have been reported.

Apparent differences between sporadic and familial IA or SAH also suggest two separate entities. Dutch, Finnish, Northern Irish and Canadian populations consistently show that familial aneurysms rupture, on average, about 5 years earlier than sporadic cases (Andrews, 1977; Lozano & Leblanc, 1987; Bailey, 1993; Ronkainen *et al*, 1995; Bromberg *et al*, 1995; Leblanc *et al*, 1995). Some report that ruptures peak in the 4^{th} decade of life in familial cases but in the 6^{th} decade for sporadic (Leblanc *et al*, 1995). Aneurysms of the anterior communicating artery are consistently twice as common in sporadic cases (Andrews, 1977; Lozano & Leblanc, 1987; Bailey, 1993; Ronkainen *et al*, 1995; Bromberg *et al*, 1995; Leblanc *et al*, 1995).

FIAs occur at the same or contralateral site in siblings twice as often as random sporadic cases (Andrews, 1977), (Lozano & Leblanc, 1987; Leblanc *et al*, 1995). Of 8 published monozygotic twin pairs, reviewed by Lozano and Leblanc, all had aneurysms at the same or contralateral site. SAH also occurs in the same decade of life more than twice as often (Andrews, 1977; Lozano & Leblanc, 1987; Leblanc *et al*, 1995).

Some studies suggest that familial aneurysms may rupture at a smaller size (Lozano & Leblanc, 1987; Ronkainen *et al*, 1995). Some studies also suggest an increased prevalence of multiple aneurysms in familial cases (Lozano & Leblanc, 1987; Bailey, 1993; Bromberg *et al*, 1995) although perhaps as many find no difference (Schievink *et al*, 1995; Leblanc *et al*, 1995; Mathieu *et al*, 1997).

Prognosis for familial SAH cases may be worse. A Dutch outcome study showed that 48% of familial cases were able to continue an independent life following SAH compared with 68% of sporadic cases at time of discharge. FIA mortality was also higher (Bromberg *et al*, 1995). Conversely, a Finnish study showed no difference in outcome, one year after discharge (Ronkainen *et al*, 1999). However, Finland is exceptional in terms of its prevalence of IA and the predominance of affected males. This may indicate different aetiology.

1.2.3: PATHOLOGY AND PATHOPHYSIOLOGY OF INTRACRANIAL ANEURYSMS:

A saccular intracranial aneurysm is a berry-shaped or multi-lobed extrusion of an artery wall, usually associated with the circle of Willis at the base of the brain. Such aneurysms are distinct in their shape and pathogenesis from atherosclerotic fusiform aneurysms; infection-derived mycotic aneurysms; and dissecting arteries, often caused by trauma (Alberts, 1999). A number of features, discussed below, are characteristic of saccular IAs. These may play a role in formation of IAs (Fig. 1.2.1).

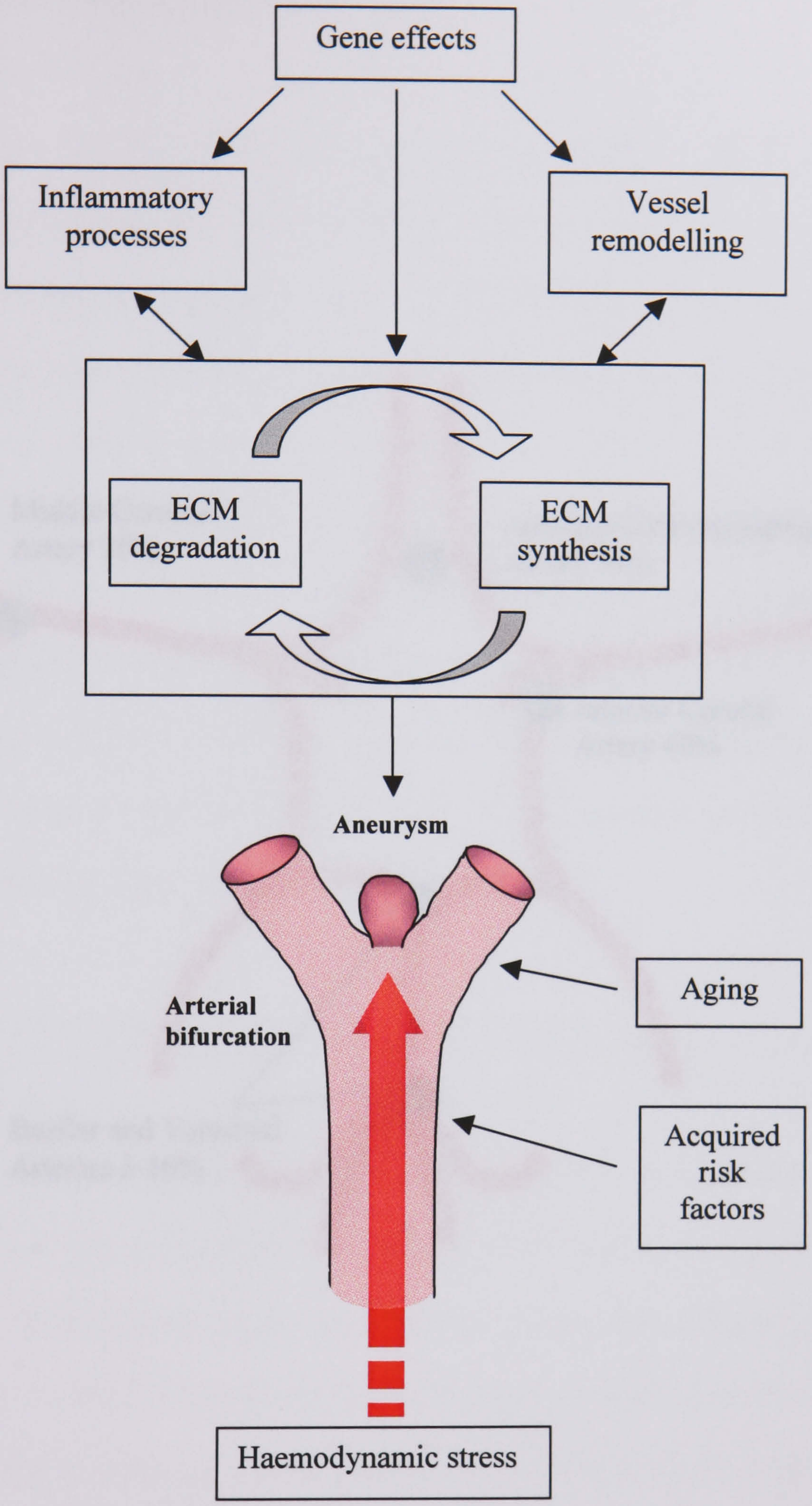


Fig 1.2.1: A schematic representation of processes which may influence arterial integrity and the formation of intracranial aneurysms. The complex aetiology may involve normal aging and stress effects exacerbated by an underlying vessel weakness. Genetic susceptibility may underlie aberrant vessel remodelling, artery wall turnover or inflammatory processes. Acquired risk factors interact with some or all components. ECM = extracellular matrix

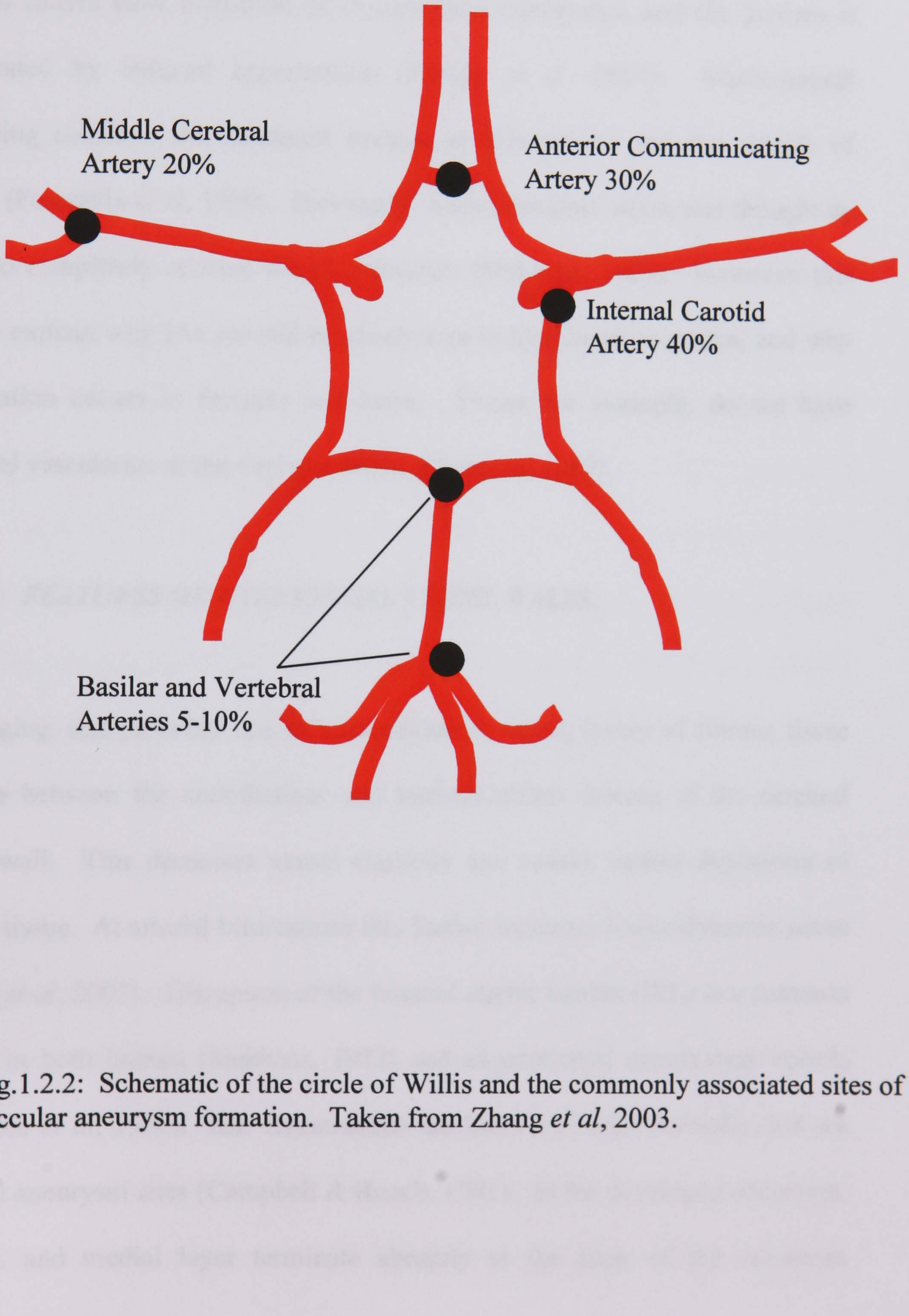


Fig.1.2.2: Schematic of the circle of Willis and the commonly associated sites of saccular aneurysm formation. Taken from Zhang *et al*, 2003.

1.2.3.1: SITE OF ANEURYSM FORMATION:

Saccular aneurysms often form at the circle of Willis because of the increased haemodynamic forces in this region (Zhang *et al*, 2003), particularly at arterial bifurcations (Leblanc, 1997) (Fig. 1.2.2). Haemodynamic stress by carotid ligation causes slow formation of experimental aneurysms, and the process is accelerated by induced hypertension (Kondo *et al*, 1997). Mathematical modelling confirms the increased stresses at bifurcations and the outside of curves (Foutrakis *et al*, 1999). Previously, haemodynamic stress was thought by some to completely account for IA formation (Stehbens, 1989). However, this fails to explain why IAs are still relatively rare in hypertensive groups, and why aggregation occurs in families and twins. Twins, for example, do not have identical vasculature at the circle of Willis (Stehbens, 1989).

1.2.3.2: FEATURES OF ANEURYSMAL VESSEL WALLS:

With aging, and probably due to haemodynamic stress, layers of fibrous tissue develop between the endothelium and internal elastic lamina of the cerebral vessel wall. This decreases vessel elasticity and causes further deposition of fibrous tissue. At arterial bifurcations this further increases haemodynamic stress (Zhang *et al*, 2003). Disruption of the internal elastic lamina (IEL) is a common feature in both human (Stehbens, 1972) and experimental aneurysmal vessels (Yamazoe *et al*, 1990). IEL fenestrations are known to exist normally, but are larger at aneurysm sites (Campbell & Roach, 1981). In the developed aneurysm, the IEL and medial layer terminate abruptly at the neck of the aneurysm

(Abruzzo *et al*, 1998; Zhang *et al*, 2003). The wall of the aneurysm shows evidence of depleted cellular elements, being little more than a fibrous layer with remnants of IEL (Stehbens, 1972). Some authors suggest a lack of fibromuscular proliferation and neointimal thickening is characteristic of such aneurysms (Abruzzo *et al*, 1998). These structural changes are more advanced in ruptured aneurysms compared with unruptured aneurysms (Kataoka *et al*, 1999).

1.2.3.3: EXTRACELLULAR MATRIX REMODELLING:

Gross architectural remodelling of extracellular matrix (ECM) has been reported in intracranial aneurysms. ECM is seen to be disorganised (Skirgaudas *et al*, 1996). Quantitatively and qualitatively abnormal reticular fibres have been reported in both aneurysm tissue specimens (Chyatte *et al*, 1990; Chyatte & Lewis, 1997) and normal cerebral arteries of affected patients (Ostergaard *et al*, 1987). Skin biopsies taken from some IA patients indicate abnormal collagen and elastic fibre morphology (Grond-Ginsbach *et al*, 2002). Some have reported reduced collagen type III content in patients' fibroblasts (van den Berg *et al*, 1997; Gaetani *et al*, 1997) or reduced collagen fibre cross-linking (Gaetani *et al*, 1996).

Expression profiling suggests that intracranial aneurysms are dynamic lesions undergoing remodelling. Peters *et al* examined an IA from a 3-year old girl and showed increased expression of several collagen subtypes, elastin, and the matrix metalloproteinase inhibitor TIMP3 (Peters *et al*, 2001). Increased expression of growth factors such as vascular endothelial growth factor (VEGF) and basic

fibroblast growth factor (bFGF) has been reported in adult lesions (Skirgaudas *et al*, 1996). A recent study reported decreased expression of bFGF, TIMP1 and components of the local renin-angiotensin system. It was suggested that a lack of vascular remodelling may prevent arterial wall thickening, in response to increased haemodynamic stress, leading to IA formation (Ohkuma *et al*, 2003). Increased expression of matrix metalloproteinases (MMP) 1, 2 and 9 and plasmin has been reported in unruptured aneurysms (Bruno *et al*, 1998). Increased MMP2 activity and reduced procollagens in serum from IA patients suggest a systemic anomaly (Chyatte & Lewis, 1997). Such observations have led to the suggestion that an imbalance in ECM turnover may be a factor in intracranial aneurysm formation.

1.2.3.4: INFLAMMATORY PROCESSES:

Some studies have shown a link between atherosclerotic profile and ruptured aneurysms (Adamson *et al*, 1994). Others have shown no increased risk (Schievink, 1997). However, there is evidence to suggest that inflammatory processes, homologous to those resulting in atherosclerotic plaques, may be involved in aneurysm development and/or rupture (Laursen *et al*, 1997). Neither atherosclerotic plaques nor intramural inflammation are necessary features of IAs (Abruzzo *et al*, 1998). However, increased infiltration of macrophages and leukocytes has been observed in ruptured compared with unruptured IAs. It has been suggested that endothelial damage encourages macrophage invasion leading to rupture (Kataoka *et al*, 1999). A study to assess markers of inflammation in unruptured IAs noted increased macrophage and T lymphocyte levels compared

with control arteries obtained at autopsy. Levels of vascular cell adhesion molecule 1 (VCAM 1) and immunoglobulins IgG and IgM were also increased (Chyatte *et al*, 1999). These cells and molecules are also involved in atherosclerosis and the macrophages are a source of MMPs.

1.2.4: ACQUIRED RISK FACTORS OF INTRACRANIAL ANEURYSMS AND SUBARACHNOID HAEMORRHAGE:

Familial IA and SAH are complex disorders; it is expected that both acquired factors and inherited genetic susceptibility underlie the increased risk in FIA/ SAH families. The majority of risk factor studies concentrate on SAH, rather than formation of IA, as an endpoint, partly due to the fact that SAH patients are more easily identified than asymptomatic IA carriers. Many studies now suggest that the primary acquired risk factors for FIA and SAH are smoking and female sex. Hypertension and alcohol consumption have been shown to be risk factors for SAH only.

1.2.4.1: SMOKING:

Smoking has been associated with increased risk of IA formation and IA growth in heterogeneous series of patients consisting of SAH survivors with additional unruptured IAs and individuals with unruptured IAs only (Juvela *et al,* 2001b). Smoking is also a risk factor for formation of multiple aneurysms, with a smoking history twice as likely in such cases (Qureshi *et al*, 1998; Ellamushi *et al*, 2001).

Many more studies have examined risk associated with SAH. Smoking has been addressed in over 20 case-control and cohort studies alone and none has yielded a non-significant association (Juvela, 2001). The association has been confirmed in Finnish, US, Australian and UK populations (Adamson *et al*, 1994; Teunissen *et al*, 1996; Weir *et al*, 1998; Leppala *et al*, 1999; Qureshi *et al*, 2001; Mhurchu *et al*, 2001; Pobereskin, 2001; Kissela *et al*, 2002). Thus, smokers are up to 5 times more common in SAH cohorts compared with the general population (Weir *et al*, 1998; Qureshi *et al*, 2001); the risk of SAH amongst smokers is increased between 3 and 9 fold (Adamson *et al*, 1994; Teunissen *et al*, 1996; Leppala *et al*, 1999; Qureshi *et al*, 2001; Kissela *et al*, 2002) and smokers, both male and female, suffer SAH perhaps as much as 10 years earlier than non-smokers (Weir *et al*, 1998; Pobereskin, 2001). The relationship is dose-dependent (Leppala *et al*, 1999; Juvela, 2001) and cessation reduces risk. However, any history of smoking increases risk (Teunissen *et al*, 1996; Qureshi *et al*, 2001; Kissela *et al*, 2002).

Smoking may promote elastin degradation by inhibition of degradation inhibitors such as serine protease inhibitor 1 which regulates extracellular matrix degradation (Qureshi *et al*, 2001). Smoking also lowers oestrogen levels which may impact collagen levels in arterial walls (Kongable *et al*, 1996).

1.2.4.2: HYPERTENSION:

Hypertension is considered neither sufficient nor necessary for formation of IAs (Alberts, 1999). Indeed, its status as a risk factor for IA formation is also in doubt. Smaller studies numbering hundreds of individuals find no increased risk of IA formation in hypertensives (McCormick & Schmalsteig, 1977; Juvela *et al*, 2001b). However, a study in 20,767 individuals with unruptured IAs found that elderly unruptured IA patients had a slightly higher prevalence of hypertension compared with the control population (Taylor *et al*, 1995). A UK study showed that hypertension increased risk of multiple aneurysms and that hypertension prevalence increased across groups having from one to five aneurysms (Ellamushi *et al*, 2001). Conversely, a similarly sized and designed US study showed no association (Qureshi *et al*, 1998). Work in animal models supports an effect since experimental aneurysm formation is accelerated if hypertension is induced by renal artery ligation and salt administration (Kondo *et al*, 1997; Morimoto *et al*, 2002). However, available evidence suggest that any increase in IA risk from hypertension is small.

A history of hypertension is an independent risk factor for SAH and indicates a poorer prognosis following SAH in both sporadic and familial cases (Ronkainen *et al*, 1999). First degree relatives of SAH patients are three times more likely to have hypertension than second degree relatives (Bromberg *et al*, 1996). There is a graded increase in risk with increase in blood pressure (Leppala *et al*, 1999). Hypertension and its sequelae are two to threefold more common in SAH cohorts than controls (Teunissen *et al*, 1996; Kissela *et al*, 2002). Blood pressure spikes.

related to normal circadian rhythm and physical exertion, coincide with rupture of cerebral aneurysms (Kleinpeter *et al*, 1995; Vermeer *et al*, 1997).

1.2.4.3: ALCOHOL CONSUMPTION:

Alcohol consumption is not described as a risk factor for IA formation (Schievink, 1997). Indeed, alcohol has also been reported to have no significant effect on risk of multiple aneurysms (Qureshi *et al*, 1998; Ellamushi *et al*, 2001) although confounding of such studies by smoking is always a problem.

With regard to risk of SAH, however, increased risk from alcohol consumption is dose-dependent like risk from smoking and hypertension. Meta-analysis estimates relative risk between 2.8 and 4.7 from longitudinal studies and odds ratio up to 1.5 for case-control studies, based on alcohol consumption of < 150 g/week and ≥ 150 g/week (Teunissen *et al*, 1996). Kissela *et al* (Kissela *et al*, 2002) reported that 2 or more drinks per day resulted in an SAH OR of 8.5 in their US case-control study.

1.2.4.4: FEMALE SEX:

Female sex is a prominent risk factor for both IA and SAH. Nearly all reported case series show an approximate 2:1 female to male ratio. This applies whether ruptured or unruptured IAs are considered (Kongable *et al*, 1996). Female sex is also a risk factor for multiple aneurysms (Qureshi *et al*, 1998; Ellamushi *et al*,

2001) independent of increased longevity (Kongable *et al*, 1996) and risk factors such as smoking, hypertension and alcohol (Teunissen *et al*, 1996).

Hormonal factors have been investigated to explain this marked and highly consistent sexual dimorphism but the case in favour is not clear-cut. Most SAH in women occurs post-menopause. Premenopausal women were reported to have an OR of 0.24 compared with age-matched postmenopausal women (Longstreth *et al*, 1994) but other studies have not found this (Mhurchu *et al*, 2001). Hormone replacement therapy (HRT) following menopause lowers SAH risk by perhaps a third or a half (Longstreth *et al*, 1994; Mhurchu *et al*, 2001) although this may be dependent on smoking history (Longstreth *et al*, 1994). Decreased oestrogen in the absence of HRT is linked to decreased collagen in skin and bone; it might be that this also affects collagen levels in arterial walls (Kongable *et al*, 1996).

Other studies suggest oestrogens are detrimental rather than protective based on their ability to stiffen blood vessels and cause hypertension (Okamoto *et al*, 2001). Results from a Japanese case-control study suggest that conditions associated with high oestrogens, such as lower gravidity or parity and greater age during first pregnancy, increased risk of SAH (Okamoto *et al*, 2001). Contrary to this, greater age at first pregnancy has also been reported to lower risk (Mhurchu *et al*, 2001). A meta-analysis concluded that use of high oestrogen content oral contraceptive pills (OCPs) was associated with a slight increase in risk (Johnston *et al*, 1998). Others have not found an effect (Teunissen *et al*,

1996; Mhurchu *et al*, 2001) even when oestrogen dosage and duration of use are examined (Schwartz *et al*, 1998).

1.2.5: EVIDENCE FOR A GENETIC COMPONENT IN INTRACRANIAL ANEURYSM FORMATION:

The nature of the genetic component of familial intracranial aneurysm is, at present, unknown. However, its existence is supported by a number of threads of evidence. Reviewers often cite three: aggregation in families has been discussed. Prevalence in monozygotic twins and the association of IAs with Mendelian disorders are also often noted. A fourth, which receives little attention, is the evidence from animal studies.

1.2.5.1: EVIDENCE FROM TWIN CASES:

A high degree of phenotypic concordance in monozygotic twins is accepted as an indicator of genetic predisposition, particularly in the presence of aggregation in other relatives (Hrubec & Robinette, 1984). No large FIA twin studies have been published but a number of individual cases have now been described (Brisman & Abassioun, 1971; Fairburn, 1973; Wilson & Cast, 1973; Schon & Marshall, 1984; Lozano & Leblanc, 1987; Hagen *et al*, 1997; Nakajima *et al*, 1998; Sharma & Brown, 2001). These are notable for the similarity of age at rupture and site of IA. These features were first described by Fairburn, who concluded that genetic factors should be considered (Fairburn, 1973). Reports of discordant twin pairs also exist, but authors suggest this may indicate reduced penetrance or a twin

who will later develop IAs (Puchner *et al*, 1994; Astradsson & Astrup, 2001). The concordance in monozygotic twins is accepted to indicate a genetic risk factor (Leblanc, 1997).

1.2.5.2: EVIDENCE FROM ANIMAL STUDIES:

Animals very rarely develop IAs and there is a lack of spontaneous animal models (Dobrin, 1999). Only a single spontaneous aneurysm in a Sprague Dawley rat, with comparable histopathology to human aneurysms, has been reported (Kim & Cervosnavarro, 1991). Thus, IA work in animals relies on intervention to induce aneurysms. Coutard and colleagues have attempted to address this deficit. They reported age and hypertension-dependent spontaneous formation of aneurysmal-like structures (ALS) in the rat testicular artery. These were regions of widened lumen lacking internal elastic lamina and medial smooth muscle cells. They showed that formation of ALS was strain-dependent and not entirely explained by blood pressure (Coutard & OsbornePellegrin, 1992; Coutard & OsbornePellegrin, 1996). Later work demonstrated strain effects on formation of induced aneurysms in Brown Norway (BN), Long Evans (LE) and Wistar Kyoto rats. The authors implicated a genetic predisposition as the underlying cause (Coutard & OsbornePellegrin, 1997). More recently, the same group showed that, on induction of hypertension, LE rats were more susceptible to IAs at the Circle of Willis. BN rats were prone to intracerebral haemorrhagic lesions. They crossbred these strains showing that F1 animals did not form IAs. However, F1 rats backcrossed to the LE strain and rats of the F2 generation developed aneurysms and more ruptured than in the parental LE strain. The

authors suggested that recessive genetic factors from the LE strain determined formation of the aneurysms, whilst arterial fragility genes from the BN strain conferred greater risk of aneurysm rupture (Coutard *et al*, 2000).

1.2.5.3: EVIDENCE FROM MENDELIAN DISORDERS:

The most widely discussed evidence for a genetic component in IA aetiology is the association of monogenic connective tissue disorders with occurrence of IA. Case reports describe cosegregation of IAs and several connective tissue disorders in individuals (Schievink, 1997). For most, there is a shortage of detailed epidemiological study. However, study has been made of autosomal dominant polycystic kidney disease, Marfan syndrome and Ehlers-Danlos syndrome type IV.

1.2.5.3.1: Autosomal Dominant Polycystic Kidney Disease:

ADPKD is characterised by the development of renal cysts on nephrons and collecting ducts, typically leading to end-stage renal failure by late middle age (Peral *et al*, 1997; Parnell *et al*, 1998). It also affects the liver, spleen, pancreas and seminal vesicles or ovaries, and features systemic arterial dissections and aneurysms. Thus, it is considered a connective tissue disorder (Schievink, 1997; Schievink, 1998). ADPKD is the only connective tissue disorder which has been conclusively linked to IAs, to date (Leblanc, 1997). Intracranial aneurysms are found in approximately one quarter of ADPKD patients at autopsy and aneurysmal rupture is the cause of death in the majority of these patients.

Conversely, ADPKD accounts for only 2% to 7% of intracranial aneurysms (Schievink, 1998).

The vast majority of ADPKD cases, perhaps 85-90%, are due to mutations in the polycystin-1 (PKD1) gene on 16p13.3 (Parnell *et al*, 1998; Torra *et al*, 1999). Most remaining cases are due to the PKD2 locus on chromosome 4q21-q22 (Torra *et al*, 1999). Nonsense and frameshift mutations have been described throughout the PKD1 (Roelfsema *et al*, 1997) and PKD2 genes (Peral *et al*, 1997; Aguiari *et al*, 1999). PKD1 encodes polycystin-1 which is a membrane-associated glycoprotein thought to act as a G-protein coupled receptor (Parnell *et al*, 1998). The product of PKD2 is polycystin-2. It is thought to be a cation channel subunit, based on sequence homology with a family of voltage-gated cation channels (Parnell *et al*, 1998) including the PCL gene, which is a channel for Ca^{2+}, K^{+} and Na^{+} when expressed in oocytes (Chen *et al*, 1999).

Polycystin-1 and polycystin-2 interact via coiled-coil domains and PKD1 may regulate PKD2 function (Qian *et al*, 1997). Studies of the *C. elegans* homologues, *lov-1 and pkd-2*, show that they are involved in the same signalling events during mating (Barr & Sternberg, 1999). This implies interactions between them but how this leads to cyst and aneurysm formation is not clear.

1.2.5.3.2: Marfan syndrome:

Marfan syndrome is an autosomal dominant disorder characterised by abnormalities of the skeleton, cardiovascular system, eye and spinal meninges (Schievink, 1997). Spontaneous aortic rupture and dissection are the most

common causes of mortality in adults (Schievink, 1997). Marfan syndrome is, clinically, highly variable (Pereira *et al*, 1994).

Marfan syndrome's association with intracranial aneurysms is a vigorously contested issue. More than 10 case reports describe Marfan syndrome patients with IAs (for example: (Higashida *et al*, 1988; Hainsworth & Mendelow, 1991; Rose & Pretorius, 1991). Association has been suggested based on IAs in two of seven patients with Marfan syndrome at autopsy (Schievink, 1997). However, no evidence of symptomatic IAs was found in 135 Dutch patients with Marfan syndrome after 4.5 year follow-up (van den Berg *et al*, 1996) and a US review of 25 Marfan autopsies found no evidence of associated IAs (Conway *et al*, 1999). In addition, no cases of Marfan syndrome were found in case series of 826 (van den Berg *et al*, 1996) and 710 individuals undergoing surgery for IAs (Conway *et al*, 1999).

Marfan is caused, possibly exclusively, by mutations in the FBN1 gene on chromosome 15q21.1 encoding the extracellular matrix protein fibrillin. Fibrillin is an integral component of microfibrils in both elastic and non-elastic tissues (Alberts, 1999). Missense mutations were first described by Dietz *et al* (Dietz *et al*, 1991) and it is now known that mutations tend to be specific to one family (Nijbroek *et al*, 1995).

Site of mutation within the FBN1 gene may explain some of the clinical heterogeneity since a number of studies have noted that neonatal and severe cases arise from mutation of the central domains of the gene (Kainulainen *et al*,

1994), (Nijbroek *et al*, 1995). This determines whether variant fibrillin-1 fibres incorporate into a microfibril and exert a dominant-negative effect. Until the relationship between mutation and phenotype is fully understood, it will be difficult to clarify the involvement of IAs in Marfan syndrome.

1.2.5.3.3: Ehlers-Danlos Syndrome type IV:

Ehlers-Danlos Syndrome type IV (EDS IV) is an autosomal dominant connective tissue disorder characterised by the spontaneous dissection, rupture or aneurysm formation of large and medium-sized arteries. The association of EDS IV with intracranial aneurysms is controversial. Case reports of cosegregation do exist (Dewazieres *et al*, 1995; DeWitte *et al*, 1997) although these are rare (Leblanc *et al*, 1990). Carotid-cavernous fistulae, derived from aneurysms, are described as common complications of EDS IV by some (Schievink *et al*, 1994) but should be considered as distinct from saccular aneurysms according to others (Leblanc *et al*, 1990). There appears to be no debate, however, that EDS IV does result in fragile cerebral arteries (Pope *et al*, 1991). However, a study in the US of 202 patients with EDS IV showed no increase in IAs compared with the general population (North *et al*, 1995).

Ehlers-Danlos syndrome type IV arises from mutations in the COL3A1 gene encoding collagen type III which is a structural protein widely distributed in blood vessels (Pope *et al*, 1996). Much of the COL3A1 gene encodes a triple helix consisting of an uninterrupted sequence of Gly-X-Y amino acid cassettes. 'X' and 'Y' are frequently proline or lysine (Pope *et al*, 1996). Mutations include point mutations, which alter Gly residues, small deletions, mutations

activating cryptic splice sites and exon-skipping mutations (Chiodo *et al*, 1995; Byers, 1995; Schwarze *et al*, 1997).

Mutations disrupting the triple helix affect folding and secretion of the protein (Narcisi *et al*, 1993). Those closest to the C terminal have the greatest effect (Mackay *et al*, 1996) and correlation between site of mutation and clinical features has been noted. For example, the region between amino acids 910 and 1021 is implicated in lethal arterial complications (Pope *et al*, 1996). It is the opinion of many that rarer type III collagen genetic mutants are associated with cerebrovascular abnormalities (Pope *et al*, 1990). Whether there is a firm link to IAs remains to be answered.

1.2.6: ROLE OF GENETICS IN TREATMENT OF INTRACRANIAL ANEURYSMS:

Overall, detection and elective surgery of IAs is preferable to emergency surgery after SAH. Meta-analysis of case series from around the world showed mortality of 1% and morbidity of about 4% for elective surgery (King *et al*, 1994). The international ISUIA study, considering the outcome of over 1,400 IA patients, estimated combined surgical morbidity and mortality at 13 to 17%. However, this still compares well with 50% and 35% respectively, post-SAH (Gaist *et al*, 2000). However, as already stated, many IAs remain asymptomatic (Schievink *et al*, 1994) and only a minority rupture. It is important to establish whether subgroups of high-risk patients are identifiable, and what the rupture risk of a particular aneurysm is (White & Wardlaw, 2003). Identifying candidates for

surgery is not trivial, requiring expensive screening which itself may carry a risk of complications. For example, conventional catheter angiography may be complicated in 1.8% and fatal in 1% of cases (van Gijn & Rinkel, 2001).

Current management guidelines of FIA families issued by the American Heart Association Stroke Council suggest screening on an 'individual basis' (Bederson *et al*, 2000). This essentially admits that no cohesive strategy exists. Several studies have attempted to model the benefits and risks of screening. Findings have suggested that screening can extend life expectancy (Leblanc *et al*, 1994), but is not beneficial in families with less than three affected individuals (Crawley *et al*, 1999). Modelling of various incidences and rupture rates led Obuchowski *et al* to recommend a compromise of one screening at age 30 or younger. However, it was noted that any screening protocol is ineffective in cases where IAs form and rupture rapidly, or form then stabilise. This will only identify low risk cases (Obuchowski *et al*, 1995). Wiebers *et al* observed that IAs over 10 mm ruptured in a series of 160 IA patients, but the average size at rupture at their institution was 7.5 mm. They concluded that IAs form and rupture at small sizes or stabilise depending on vessel wall elasticity. If they stabilise, they rupture when larger than 10 mm (Wiebers *et al*, 1987). The size dependency of rupture has been noted by others (Rinkel *et al*, 1998; Raaymakers *et al*, 1999; Wiebers *et al*, 2003).

Many authors recommended screening in families with a strong history, based on their clinical experience (Schievink *et al*, 1991; Alberts *et al*, 1994; Ronkainen *et al*, 1994). Ronkainen *et al* advocated a dynamic individual approach based on

the history of the family, for example screening at the same age as a patient's sibling or relative ruptured (Ronkainen *et al*, 1996). However, retrospective data from the ISUIA study suggested that annual rupture rates of IAs are lower than was previously believed; 0.05% for IAs less than 10 mm and 0.5% for IAs over 10 mm. Risk of rupture was dependent on personal history of SAH, location and size of IA (Wiebers *et al*, 1998). The same study recently published the first prospective data on IAs which supported the retrospective findings (Wiebers *et al*, 2003). Overall, many now argue that there is a strong case against screening for IAs (Kirkpatrick & McConnell, 1999; Crawley *et al*, 1999; White *et al*, 1999). This is based on low rupture rates, the fact that screening is expensive and will identify low-risk lesions, and that risks of surgical intervention in such low-risk cases outweigh the reduction of rupture risk.

One way forward from this conundrum is to identify which individuals are most at risk. More information is needed on the genetic basis and inheritance patterns of IA and SAH (White *et al*, 1999). An understanding of the genetics of FIA formation and rupture may open the door to cheap and simple testing, and prophylactic treatment. This would reduce the risk and expense of screening and, importantly, surgical intervention. The knowledge might also be extrapolated to sporadic IA and SAH treatment.

1.3: STRATEGIES FOR THE DISSECTION OF COMPLEX TRAITS:

The challenge of dissecting complex disorders is immense due to small individual gene effects, gene-gene interactions and gene-environment interactions, genetic heterogeneity, phenocopy and incomplete penetrance (Lander & Schork, 1994; Tabor *et al*, 2002). The underlying gene variants may be common – the 'common disease, common variant' hypothesis – as seen for the ApoE4 allele in Alzheimer's disease and the insulin gene minisatellite in type 1 diabetes. Others argue a larger pool of less common variants may be more likely. The truth may lie somewhere between these extremes (Johnson & Todd, 2000). Genetic strategies evolve continually in the face of this challenge. In humans, these include study of rare Mendelian disorders, genome-wide scanning strategies and candidate gene approaches (O'Shaughnessy, 2001). In animal models, genome-wide scans have been confirmed by congenic strain production leading to target genomic regions (Stoll *et al*, 1999; Dominiczak *et al*, 2000). Mendelian disorders relevant to hypertension and intracranial aneurysms are described in Sections 1.1 and 1.2. The other strategies will now be discussed.

1.3.1: GENOME-WIDE SCANS:

Genome-wide scans (GWS) allow screening of the entire genome in the absence of any *a priori* hypothesis regarding location or function of causative genes. Traditionally, GWS studies have involved linkage analysis which detects cosegregation of a marker and phenotype in relatives. Such studies reveal regions of linkage which provide targets for further detailed study (Dominiczak

et al, 1998). In studies examining continuous traits, these regions of linkage are known as quantitative trait loci (QTL). Linkage studies originally used single restriction fragment length polymorphisms (RFLPs) in Mendelian diseases (Botstein *et al*, 1980). This expanded to encompass heterogeneous disorders, then the entire human genome (Lander & Botstein, 1986), before adaptation to experimental models (Lander & Botstein, 1989). RFLPs were soon replaced by microsatellite markers which are more common and more suitable to high throughput systems.

There are now many reported genome-wide scans of complex traits (Altmuller *et al*, 2001) and it is well recognised that agreement between them is limited. Their widespread use has resulted in great debate regarding interpretation of results and issues of statistical significance. Lander and Kruglyak were the first to suggest guidelines for reporting linkage (Lander & Kruglyak, 1995). They set stringent thresholds of significance, dependent on study design. For sib pair studies, for example, these were logarithm of odds (LOD) ≥ 3.6; $Z \geq 4.1$; $P \leq 2x10^{-5}$, equating to a corrected 95% confidence level. Others argued for lower stringency, used a less demanding model to reduce false negatives, and suggested $P \leq 0.0023$ as significant (Rao & Province, 2000). What has become clear is that reproduction in different studies is key (Krushkal *et al*, 1999).

1.3.1.1: GENOME-WIDE SCANS FOR ESSENTIAL HYPERTENSION:

At present, there are more than 25 genome-wide scan studies relating to blood pressure (Table 1.3.1). Most report nominal or suggestive linkages with only 4

(Levy *et al*, 2000; Allayee *et al*, 2001; Kristjansson *et al*, 2002; Angius *et al*, 2002) reaching the stringent genome-wide significance levels of Lander and Kruglyak (Lander & Kruglyak, 1995). It is notable that two of these studies were in relatively isolated populations (Kristjansson *et al*, 2002; Angius *et al*, 2002), although Angius *et al* only reached genome-wide significance on increasing marker density across a suggestive locus. The Framingham study is unique in its use of a longitudinal blood pressure phenotype (Levy *et al*, 2000).

The diversity of the studies is emphasised by Table 1.3.1. The first reported GWS (Krushkal *et al*, 1999) analysed SBP using a discordant sib pair design, sampling individuals from the top and bottom quintiles of blood pressure distribution in the US. Since then, studies in unselected sib pairs, affected sib pairs, nuclear families, extended families and large single pedigrees have also been reported. These have been selected from families ascertained by blood pressure alone, or with diabetes (Hsueh *et al*, 2000), obesity (Rice *et al*, 2000), familial hyperlipidaemia (Allayee *et al*, 2001), early-onset hypertension (von Wowern *et al*, 2003) or early-onset cardiovascular disease (Camp *et al*, 2003). South East Asian, Northern European, Southern European, African, and Hispanic ethnic groups are represented. Outbred populations predominate, but isolated communities such as the Old Order Amish (Hsueh *et al*, 2000), Sardinian villagers (Angius *et al*, 2002) and rural Chinese individuals (Gong *et al*, 2003) have been studied. Phenotypes tested encompass hypertension status and quantitative phenotypes for SBP, postural SBP, DBP and pulse pressure (PP). Some studies also examined effects of fitness training (Rankinen *et al*, 2001; Rice *et al*, 2002).

Table 1.3.1: Genome-wide scan studies of hypertension and blood pressure phenotypes

Study	Phenotype	Correction	Design	N	Ethnicities	Markers: N (Set)	Multipoint linkage method	Significance level	Primary QTL	Reference
FBPP GENOA	SBP		EDSP (top/ bottom 20%) + relatives	427 indivs (69 EDSPs)	US	359 (Weber v6/v8)	Hidden Markov model	P<0.01	2p22, 5q33, 6q22, 15q23	Krushkal et al, 1999
HACP	SBP/ DBP		EDSP/ ECSP (top/bottom 10%) + parents	360 SPs	Chinese	367 (Weber v9)	ASPEX & Mapmaker/Sibs	Simulated P<0.05 = LOD > 2.9	3p26, 11q12, 15q25, 16q11.2, 17p11.2	Xu et al, 1999
AFDS	SBP/DBP/		Type II diabetics + relatives	661indivs	US OOA	357 (ABI LMS)	SOLAR	Simulated P<0.05	2q32	Hsueh et al, 2000
Finnish Twin Cohort	HTN		ASPs (>140/80 mmHg or treated) + relatives	11 SPs 36 DZ twin pairs	Finnish	399 (Weber v9)	Mapmaker/Sibs	N/A	1q23, 3q24, 22q12, Xp11.2	Perola et al, 2000
Framingham	Longitudinal SBP/DBP	Age, sex, BMI, Treatment	Extended families	1702 indivs (332 families)	US white	400 (Weber v8A)	SOLAR	LOD > 3.3	17q21, 17q23, 18p11.3	Levy et al, 2000
Quebec Family	SBP/DBP	Age, sex, Generation	Obese/ unselected extended families	700 indivs	Canadian	400 (Weber v8A)	Segpath	P<0.0023 = LOD>1.75	2p11.2, 2q11.2, 1p11, 5p11, 5p14, 19p13.3, 7q31, 8q12	Rice et al, 2000
FBPP HyperGEN	Postural SBP	Age, sex, Treatment	Unselected SP	636 indivs	US white	387 (Weber vN/A)	Segpath	LOD > 3.3	18q21, 6p21.1	Pankow et al, 2000
Cambridge, UK	HTN		ASP (treated for HTN)	169 SPs	UK white	262 (MRC set)	Genehunter 1.1	P<0.05	No multipoint results, 11q23 (2-point)	Sharma et al, 2000
SAFHS	SBP/ DBP	Age, sex, BMI, Treatment	Extended families	637 indivs (10 families)	Mex Am	399 (Weber v6/v8)	2-point only (PAP v4)	LOD > 3.3	2p11.2, 8q24.3,18q23 21q2	Atwood et al, 2001

Table 1.3.1 continued.

Study	Phenotype	Correction	Design	N	Ethnicities	Markers: N (Set)	Multipoint linkage method	Significance level	Primary QTL	Reference
HERITAGE	Exercising SBP/ DBP/ post-20 wk training	Sex, age, Generation	Unselected SP	102 black SPs 344 white SPs	US black, white	344 (Weber 8a)	Segpath	P<0.0023	Whites: 18q24.3, 10q23, Blacks: 18q21, 11q13, 10q22	Rankinen et al, 2001
Shanghai, China	HTN		ASP (>150/95 mmHg or treated)	283 ASPs	Han Chinese	240 (ABI LMS)	Genehunter	P<0.0001	2q21	Zhu et al, 2001
Los Angeles, US	SBP/DBP /MAP	Sex	Extended families	390 indivs (77 families)	Hispanic	387 (Weber v9)	SOLAR	Simulated P<0.05	7q31	Cheng et al, 2001
Utrecht, Netherlands	SBP/DBP	Age, sex	SP from hyperlipidaemic families	322 indivs	Dutch	399 (Weber v6)	Mapmaker/Sibs	LOD > 3.3	4p15.3, 19p13.3, 6q14, 8p11.2	Allayee et al, 2001
Talana, Sardinia	HTN		Affected relatives, single pedigree (>150/95 mmHg)	36 indivs	Sardinian isolate	400 (ABI LMSv2)	SimWalk2	Simulated P<0.05 = LOD>1	2p25	Angius et al, 2002
Victoria Family	SBP/DBP	Age, sex, height, weight, treatment	Unselected SPs	274 SPs	Aus white	400 (ABI LMSv2)	Genehunter II	Suggestive: Z > 3.1	1p32, 4q22, 16p11.1, Xp11.4	Harrap et al, 2002
HERITAGE	SBP/DBP/ 20 week training effect	Age, sex, BMI, race	Nuclear families, healthy	317 blacks (114 families) 519 whites (99 families)	US black, white	509 (selected)	Segpath	P < 0.0023 = LOD>1.75	Whites:3p26.3, 3q28, 11q13 Blacks: 2p14, 19p13, 12q21	Rice et al, 2002
NHLBI Family Heart	SBP/DBP/ HTN	Age, sex, BMI, study centre	Extended families	2959 indivs (500 families)	US white	402 (Weber v10)	Genehunter 2, Allegro (HTN)	Not considered	1q23, 7p12, 7q3, 12q14, 15q25, 6q14	Hunt et al, 2002
ICSHIB	SBP/DBP	Age, sex, BMI	Unselected nuclear families	792 indivs (196 families)	Nigerian	378 (Weber v9)	Genehunter 2	P < 0.0023 = LOD > 1.75	19p13.12, 19q13.3, 2p11.2, 3p24, 5q15, 7q11.2, 7q2, 10q11.2	Cooper et al, 2002
deCODE Genetics	HTN		Extended families (>160/95 mmHg)	490 indivs (120 families)	Icelandic	904 (deCODE, Weber map)	Allegro	LOD > 3.6	18q21	Kristjansson et al, 2002

Table 1.3.1 continued.

Study	Phenotype	Correction	Design	N	Ethnicities	Markers: N (Set)	Multipoint linkage method	Significance level	Primary QTL	Reference
FBPP HyperGEN	HTN		ASPs, (>160/100 mmHg)	650 black ASPs 915 white ASPs	US black, white	391 (Weber v8)	Mapmaker/Sibs	LOD > 3.6	Blacks: 2p16	Rao et al, 2003
FBPP GenNET	SBP/DBP	Sex, age	Unselected SP	514 black SPs 394 white SPs	US black, white	372 (Weber v8)	Genehunter 2, Sibpal2	LOD > 3.6	Whites: 11q13, 1q21, 3q12	Thiel et al, 2003
FBPP GENOA	HTN		ASPs (>140/80 mmHg)	450 black ASPs 539 white ASPs	US black US white	338 (Weber v6) 381 (Weber v9)	Genehunter 2	LOD > 3.6	1q11	Kardia et al, 2003
FBPP SAPPHIRe	HTN/ low BP		EDSP, ECSP (top 20%, bottom 30%)	1347 Chin SPs 302 Jap SPs	US Chin, Jap	388 (Weber v9)	ASPEX sib_phase	LOD > 3.6	9q34, 10p14, 14q23	Ranade et al, 2003
FBPP meta analysis	HTN		Meta analysis	6000 indivs	US white, black, Chin, Jap	387 (Weber v8)	N/A	LOD > 3.6	10p12	Province et al, 2003
Beijing, China	HTN		Extended pedigree + 32 nuclear families	94 indivs + 174 indivs	Rural, isolated Chinese	386 (Genethon)	Parametric, Genehunter 2.1	Not quoted	12p11.2	Gong, et al, 2003
Malmo, Sweden	Early-onset HTN		ASP, >160/>90 mmHg at < 50 yrs	243 indivs, 91 families	Swedish/ Finnish	362 (ABI MD10)	Genehunter 2	Simulated P<0.05 = LOD>2.7	2q14.1, 14q12	Von Wowern et al, 2003
Utah, US	PP	Sex, age, age^2, BMI	Extended pedigrees with early-onset CVD history	1454 indivs 26 families	Utah, US white	Not quoted (Weber v10)	Parametric, MCLINK	Calculated, LOD > 3.8	8p11.2, 12q23	Camp et al, 2003
BRIGHT	HTN		ASP (top 5% = >150/100 mmHg)	2010 ASPs	UK white	400 (ABI MD10)	Merlin & MLSix	Simulated P<0.05 = LOD>3.1	2q22, 5q13, 6q27, 9q33	Caulfield et al, 2003

FBPP = Family Blood Pressure Program; HACP = Harvard-Anhui Collaborative Project; AFDS = Amish Family Diabetes Study; SAFHS = San Antonio Family Heart Study; BRIGHT = British Genetics of Hypertension Study; ICSHB = International Collaborative Study on Hypertension in Blacks; OOA = Old Order Amish; Mex Am = Mexican Americans; SP = sib-pairs; ED = extreme discordant; EC = extreme concordant; ASP = affected SP. HTN = Hypertension; SBP = systolic blood pressure; DBP = diastolic blood pressure; PP = pulse pressure; BMI = body mass index; CVD = cardiovascular disease; LOD = logarithm of odds score. QTL in bold are significant by the authors criteria. QTL in red are significant by the criteria of Lander & Kruglyak (see text).

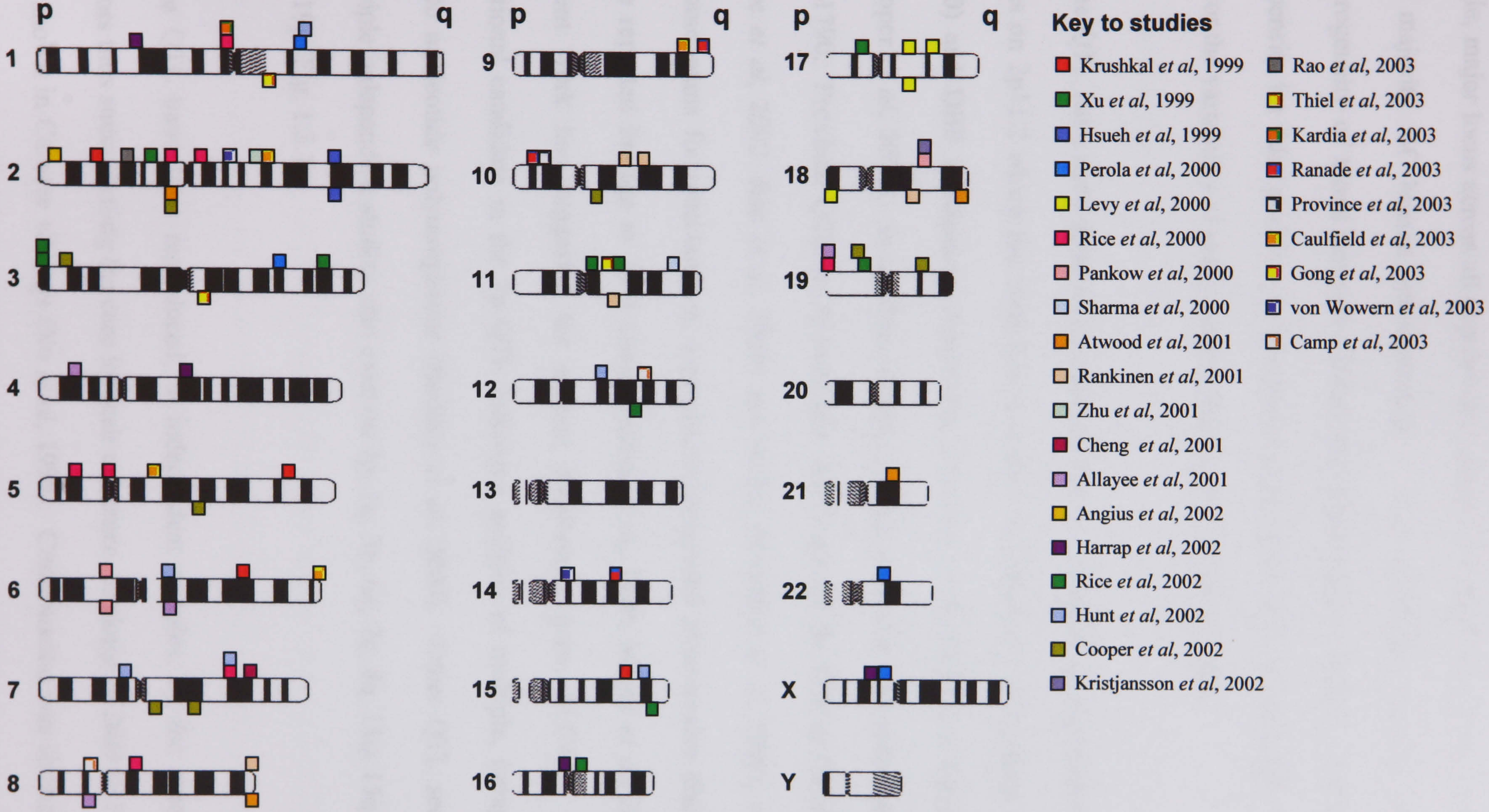


Fig. 1.3.1: A schematic summary of primary QTL of blood pressure and hypertension genome-wide scans. Boxes above chromosomes represent linkage to SBP, PP or hypertension phenotypes. Those below represent linkage to DBP phenotypes.

The major QTL from each study are shown in Figure 1.3.1. There is clearly no single, major locus across all populations. Areas of linkage have been found on the majority of human chromosomes. This probably reflects genetic heterogeneity of blood pressure control and hypertension (Samani, 2003), the propensity for false positives in genome-wide scans (Lander & Kruglyak, 1995) and/or the variability of study design (Munroe & Caulfield, 2000).

Some QTL have been detected in multiple studies. Primary among these is the locus on 2p11.2 which has been linked to SBP in Canadian whites (Rice *et al*, 2000) and DBP in Mexican Americans (Atwood *et al*, 2001) and Nigerians (Cooper *et al*, 2002). In all three studies, linkage peaked at the same marker, D2s1790. Proximal QTL on 2p have also been reported for SBP in US blacks (Rice *et al*, 2002; Rao *et al*, 2003) and whites (Krushkal *et al*, 1999), whilst genome scans for preeclampsia, a pregnancy-associated hypertensive disorder, have reported linkage to 2p13 (Arngrimsson *et al*, 1999; Moses *et al*, 2000). Recent work has suggested the sodium bicarbonate gene, SLC4A5, as a positional candidate in the 2p QTL following analysis of multiple, intragenic single nucleotide polymorphisms (Barkley *et al*, 2004). Other QTL seen in multiple independent studies also exist on 1p, 1q, 3p, 6q, 7q, 8q, 11q, 15q, 18q and 19p (Fig. 1.3.1).

Some QTL have been reproduced, in independent samples, by the reporting authors thus strengthening the case for their existence. Linkage of DBP to 15q25 was shown in Chinese siblings (Xu *et al*, 1999). Confirmation was shown in a second independent cohort of low DBP siblings, with the LOD score increasing

from 2.7 to 3.2 at the same marker, D15s657. Increasing the marker density in this locus from an average spacing of 10 cM to 2 cM resulted in a LOD score of 3.77 (Xu *et al*, 1999). Zhu *et al* (Zhu *et al*, 2001) also used a fine mapping approach in an independent sample of Han Chinese. A marker spacing of 2 cM resulted in a multipoint Z score of 3, confirming linkage of hypertension to 2q21.

The heterogeneity of hypertension and blood pressure control is a major hurdle for GWS studies. The lack of agreement between studies has led some to suggest, retrospectively, that analysis of subgroups may improve this. It is argued that a more homogeneous study sample may improve power, where simply increasing numbers of subjects has failed (Samani, 2003; Lalouel, 2003; Harrap, 2003). This notion is strengthened by data from the Family Blood Pressure Program (FBPP) meta-analysis (Province *et al*, 2003). Analysis of over 6,000 individuals pooled from black, white, Hispanic and Asian cohorts failed to improve on LOD scores obtained in the individual groups (Province *et al*, 2003; Lalouel, 2003).

Some studies have already attempted to address the issue of heterogeneity by stringent recruitment policies. The best example is the BRIGHT Study from the UK which is also the largest single hypertension GWS to date (Caulfield *et al*, 2003). BRIGHT recruited phenotypically-extreme affected sib pairs in the top 5% of blood pressure distribution from the Caucasian population. Thus, subjects had blood pressures over 150/100 mmHg as determined at a single screening. In addition, age at onset of hypertension was below 60 years. The study aimed to recruit individuals with BMI less than 30 kg m^{-2}, and was 80% successful in this.

Those with high alcohol consumption, diabetes or renal disease were excluded. This stringent recruitment enriched the sample for linkage information to hypertension loci, reduced heterogeneity and maximised power for the 2,010 sib pairs analysed. With this rigorous design and large size, the maximum LOD score obtained was 3.21 at 6q27 (Caulfield *et al*, 2003).

1.3.1.2: GENOME-WIDE SCANS FOR FAMILIAL INTRACRANIAL ANEURYSM AND SUBARACHNOID HAEMORRHAGE:

There are inherent problems in performing GWS studies of familial IA or SAH due to the high mortality, difficulties of determining affection status and potential for misclassification. Thus, any GWS in FIA or SAH is a major undertaking and must rely on an affected patient strategy. At present two GWS have been reported. Onda *et al* (Onda *et al*, 2001) used a suitably focused strategy in an, inevitably, small study of 104 Japanese affected sib pairs, most of whom had suffered IA ruptures. Two-point linkage highlighted three regions on 5q22, 7q11 and 14q22 for fine mapping. Multipoint analysis gave LOD scores of 2.24, 3.22 and 2.31 respectively. Yamada *et al* aimed, but failed, to reproduce the results of Onda (Yamada *et al*, 2003). However, with mostly unruptured cases and different markers this is difficult to interpret. A recent US study showed linkage to 7q11 in 13 extended Utah families with 39 IA cases. Multipoint analysis of three markers across the region gave LOD = 2.34 (Farnham *et al*, 2004).

Olson *et al* (Olson *et al*, 2002) used 48 Finnish affected sib pairs for their GWS. They also focused fine mapping efforts by initial linkage analyses. Of seven

regions, which were fine mapped, the most promising multipoint LOD score of 2.6 was obtained for chromosome 19q. No QTL overlay those of the Japanese study. However, the two studies were performed in distinct ethnic groups and Finnish IA cases may be particularly atypical. SAH history in the Finnish patients was also disregarded.

1.3.2: CANDIDATE GENE STUDIES:

1.3.2.1: EVIDENCE-BASED CANDIDATE GENE SELECTION:

In contrast to the genome-wide approach, candidate genes studies are entirely based on *a priori* hypotheses. Lack of reproducibility of candidate gene studies in complex traits has led to recommendations for a multifaceted selection strategy. Thus, type of phenotype and risk factors; heritability; mode of inheritance; penetrance; tissue-specific expression studies and animal model evidence may be used when assessing the potential pathophysiological role of a gene (Tabor *et al*, 2002).

Useful leads can be derived from many sources. Study of Mendelian disease suggests physiological pathways involved in the regulation of complex traits (Dominiczak *et al*, 1998). Study of complex traits and intermediate phenotypes increases knowledge of interacting systems and possible pathophysiological processes (Dominiczak *et al*, 1998). Genome-wide scans implicate genomic regions and the positional candidate genes within them. QTL may be large (Lee *et al*, 2000), and contain many genes, but narrow the field considerably. Rodent

QTL can be transferred to the equivalent 'syntenic' chromosomal region(s) in humans – so-called 'comparative mapping' (Gauguier *et al*, 1999; Stoll *et al*, 2000). Candidate gene and biochemical studies in animals, and knock-out or knock-in mouse models, implicate homologous genes in humans (Dominiczak *et al*, 1998). Increasingly, microarray technology is being used to provide genome-wide gene expression data (Aitman *et al*, 1999; McBride *et al*, 2003).

1.3.2.2: ASSOCIATION, LINKAGE DISEQUILIBRIUM & HAPLOTYPE TAGS:

A candidate gene study most often tests for association of polymorphic markers with trait, although linkage analysis may also be used. Association to a trait is demonstrated by allele-specific frequency differences between affected and unaffected samples. This is distinct from linkage which compares the inheritance of a marker and a trait amongst relatives and is not allele-specific. Association is strongest when the polymorphism being tested is the causative locus underlying the trait (Risch & Merikangas, 1996). Otherwise, any marker tested is a surrogate for the disease locus and must be in linkage disequilibrium (LD) with the disease locus.

LD is a short-range effect compared to linkage (Kruglyak, 1999). It is a non-random association of alleles at adjacent loci (Ardlie *et al*, 2002), for example a marker locus, M, and a disease mutation locus, D. LD is proportional to distance between loci at distances over 60kb; below this the relationship is non-linear (Wright *et al*, 1999; Drysdale *et al*, 2000). LD is said to be high if the allele at M predicts the allele at D. If there is little or no relationship between M and D, LD

is low (Remm & Metspalu, 2002). LD is affected by recombination, the number of original founder mutations, natural selection, population structure and allele frequency (Weiss & Clark, 2001; Zondervan & Cardon, 2004).

Simulations by Risch and Merikangas showed that the power of association to detect small gene effects exceeds that of linkage (Risch & Merikangas, 1996). Therefore, association methods have been applied to microsatellite markers and, more commonly, single nucleotide polymorphisms (SNPs) to interrogate candidate genes. SNPs are now more commonly used because they are the most common genomic variation in species (Jordan *et al*, 2002; Gabriel *et al*, 2002), may affect gene function and are well suited to high-throughput genotyping (Syvanen, 2001).

As a rule, SNPs within candidate genes have been studied as individual loci (Sharma & Jeunemaitre, 2000). However, haplotype tagging approaches have recently been developed (Johnson *et al*, 2001). These make use of the fact that small blocks of the genome appear to undergo relatively little recombination, and SNPs within these regions segregate into distinct haplotypes. These 'haplotype blocks' of 1 Kb to 100 Kb have been reported by a number of studies (Daly *et al*, 2001; Dawson *et al*, 2002; Gabriel *et al*, 2002) and are separated by 'hot spots' of recombination (Maniatis *et al*, 2002). Common haplotypes can be determined by genotyping specific haplotype tag SNPs and can allow interrogation of regions with the minimum of genotyping. However, this requires prior knowledge of LD within the local region (Johnson *et al*, 2001) and detailed haplotype and LD map construction is underway (Gibbs *et al*, 2003). Such

haplotype tagging methods have been used recently in common disorders to investigate small regions and specific candidate genes (Drysdale *et al*, 2000; Rioux *et al*, 2001; Gretarsdottir *et al*, 2003).

1.3.2.3: CANDIDATE GENE STUDIES FOR ESSENTIAL HYPERTENSION AND BLOOD PRESSURE PHENOTYPES:

Many genes, to a greater or lesser extent, have been investigated as hypertension candidates. In general, lack of reproducible evidence means that most are still candidates but no more. Some examples are discussed below.

1.3.2.3.1: Angiotensinogen:

Angiotensinogen (AGT) is the precursor of angiotensin I and, ultimately, angiotensin II (Ang II). Ang II regulates aldosterone production by aldosterone synthase which determines sodium reabsorption via ENaC in the distal tubule (Morgan *et al*, 1996) (See Fig. 1.1.4). Jeunemaitre *et al* (Jeunemaitre *et al*, 1992b) demonstrated linkage of a microsatellite in the 3' region of the AGT gene to hypertension, association of the M235T polymorphism to hypertension, and variation of circulating AGT levels with M235T genotype in French and US sibling pairs. Caulfield *et al* (Caulfield *et al*, 1994) examined a proximal dinucleotide repeat and the intragenic M235T and T174M polymorphisms in 63 white, European families. Linkage and association of hypertension to the dinucleotide repeat, but not the other polymorphisms, was shown. Later work in African Caribbean sib pairs similarly showed linkage and association to the dinucleotide repeat only (Caulfield *et al*, 1995).

Subsequent studies of AGT polymorphisms have found no association with hypertension (Corvol *et al*, 1999), nor linkage to hypertension even in the phenotypically extreme resource of the BRIGHT Study (Munroe *et al*, 2000). However, meta-analyses of the M235T polymorphism across multiple ethnicities (Staessen *et al*, 1999), Japanese studies (Kato *et al*, 1999) and Caucasian studies (Kunz *et al*, 1997) all demonstrate a weak, but significant, association of the T allele with hypertension. It is possible that the effect of the AGT locus is more pronounced in white and younger individuals (Staessen *et al*, 1999). The M235T locus is in linkage disequilibrium with a promoter substitution, A(-6)G, known to enhance gene transcription (Inoue *et al*, 1997). Elegant observations in mice show that blood pressure increases as AGT gene copy number, and expression, increase (Kim *et al*, 1995).

1.3.2.3.2: Angiotensin converting enzyme:

The angiotensin converting enzyme (ACE) locus has been the target of intense study. It catalyses the conversion of Ang I to Ang II (Morgan *et al*, 1996) (See Fig. 1.1.4), affects vascular reactivity and morphology (Lee *et al*, 2000), and is the target of ACE inhibitor anti-hypertensive therapy (Black *et al*, 1997). Studies in Europeans initially suggested no linkage of the ACE locus to essential hypertension (Jeunemaitre *et al*, 1992a). However, the Rochester Family Heart study later showed linkage (Fornage *et al*, 1998) and the Framingham study showed linkage and association to blood pressure (O'Donnell *et al*, 1998) in white, male US population samples. Framingham found that the D allele of the I/D polymorphism in intron 16 of the ACE gene increased hypertension risk in men only (O'Donnell *et al*, 1998). The D allele also associated with hypertension

in Japanese men (Higaki *et al*, 2000). These significant results contrast with an earlier meta-analysis of 23 I/D studies which comprised nearly 7,000 pooled subjects for hypertension (Staessen *et al*, 1997) and showed no association to the D allele. Meta-analysis of quantitative BP traits in over 12,000 individuals from 46 studies also showed no association (Staessen *et al*, 1997). The variability of results from studies of the AGT and ACE loci suggests, then, that any effect on hypertension risk and blood pressure variation is probably small, and in the case of ACE, may be more influential in men.

1.3.2.3.3: Epithelial sodium channel subunits:

AGT and ACE have been studied *ad infinitum*, but systematic assessment of Mendelian blood pressure genes, for example the epithelial sodium channel subunits, is less common (Lifton *et al*, 2001). Linkage analyses of β and γ-ENaC subunit loci, implicated by their role in Liddle syndrome (Hansson *et al*, 1995; Hansson *et al*, 1995), have proved non-significant in Chinese hypertensive sib pairs (Niu *et al*, 1999), but positive in white Australian families (Wong *et al*, 1999). A case-control study of 206 hypertensives and 142 controls suggested association of a T594M variant in the β-subunit with hypertension in UK blacks (Baker *et al*, 1998) who are also more prone to salt-sensitive hypertension (Kornitzer *et al*, 1999). However, coding regions of the β subunit gene were sequenced in whites and blacks but functional effects of the seven polymorphisms found were small and non-significant in *Xenopus* oocytes. There was also no evidence of association of a G589S variant, which had the greatest effect on ENaC activity, and hypertension in a single large family (Persu *et al*, 1998).

1.3.2.3.4: Lysine-deficient protein kinase 4:

Another direct consequence of findings in Mendelian disorders is the recent interest in the role of lysine-deficient protein kinase 4 (WNK4) in essential hypertension. This follows its implication in PHA II (Wilson *et al*, 2001) (See Section 1.1). Recently, a case-control study showed association of hypertension with a single SNP in intron 10 of WNK4 in whites but not blacks (Erlich *et al*, 2003). Linkage studies of the genomic region have also been positive in whites but not blacks (Julier *et al*, 1997; Baima *et al*, 1999). The primary finding of the Framingham GWS study was a QTL for longitudinal SBP on chromosome 17q21, the location of WNK4 (Levy *et al*, 2000). To complete the picture, linkage in the stroke-prone spontaneously hypertensive rat model to chromosome 10 has also been demonstrated (Jacob *et al*, 1991), although recent sequencing and transcription studies of rat Wnk4 have not supported its role (Monti *et al*, 2003).

1.3.2.4: CANDIDATE GENE STUDIES FOR FAMILIAL INTRACRANIAL ANEURYSMS AND SUBARACHNOID HAEMORRHAGE:

The cosegregation of IAs and connective tissue disorders, and expression profiling in IA patients, have concentrated candidate gene studies on components of vessel structure and remodelling.

1.3.2.4.1: Elastin:

Elastin and its primary protease, elastase, are key to the elastic properties of the artery. Reduced elastin in aortic aneurysms has been reported (Minion *et al*.

1994). Aortic or intracranial aneurysms develop in transgenic mice with compromised elastin genes or lysyl oxidase, the latter being required for correct elastin synthesis (Sechler *et al*, 1995; Coutard, 1999). Inhibition of elastin crosslinking by β-aminopropionitrile leads to experimental IA formation (Yamazoe *et al*, 1990).

The Japanese GWS by Onda *et al* implicated the elastin locus at 7q11 (Onda *et al*, 2001). They followed this with haplotype analysis of SNPs in the gene, finding a strong association of a 2-locus haplotype with intracranial aneurysms. This equated to an odds ratio of 4.39 for homozygous carriers of this haplotype. A European study was unable to demonstrate a similar association in a case-control study using IA patients with mostly unknown family history (Hofer *et al*, 2003). Further clarification of the role of elastin in aneurysm development is needed (Zhang *et al*, 2003).

1.3.2.4.2: Collagen type I α2:

The COL1A2 gene is located on 7q22.1 and encodes collagen type I α2, one of the most abundant structural proteins in vertebrate connective tissue. Overexpression in IA tissue has been reported (Peters *et al*, 2001). On the strength of this and its location, the COL1A2 gene was investigated by the Japanese GWS group as a second positional candidate near 7q11 (Yoneyama *et al*, 2004). Association was seen to an Ala → Pro polymorphism in exon 28 in 260 cases and 293 controls. Functional studies suggested that this affected the melting temperature of the triple helix. However, with an 8% frequency in familial IA cases it may only account for a proportion of IA (Yoneyama *et al*,

2004). Confirmation of this is needed but there is, at present, no other study for IA and only one in aortic aneurysmal disease which showed no linkage to the locus in sib pairs (van Vlijmen-van Keulen *et al*, 2003).

1.3.2.4.3: Matrix metalloproteinases and their inhibitors:

Matrix metalloproteinases (MMPs) have wide-ranging roles in protease activation, structural protein degradation and tissue remodelling (Nagase, 1997). Their increased expression in IAs has been shown in several studies (Chyatte & Lewis, 1997). Peters *et al* (Peters *et al*, 1999) showed association of a dinucleotide repeat in the promoter of MMP9 in a US case-control study. Interestingly, the polymorphism affected gene expression in fibroblasts. However, only 10% of cases had a confirmed family history of IA. A Finnish study using 57 familial cases (Yoon *et al*, 1999), and a UK study with 92 mixed cases (Zhang *et al*, 2001), were also negative. Promoter polymorphisms of MMP1, 3 and 12 also showed no association in the latter study.

Tissue inhibitors of metalloproteinases (TIMPs) regulate the activity of MMPs (Nagase, 1997). Their elevated expression has also been demonstrated in IA tissue (Kim *et al*, 1997). Krex *et al* (Krex *et al*, 2003) studied TIMPS 1, 2 and 3 in 44 cases and controls. Polymorphisms were discovered by sequencing of the coding and promoter regions of the three genes, but there was no association to any of the loci. This agreed with earlier work on TIMP 1 and 2. A SNP in each gene, discovered by sequencing, showed no association in a small number of IA patients, although an nt573 polymorphism in TIMP2 was more common in aortic aneurysm patients (Wang *et al*, 1999).

1.3.3: REDUCING THE COMPLEXITY: USE OF EXPERIMENTAL MODELS:

Use of animal models in complex disease genetics allows tight control of environmental factors, rapid breeding and large litters. Late onset disorders, taking decades in humans, present within weeks in animal models. Mating pairs can be selected for informativeness and inbred strains simplify the genetics of the experimental trait under investigation (Dominiczak *et al*, 2000).

1.3.3.1: RAT MODELS OF GENETIC HYPERTENSION:

Traditionally, the rat has been the model of choice in essential hypertension research. Multiple inbred strains now exist which were selectively bred for blood pressure phenotypes (Table 1.3.2). It is important to note that these experimental strains develop a continuously distributed, complex hypertensive trait under oligo- or polygenic control (Rapp, 2000).

1.3.3.1.1: Genome-wide scans in rat models of hypertension:

Genome-wide scans have been widely published in rat models (Jacob *et al*, 1991; Schork *et al*, 1995; Clark *et al*, 1996; Kovacs *et al*, 1997). The concept is very similar to that in human populations. Essentially, two inbred strains discordant for the trait of interest are bred together to generate an F1 population which is then brother x sister mated to produce an F2 population. In the case of a quantitative trait, like blood pressure, this F2 population will represent the full distribution of that trait between the normotensive and hypertensive rat strains

used (Lee *et al*, 2000). Linkage analysis of microsatellites across the genome leads to the identification of QTL as in the human paradigm. QTL for blood pressure phenotypes of 20-30 cM (Dominiczak *et al*, 2000) have been reported on the majority of rat chromosomes through use of different inbred crosses (Lee *et al*, 2000) (Table 1.3.3). Since all rats of an inbred strain are, by definition, genetic clones, modelling of the genetic heterogeneity inherent in human populations is achieved by use of various crosses.

1.3.3.1.2: Congenic rat strains:

It is once the GWS has been completed and thus when QTL need to be confirmed, and genes positionally cloned, that rat models come into their own. The effect of a QTL can be directly proved by the production of congenic strains achieved by breeding parental strains together and then selectively back-crossing the offspring to one or other of the parental strains. The resultant congenic strains are homozygous for a specified chromosomal segment from strain A in a genome otherwise derived from strain B. Two lines can be made, one in which the QTL from the hypertensive strain is introgressed into the normotensive background and a reciprocal strain in which the converse is true. If the QTL is genuine, BPs of these two congenic strains should be higher than the normotensive strain and lower than the hypertensive strain, respectively. This provides proof-positive of the existence of relevant genes in the QTL (Rapp, 2000), an option not available in human studies.

By breeding of substrains, congenic regions can be narrowed, ultimately allowing more precise positioning of causative genes. Theoretically, the

Table 1.3.2: Rat strains selectively bred for blood pressure

Rat Strain	Lines	Original stock	Reference
Genetically hypertensive (GH)	H, C	Wistar-derived	Smirk & Hall, 1958
Dahl salt-sensitive (S) and Dahl salt-resistant (R)	H, L	Sprague Dawley	Dahl *et al,* 1962
Spontaneously hypertensive rat (SHR)	H	Wistar-derived	Okamoto & Aoki, 1963
Spontaneously hypertensive rat, stroke-prone (SHRSP)	H	Wistar-derived	Okamoto *et al*, 1974
DOCA salt-sensitive (SBH) and resistant (SBN)	H, L	Unknown	Ben-Ishay *et al*, 1972
Lyon hypertensive (LH), Lyon normotensive (LN), and Lyon low blood pressure (LL)	H, C, L	Sprague-Dawley	Dupont *et al*, 1973
Milan hypertensive (MHS) and Milan normotensive strains (MNS)	H, C	Wistar	Bianchi *et al*, 1974
Fawn-hooded hypertensive (FHH) and fawn-hooded low blood pressure (FHL)	H, L	Greman brown x white Lashley	Kuijpers & Gruys, 1984
Inherited stress-induced arterial hypertension (ISIAH)	H	Wistar-derived	Markel, 1985
Prague hypertensive rat (PHR), Prague normotensive rat (PNR)	H, L	Wistar-derived	Heller *et al*, 1993

Lines as follows: H = line selected for high blood pressure; C = control line, unselected, random bred; L = line selected for low blood pressure. Taken from Rapp, 2000.

Table 1.3.3: Quantitative trait loci for blood pressure in rat genome-wide scans

Chr	Rat strains	Phenotype	Central Marker/ gene	LOD score
1	WKY_{Hd} x $SHRSP_{Hd}$	SBP after salt	Sa	N/A
	Lewis x Dahl SS	SBP	D1Mco1, Cytp450	3.5
	Lewis x Dahl SS	SBP	Sa	2.5
	WKY x SHR	SBP, DBP	Sa	4.5, 3.8
	WKY x SHR	SBP	Mt1pa	3.0
	Sabra HR x Sabra HP	SBP after salt	D1Mit2, Sa	4.9
	Sabra HR x Sabra HP	SBP after salt	D1Mit1, Cytp450	4.7
2	WKY_{gla} x $SHRSP_{gla}$	SBP, DBP after salt	D2Mit6, D2Mit14	3.3, 3.1
	WKY x Dahl SS	SBP after salt	Nakα1	3.4
	MNS x Dahl SS	SBP after salt	Camk	2.6
	Lyon N x Lyon H	PP	Cpb	7.0
	BN x SHR	SBP	Mt1pb	3.0
	BN x SHR	SBP	Gca	6.3
	BN x SHR	SBP	R598	3.0
3	WKY_{gla} x $SHRSP_{gla}$	SBP after salt	D3Mgh16	5.6
	WKY_{izm} x $SHRSP_{izm}$	SBP	D3Mgh12	6.2
4	RI(BN x SHR)	MAP	Il6	N/A
	WKY x SHR	SBP, DBP, MAP	Npy, Spr	N/A
5	LEW x Dahl SS	SBP after salt	Glutb, Et-2	N/A
	WKY x SHR	SBP	Mitr1678, Anf, Bnf	4.2
10	WKY x SHRSP	SBP, DBP after salt	Ace	N/A
	WKY x $SHRSP_{Hd}$	SBP after salt	Ace	5.1
	MNS x Dahl SS	SBP after salt	Nos2	6.3
	MNS x Dahl SS	SBP after salt	Ace	4.8
13	Lyon-N x Lyon-H	DBP	Ren	5.6
	WKY x SHR	SBP, DBP	D13Mit2	5.7
16	BN x SHR	SBP	R220	4.3
17	Sabra HR x Sabra HP	SBP after salt	D17Mgh5	3.4
18	WKY x $SHRSP_{Hd}$	SBP	Rr1094	3.2
19	RI(BNxSHR)	SBP	D19Mit7	N/A
20	RI(BNxSHR)	SBP	Hsp70	N/A
X	WKY x $SHRSP_{Hd}$	SBP	Per-ha2, Per-ha7	N/A
Y	WKY x SHR	SBP	N/A	N/A

WKY = Wistar-Kyoto; SHRSP = stroke-prone spontaneously hypertensive; izm = Izumo; Hd = Heidelberg; gla = Glasgow; Dahl SS = Dahl salt-sensitive; Sabra HR = Sabra hypertension-resistant; Sabra HP = Sabra hypertension-prone; Lyon-N = Lyon normotensive; Lyon-H = Lyon hypertensive; BN = Brown Norway; MNS = Milan normotensive; RI(BNxSHR) = recombinant inbred strain from BN x spontaneously hypertensive. N/A = LOD score not quoted. Taken from Lee *et al*, 2000.

congenic strategy was thought to allow QTL mapping to a 1 cM segment, small enough to allow sequencing. In reality, it seems that even greater resolution is possible with the report of a Dahl S x Dahl R congenic having an introgressed segment < 177 kb in size on chromosome 7 (Garrett & Rapp, 2003). Many other congenic strains have confirmed QTL found by GWS on most rat chromosomes (St.Lezin *et al*, 1999; Jeffs *et al*, 2000; Frantz *et al*, 2001; Yagil *et al*, 2003). Locus-locus interactions have also been demonstrated by so-called 'double' congenics having two introgressed regions. Rapp *et al* (Rapp *et al*, 1998) showed an interactive effect on blood pressure of QTL on chromosomes 2 and 10 in a Dahl R x S cross by this method.

1.3.3.1.3: Comparative mapping: the application of animal data in humans:

Observations in animal models are only useful if they can be translated to humans. There are limited examples of this, at present (Corvol *et al*, 1999), but the following two examples demonstrate the potential.

1.3.3.1.3.1: Comparative mapping of a rat blood pressure QTL to human:

The rat chromosome 10 blood pressure QTL has already been mentioned in the context of ACE and WNK4 studies. It is also the only example of prospective mapping of a common rat blood pressure QTL to human. The rat chromosome 10 QTL was first reported in SHRSP x WKY experimental crosses (Hilbert *et al*, 1991; Jacob *et al*, 1991), confirmed in other strains and proven by congenic strain production (Rapp, 2000). On the strength of the evidence in rat, Julier *et al* (Julier *et al*, 1997) investigated the syntenic region on human chromosome 17 and showed linkage to hypertension in French and UK sib pairs. Baima *et al*

(Baima *et al*, 1999) offered some confirmation in a US sample. However. studies of Afro-Caribbeans (Knight *et al*, 2000) and African Americans (Baima *et al*, 1999) have proved negative as have studies in Caucasian populations excluding diabetics (Knight *et al*, 2001; Rutherford *et al*, 2001). The chromosome 17 locus may, therefore, be relevant only in a subset of hypertensives (Knight *et al*, 2003). However, the study by Julier *et al* illustrates the application of comparative mapping to complex disease.

1.3.3.1.3.2: α-Adducin:

Adducin is a heterodimeric protein consisting of combinations of α, β or γ subunits. It regulates cell-signal transduction by changes in the actin skeleton (Cusi *et al*, 1997). The membrane skeleton was implicated in membrane Na^+ transport differences in Milan hypertensive (MHS) and normotensive (MNS) rats. Adducin was identified as a potential candidate and non-synonymous Tyr to Phe and Gln to Arg polymorphisms were found α and β subunits, respectively (Tripodi *et al*, 1991). These affected protein phosphorylation, and genotypes at the loci correlated with blood pressure in an MNS x MHS F1 cross, accounting for 43% of SBP difference between strains (Bianchi *et al*, 1994). The same group translated these findings to humans, with case-control evidence of association of hypertension to markers around the α-adducin locus in Italians (Casari *et al*, 1995), and confirmation in independent Italian and French samples (Tripodi *et al*, 1991). Study of a Gly460Trp polymorphism showed that the Trp allele was associated with hypertension, greater sensitivity to body sodium levels, and greater response to diuretic treatment (Cusi *et al*, 1997). Association of the Trp allele to hypertension was supported by the US HyperGEN study in

whites. However, their findings in blacks suggested no effect of the α-adducin locus (Province *et al*, 2000). Ethnic differences may be important, given other negative case-control studies in Japanese (Kato *et al*, 1998; Ishikawa *et al*, 1998; Ranade *et al*, 2000) and mixed South East Asian samples (Ranade *et al*, 2000). The involvement in whites may also be complex since studies of blood pressure in white US populations report no association of Gly460Trp (Schork *et al*, 2000; Bray *et al*, 2000a).

1.3.3.1.4: Mouse models in hypertension research:

The ability to produce knock-in and knock-out mouse genetic models has enabled functional studies of genes in blood pressure regulation. The study of Kim *et al* (Kim *et al*, 1995) has already been mentioned in the context of angiotensinogen. This very elegant work demonstrated an almost linear relationship between blood pressure and 0 to 4 functional copies of the murine *agt* gene expressing in mice. The interaction of renin and angiotensinogen has also been demonstrated in mice expressing the human homologues. Both human AGT and REN were required for an effect on blood pressure because murine renin is ineffective against human angiotensinogen (Murakami & Fukamizu, 1999). Transgenic approaches have proved informative in the study of many components of blood pressure control including enzymes, vasoactive peptides, receptors and ion channels (Cvetkovic & Sigmund, 2000).

The genome-wide approaches used in rat are not as widespread in mouse, partly due to difficulties of phenotyping such small animals (Fitzgerald *et al*, 2003). However, at least two GWS have now been reported using F_2 crosses of different

hypertensive and normotensive mouse strains (Wright *et al*, 1999; Sugiyama *et al*, 2001). Both report a number of significant loci although none are present in both studies. However, the latter noted that, of six QTL, five were concordant with rat QTL, and four were concordant with human QTL (Sugiyama *et al*, 2001).

1.3.3.2: EXPERIMENTAL MODELS FOR INTRACRANIAL ANEURYSMS:

1.3.3.2.1: Rat models:

The dearth of genetic rat models of aneurysm formation has been discussed in Section 1.2. As noted, with the exception of the work by Coutard and colleagues, there has been no genetic study of IAs in rats due, quite simply, to the fact that they do not occur spontaneously (Coutard *et al*, 2000). Induction of aneurysms by renal hypertension and carotid ligation is common, and has been used to show, for example, changes in vessel wall structure (Kondo *et al*, 1998). Aneurysms have been induced in animals by infusion of large doses of elastase which underlines the importance of the extracellular matrix, and elastase itself (Miskolczi *et al*, 1998; Short *et al*, 2001). However, these models are intended for evaluation of surgical devices and are not physiological.

1.3.3.2.2: Mouse models:

As with hypertension, mouse models can provide useful data on gene function. Knockout mice, which develop arterial aneurysms and ruptures, may suggest candidate genes for IA formation. For example, expression of truncated rat elastin genes in mice resulted in an emphysematous phenotype for genes lacking

exon 33, but aortic rupture in mice lacking exons 19-31 (Sechler *et al*, 1995). This suggests potential for considerable variety in effects of elastin mutations, including haemorrhagic arteriopathies. Mice null for the lysyl oxidase gene, which initiates crosslinking of collagen and elastin fibres, also develop aortic aneurysms (Maki *et al*, 2002). The Blotchy mouse, which has a defective lysyl oxidase gene, develops cerebral and peripheral aneurysms with greater ease than controls following carotid artery ligation (Coutard, 1999). In mice it has also been shown that knockout of matrix metalloproteinase 9 reduces aortic aneurysm formation by elastase infusion or application of $CaCl_2$ (Pyo *et al*, 2000; Longo *et al*, 2002). Conversely, disruption of TIMP1 function increases aortic aneurysm formation in mice (Silence *et al*, 2002). As has been described, genetic studies to follow up such leads have been performed in humans. Unfortunately, this is the only option with the present lack of genetic models.

1.4: AIMS OF THE STUDY:

The overall aim of this work was to apply current genetic thinking and strategies to the dissection of two complex disorders, essential hypertension and familial intracranial aneurysm, in human study samples. Linkage and association mapping methods were used to accomplish this, taking into account current opinions on best practice. There were three specific aims:

1. To examine two-point and multipoint linkage of a discrete region on the distal arm of chromosome 5 to blood pressure and essential hypertension in the Silesian Hypertension Study resource, with confirmation in a Scottish population sample from the MIDSPAN Family Study.

2. To follow up linkage evidence by studying cardiovascular candidate genes within the 5q linkage region. This was achieved by linkage and association study of known functional polymorphisms within the β_2-adrenergic receptor gene, and sequencing and study of polymorphic loci in the fibroblast growth factor 1 gene.

3. To perform a pilot study of candidate genes of familial intracranial aneurysm, concentrating on collagen type III, polycystin I, fibrillin I and serine protease inhibitor A1. Linkage and association analyses were applied to a small sample of Scottish families. Studies of the serine protease inhibitor A1 locus were expanded to a combined group including a small number of families from the south of England.

CHAPTER 2

MATERIALS & METHODS

2.1: GENERAL LABORATORY PRACTICE:

The procedures detailed herein were conducted with appropriate regard for safety. Routinely, lab coat and powder-free latex gloves were used as a minimum. Laboratory spectacles, facemasks and fume hoods (Holliday, Fielding & Hocking, UK) were used as required when suggested by hazard labelling on reagents.

Instrumentation was used following appropriate training. Instruments were maintained under contract and calibrated, in line with manufacturer's guidelines, where necessary. Software used for collecting and processing data was as supplied by manufacturers for the requisite instrument and application. Computing equipment was sourced from reputable manufacturers and data backed up to appropriate external storage media.

Reagents were of the highest quality available and sourced from specialist suppliers. Aqueous solutions were made using autoclaved, deionised water. Plasticware was disposable and either sterilised by irradiation or autoclaving. Glassware was washed in Decon 75 detergent, rinsed with distilled water and dried in an oven at 37°C. Manual liquid handling of volumes from 0.1-5,000 µl was performed using calibrated Finnpipettes (Life Sciences International, UK) and autoclaved tips (Alpha Labs, UK). Automated liquid handling of 5–1,000 µl volumes was performed by a TECAN Genesis RSP 500 workstation (TECAN, UK) using supplied conductive tips.

2.2: STUDY RECRUITMENT AND PHENOTYPING:

The work detailed in Chapters 3 to 6 used DNA resources collected for three separate studies. The studies described were conducted with the approval of the appropriate local ethics committees. All recruitment to the studies was with the informed consent of the participants. All data collected were anonymised before being made available to laboratory staff.

2.2.1: SILESIAN HYPERTENSION STUDY:

The Silesian Hypertension Study was based on recruitment of probands with diagnosed essential hypertension and their available parents and siblings. Recruitment strategies for the study were designed by Dr Maciej Tomaszewski, clinical researcher at the Silesian School of Medicine, Poland. Participants were recruited through probands attending hypertension clinics in the Silesian region of southern Poland by Drs Tomaszewski and Lacka during 1999 to 2000. In 2001, Dr Tomaszewski received the International Society of Hypertension Fellowship based in Professor Dominiczak's laboratory at the University of Glasgow in order to learn genetic techniques and apply these to the Silesian Hypertension Study. He brought with him to Glasgow all DNA samples. I have set up the relevant laboratory techniques and trained Dr Tomaszewski in all relevant genotyping methods. Subsequently, he and I analysed all samples. This involved 638 DNA samples tested for 11 microsatellite markers and 6 SNPs and required several months of set-up work and genotyping from both of us. It is routine, in our laboratory, that genotyping of large clinical studies is performed

by several research and technical personnel working together. The data have been analysed and written up (Tomaszewski *et al*, 2001; Charchar *et al*, 2002) as a joint effort under overall supervision of Professor Dominiczak.

In the Silesian Hypertension Study, hypertension was defined as systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure greater than 90 mmHg on three separate occasions and/or anti-hypertensive treatment. Clinical history, anthropometric data and cardiovascular phenotypes were recorded. Subjects with secondary hypertension were excluded. All other individuals with essential hypertension and their relatives were included. Clinical histories indicated only two cases of type II diabetes in parents.

Height and weight were measured under standard conditions to allow calculation of body mass index (BMI). Blood pressures were measured by mercury sphygmomanometer with subjects seated and rested for 20 minutes. Systolic blood pressure (SBP) was recorded at the return of arterial sounds (Korotkoff phase I) and diastolic blood pressure (DBP) at the disappearance of arterial sounds (Korotkoff phase V). Three consecutive readings were averaged for both SBP and DBP. Blood samples were collected for DNA extraction and to provide serum samples for freezer storage. Biochemistry and electrolyte assays were not routinely performed.

For subjects receiving hypertensive treatment, the recorded blood pressures are right-censored values. With such a high percentage of treated hypertensives in the sample, a correction for this was necessary. We employed a non-parametric

method developed by the Framingham Study (Levy *et al*, 2000). Separate adjustments were conducted for men and women. Cubic regression models were fitted to account for age effects. Residuals were then sorted from highest to lowest within age groups (<35, 35 to 44, 45 to 54, 55 to 64, 65 to 74, and 75+) and a correction applied within each age group. For treated individuals, the algorithm used the expected value of the untreated residual conditioned on the distribution of other residuals of greater or equal magnitude, as the adjusted residual for a treated measurement. The adjusted residual equals the average of that individual's residual and all larger residuals. Adjusted BP equals the measured BP plus the difference between the adjusted residual and the initial residual. This approach takes into account an individual's position within the distribution of BP for his age/ sex group. This is perhaps a more individualised approach than an adjustment using an estimated age-specific treatment effect derived from a regression on age and treatment. The latter would result in a standard adjustment for all treated persons. The Framingham approach tailors the adjustment to the individual by using residuals from untreated individuals of the same sex and similar age to derive an expected untreated blood pressure. Thus, patients who have had their blood pressure lowered considerably by intervention will be corrected upwards to a greater extent than individuals who are treated but still remain hypertensive. It should be noted, however, that all such adjustments are inexact since the magnitude of response of an individual to a specific treatment regime is impossible to determine using the available data.

2.2.2: MIDSPAN FAMILY STUDY:

The MIDSPAN Family Study was implemented and recruited by clinical staff of the Department of General Practice, University of Glasgow. Between 1972 and 1976, all residents aged 45-64 living in the large burghs of Renfrew and Paisley situated in the west of Scotland were invited to participate. Those willing were asked to complete a questionnaire and invited to attend for screening examination at clinics set up nearby. At screening, detailed data were collected on seated blood pressures and other clinical, anthropometric and socio-economic characteristics. In 1993-1994, the offspring of the original cohort were traced and those aged 30-59 years were invited to join the study. A total of 2,338 offspring participated consisting of 869 singletons and 1,469 individuals derived from 608 sibships ranging in size from 2 to 8 members. Data collected on the offspring cohort included blood pressures, history of cardiovascular events and diabetes, smoking history and details of medication. Blood samples were taken for DNA extraction and measurement of serum levels of potassium, sodium, urea and creatinine. Glucose and total cholesterol were also measured although most subjects were not fasted. All data were stored in a Microsoft Access database.

2.2.3: FAMILIAL INTRACRANIAL ANEURYSM PILOT STUDY:

Recruitment of the FIA pilot study was funded by the McFeat Bequest administered by North Glasgow University Hospitals NHS Trust. Recruitment was carried out during 1998 to 1999 by a research nurse, Sister Mairi McDade, in Glasgow. The study was originally conceived as an affected sib-pair study, a

design since used by genome-wide scans of FIA. All families were under the care of Mr Ken Lindsay, a neurosurgeon at Glasgow's Southern General Hospital. Initial contact was made with a proband by the nurse. Further recruitment within a family was via the proband. After 6 months of recruitment, it was clear that affected sib-pairs were not easily available. I then focused recruitment on obtaining as many affected relatives as possible and on recruitment of parents of affected individuals, or unaffected siblings if two parents were not available. Relatives not known to be affected were recruited when possible. This rationale was based on the requirements of the increasingly popular transmission-disequilibrium statistical approaches of the time (discussed further in Chapter 5), the classic approach for which required clinical phenotype to be known for an affected child only. This was an advantage given the difficulties of establishing a genuine unaffected status in individuals (discussed further in Chapter 5).

No distinction was made between individuals with unruptured IA or those who had survived aneurysmal SAH. The scale of this single centre study combined with the relative paucity of IA/ SAH patients did not allow us the luxury of studying subgroups. For the same reason, phenotypes were not differentiated in analyses. Presently, the largest published studies of FIA genetics also face this problem. For example, a recently published genome-wide scan in 104 Japanese sib-pairs also combined ruptured with unruptured patients (Onda *et al*, 2001).

Autosomal dominant polycystic kidney disease (ADPKD) is a rare disorder which is firmly linked with occurrence of intracranial aneurysms. We therefore

wished to exclude cases of ADPKD from the study. To this end, renal ultrasound (RUS) can be used to detect the characteristic renal cysts. RUS was offered to all participants and accepted by 58 individuals. Family history and individual serum creatinine levels were also examined. Of 80 study participants, RUS and family history eliminated ADPKD in 58 individuals. RUS was refused by 22 individuals but family history and serum creatinine levels did not indicate ADPKD.

Participating subjects visited the hospital where the nurse administered a standard questionnaire. This included history of hypertension, smoking, medication, personal and family history of intracranial aneurysms and subarachnoid haemorrhages, and angiography. Blood pressure was measured in a seated position and blood samples for DNA extraction and blood chemistry assays were collected. Affected status was confirmed by reference to case notes. Data were transcribed into a Microsoft Access database. Transcription of the data was checked by a second person to ensure accuracy.

2.3: DNA EXTRACTION, QUANTITATION AND DILUTION:

Extraction methods for obtaining genomic DNA from whole blood varied by study. Extraction of DNA from Silesian blood samples was performed by technical staff at the Silesian School of Medicine. Extractions were performed using MasterPure DNA purification kits (Epicentre Technologies, US) following the manufacturer's protocol.

MIDSPAN Study DNA samples were extracted by technical staff at the University of Glasgow under the direction of Dr. Gordon Inglis using a standard phenol-chloroform method adapted from Miller *et al* (Miller *et al*, 1988). Briefly, cells were lysed with buffer containing 0.32 M sucrose, 10 mM Tris, 5 mM $MgCl_2$, and 1% Triton X-100, pH 7.5. Samples were mixed by inverting and centrifuged at 1,500g for 10 min. Nuclei were lysed by suspension of pellets in 3 ml of buffer containing 10 mM Tris, 0.4 M NaCl, 2 mM EDTA, pH 8.2. Addition of 0.6% (v/v) SDS and 200 µl of Proteinase K (Sigma, UK) was followed by 18 hr incubation at 37°C. 1 ml of saturated NaCl and 5 ml of phenol/chloroform solution were added followed by centrifugation at 1,200g for 10 min. The aqueous layer was washed with 99% ethanol. Precipitated DNA was resuspended in 400 µl of autoclaved, deionised water. Samples were stored at -70°C until use.

Extraction of DNA samples for the FIA study was performed by technical staff at the University of Glasgow using Wizard Genomic DNA Purification kits (Promega, UK). Extractions were performed using the manufacturer's recommended protocol.

Quantitation of all Silesian and MIDSPAN DNA was by Picogreen dsDNA Quantitation kit (Molecular Probes, Netherlands). Genomic DNA was diluted 1000 fold by two serial dilutions of 1:10 followed by 1:100. The manufacturer's protocol using a high concentration standard curve was followed. Samples were read in black 96-well plates on a Wallac Victor^2 microplate reader (Perkin Elmer, UK).

Quantitation of DNA for the FIA study was performed using a Biotech Ultraspec 2000 spectrophotometer (Amersham Biosciences, UK). 5 µl of genomic DNA was added to 995 µl of deionised water in a quartz cell and mixed. Absorbance at 260 and 280 nm, and concentration in ng/µl, were recorded. The reading was then repeated and the results averaged.

Following quantitation, genomic DNA samples were diluted into deep well plates (Beckman Coulter, UK) at the appropriate concentration for the downstream application. Water blanks and control DNA were included on all plates. Control DNA was obtained from Coriell Cell Repositories.

2.4: MICROSATELLITE MARKER GENOTYPING:

2.4.1: POLYMERASE CHAIN REACTION AMPLIFICATION:

Primer sequences for PCR amplification of microsatellite markers were obtained from the Genome Database (GDB, http://gdbwww.gdb.org/), National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/), Applied Biosystems Linkage Marker Set 2 (Applied Biosystems, UK) or the literature as specified. Primer sequences are given in Appendix 3.

Primers were manufactured by either MWG Biotech or Applied Biosystems. To enable fluorescent genotyping, forward primers were synthesised with 5' fluorescent dyes. To improve electrophoretic resolution and peak morphology

reverse primers were synthesised with a 7 base tail at the 5' end, the sequence of which was GTTTCTT. This has been shown to improve peak morphology which gives cleaner data and more accurate genotyping (Magnuson *et al*, 1996).

PCR was performed using HotStar Taq (Qiagen, UK) with 25 ng DNA; 2 µl of 10x buffer with 15 mM $MgCl_2$; 200 mM of each dNTP; 20 pmol primers and 0.2 U of enzyme in a total volume of 20 µl. Reactions were set up in 96-well microplates (ABgene, UK) and cycled on PCT-225 thermal cyclers (MJ Research, US). A PCR program was used which maximised addition of 'plus-A' bases at the 3' end of amplification products to facilitate genotyping. This was as follows:

1) 95°C for 15 min
2) 94°C for 1 min
3) *x*°C for 1 min where *x* = annealing temperature
4) 72°C for 1 min
5) Steps 2-4 repeated 34 times
6) 60°C for 30 min
7) Cool to 12°C

Cycled PCR reactions were stored at -20°C until used.

2.4.2: MICROSATELLITE MARKER GENOTYPING:

2.4.2.1: ABI 377 SEMI-AUTOMATED DNA ANALYSER:

Genotyping of microsatellites for the Silesian Study, the MIDSPAN Study and the FIA pilot study was performed on an ABI 377 (Applied Biosystems, UK). The process of genotyping on the ABI 377 is presented in schematic format in Figure 2.4.1. Electrophoresis was performed through 5% denaturing

polyacrylamide gels. Gels were cast by hand using 50 ml of Long Ranger 5% acrylamide gel solution (FMC Bioproducts, US) containing 6 M urea, 250 µl of 10% APS (Amresco, US) and 25 µl of TEMED (Amresco, US). They were allowed to polymerise for a minimum of one hour and a maximum of overnight. Microsatellite PCR products were pooled together and combined with a loading cocktail containing ultrapure deionised formamide (Amresco, US), GS350TAMRA or GS350ROX size standard (Applied Biosystems, UK) and Dextran-EDTA loading buffer (Applied Biosystems, UK). Samples were denatured for 2 min at 95°C and resolved on the ABI 377 (AppliedBiosystems, UK) using the manufacturer's recommended protocol. On completion of electrophoresis, raw data were extracted with GeneScan v3.1 and genotyped with Genotyper v2.1 or v2.5 (Applied Biosystems, UK). All gentotypes were checked by two individuals who were unaware of phenotypes. Positive control DNA was included on all plates of samples to ensure consistency from run to run. This was obtained commercially (Coriell Cell Repositories, US) and derived from a stable cell line from an individual of the CEPH resource.

For samples where one or more genotypes were not retrieved, due to failure or ambiguity, the entire pool containing the problem marker was re-amplified by PCR and re-genotyped. Potentially the greatest source of genotyping error comes from the compilation of plates of the various DNA samples to be re-genotyped. This has to be done manually and there is potential for human error. By following the rigorous approach of re-genotyping an entire pool of markers, rather than just those that were lacking, we were able to monitor two things. Firstly, we could confirm that the correct DNA sample was being re-genotyped .

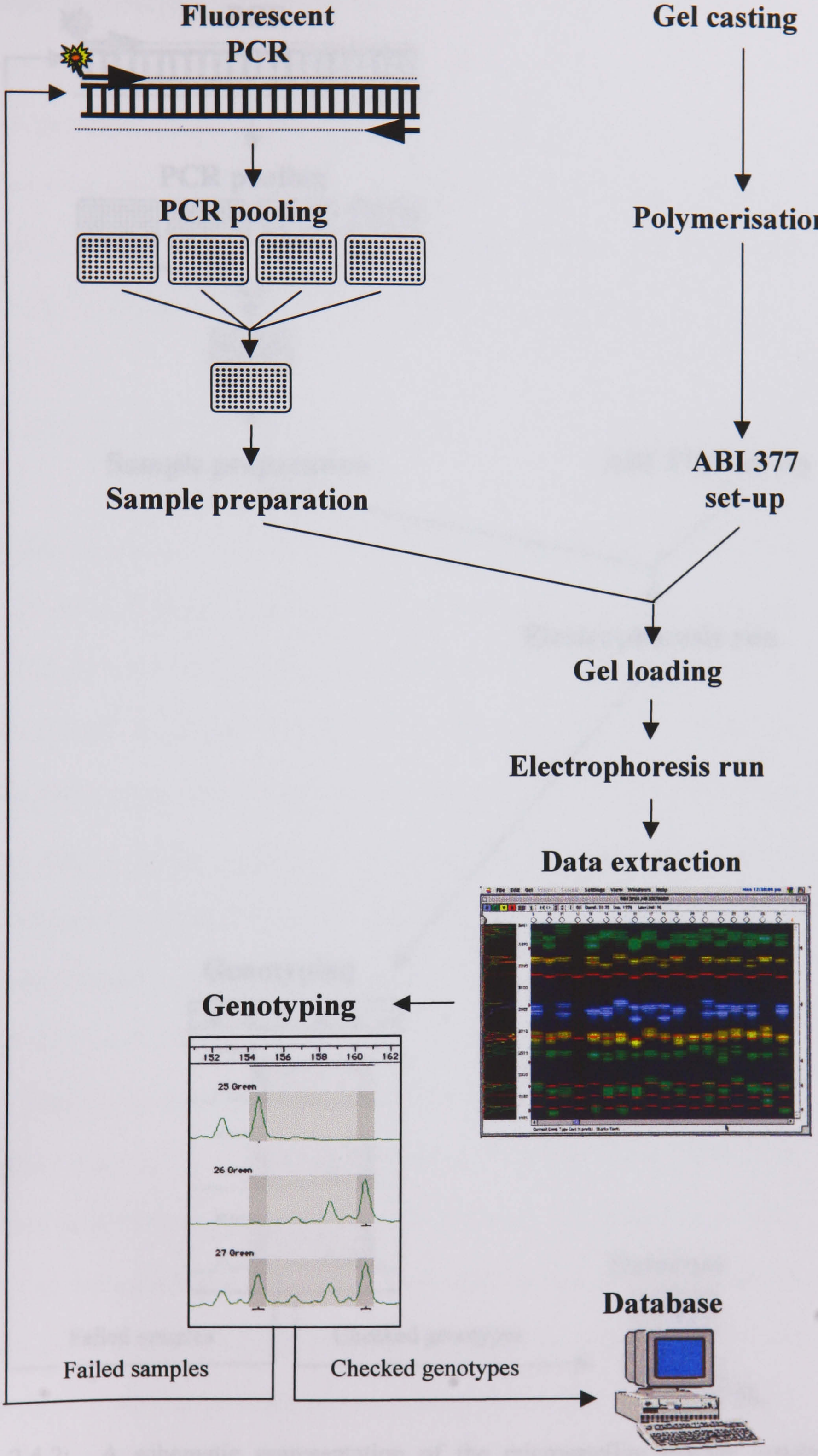


Fig. 2.4.1: A schematic representation of the microsatellite marker genotyping process using the ABI 377 semi-automated DNA analyser. Steps requiring human intervention are shown.

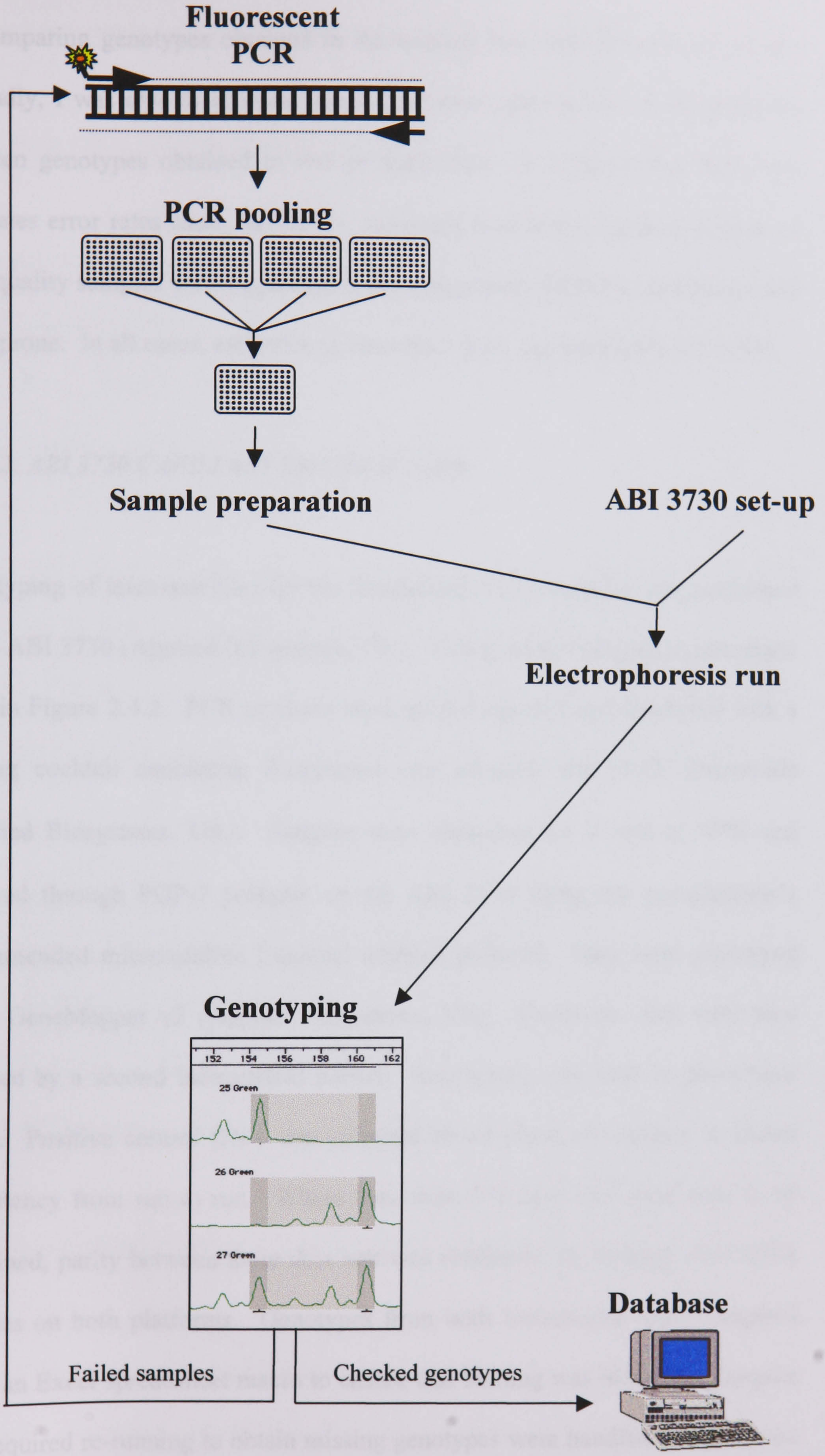


Fig. 2.4.2: A schematic representation of the microsatellite marker genotyping process using the ABI 3730 automated DNA analyser. Considerably less intervention is required and run times are shorter.

by comparing genotypes obtained in the original run with those in the re-run. Secondly, I was able to estimate genotyping error rates based on disagreement between genotypes obtained in two or more runs. It is likely that this over-estimates error rates since, inevitably, estimates tend to be based on re-runs of poor quality samples where genotyping is perhaps more difficult, ambiguous and error-prone. In all cases, estimates of error rates were approximately 0.3-0.5%.

2.4.2.2: ABI 3730 CAPILLARY DNA ANALYSER:

Genotyping of microsatellites for the Southampton FIA samples was performed on an ABI 3730 (Applied Biosystems, UK). This process is shown in schematic form in Figure 2.4.2. PCR products were pooled together and combined with a loading cocktail containing fluorescent size standard and Hi-Di formamide (Applied Biosystems, UK). Samples were denatured for 2 min at 95°C and resolved through POP-7 polymer on the ABI 3730 using the manufacturer's recommended microsatellite fragment analysis protocol. Data were genotyped using GeneMapper v3 (Applied Biosystems, UK). Genotypic data were then checked by a second independent person. Genotyping was blind to phenotypic status. Positive control DNA was included on all plates of samples to ensure consistency from run to run. Where data from 377 and 3730 runs were to be combined, parity between these data sets was confirmed by running some DNA samples on both platforms. Genotypes from both instruments were compared using an Excel spreadsheet macro to ensure that binning was identical. Samples that required re-running to obtain missing genotypes were handled in exactly the same way as ABI 377 genotyping. Error rates were similarly low.

2.5: β_2-ADRENERGIC RECEPTOR GENE SNP GENOTYPING:

2.5.1: PCR OF β_2-ADRENERGIC RECEPTOR SNPs:

Three polymorphisms within the ADRB2 gene were chosen for genotyping due to their reported functional significance. These were Arg16Gly, Gln27Glu and Thr164Ile. Amplification of fragments containing the single nucleotide polymorphisms (SNPs) of interest was accomplished by PCR with HotStar Taq (Qiagen, UK). Primer sequences are given in Appendix 3. Total reaction volume was 20 µl and included: 25 ng genomic DNA, 2 µl 10x PCR buffer containing 15 mM $MgCl_2$, 200 µM each dNTP, 20 pmol of primers and 0.2 U of enzyme. Reactions were set up in 96-well microplates (ABgene, UK) and cycled on PCT-225 thermal cyclers (MJ Research, US). The following program was used:

1) 95°C for 15 min
2) 94°C for 1 min
3) *x*°C for 1 min where *x* = annealing temperature
4) 72°C for 1 min
5) Steps 2-4 repeated 34 times
6) 60°C for 30 min
7) Cool to 12°C

Cycled PCR reactions were stored at -20°C until used.

2.5.2: GENOTYPING OF β_2-ADRENERGIC RECEPTOR SNPs:

Each of the three polymorphisms introduces a restriction site into the genomic sequence. Therefore, genotyping was performed by restriction digest and gel resolution. The Arg16Gly SNP was genotyped by digestion of the PCR products

with 2 U of *Bsr*DI enzyme (New England Biolabs, UK) at 60°C for 6 hours. The restriction products were resolved on 3% Agarose-1000 (Life Technologies, UK) gel containing ethidium bromide. Bands were visualised using a Fluor-S Multi-Imager (Biorad Laboratories, UK). Gels were scored, by eye, by two individuals.

The Gln27Glu SNP was genotyped by digestion of PCR products with 1.5 U of *Ita*I restriction enzyme (Roche, Switzerland) at 37°C for 20 hours. Digestion products were resolved on 2.5% Agarose-1000 gels containing ethidium bromide. Visualisation was performed on the Fluor-S Multi-Imager. Gels were scored, by eye, by two individuals.

The PCR fragments containing the Thr164Ile SNP were digested with 2 U of *Mnl*I restriction enzyme (New England Biolabs, UK) at 37°C for 5 hours. Resolution on 2% Ultra Pure agarose (Life Technologies, UK) gels containing ethidium bromide was followed by visualisation on the Fluor-S Multi-Imager. Genotypes were scored by two individuals.

Accuracy of genotyping and reliability of the restriction digests were verified by direct sequencing of 15 unrelated individuals performed on the ABI 377 using a standard BigDye Terminator protocol (Applied Biosystems). All genotypes were found to agree with the sequence data.

2.6: FIBROBLAST GROWTH FACTOR 1 SNP IDENTIFICATION AND GENOTYPING:

Within the FGF1 gene, no known coding region polymorphisms were found in the literature or on-line SNP databases such as NCBI (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org). To confirm the presence or absence of coding region polymorphisms, in the Silesian population, the coding exons, flanking intronic regions and 3' UTR of FGF1 were amplified by PCR and sequenced.

2.6.1: FIBROBLAST GROWTH FACTOR 1 PCR AMPLIFICATION:

Twenty unrelated individuals from the Silesian Hypertension Study group were selected for sequencing. Our rationale was to select a heterogeneous group of male and female hypertensives and normotensives. Thus, of 20 selected, ten individuals were hypertensive and ten individuals were normotensive. Within these subgroups, half were male and half female. Sequencing of these 40 chromosomes was expected to give 99% probability of detecting polymorphisms with minor allele frequency of 10% and nearly 90% power to detect polymorphisms with 5% minor frequency.

PCR primers were designed to flanking intronic sequences to allow amplification of exons and exon/intron boundaries. Primers for amplification of the 3' UTR were also designed. Sequences of these primers are given in Appendix 3. The target genomic sequence was taken from the National Centre for Biotechnology Information (NCBI; Accession no: NT_029289) and exon sequences were taken

from published cDNA sequences from NCBI and the literature (Payson *et al*, 1993). PCR was performed using HotStar Taq (Qiagen, UK) to amplify template DNA for sequencing. Reaction volume was 20 µl and consisted of: 2 µl of 10x PCR buffer containing 15 mM $MgCl_2$, 200 mM of each dNTP, 20 pmol of primers, 25 ng genomic DNA and autoclaved deionised water to volume.

Reactions were set up in 96-well microplates (ABgene, UK) and cycled on PCT-225 thermal cyclers (MJ Research, US). The following program was used:

1) 95°C for 15 min
2) 94°C for 1 min
3) *x*°C for 1 min where *x* = annealing temperature
4) 72°C for 1 min
5) Steps 2-4 repeated 34 times
6) 60°C for 30 min
7) Cool to 12°C

Cycled PCR reactions were stored at -20°C until used.

2.6.2: FIBROBLAST GROWTH FACTOR 1 SEQUENCING:

PCR products were purified prior to sequencing to remove unincorporated dNTPs and primers. Nucleofast-96 plates (Macherey-Nagel, Germany) were used which work on the principal of size exclusion. PCR products are retained but small primers and dNTPs pass through a porous membrane to waste. The manufacturer's protocol for centrifuge filtration was followed with products being re-suspended in 50 µl of autoclaved water.

Purified PCR products were sent to MWG Biotech for direct sequencing. Forward sequences were obtained using the forward PCR primers. Sequence

data obtained were aligned to a reference sequence using Vector NTI v8 (Informax, US). Potential polymorphic sites were identified following alignment and confirmed by reference to electropherograms from the MWG sequencing runs.

2.6.3: FIBROBLAST GROWTH FACTOR 1 SNP GENOTYPING:

Genotyping of polymorphisms within the FGF1 gene was done by the MRC Gene Service (Cambridge, UK). Genomic DNA at 5 ng/µl was provided. PCR primers were identical to those used during sequencing of the FGF1 gene. Genotyping was performed on an ABI 7900HT (TaqMan) instrument platform (Applied Biosystems, UK). Allelic recognition probes were labelled with the fluorescent dyes VIC or FAM. Their sequences are provided in Appendix 3.

2.7: STATISTICAL ANALYSES:

All genotypic data were checked with PEDCHECK (O'Connell & Weeks, 1998) to detect genotyping errors and misspecification of relationships. Inconsistencies were corrected, if possible, or individuals were removed from the dataset.

2.7.1: LINKAGE ANALYSES:

Linkage analyses were conducted on genotype data derived from the Silesian and MIDSPAN study groups. All genotyped samples, which were passed by PEDCHECK, were included in analyses. Whilst this, potentially, leads to

analysis of a more heterogeneous sample, neither resource was large enough to sensibly address hypertensive subgroups. Different statistical approaches were used in each study sample owing to the differing ascertainment criteria and family structures.

Multiple methods were employed for analysis of the Silesian data as consistency between different methods provides confidence in the result. Haseman-Elston (H-E) regression as implemented in SIBPAIR was used for identity by descent (IBD) two-point linkage analyses of hypertension status and systolic and diastolic blood pressures (Duffy, 1999). The merit of this test lies in its applicability to both quantitative and qualitative traits. Analysis of both is preferable to give confidence in results and allows direct comparison of them. The H-E non-parametric method regresses a function of squared trait sums and differences between siblings on the proportion of alleles identical by descent. The test essentially determines whether siblings, who are phenotypically alike, are also more genotypically alike than would be expected at a given locus. The identity-by-descent of siblings reflects the sharing of 0, 1 or 2 alleles which derive from a common ancestral chromosome. IBD was estimated within SIB-PAIR based on sib pair and parental genotypes. Parental genotypes were not available in 30% of families and in these cases IBD was estimated using the sum of sharing for each possible set of parental genotypes weighted by their likelihood based on all children in the sibship. Allele frequencies were calculated within SIB-PAIR using founders of pedigrees.

SPLINK v1.08 was used to run a second confirmatory IBD two-point analysis of hypertension status (Clayton, 2000). This is an affected sib pair analysis which uses a maxmimum likelihood approach to estimate the probability that two affected sibs share 0, 1 or 2 autosomal marker alleles IBD. Allele frequencies are also estimated by a maximum likelihood method for use in IBD estimation in absence of informative parental genotypes. SPLINK has the advantage that it does not use the unaffected siblings other than to assist IBD estimation. Given that some of the normotensive siblings may, later, develop hypertension, and therefore are misclassified, an affected sib pair analysis provides useful confirmation in a sample with unambiguous disease status.

A two-point χ^2 identity by state (IBS) affected sibpair method in SIB-PAIR was also used to test linkage to hypertension as confirmation of the H-E results. Whilst of lower power than IBD analyses, the IBS approach does not rely on estimation of IBD and therefore circumvents any bias arising from such estimation. Identity-by-state is determined by the number of alleles shared, regardless of their ancestral origin. Observed IBS statistics are compared, by χ^2 test, with expected IBS which are derived from allele frequencies in the whole sample. Missing parental genotypes have no effect on the test.

MAPMAKER/SIBS was used for non-parametric multipoint analyses of quantitative traits since these are expected to be more informative than analysis of arbitrarily defined qualitative traits such as hypertension. For this reason, multipoint SPLINK analysis was not performed. MAPMAKER/SIBS calculates the full IBD probability distribution by a maximum likelihood method. The non-

parametric test ranks sib pairs by their absolute value of phenotypic difference. For each location across the region under investigation, a statistic is defined to relate the rank to number of alleles shared IBD and thus linkage may be demonstrated or refuted. Of the available tests in MAPMAKER/SIBS, the non-parametric option was preferred since it may be more powerful than other available options such as multipoint Haseman-Elston. It also makes no assumptions about distribution of phenotypic noise and therefore provides a robust confirmation of two-point results (Kruglyak and Lander, 1995). Allele frequencies for use in the multipoint analysis were taken from SIB-PAIR which calculates frequencies based on founders of pedigrees only.

Both two-point and multipoint analyses in the MIDSPAN Study used the variance components methodology of SOLAR (Almasy & Blangero, 1998). SOLAR was selected following the example of other studies in population samples, for example the Framingham Study genome-wide scan (Levy *et al*, 2000). Unlike the Silesian Study sample, which consists of small family units of consistent size, the MIDSPAN sample contains sibships ranging between 2 and 8 individuals. An advantage of the variance components approach is that it treats each family as a unit, explicitly allowing for non-independence among sibs. This pedigree approach is more powerful and less liable to type I error, when using larger sibships, than are statistics based on pairs of relatives (Levy *et* al, 2000; Marlow, 2002). This made SOLAR a more appropriate choice than the sib-pair approach of MAPMAKER/SIBS. Variance components methods assume normally distributed phenotypes and so log10-based transformations of SBP, DBP and MAP, and the BMI covariate, were performed. Normal distribution

was confirmed by a Kolmogorov-Smirnov test using Minitab v. 12 (Minitab Inc.). In the absence of parents in the MIDSPAN sample, IBD was calculated by SOLAR using a maximum likelihood method. This was based on allele frequencies which were also calculated internally by the program, again using maximum likelihood. To improve these estimates, singletons from the MIDSPAN sample were genotyped and included in the input. Two-point analysis was also repeated with all treated individuals omitted from the data set to ensure that inclusion of treated blood pressures did not adversely skew the analysis. The correction used in the Silesian sample was not applied as this, of course, cannot accurately derive untreated BP. Since the percentage of treated individuals in the MIDSPAN sample was so small, exclusion of these was more appropriate, rather than replacing treated blood pressures with estimates.

2.7.2: BLOOD PRESSURE CANDIDATE GENE ANALYSES:

2.7.2.1: β_2-ADRENERGIC RECEPTOR ANALYSES:

Analysis of the ADRB2 SNPs was conducted in line with analyses by Bray *et al*, whose study provided the initial motivation to examine the chromosome 5q region and the ADRB2 gene (Bray *et al*, 2000). They used TDT analysis in their families, and logistic regression in the parental generation of their sample. We therefore did likewise to make our findings as comparable as possible.

Transmission disequilibrium testing (TDT) was employed to test for linkage in the presence of association (Spielman *et al*, 1993) using two parent-one child

trios. TDT detects preferential transmission of an allele (greater than 50%) from parents to affected offspring compared with untransmitted alleles which effectively provide the control genotype. Thus, TDT examines both linkage and association at once. At least one heterozygous parent is required for a family trio to be informative. TDT and similar family-based tests were devised to avoid the problem of population stratification which may give false positive results in case-control association studies.

The results of TDT were confirmed by the empirical variance family-based association test (EV-FBAT), a method which was unavailable to Bray *et al*. EV-FBAT tests the null hypothesis that linkage is present but there is no association (Lake *et al*, 2000). This was appropriate because we were testing SNPs within a region already showing evidence of linkage to the trait. Unlike TDT, EV-FBAT does not require parental genotypes as it is not based on treating non-transmitted alleles as controls. Rather, the distribution of offspring genotypes is important and this can be calculated from genotypic data for all offspring within the family, in the absence of parents. Thus, it does not suffer from the costly reduction in sample size caused by missing parental data that is inherent with approaches that condition on sibling IBD patterns. EV-FBAT determines the expected value of an association test statistic by conditioning on the minimal sufficient statistic under the null hypothesis and uses an empirical variance-covariance estimator which is unaffected by correlation of sibling genotypes. At the time of the ADRB2 analyses, EV-FBAT was a relatively unknown method, and we therefore preferred the widely reported and validated TDT method as a primary approach, as used by Bray *et al*. However, in its favour EV-FBAT allowed us to make

better use of families which contained more than one genotyped child. FBAT was designed to handle more complex families, and decomposes such families into nuclear units for analysis. TDT, however, is completely restricted in its use of trios and makes no use of genotyping in extra siblings.

In concordance with Bray *et al*, binary logistic regression analysis was performed in the parental generation to test for increased risk of hypertension associated with each tested genetic variant of ADRB2. Correction was made for age, sex and body mass index. Finally, SNP haplotypes were assessed using a modified TDT method implemented in TRANSMIT. This is a likelihood method which allows for missing parental data and haplotype phase uncertainty and uses genotype data from available parents and offspring to efficiently determine phase. A score vector is generated which is averaged for all possible configurations of haplotypes and transmissions from parents to offspring consistent with the observed data. Additional offspring who are of unaffected or unknown status are used to narrow the options for parental haplotypes. The method is robust against population stratification. Again, this was a methodology not available to Bray *et al*, but haplotype analyses are expected to be more informative than single locus anlyses. This is particularly true where the combined effect of functional loci can be considered rather than examining each without regard to positive or negative influences from others. We felt this would be a more categorical analysis to confirm or refute the previous findings of ADRB2 studies.

2.7.2.2: FIBROBLAST GROWTH FACTOR 1 ANALYSES:

Analysis of FGF1 SNPs was conducted using EV-FBAT (Lake *et al*, 2000) and the recent extension of this methodology, Haplotype FBAT. TDT was not used for FGF1 analyses since, by this point, EV-FBAT was more widely reported and considered reliable. EV-FBAT was felt to be a more useful analysis since it is appropriate for both qualitative and quantitiative traits, unlike classical TDT. In addition, as explained above, it extracts all possible information from complex families regardless of missing parents and multiple offspring. This was more appropriate for a study group that contained genotypes in additional siblings. As stated, classical TDT is totally restricted to trios and dichotomous traits. At the time of the ADRB2 analyses, Haplotype-FBAT had not been developed. However, its availability for analysis of FGF1 allowed consistency between the single locus and haplotype analyses of this gene and made it the preferred approach rather than TRANSMIT. It allowed analysis of the three locus haplotype across all SNPs gemotyped in FGF1. This is discussed further in Chapter 4.

2.7.3: FAMILIAL INTRACRANIAL ANEURYSM CANDIDATE GENE ANALYSES:

The variable structure of the families recruited to the FIA pilot study and difficulties of defining individuals as unaffected meant that no single analysis method used the study sample to its full extent. I therefore applied multiple methods in an effort to detect linkage and/or association, with the aim of using as

many of the families as possible. Given the small size of the study, my primary aim of analysis was to identify trends across different analytical methods.

IBS linkage between disease status and markers was assessed by the Affected Pedigree Member (APM) method (Weeks & Lange, 1988). IBS methods test for sharing of alleles between relatives. However, this is done in such a way as to disregard whether these shared alleles originated from the same ancestral chromosome. This makes IBS methods less powerful than IBD methods which are based on estimation of allele sharing where such alleles are derived from the same founder chromosome. Not all parents were available in families suitable for the affected pedigree member analysis. In the absence of parents, IBD statistics are estimated by a likelihood approach. However, the very small number of individuals suitable for the APM analysis would have made such estimates highly speculative and unreliable. The more simplistic IBS approach is therefore more appropriate, although a larger study would have benefited from IBD analysis.

Linkage and association were assessed using the combined Transmission Disequilibrium Test (TDT) (Spielman *et al*, 1993) and sibling TDT (S-TDT) (Spielman & Ewens, 1998) which was described in Section 2.7.2.1. Tests of association and linkage within extended families were done with FBAT and EV-FBAT (Horvath *et al*, 2001). FBAT tests the null hypothesis of no linkage and no association. It is also based on calculation of the expected value of an association test statistic conditioned on the minimal sufficient statistic, and again

was designed to handle complex pedigrees and information from additional siblings. EV-FBAT was previously described in Section 2.7.2.1.

A case-control paradigm was also used. The existence of Hardy-Weinberg equilibrium in controls was first confirmed by Fisher's exact test using GDA v1.0 (Lewis, P. O., and Zaykin, D. 2001; http://lewis.eeb.uconn.edu/lewishome /software.html). Frequencies of genotype in case and control groups were compared by an exact χ^2 test using STATXACT-TURBO v2.1 (Cytel Software Corporation, USA). Genotype frequencies were compared since they do not make the assumption of an additive affect, which underlies the commonly used comparison of allele frequencies.

CHAPTER 3

LINKAGE OF BLOOD PRESSURE AND HYPERTENSION TO HUMAN CHROMOSOME 5q31.1-q33 IN TWO POPULATIONS

3.1: INTRODUCTION:

Essential hypertension is an important risk factor for cardiovascular disease (Lifton *et al*, 2001). It is a multifactorial, complex polygenic or oligogenic trait and, as such, has seen the application of various complementary genetic strategies to clarify the underlying genes. Work has been conducted along three lines. Firstly, study of Mendelian hypertensive diseases has resulted in elucidation of a number of disorders, such as Liddle syndrome (Shimkets *et al*, 1994), glucocorticoid-remediable aldosteronism (GRA) (Lifton *et al*, 1992a) and syndrome of apparent mineralocorticoid excess (AME) (Mune *et al*, 1995). Such advances have clarified the role of various pathways in blood pressure regulation but not contributed greatly to the public health impact of hypertensive disease. Secondly, genome-wide scan (GWS) linkage approaches have implicated quantitative trait loci (QTL) in essential hypertension, but have not definitively implicated a common QTL or, therefore, causative genes (Samani, 2003). Thirdly, candidate gene approaches have been used to examine genes implicated by Mendelian disorders, genes in QTL and other plausible biological candidate genes. Whilst a potentially powerful approach, results have been conflicting and, as such, difficult to interpret (Risch, 2000; Tabor *et al*, 2002).

A targeted chromosomal region approach to complex genetics, essentially amalgamating the genome-wide and candidate gene rationales, has also been postulated (Krushkal *et al*, 1998). A small chromosomal region, selection of which is based on genome-wide evidence and the presence of suitable

physiological candidate genes, allows denser saturation with markers and evidence of linkage can be followed by positional analysis. The 5q31-qter region of human chromosome 5 is an excellent target region for such a study. A number of genes, known to be important regulators of blood pressure or implicated in cardiovascular phenotypes, cluster here (Fig. 3.1.1). These include the β_2- and α_{1B}-adrenergic receptors; the dopamine receptor type 1A; glucocorticoid receptor and glutathione peroxidase. Owing to the presence of such genes, the 5q31-qter region has been focused on before.

Evidence of linkage in the 5q31-qter region to systolic blood pressure (SBP) was reported in young, discordant US sib pairs (Krushkal *et al*, 1998). Multipoint linkage was maximal at 172 cM from the pter, following genotyping of 8 microsatellite markers across the region at an average density of 4.5 cM. Transmission disequilibrium testing (TDT) also showed greatest linkage and association at this locus. Maximal linkage at this same marker was later reported in a genome-wide scan of the same sibships using a 10 cM marker set (Krushkal *et al*, 1999). More recent genome-wide scans have also reported multipoint linkage peaks in the region at 189 cM (Hunt *et al*, 2002) and 160 cM (Kristjansson *et al*, 2002) when investigating hypertension, and proximal to the ADRB2 locus for exercise-induced change in diastolic blood pressure (Rankinen *et al*, 2001). These have all been in Caucasian populations.

With the exception of Krushkal's studies, the evidence of 5q linkage in the genome-wide scans has largely been overlooked. In all cases, greater logarithm

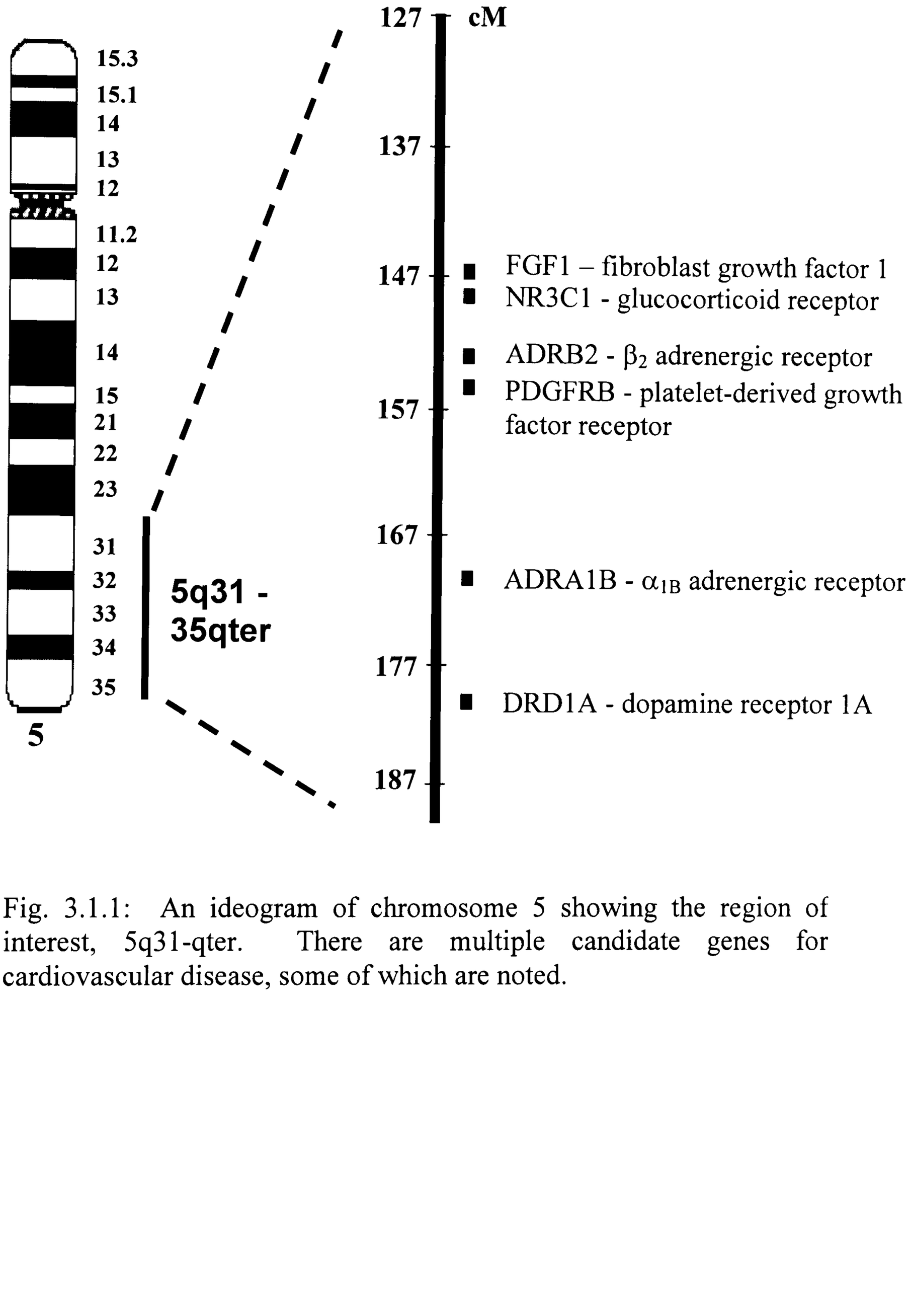


Fig. 3.1.1: An ideogram of chromosome 5 showing the region of interest, 5q31-qter. There are multiple candidate genes for cardiovascular disease, some of which are noted.

of odds (LOD) scores were obtained on other chromosomes and emphasis was placed on these. This is due, to a large extent, to the importance placed on absolute magnitude of LOD scores by Lander and Kruglyak (Lander & Kruglyak, 1995). However, the majority of genome-wide scans for complex traits have failed to satisfy these standards leading to calls for less stringent levels of significance (Rao & Province, 2000). Lander and Kruglyak did, however, stress that linkage falling short of their genome-wide threshold may, nonetheless, be genuine.

Such ambiguity has led to the suggestion that combination of evidence from several studies may be needed to localise genes with modest effect (Altmuller *et al*, 2001). To this end, linkage studies in two independently ascertained human populations are described here. The Silesian Hypertension Study is a sample of Polish nuclear families with aggregation of hypertension. Linkage of quantitative and qualitative blood pressure traits to the 5q31-qter region was assessed in this resource. The MIDSPAN Family Study, a large, general population sample from Scotland, was used to determine whether findings in the Silesian Study were applicable outwith this hypertensive sample.

3.2: METHODS:

3.2.1: STUDY DESIGN AND RECRUITMENT:

3.2.1.1: SILESIAN HYPERTENSION STUDY:

The Silesian Hypertension Study recruited hypertensive nuclear families from the region of Silesia in southern Poland as detailed in Section 2.2.1 using probands in hypertension clinics as a first contact. Subjects with secondary hypertension were excluded. A total of 638 individuals was recruited from 212 families with clustering of essential hypertension. Hypertension was defined as systolic and/or diastolic blood pressure over 140/90 mmHg, respectively, on 3 separate occasions and/or treatment for hypertension. Clinical history, anthropomorphic data and cardiovascular phenotypes were recorded for a total of 635 individuals from 210 families. Blood samples for DNA extraction were taken.

3.2.1.2: MIDSPAN FAMILY STUDY:

The MIDSPAN Family study represents a sample of the general population from the west of Scotland consisting of sibships ranging, in size, from singletons to octets. These individuals were recruited in a second phase of the MIDSPAN Study as detailed in Section 2.2.2. A total of 2,338 individuals were recruited from 1,477 pedigrees. This comprised 869 singletons and 1,469 individuals from 608 sibships. These individuals completed questionnaires on diverse lifestyle

and clinical parameters and were phenotyped for blood pressure as well as anthropometric and other data. Blood samples for DNA extraction and serum assays were taken.

3.2.2: MICROSATELLITE MARKER STUDIES:

3.2.2.1: SELECTION OF MICROSATELLITE MARKERS:

Initially, 8 microsatellite markers spanning the 36 cM from 5q31.1 to qter were genotyped. These were taken from the original study of this region (Krushkal *et al*, 1998). Following initial analyses, 3 additional markers spanning 19 cM proximal to the original markers were added to further define the linkage region. These were selected for their high reported heterozygosity and inter-marker spacing. Thus a total of 55 cM was investigated with 11 markers at an average distance of 5 cM (2.67 to 7.94 cM). Map distances were taken from the Marshfield sex-averaged genetic map as quoted by the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). The Marshfield genetic map was compared with the physical genomic sequence map to confirm that marker order was correct.

3.2.2.2: MICROSATELLITE MARKER GENOTYPING:

Genomic DNA was extracted from whole blood samples as described in Section 2.3. DNA for both Silesian and MIDSPAN studies was diluted to 5 ng/µl in sterile deep well microplates (Beckman Coulter UK Ltd, UK). 25 ng DNA per

PCR reaction was used. PCR was performed as detailed in Section 2.4.1 using Qiagen HotStar Taq (Qiagen Ltd, UK). Forward primers used were 5'-labelled with either the fluorescer 6-carboxyfluorescein (FAM) or its analogues TET or HEX. The size range of marker alleles and pooling considerations dictated the specific label used. PCR products for an individual's 11 markers were combined into 3 pre-determined pools for resolution on the ABI 377 DNA Analyser. Resolution and genotyping proceeded as per Section 2.4.2.1. Genotypes were scored by two independent observers blinded to phenotype.

3.2.3: STATISTICAL ANALYSES:

Genotypes from both Silesian and MIDSPAN studies were checked for Mendelian inconsistencies using the PEDCHECK program (O'Connell & Weeks, 1998). Genotypes were confirmed for individuals flagged by the software, corrected if appropriate or individuals removed from the dataset.

Blood pressures of the Silesian Hypertension Study sample were corrected for anti-hypertensive treatment effect as described in Section 2.2.1. Multiple methods were then used for linkage analyses of the Silesian Hypertension Study dataset. Haseman-Elston regression analysis was used to assess two-point identity by descent (IBD) linkage of markers to quantitative blood pressure phenotypes. A function of squared trait sums and differences between siblings' blood pressures was regressed on number of alleles shared IBD. Two-point linkage of markers to hypertension status was assessed by an IBD approach implemented in SPLINK v1.08 (Clayton, 2000). Two-point linkage to

hypertension was confirmed by an identity by state (IBS) χ^2 test, with alleles shared IBS compared with a random distribution. Both the IBD Haseman-Elston test and IBS χ^2 test were implemented in the SIB-PAIR software package (Duffy, 1999). Multipoint non-parametric IBD linkage analysis was performed using MAPMAKER/SIBS for quantitative blood pressure phenotypes. Analyses are discussed in detail in Section 2.7.1.

The MIDSPAN dataset was analysed for both two-point and multipoint IBD linkage to quantitative blood pressure phenotypes using the variance-components methodology of SOLAR (Almasy & Blangero, 1998). SOLAR is particularly suitable given the variable size of sibships within the MIDSPAN cohort, as it intrinsically corrects for dependence of multiple sib pairs derived from the same pedigree (Marlow, 2002). This pedigree-based approach of SOLAR is also more powerful than sib pair analysis when data on larger sibships are available. For this reason it has been the method of choice for other large linkage studies of general population samples (Levy *et al*, 2000). Traits were transformed to a normal distribution by a log10 transformation, and this distribution cofirmed by Kolmogorov-Smirnov test. With only 5% of individuals treated, a correction was not done since this would only estimate untreated pressures. However, a second twopoint analysis was also performed with all treated individuals omitted from the dataset to confirm that these did not skew the result. Analytical methods are discussed in detail in Section 2.7.1.

Since IBD estimation in the absence of parental genotypes is reliant upon estimated allele frequencies, the 868 singletons were included in analysis. This

improves both allele frequency and phenotypic distribution estimates. Allele frequencies were calculated internally by SOLAR using an maximum likelihood estimation. SOLAR also modelled the contribution of age, sex and normalised BMI within the MIDSPAN cohort. These were retained by the model and thus included as covariates.

3.3: RESULTS:

3.3.1: STUDY SAMPLES:

In the Silesian Hypertension Study, 629 individuals from 207 families were included in analyses. These included 207 hypertensive probands (57% male; 43% female), 174 parents (130 fathers; 144 mothers) of whom 53% had essential hypertension and 148 siblings (43% male; 57% female) of whom 32% had essential hypertension. Demographic and clinical characteristics are shown in Table 3.3.1.

The MIDSPAN Family cohort included 869 singletons and 1,469 siblings from a total of 1,477 families of whom 45% were male. Of the 2,338 individuals, 22.3% were hypertensive at time of recruitment and 26% of the hypertensives (5.8% of the cohort) reported being on anti-hypertensive treatment. Mean blood pressures were normotensive and body mass index was within normal limits, specifically: SBP was 127 ± 15.9 mmHg; DBP was 74.6 ± 11.2 mmHg and BMI was 26.1 ± 4.53 kg m^{-2}. Demographic and phenotypic characteristics are described in Table 3.3.2, sub-categorised by size of sibship.

Table 3.3.1: Demographic and phenotypic characteristics of subjects of the Silesian Hypertension Study

	Probands (N=207)	Mothers (N=144)	Fathers (N=130)	Siblings (N=148)
Gender, M/F (%)	57 : 43	0 : 100	100 : 0	43 : 57
Age, y	36.2 ± 15.6	54.2 ± 10.8	55.8 ± 10.0	42.4 ± 14.8
Hypertensives, N (%)	207 (100)	85 (59)	61 (47)	48 (32)
SBP, mmHg	143 ± 14.0	139 ± 23.3	139 ± 19.8	132 ± 21.8
DBP, mmHg	91.2 ± 10.6	88.7 ± 13.6	87.2 ± 11.6	83.7 ± 11.1
BMI, kg m^{-2}	26.6 ± 4.6	27.5 ± 5.1	27.6 ± 4.1	25.7 ± 4.6

Values are mean ± SD. SBP = systolic blood pressure; DBP = diastolic blood pressure; BMI = body mass index.

Table 3.3.2: Demographic and phenotypic characteristics of individuals in the MIDSPAN Family Study categorised by size of sibship.

Size of sibship (N, Individuals)	Singletons (N=869)	Pairs (N=872)	3 (N=342)	4 (N=168)	5 (N=60)	6 (N=12)	7 (N=7)	8 (N=8)
Gender, M/F (%)	44 : 56	44 : 56	44 : 56	47 : 53	52 : 48	50 : 50	43 : 47	25 :75
Age, y	45.9 ± 5.90	45.0 ± 6.16	44.2 ± 6.40	44.3 ± 6.60	43.0 ± 7.04	40.8 ± 4.89	43.1 ± 7.36	42.4 ± 5.63
Hypertensives, N (%)	207 (24)	202 (23)	73 (21)	32 (19)	8 (13)	3 (25)	0 (0)	3 (38)
SBP, mmHg	128 ± 15.5	127 ± 16.2	126 ± 16.3	126 ± 14.9	122 ± 8.3	127 ± 13.7	126 ± 10.0	124 ± 15.5
DBP, mmHg	75.0 ± 10.9	74.6 ± 11.6	74.4 ± 11.3	73.8 ± 10.8	72.0 ± 11.1	79.1 ± 9.57	73.9 ± 8.09	67.3 ± 8.66
BMI, kg m^{-2}	26.2 ± 4.67	26.1 ± 4.44	26.3 ± 4.46	26.3 ± 4.90	25.6 ± 4.41	23.7 ± 4.57	26.5 ± 1.59	29.7 ± 4.34

Values are mean ± SD. SBP = systolic blood pressure; DBP = diastolic blood pressure; BMI = body mass index.

Table 3.3.3: Map positions, number of alleles and observed heterozygosities of 5q31.1-qter microsatellite markers in the study populations.

		Silesian Hypertension Study		MIDSPAN Study	
Marker	Position (cM)	Alleles (N)	H	Alleles (N)	H
D5s494	127.93	15	0.75	16	0.76
D5s642	134.72	11	0.72	11	0.72
D5s500	140.72	11	0.81	14	0.84
D5s1480	147.49	9	0.82	9	0.82
D5s636	153.17	12	0.76	15	0.80
D5s820	159.77	8	0.77	9	0.80
D5s2093	164.19	9	0.66	10	0.69
D5s1471	172.13	14	0.77	15	0.75
D5s1456	174.80	9	0.78	11	0.80
D5s462	178.75	8	0.58	8	0.62
D5s211	182.89	8	0.74	13	0.75

Map positions are taken from the Marshfield sex-averaged genetic map and are quoted as distance from the pter. H = observed heterozygosity.

3.3.2: LINKAGE STUDIES OF 5q31.1-qter:

Eight microsatellite markers were genotyped, initially, with the subsequent addition of three markers. Thus, 11 markers across 55 cM of the distal portion of chromosome 5q were ultimately genotyped. All markers had high observed heterozygosity in both populations studied. Marshfield map positions, numbers of alleles and observed heterozygosities are shown for both Silesian and MIDSPAN study populations in Table 3.3.3.

Linkage analysis was conducted, initially, in the Silesian study sample. Two-point linkage results are shown in Table 3.3.4. Two-point analysis of the 8 distal microsatellite markers by Haseman-Elston regression showed significant linkage of D5s1480 to hypertension (P=0.001). This result was corroborated by SPLINK IBD and SIB-PAIR IBS two-point analyses. No other microsatellite marker demonstrated significant linkage. Results of linkage to hypertension status were supported by analyses of quantitative phenotypes, with systolic (SBP) and diastolic blood pressure (DBP) both adjusted for anti-hypertensive treatment effect. D5s1480 again showed significant linkage to both phenotypes as shown in Table 3.3.4.

Multipoint linkage testing followed two-point analyses. Results of two-point analyses were apparently confirmed by the multipoint Z scores, maximal at D5s1480, with the Z statistic still increasing as the limit of the marker map was reached. D5s1480 marked the proximal margin of the marker map and it is possible that artefactual multipoint linkage may arise at the edges of marker sets.

To eliminate this possibility, the marker set was extended proximally with three additional microsatellites which were genotyped and analysed. Two-point Haseman-Elston analysis of linkage to essential hypertension implicated the two markers closest to D5s1480. Of the two, D5s500 was closest and most significant with t=2.45 (P=0.008) whilst for D5s642 t=1.96 (P=0.03). Two-point linkage of D5s500 also reached borderline significance for SBP (P=0.09) and DBP (P=0.06).

Multipoint linkage results of SBP using the extended marker map are shown in Fig. 3.3.1. Multipoint analysis showed that linkage to SBP was maximal proximal to D5s1480 (Z = 2.3; P = 0.01). This implicated a 7 cM region between D5s1480 and D5s500, and confirmed that the previously observed multipoint linkage had not been an artefactual effect due to the marker map. Analysis of DBP showed an equivalent linkage peak with a maximal Z score of 1.8 (P = 0.03) proximal to the D5s1480 locus.

Further genotyping and linkage analysis were subsequently conducted in the MIDSPAN cohort with a view to following up the results in the Silesian sample. All analyses were conducted using SOLAR with age, sex and BMI as covariates. Two-point linkage analysis again implicated D5s1480, with linkage to all quantitative phenotypes greatest at this locus. The highest two-point LOD score was obtained for mean arterial pressure (MAP) (Table 3.3.5). Furthermore, when multipoint analyses were performed, a linkage peak maximal at the

Table 3.3.4: Results of two-point linkage of essential hypertension, systolic and diastolic blood pressures to microsatellite markers spanning the 5q31.1 to 5qter region of human chromosome 5 in the Silesian study group.

	EHT					SBP		DBP	
	H-E		SPLINK	IBS χ^2		H-E		H-E	
Marker	*t*	P	P	χ^2	P	*t*	P	*t*	P
D5s1480	3.1	0.001	0.03	8.2	0.01	2.5	0.006	2.4	0.01
D5s636	1.2	0.12	0.58	0.8	0.67	1.2	0.11	0.7	0.25
D5s820	1.3	0.11	0.40	0.6	0.74	1.3	0.09	1.8	0.03
D5s2093	1.2	0.12	0.37	0.6	0.73	1.4	0.08	1.1	0.13
D5s1471	-0.9	0.81	0.57	0.4	0.81	-0.4	0.66	-0.8	0.79
D5s1456	0.9	0.17	0.57	1.1	0.58	1.2	0.11	1.2	0.11
D5s462	1.2	0.11	0.39	6.4	0.04	1.4	0.07	1.2	0.11
D5s211	1.3	0.09	0.14	3.7	0.16	0.5	0.31	0.8	0.21

EHT = essential hypertension; H-E = Haseman-Elston regression analysis; SBP = systolic blood pressure; DBP = diastolic blood pressure. SBP and DBP had been adjusted for effect of anti-hypertensive treatment.

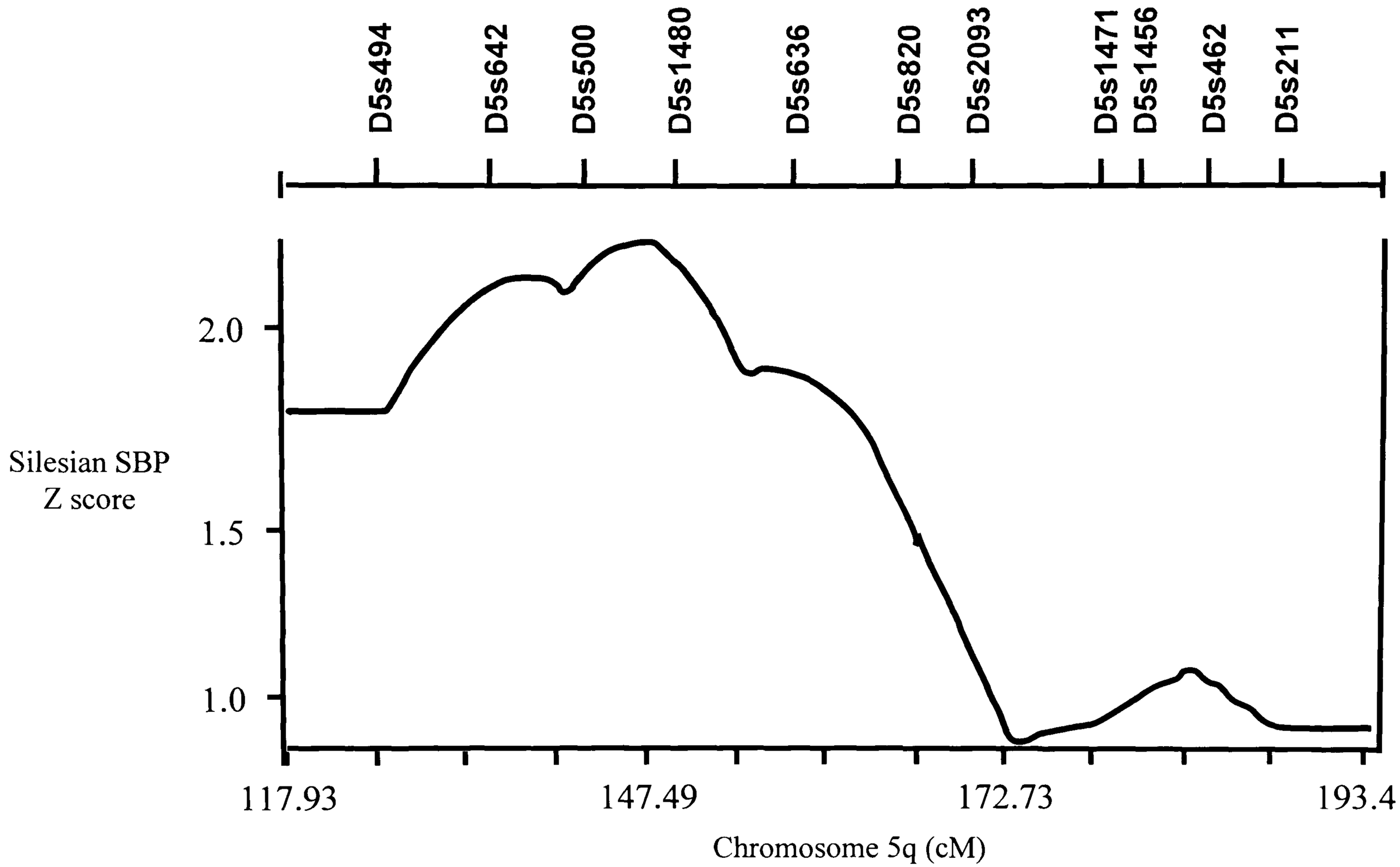
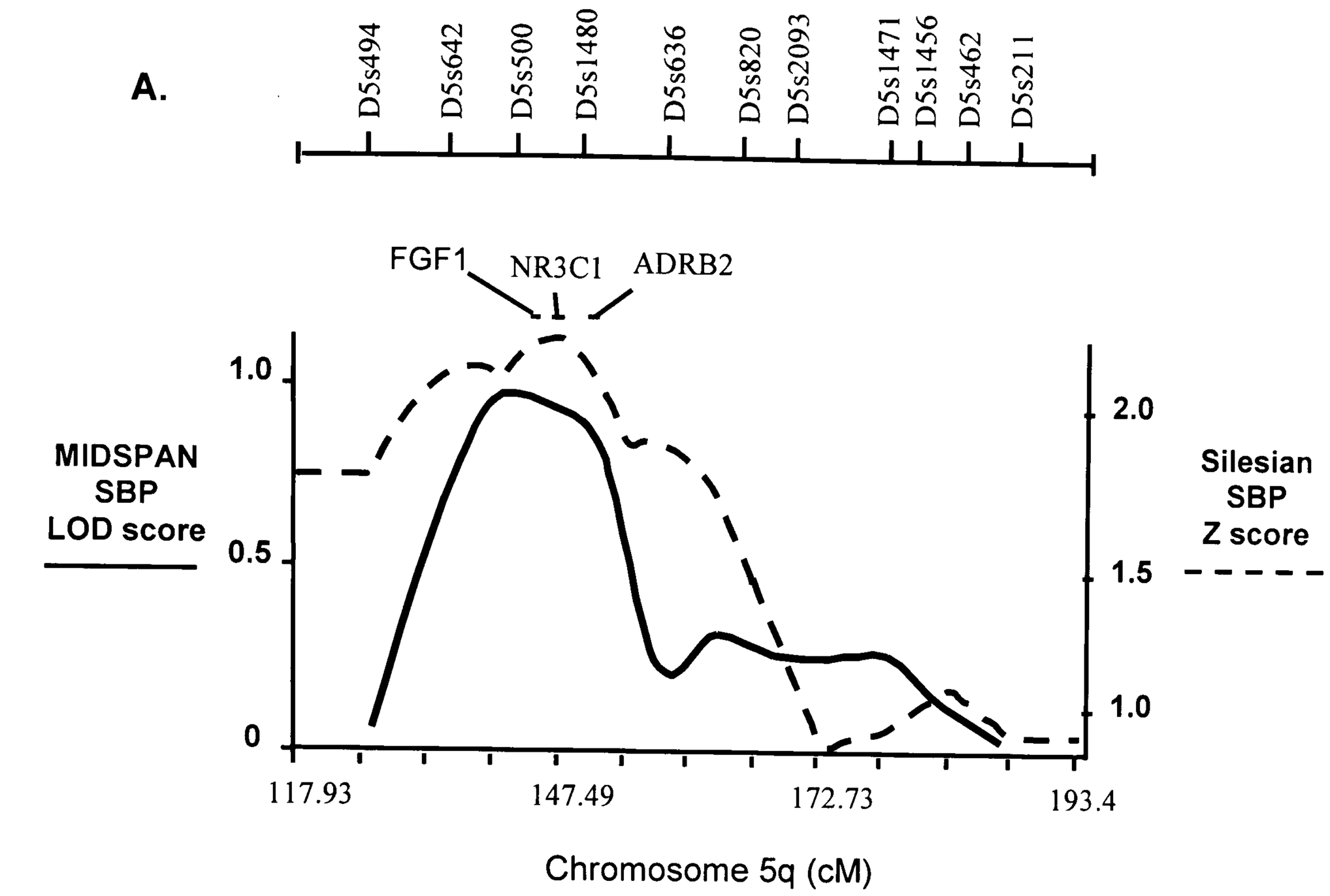


Fig. 3.3.1: Results of multipoint linkage analysis for SBP in the Silesian Hypertension Study group. Eleven microsatellite markers spanning 55 cM of 5q31.1 to 5qter were assessed for linkage to systolic blood pressure, after adjustment for treatment, using MAPMAKER/SIBS.

Table 3.3.5: Results of two-point linkage of systolic, diastolic and mean arterial blood pressures to microsatellite markers spanning the 5q31.1 to 5qter region of human chromosome 5 in the MIDSPAN study group.

	SBP	DBP	MAP
Marker	LOD	LOD	LOD
D5s494	0.00	0.13	0.03
D5s642	0.90	1.05	0.90
D5s500	0.71	0.63	1.02
D5s1480	**1.19**	**1.18**	**1.57**
D5s636	0.06	0.00	0.00
D5s820	0.61	0.63	1.12
D5s2093	0.00	0.00	0.00
D5s1471	0.28	0.05	0.13
D5s1456	0.42	0.00	0.05
D5s462	0.02	0.00	0.05
D5s211	0.06	0.32	0.09

SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; LOD = logarithm of odds score.



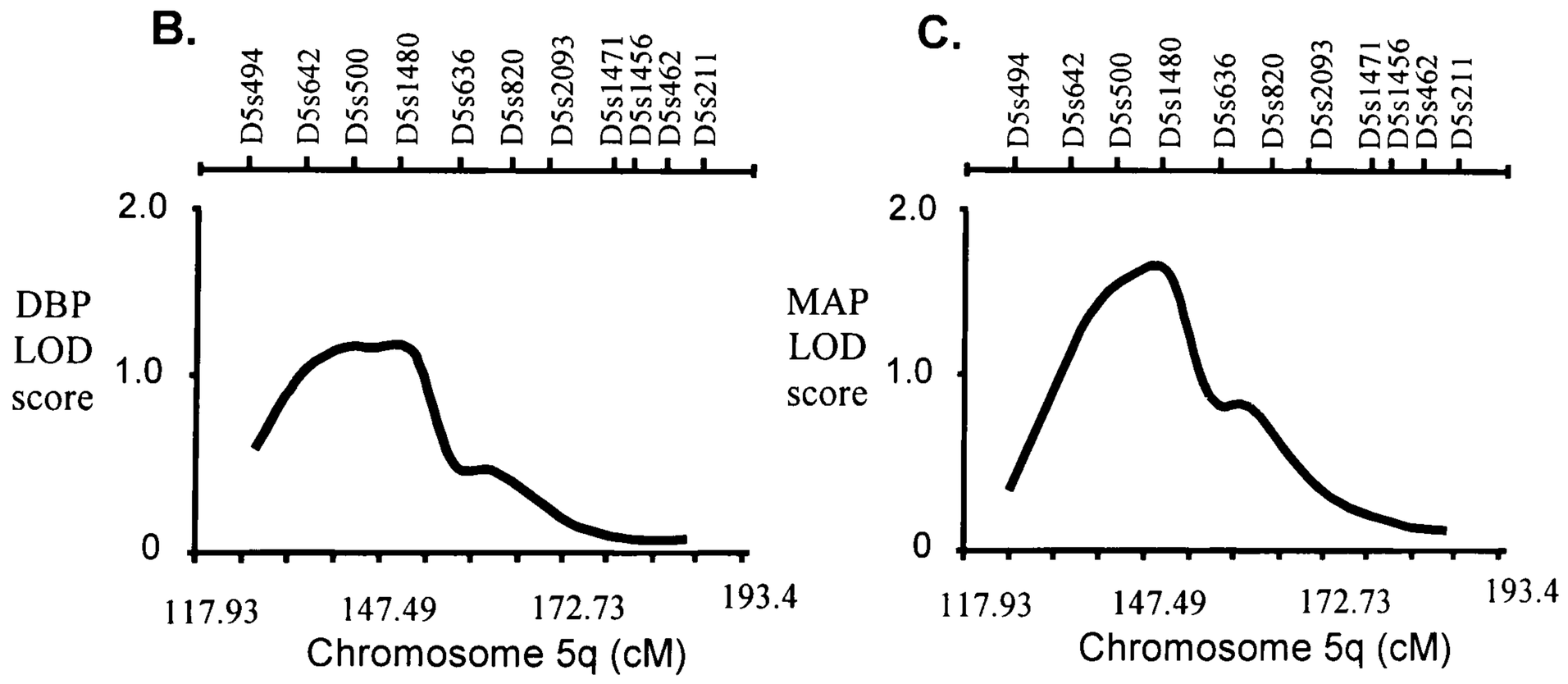


Fig. 3.3.2: Results of multipoint linkage analysis of quantitative blood pressure phenotypes to 11 microsatellite markers across the 5q31.1-qter region in the MIDSPAN study group. Covariates of age, sex and BMI were included in the analyses performed with SOLAR. (A) Comparison of multipoint results from the MIDSPAN and Silesian study groups shows the close agreement of linkage in the two populations. Location of the genes FGF1, NR3C1 and ADRB2 are shown. (B) Maximal linkage to DBP and (C) MAP was also seen at the same locus. LOD = logarithm of odds

D5s1480 locus was present whether SBP, DBP or MAP was assessed. Maximal multipoint LOD was 1.65 for MAP. Both location and morphology of the linkage peaks were highly comparable between the Silesian and MIDSPAN study groups. Results of these multipoint analyses are shown in Fig. 3.3.2. To confirm that inclusion of treated MIDSPAN individuals did not materially affect the result, a second two-point analysis was performed with treated individuals removed from the dataset. Two-point LOD scores were maximal at D5s1480 for MAP and DBP (1.68 and 1.11, respectively). LOD scores for SBP were 1.26 at the D5s1480 locus but maximal at the flanking D5s500 locus (LOD = 1.29). This mirrors the broader multipoint peak seen for SBP (Fig. 3.3.2 A).

3.4: DISCUSSION:

A targeted approach has been used, focusing on the 5q31.1 to qter chromosomal segment, to examine linkage to blood pressure phenotypes in Caucasian study samples. The decision to focus on this small chromosomal region was based on previous linkage studies, which have implicated this chromosome segment (Krushkal *et al*, 1998; Krushkal *et al*, 1999), and the presence of putative cardiovascular candidate genes which provide scope for subsequent positional analysis if appropriate. Suggestive linkage of a 7 cM region to blood pressure and hypertension status was found in the Silesian Hypertension Study (Tomaszewski *et al*, 2002). A peak of suggestive linkage to quantitative BP traits was also seen at the same locus in the MIDSPAN Family Study cohort.

The Silesian Hypertension Study comprised nuclear families that were recruited based on their family history of essential hypertension from the Silesian region of Poland. Consequently, nearly 64% of individuals were hypertensive and many were being treated for their hypertension. Therefore, an adjustment for treatment was applied to blood pressures (Tomaszewski *et al*, 2002). The MIDSPAN Family Study comprised unselected sibships from the general population in the west of Scotland. Just over one fifth of participants were hypertensive at recruitment. This agrees with prevalence of hypertension reported by large epidemiological studies in Western populations (Burt *et al*, 1995).

Linkage to the same locus, proximal to D5s1480, was demonstrated for both quantitative blood pressure traits and hypertension status in the study groups. The idea that the same genes underlie both normal population distribution of blood pressure and the pathological development of high blood pressure and hypertension is often tacitly assumed (Altmuller *et al*, 2001). Indeed, it is rare for linkage studies to analyse linkage to hypertension status and blood pressure. This is probably driven by two factors. Firstly, general population samples suitable for blood pressure linkage analysis often lack sufficient hypertensives to contribute power to such an endeavour; secondly, there is a widely held opinion that quantitative traits provide more power than qualitative traits which, as with hypertension, may be somewhat arbitrary in their definition. Interestingly, a recent review of 101 genome-wide scans across 31 different complex diseases found no difference in rate of linkage detection between quantitative and qualitative trait scans (Altmuller *et al*, 2001). However, statistical studies using

simulated datasets have suggested that quantitative traits are more informative (Duggirala *et al*, 1997).

The ideal approach to analysis of complex disorders may be to analyse both qualitative and quantitative traits jointly, if study samples lend themselves to this, since this may increase power to detect effects (Williams *et al*, 1999). Since the Silesian study sample consists of a high percentage of hypertensive individuals, this provided the opportunity to analyse both blood pressure and hypertensive status. The results from these analyses suggest that, at least in the QTL proximal to D5s1480, the same genes may be involved in blood pressure control and hypertension development. However, this may not necessarily be the case for all QTL. Linkage to both hypertension and blood pressure has been examined in the same sample in at least one genome-wide scan. In this case the separate analyses gave very different results (Hunt *et al*, 2002).

The major strength of this study is that the two study groups are very different yet the linkage to blood pressure at D5s1480 is present in both. Consistency of linkage evidence to blood pressure or hypertension is rare across the many studies now published (Samani, 2003; Harrap, 2003). A few studies have replicated their initial findings in an independent sample (Xu *et al*, 1999; Zhu *et al*, 2001) but this is the exception rather than the rule. As such this has caused concern and raised the question of the feasibility of finding blood pressure and hypertension genes by such methods (Harrap, 2003).

Much of the conjecture surrounding the successfulness of linkage approaches in complex diseases has been based around the lack of genome-wide significant results. This concept, first proposed by Lander and Kruglyak (Lander & Kruglyak, 1995), was based on their realisation that testing of hundreds of markers would certainly result in false positive results. However, in their argument for stringency these authors also left room for smaller scale studies which, unlike genome-wide scans, are partly hypothesis-driven. Given that such a targeted approach, as employed here, does not necessitate the use of many markers, it also does not require such stringent levels of significance.

Since Lander and Kruglyak's guidelines were published, the practicality of their suggestions has been tested empirically. Logarithm of odds (LOD) scores of 3.6 or over in complex disease studies are now felt by many to be too stringent (Rao & Province, 2000). Thus the thin red line of significance has given way to a broader, interpretative approach. Often this involves simulation methods to empirically derive significance levels (Cheng *et al*, 2001; Angius *et al*, 2002; Caulfield *et al*, 2003). Other studies use lower fixed thresholds of significance (Rankinen *et al*, 2001; Rice *et al*, 2002; Cooper *et al*, 2002). No approach to significance determination represents a panacea, and interpretation of linkage results, particularly genome-wide data, is still very much a grey area. Indeed, it was acknowledged from the outset that such interpretation was not trivial and that high LOD scores could be false just as low LOD scores could be genuine (Lander & Kruglyak, 1995).

In light of the difficulties with interpreting linkage evidence, combination of evidence from multiple samples remains essential (Altmuller *et al*, 2001). Replication is one of the few ways to increase confidence in a linkage result, in the absence of a method for definitively proving the existence of a QTL. It also provides some evidence for broader applicability of the QTL than simply to the original sample studied. With respect to the identification of genes relevant to hypertension development at the population or global level, this is obviously important. Replication of our 5q QTL thus provides confidence in the result and may indicate a broader relevance to Caucasian populations.

The expectation from simulation studies is that study samples with more extreme phenotypes provide greater power to detect genetic effects (Risch & Zhang, 1995; Gu *et al*, 1997). This philosophy has been applied in some hypertension scans which have recruited individuals at the extremes of blood pressure distribution (Xu *et al*, 1999; Caulfield *et al*, 2003). From this, the expectation with our study samples is that the Silesian group should provide more power to detect blood pressure linkage given the majority of hypertensive individuals. That the same QTL with comparable linkage has also been detected in the normal, healthy MIDSPAN group is probably a consequence of the much greater size of this population. Certainly sample size has been identified as a major factor for success in linkage studies (Altmuller *et al*, 2001).

The QTL described in our two populations is approximately 30 cM from that of Krushkal *et al* (Krushkal *et al*, 1999). QTL separated by such a distance would normally be considered to indicate different linkage effects. It is interesting,

however, that further work by the US group could only implicate the ADRB2 gene, which is within the linkage region identified in our study (Bray *et al*, 2000b). Subsequent studies have reported linkage in the region proximal to ADRB2. A US genome-wide scan of 344 healthy, white sib pairs assessed the effect of a 20 week exercise program on ΔDBP. A multipoint LOD score of 1.38 at the ADRB2 locus was reported (Rankinen *et al*, 2001). A GWS in Icelandic families reported a LOD score of 0.95 for hypertension close to the ADRB2 locus (Kristjansson *et al*, 2002). A population-based study in the US reported linkage to hypertension but not to SBP or DBP at the qter in Caucasian families (Hunt *et al*, 2002). Between these previously published studies, the entire 5q31.1 to qter region has been implicated. However, with the reproducible linkage found in our populations, and in light of the findings by others proximal to the ADRB2 locus, the region around 5q31.1 to q33 is now highlighted as a promising region (Tomaszewski *et al*, 2002).

Classically, QTL may be tightened by addition of additional microsatellite markers to increase map density. We chose not to do this since our study is an amalgamation of linkage study and candidate gene study rationales. Our prior hypothesis is that any linkage detected within the 5q region may be related to variants of the ADRB2 gene encoding the β_2-adrenergic receptor given the findings of the US studies (Bray *et al*, 2000b). At a 5 cM resolution, we are already working with a map which is at least twice as dense as GWS studies. The observations of Krushkal *et al* and Bray *et al* (Krushkal *et al*, 1999; Bray *et al*, 2000b) also indicate that linkage maxima may not coincide with the gene locus in this region. Finally, the linkage region has a lower gene density than the

genomic average, with only a few sensible cardiovascular candidates. A candidate gene approach from this point therefore seems reasonable although grid tightening is a future option.

Evidence from experimental models further strengthens the case for the linkage region found in our populations. A region of human chromosome 5q of approximately 13 Mbp is syntenic with rat and mouse chromosome 18. This syntenic block extends from the centromeric flank of D5s494 to the distal flank of D5s636, thus encompassing our entire linkage region. Genome-wide scanning of a stroke-prone spontaneously hypertensive (SHRSP) x Wistar-Kyoto (WKY) F2 cross detected a QTL for baseline and salt-loaded DBP on rat chromosome 18 (Jacob *et al*, 1991). This QTL, denoted BP2, extended distally to the rat glucocorticoid receptor gene, Grl, the human homologue of which (NR3C1) is located under our peak of linkage on human chromosome 5q (see Fig. 3.3.2A). Linkage in this region of rat chromosome 18 has since been reported by other genome-wide scans in rat models; specifically, SBP in a cross of spontaneously hypertensive rat (SHR) x BioBreeding OK substrain (BB/OK) (Kovacs *et al*, 1997) and salt-loaded blood pressures in Brown Norway (BN) x Dahl salt-sensitive (SS) (Cowley *et al*, 2000). This QTL is absent in other strains, notably our own Glasgow WKY x SHRSP cross (Clark *et al*, 1996). In mice, a QTL for SBP in a cross of hypotensive BP/1 mice with normotensive *M. spretus* was also reported on mouse chromosome 18, at the proximal limit of the region syntenic to human chromosome 5q (Wright *et al*, 1999).

Our QTL in the 5q31.1-q33 region contains several promising candidate genes. The previous work implicating the ADRB2 gene (Bray *et al*, 2000b), has implicated this as a prime positional candidate and physiological candidate with its involvement in regulation of vascular tone and dilatation (Wood, 2003). With regard to the wider cardiovascular picture, the β_2-adrenergic receptor is known to mediate thermogenesis and lipolysis (Lowell & Bachman, 2003). It can be hypothesised to connect the vascular and metabolic abnormalities often cosegregating in hypertensive patients (Reaven, 2003). Functional relevance of ADRB2 polymorphisms is relatively well understood due, in part, to the interest of asthma researchers in ADRB2-mediated bronchial dilatation (Drysdale *et al*, 2000). The ADRB2 locus has been studied, quite extensively, as a candidate gene in blood pressure and hypertension. Some studies imply an effect in Caucasian (Gratze *et al*, 1999; Busjahn *et al*, 2000; Bengtsson *et al*, 2001) and black populations (Kotanko *et al*, 1997), whilst others show no association (Jia *et al*, 2000). Our own findings with regard to ADRB2 (Tomaszewski *et al*, 2002) are described in Chapter 4.

A novel cardiovascular candidate gene, which is located within the middle of the linkage region, is the acidic fibroblast growth factor gene, FGF1. Following comparative mapping of QTL between rat and the human 5q31-qter region, FGF1 was suggested as a positional candidate for blood pressure (Stoll *et al*, 2000). Despite this, relatively few studies have assessed the potential of this gene as a factor in blood pressure control and hypertension. However, as a potent endothelial cell proliferator (Powers *et al*, 2000), with proven effects on blood pressure in animal models (Cuevas *et al*, 1991; Wu *et al*, 1996) and a

possible role in defence against oxidative stress (Yang & deBono, 1997), FGF1 suggests itself as a biological candidate whose involvement in the pathogenesis of hypertension could be explained by a number of mechanisms.

The glucocorticoid receptor (GR), encoded by the NR3C1 gene, is also located within the linkage region. As a regulator of gene transcription, GR affects immune, metabolic, behavioural, cell-growth and vascular processes (De Rijk *et al*, 2002). Mutations affecting ligand binding or receptor regulation cause corticosteroid resistance, one feature of which is hypertension (Ruiz *et al*, 2001). The locus has been implicated in hypertension by some (Watt *et al*, 1992) but excluded by others (Lin *et al*, 1999; Takami *et al*, 1999; Dobson *et al*, 2001) using both association and linkage methods.

The work described here, then, has suggested evidence of linkage of a 7 cM region of human chromosome 5q31.1-q33 to blood pressure and hypertension. The linkage evidence to blood pressure phenotypes has been seen in two Caucasian study populations despite different ascertainment criteria. Previous targeted studies and genome-wide scans had, between them, implicated a broad region of the distal 5q arm. The QTL described here provides a more focused area for further efforts. The implication of the proximal segment of the 5q31.1-qter region is also reasonable in the context of work in rat and mouse models. Physiological positional candidates for cardiovascular disease exist within the QTL and will provide a focus for further work to dissect this locus.

CHAPTER 4

GENETIC STUDIES OF THE POSITIONAL AND PHYSIOLOGICAL CANDIDATE GENES ADRB2 AND FGF1 IN THE HUMAN 5q31.1- q 33 BLOOD PRESSURE QTL

4.1: INTRODUCTION:

The role of essential hypertension as a risk factor for stroke and coronary heart disease, and its 30-60% genetic determination (Ward, 1990), have made it the focus of Herculean efforts by the research community (Kannel, 2000). However, its complex, multifactorial, oligo- or polygenic nature (Lander & Schork, 1994; Caulfield *et al*, 2003) combined with small individual gene effects (Stoll *et al*, 2000) has consistently hampered advances in understanding. There are, inevitably, inherent difficulties in studying heterogeneous human populations with their variation in environment and genetic factors (Lander & Schork, 1994).

Dissection of the complex genetics of essential hypertension necessitates combination of complementary approaches (Risch, 2000). Thus we began with a targeted linkage study concentrating on the human 5q31.1 to q33 chromosomal region. We confirmed the presence of linkage to blood pressure in this region in two independent study samples (Tomaszewski *et al*, 2002). Part of the rationale for focusing on the distal segment of chromosome 5q was the presence of a number of good candidate genes for blood pressure control and cardiovascular disease. In addition, this region had been implicated by other linkage studies of blood pressure (Krushkal *et al*, 1999). By identifying linkage to a 7 cM region we were able to narrow the field of candidate genes to those which were both physiological and positional candidates. The natural progression of this work, then, is the application of candidate gene strategies to assess the contribution of such genes to the blood pressure linkage.

Simulation studies suggest that association approaches should be more powerful in detecting small to moderate gene effects than linkage approaches (Risch & Merikangas, 1996). Such approaches lend themselves to interrogation of candidate genes and are, potentially, very powerful but can also be problematic. Analysis of candidate genes has been criticised on two fronts: firstly, the significant findings of association in many candidate genes studies have not been replicated when followed by subsequent studies. Secondly, candidate gene studies are based on the ability to predict functional genes and variants. Some commentators believe that current knowledge is insufficient to allow this (Tabor *et al*, 2002).

Many of the pitfalls of candidate gene studies result from the methodologies used. The most commonly used paradigm, the case-control study, can give false positive and negative results due to population stratification (Cardon & Palmer, 2003; Ziv & Burchard, 2003). This has led to the development of family-based methods in an effort to control for such population effects (Spielman & Ewens, 1996; Rabinowitz & Laird, 2000). In addition, studies are commonly based on the genotyping of a single polymorphism within genes which may span hundreds of kilobases. The polymorphism genotyped may have no functional relevance itself in which case it must be in linkage disequilibrium with any functional polymorphisms for association with disease to be detected (Sharma & Jeunemaitre, 2000).

Current guidelines recommend a hierarchical selection policy for polymorphisms based on their potential for functional effects (Tabor *et al*, 2002). In addition, it is recommended that multiple polymorphic sites within a gene are genotyped (Sharma & Jeunemaitre, 2000). Here these policies are applied, in conjunction with family-based analysis methods, to the investigation of two candidate genes which are both biological and positional candidates following the previously described linkage studies. These candidates are the β_2-adrenergic receptor and the acidic fibroblast growth factor genes, located within the 5q31.1-q33 blood pressure QTL.

ADRB2, encoding the β_2-adrenergic receptor, is well recognised as a potentially important cardiovascular candidate gene. The gene has a simple structure consisting of a promoter region of approximately 300–500 bp followed by a single coding exon (Emorine *et al*, 1987). The protein is a 413 residue peptide with 7 transmembrane domains (Fig 4.1.1). Expressed by multiple tissues including epithelial cells, adipose tissue, vascular endothelium and bronchial smooth muscle, ADRB2 is a G protein-coupled receptor (GPCR) which mediates signalling by catecholamines such as adrenaline (Liggett, 1997). Binding of ligand results in G_s-dependent stimulation of adenylyl cyclase and production of cAMP. In the context of blood pressure regulation, this signalling pathway mediates vasodilatory responses through receptors expressed in the vessel wall, but the role of the ADRB2 receptor may be considerably more complex. Recent evidence has suggested that the ADRB2 receptor complexes with, and regulates signalling through, other GPCRs including the angiotensin II type I receptor (AT_1R). Antagonism of ADRB2 in mice was recently shown to

also reduce signalling via the AT_1R (Barki-Harrington *et al*, 2003). ADRB2 is also known to be integral to metabolic processes *in vivo* and the β-adrenergic receptors, generally, mediate sympathetically driven thermogenesis as well as lipolysis and glucose homeostasis (Lowell & Bachman, 2003).

ADRB2 has received much interest from the asthma community owing to its role in dilatation of the bronchi and the known therapeutic benefits of β-agonists. Consequently, polymorphisms within the gene are well known and some have been functionally characterised (Liggett, 1997). This prior knowledge allows the selection of known functional polymorphisms. Family-based methods were used to investigate association of three functional gene polymorphisms as single loci and haplotypes to hypertension in the nuclear families of the Silesian Hypertension Study.

FGF1, encoding the acidic fibroblast growth factor, is a member of the FGF gene family encoding at least 22 ligands. These mediate a variety of signalling events via four widely expressed fibroblast growth factor receptors (FGFRs). FGF1 itself has kidney and brain-specific promoters, and additional promoters controlling expression in various tissues including vascular smooth muscle (VSM) (Fig. 4.1.2). It is unusual in that it binds with high affinity to all known FGFR isoforms, unlike other FGFs (Chiu *et al*, 2001). FGF1 is released from cells to bind to cell-surface FGFRs which triggers a signalling cascade and stimulates gene expression. It has wide-ranging roles in development, wound healing, tumour development and progression, and angiogenesis. FGF1 is also a potent proliferator of endothelial cells (Powers *et al*, 2000). Systemic

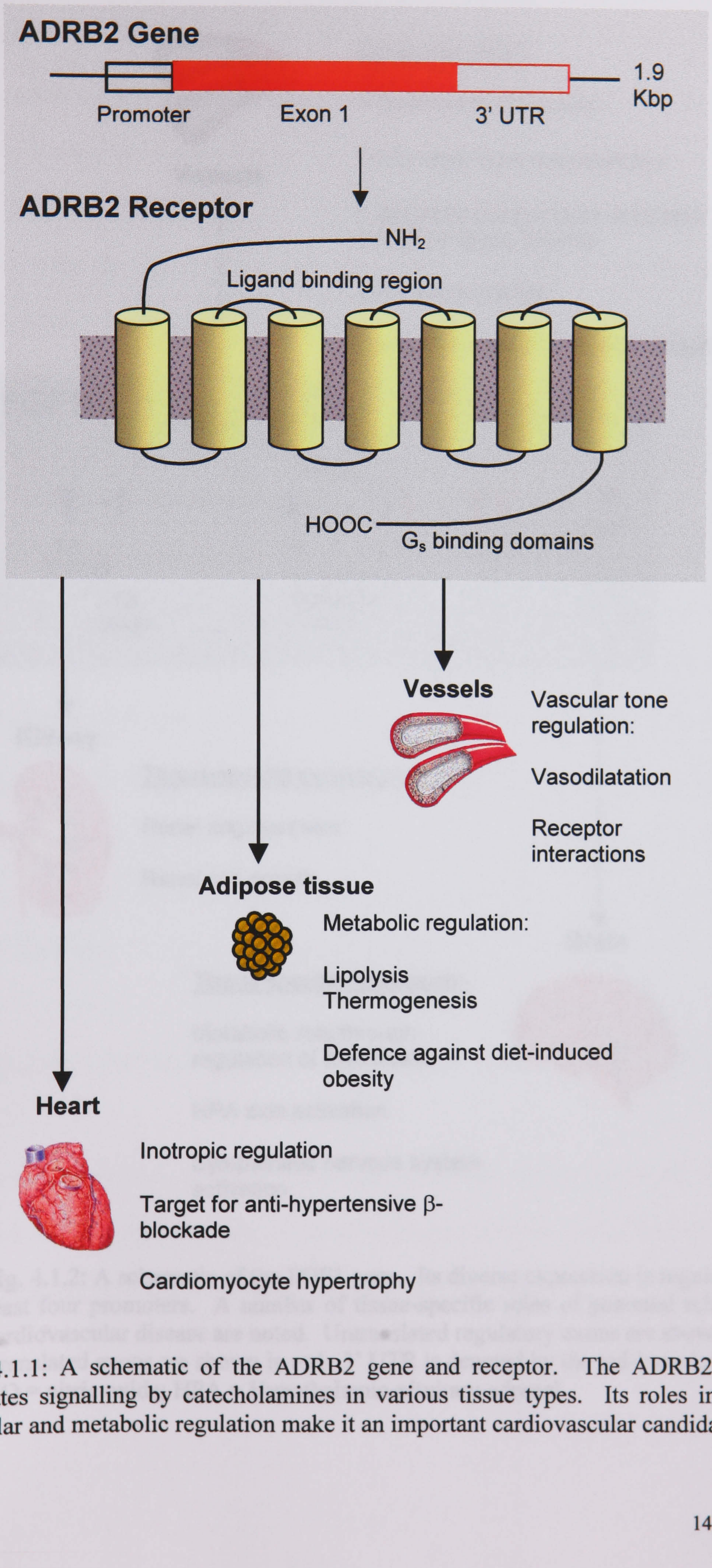
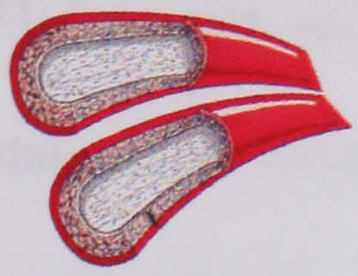


Fig. 4.1.1: A schematic of the ADRB2 gene and receptor. The ADRB2 receptor mediates signalling by catecholamines in various tissue types. Its roles in cardiac, vascular and metabolic regulation make it an important cardiovascular candidate gene.



Vessels

Circulating FGF1:

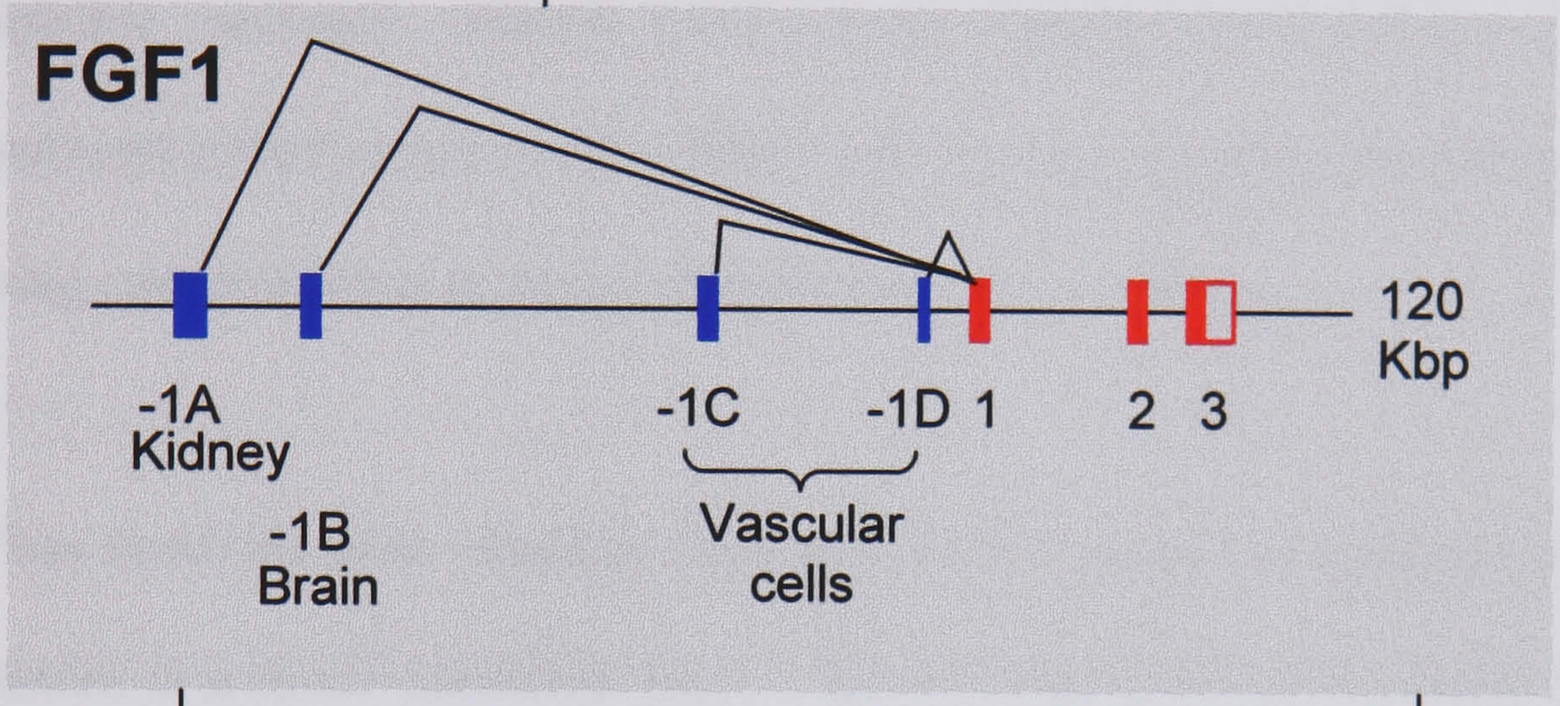
↑ Endothelial cell growth

↑ NO-dependent vasodilatation

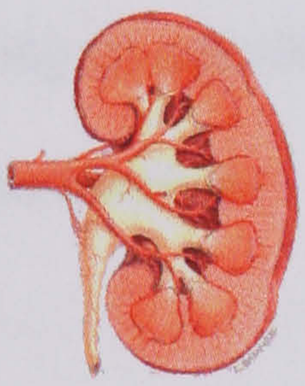
↑ glutathione peroxidase-mediated oxidative stress defence

HPA axis activation

Sympathetic nervous system activation



Kidney



Tissue-specific expression:-

Renal angiogenesis

Renal cell growth

Brain

Tissue-specific expression:-

Metabolic role through regulation of food intake

HPA axis activation

Sympathetic nervous system activation

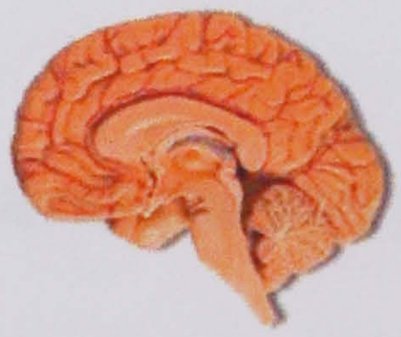


Fig. 4.1.2: A schematic of the FGF1 gene. Its diverse expression is regulated by at least four promoters. A number of tissue-specific roles of potential relevance to cardiovascular disease are noted. Untranslated regulatory exons are shown in blue. Translated exons are shown in red. 3' UTR is denoted by the red-boxed area.
NO = nitric oxide; HPA = Hypothalamic-pituitary-adrenal.

administration of FGF1 into rats causes a dose-dependent decrease in blood pressure, arteriolar dilatation and increased vascular flow. Nitric oxide synthase inhibitors or endothelial denudation abolish these effects (Cuevas *et al*, 1991; Wu *et al*, 1996). FGF1 may also increase the endothelium's resistance to oxidative stress by increase of intracellular reduced glutathione (Yang & deBono, 1997). Infusion into rats has also been shown to stimulate the hypothalamic pituitary adrenal (HPA) axis, leading to increased plasma corticosterone (Matsumoto *et al*, 1998b), and the sympathetic nervous system, increasing catecholamine secretion (Matsumoto *et al*, 1998a).

FGF1 has received little attention from the cardiovascular community, and in this context is a novel candidate gene. To investigate this novel locus, FGF1 sequencing and SNP locus and haplotype studies are presented as first steps in assessing its contribution to blood pressure variation and hypertension risk in the Silesian families.

4.2: METHODS:

4.2.1: SILESIAN HYPERTENSION STUDY DESIGN AND RECRUITMENT:

The Silesian Hypertension Study was used in linkage studies described in Chapter 3. A detailed description of the ascertainment strategy, recruitment and phenotyping can be found in Sections 2.2.1 and 3.2.1.1.

4.2.2: β_2-ADRENERGIC RECEPTOR CANDIDATE LOCUS STUDY:

4.2.2.1: GENOTYPING OF β_2-ADRENERGIC RECEPTOR SNPS:

Three non-synonymous functional SNPs were genotyped in the Silesian sample: Arg16Gly, Gln27Glu and Thr164Ile. PCR for each was performed as detailed in Section 2.5.1 in a volume of 20 µl using HotStar Taq (Qiagen Ltd, UK). Each polymorphism introduces a restriction site into the sequence. Therefore, genotyping was done by restriction enzyme digest as described in Section 2.5.2. Arg16Gly was digested with *Bsr*DI (New England Biolabs (UK) Ltd, UK); Gln27Glu was digested with *Ita*I (Roche, Switzerland); Thr164Ile SNP was digested with *Mnl*I (New England Biolabs (UK) Ltd, UK). Digestion products were resolved on agarose gels and genotypes were scored visually by two individuals. Digestion products have been described previously (Large *et al*, 1997). Examples are shown in Appendix 4. Arg16Gly resulted in fragments as follows: 14, 56, and 131 bp for Arg homozygotes; 14, 23, 56 and 108 bp for Gly homozygotes. Heterozygotes were distinguished by the presence of 108 and 131 bp fragments. Gln27Glu digestion gave the following fragments: 27, 55, 97 and 174 bp for Gln homozygotes; 27, 97 and 229 bp fragments for Glu homozygotes. Heterozygotes were distinguished by the presence of 174 and 229 bp fragments. Thr164Ile gave 38, 114 and 206 bp fragments in Thr homozygotes; 38 and 320 bp fragments in Ile homozygotes and 38, 114, 206 and 320 bp fragments in heterozygotes.

4.2.3: FIBROBLAST GROWTH FACTOR 1 CANDIDATE LOCUS STUDY:

4.2.3.1: FIBROBLAST GROWTH FACTOR 1 SEQUENCING:

Single nucleotide polymorphism (SNP) discovery within the FGF1 locus was performed by sequencing coding, flanking intronic and 3' untranslated regions (3' UTR) of the gene as described in Sections 2.6.1 and 2.6.2. Three coding exons are known at present, as detailed in Fig. 4.1.2. PCR primers were designed to intronic sequences flanking each exon based on genomic sequences in the NCBI database (www.ncbi.nlm.nih.gov; Accession no: NT_029289) and FGF1 cDNA sequences from the same database and the literature (Payson *et al*, 1993). Primer sequences are given in Appendix 3.

Twenty unrelated Polish individuals were selected for sequencing. A deliberately heterogeneous group was used. Thus, ten individuals were male, ten female. Of these two subgroups, half were hypertensive and half normotensive. HotStar Taq (Qiagen Ltd, UK) was used to amplify intron/exon and 3' UTR sequences as described in Section 2.6.1. PCR products were purified by Nucleofast-96 plates (Macherey-Nagel, Germany). Direct sequencing of the purified PCR products from the 20 individuals was done by MWG Biotech using the forward PCR primers. Data from MWG were aligned to a reference sequence using Vector NTI v8 (Informax Inc, US). Putative polymorphic sites were confirmed by visual inspection of the electropherograms.

4.2.3.2: FIBROBLAST GROWTH FACTOR 1 SNP GENOTYPING:

Genotyping of polymorphisms within the FGF1 gene was done by the MRC Gene Service (Cambridge, UK). Genomic DNA at 5 ng/μl was provided. PCR primers were identical to those used during sequencing of the FGF1 gene. Genotyping was performed on an ABI 7900HT (TaqMan) instrument platform (Applied Biosystems, UK). Allelic recognition probes were labelled with the fluorescent dyes VIC or FAM. Probe sequences are provided in Appendix 3.

4.2.4: STATISTICAL ANALYSES:

Genotypic data were checked for Mendelian inconsistencies using the PEDCHECK program (O'Connell & Weeks, 1998). Genotypes were confirmed for individuals flagged by the software, corrected if appropriate or individuals removed from the dataset.

Blood pressure measurements were corrected for the effect of anti-hypertensive treatment using a method developed by the Framingham Study (Levy *et al*, 2000) and described in Section 3.2.

4.2.4.1: ANALYSIS OF THE β_2-ADRENERGIC RECEPTOR LOCUS:

A detailed description of analytical methods used and reasons for their selection can be found in Section 2.7.2.1. Briefly, family-based tests of association and/or linkage were used to assess the SNPs genotyped in the ADRB2 gene and

hypertension status. Transmission disequilibrium testing (TDT) was employed to test for linkage and association (Spielman *et al*, 1993). This was originally devised as a test for linkage in the presence of association and uses simplex families consisting of parents and an affected child. Transmission of alleles from parent to child at a polymorphic locus is compared to non-transmitted alleles. This test is robust to the effects of population stratification since the non-transmitted alleles provide the control genotype. To test for association with hypertension, assuming linkage, the empirical variance option of FBAT (EV-FBAT) was used. The null hypothesis of linkage but no association is tested by this method (Lake *et al*, 2000). FBAT is robust with regard to pedigree structure, population admixture, phenotype distribution and ascertainment methods and uses all individuals in a pedigree unlike TDT.

ADRB2 SNP haplotypes were assessed using a modified TDT method implemented in TRANSMIT (Clayton, 1999). This is a likelihood method which allows for missing parental data and haplotype phase uncertainty and uses genotype data from available parents and offspring to efficiently determine phase. The method is robust against population stratification.

Binary logistic regression analysis was performed in the parental generation to test for increased risk of hypertension with genotype at the three polymorphic loci. Covariates of age, sex, and body mass index were included.

4.2.4.2: ANALYSIS OF THE FIBROBLAST GROWTH FACTOR 1 LOCUS:

Genotypes derived from SNP genotyping of the FGF1 locus in the Silesian Hypertension Study group were assessed for association to quantitative and qualitative blood pressure traits. The software package FBAT was used to perform a family-based association test of SNPs to phenotypes (Horvath *et al*, 2001). The empirical variance option (EV-FBAT) was used to investigate the null hypothesis of linkage but no association (Lake *et al*, 2000). In addition, haplotype analyses were performed using Haplotype-FBAT (H-FBAT) in which genotypes at all polymorphic loci were assessed jointly. The method handles missing parental genotypes and unknown haplotype phase.

4.3: RESULTS:

4.3.1: STUDIES OF THE β_2-ADRENERGIC RECEPTOR LOCUS:

ADRB2 was examined as a positional and physiological candidate which has been implicated by other studies of blood pressure and hypertension. Three non-synonymous, functional single nucleotide polymorphisms within the ADRB2 gene were genotyped and analysed. These were Arg16Gly, Gln27Glu and Thr164Ile. TDT analysis was used to assess linkage in the presence of linkage disequilibrium to essential hypertension. Analysis was performed in trios consisting of two parents, one child or trios of one parent, one affected child, one unaffected child. Arg16Gly and Gln27Glu were not significantly

Table 4.3.1: Results of TDT and EV-FBAT analyses of three SNPs within the ADRB2 candidate gene and association to hypertension status.

SNP	Allele	Transmissions N (%)	TDT		EV-FBAT
			χ^2	P	P
Arg16Gly	Arg	54 (54.5)			
	Gly	45 (45.5)	0.8	0.37	0.67
Gln27Glu	Gln	62 (49.2)			
	Glu	64 (50.8)	<0.1	0.86	0.55
Thr164Ile	Thr	4 (80)			
	Ile	1 (20)	ND	ND	ND

ND = Not done due to the rare occurrence of the Ile allele.

Table 4.3.2: Results of TRANSMIT haplotype analyses of a 3 locus haplotype within the ADRB2 candidate gene and association to hypertension status.

H	Haplotype alleles			Estimated frequency	Observed transmissions	Expected transmissions	χ^2	P
	Arg16Gly	Gln27Glu	Thr164ile					
B	Gly	Glu	Thr	42.2%	217	211	0.52	0.47
D	Arg	Gln	Thr	34.8%	178	174	0.59	0.44
F	Gly	Gln	Thr	20.4%	95	103	2.03	0.15

H = haplotype.

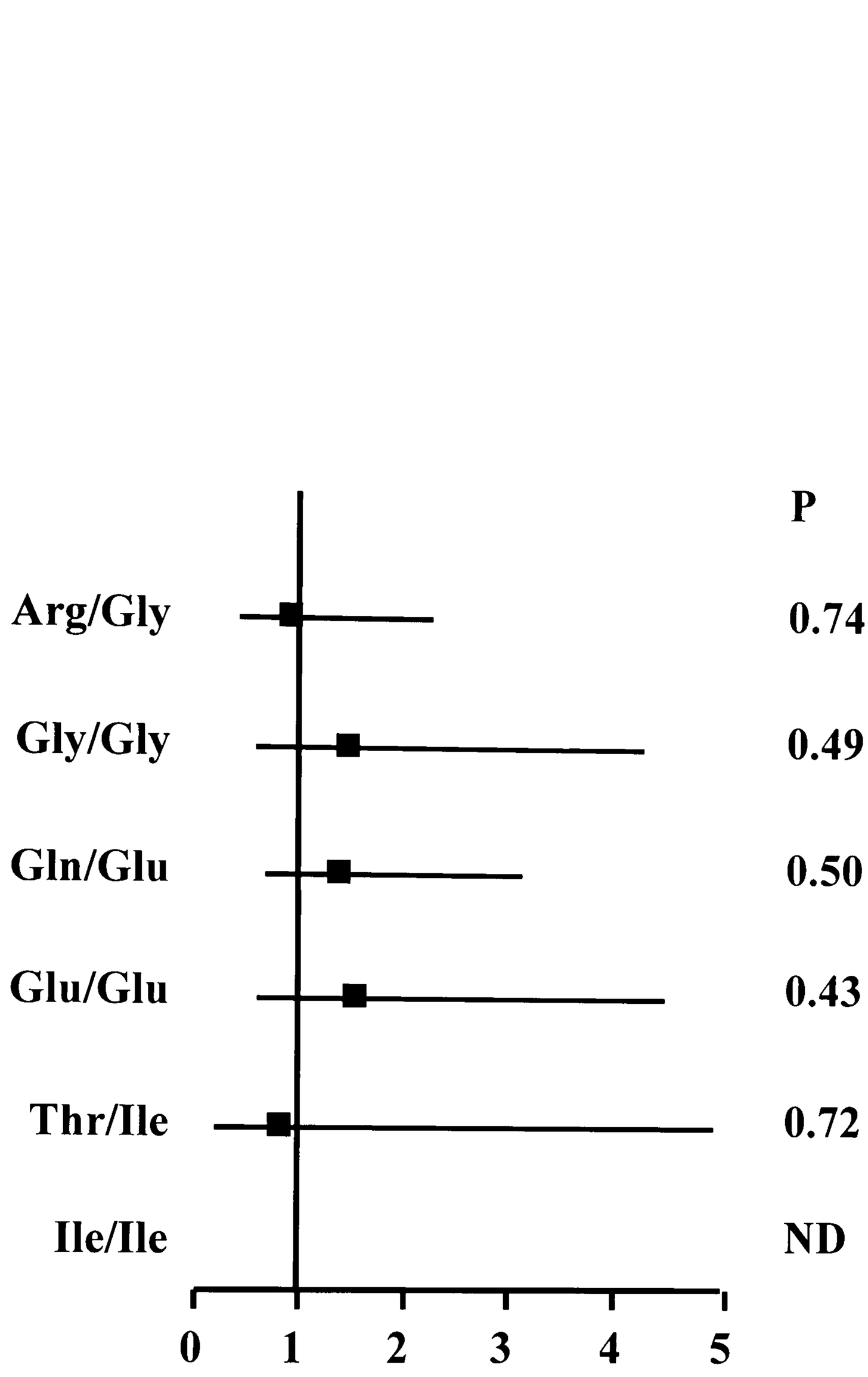


Fig. 4.3.1: Odds ratios (OR) and 95% confidence intervals for binary regression modelling of essential hypertension risk. Confounders of age, sex and BMI were included. OR was calculated for heterozygous or homozygous variant individuals with respect to wild type homozygotes.
ND = not done due to the rarity of the Ile allele.

linked to hypertension with P values greater than 0.05. The allele frequencies of 164Ile wer einsufficient to allow a vlid test of this locus (Table 4.3.1).

The EV-FBAT method confirmed the TDT results. No test could be performed for the Thr164Ile polymorphism due to the rarity of the Ile allele with only 17 occurrences in the study group. However, tests for Arg16Gly and Gln27Glu agreed with the findings of the TDT analysis. Neither polymorphism was significantly associated with essential hypertension (Table 4.3.1).

Of 8 possible haplotypes, 7 were observed in our population. These were designated A to G. Haplotypes B, D and F were most common and accounted for 97.4% of observations (Table 4.3.2). Transmissions of each haplotype were compared with expected number of transmissions using TRANSMIT. The observed transmissions from heterozygous parents to hypertensive offspring were not significantly different from the expected. Haplotype analysis therefore supported the single locus analyses, indicating no association of the polymorphisms to essential hypertension (Table 4.3.2).

Binary regression modelling, including confounders of age, sex and BMI, also confirmed the single locus and haplotype TDT analyses. Presence of one or two variant alleles at any SNP locus did not significantly increase risk of hypertension when compared to common individuals. Odds ratios for heterozygotes and variant homozygotes were: 0.85 (95% CI, 0.3 to 2.2); P=0.74) and 1.46 (95% CI, 0.5 to 4.2; P=0.49) for Arg16Gly; 1.33 (95% CI, 0.6 to 3.1;

P=0.5) and 1.54 (95% CI, 0.5 to 4.4; P=0.43) for Gln27Glu; and 0.7 (95% CI, 0.1 to 4.9; P=0.72) for Thr164Ile respectively (Fig. 4.3.1).

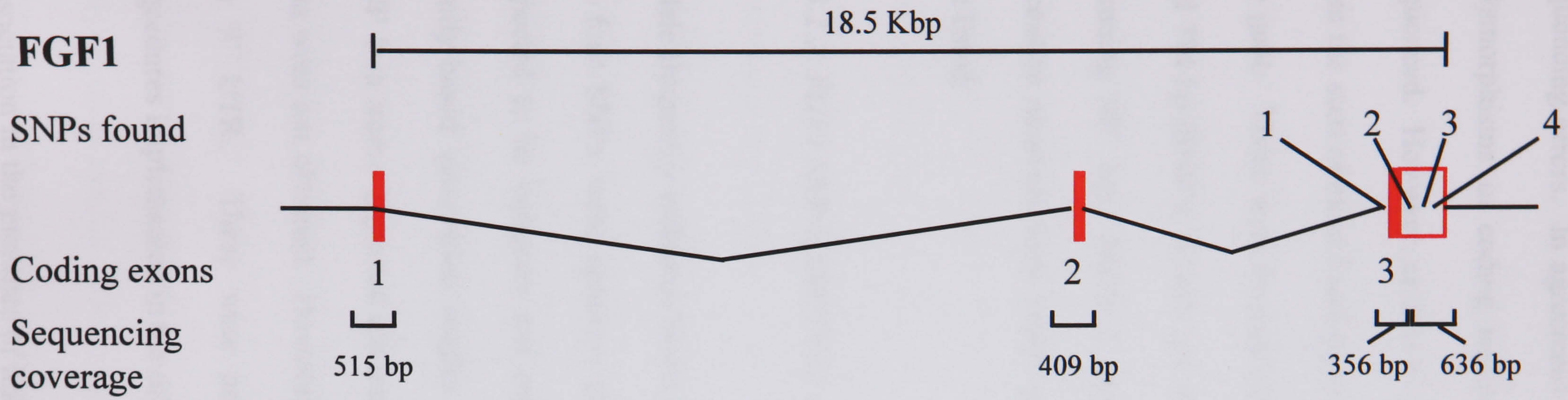
4.3.2: STUDIES OF THE FIBROBLAST GROWTH FACTOR 1 LOCUS:

FGF1 was investigated next as a second putative physiological candidate which lay under the maximal multipoint linkage region. SNP hunting and association analyses were performed in the Silesian hypertensive family resource.

4.3.2.1: FIBROBLAST GROWTH FACTIOR 1 SEQUENCING AND SNP DETECTION:

No SNPs within coding exons were listed in on-line databases such as NCBI (http://www.ncbi.nlm.nih.gov/) so coding exons, flanking intronic regions and the 3' UTR of the gene were sequenced to allow identification of polymorphisms in the Silesian population. Sequencing was performed with genomic DNA from 20 unrelated individuals, half of whom were hypertensive. This sample of 40 chromosomes gave an 87% probability of finding polymorphisms with minor allele frequencies of 5% and 99% probability of finding polymorphisms with 10% minor allele frequency.

Regions covered, and results obtained by sequencing, are shown in Fig. 4.3.1. Sequence data obtained were aligned to reference sequences from the NCBI database. Polymorphic sites were identified which differed from the reference sequence. These were confirmed by a visual check of the sequencing



	Alleles	Location	Estimated minor allele frequency
SNP 1	T → G	Intron 2	0.4
SNP 2	C → A	3' UTR	0.4
SNP 3	G → A	3' UTR	0.2
SNP 4	G → C	3' UTR	0.3

Fig. 4.3.2: A schematic of the coding region of FGF1 indicating the regions sequenced, length of sequence obtained and location of SNPs found. Coding exons are denoted by the red bars. 3' UTR is denoted by the red boxed area. These regions were sequenced in 20 unrelated individuals from whom estimates of minor allele frequencies were obtained for the Silesian population. These are shown with the base change for each locus in the accompanying table.

electropherograms. The majority of polymorphisms were eliminated as sequencing errors. In agreement with available information, no SNPs or other polymorphisms in coding regions were found in the 20 Polish individuals sequenced. However, an intron 2 SNP (SNP 1, T→G) was found 19 basepairs from the start of exon 3 and three further SNPs were identified in the 3' UTR of the gene. These were located 284 bp (SNP 2, C→A), 323 bp (SNP 3, G→A) and 774 bp (SNP 4, G→C) into the 3' UTR. In total, then, 4 SNPs were found spanning 987 bp. SNPs 2 and 4 were listed in public databases; dbSNP accession numbers were 34002 and 2278688, respectively. SNPs 1 and 3 were not listed.

4.3.2.2: FGF1 SNP GENOTYPE ANALYSIS:

Allele frequency estimates based on sequencing 40 chromosomes suggested that all four SNPs were common (Fig. 4.3.1). Given this, heterozygotes were expected to be common and each SNP was expected to be informative for family-based association studies. Therefore, all four SNPs were genotyped. SNP 1 in intron 2 proved intractable to the hybridisation method used, and so data were not obtained. However, genotypes were obtained for SNPs 2 to 4 in the 3' UTR. These were analysed by family-based association testing procedures implemented in the software FBAT.

Association in the presence of linkage of each individual SNP and hypertension was tested using the empirical variance FBAT (EV-FBAT) test. This method was also used to test for association to SBP, DBP and pulse pressure (PP). EV-

FBAT was preferred over TDT for FGF1 analyses since it makes more thorough use of available genotyped individuals, whilst TDT is restrictive in its use of simplex families. Use of TDT as the primary method for ADRB2 analyses had been prescribed by our desire to follow-up the ADRB2 studies of Bray *et al* (2000b). This decision meant we concentrated on analysis of hypertension status for ADRB2. However, because we subsequently adopted FBAT as our primary analysis for FGF1, we were able to assess SBP, DBP and pulse pressure and compare them directly. In FGF1, SNP 2 proved to be associated with hypertensive status, pulse pressure and systolic blood pressure at the 95% confidence level. SNPs 3 and 4 showed no evidence of such association ($P > 0.05$). No loci were associated with DBP.

Association of haplotypes across the three SNP loci and hypertension status was considered using the haplotype extension of FBAT (H-FBAT). This methodology had not been developed when ADRB2 was studied. However, its availability for FGF1 allowed us to examine single loci and haplotypes in a concerted manner. Of a possible 8 haplotypes, 7 were observed, denoted A to G. Three of these, haplotypes A, B and C, accounted for 96.9% of all observations (Table 4.3.4). These were common enough to be informative in the study group. Haplotype A, consisting of the alleles C, G and G at SNPs 2, 3 and 4, respectively, was significantly associated with hypertension status in the Silesian study sample. Neither haplotype B nor C was significantly associated. It appears that association of haplotype is dependent on the allele at SNP 2. All other haplotypes having allele C at SNP 2 had frequencies of 0.5% so were

Table 4.3.3: Single locus analysis of three SNPs within the 3' UTR of the FGF1 gene by Family-Based Association Test

		SBP		DBP		PP		EHT	
SNP #	Allele	z	P	z	P	z	P	z	P
2	A	-2.0	**0.045**	-1.3	0.177	-2.3	**0.019**	-2.1	**0.038**
	C	2.0		1.3		2.3		2.1	
3	A	-0.3	0.785	-0.32	0.749	-0.2	0.848	-1.7	0.091
	G	3.0		0.32		0.2		1.7	
4	C	-0.2	0.868	-0.37	0.710	-0.1	0.939	-1.5	0.138
	G	0.2		0.37		0.1		1.5	

SBP = systolic blood pressure; DBP = diastolic blood pressure; PP = pulse pressure; EHT = essential hypertension. Quantitative traits were corrected for hypertensive treatment effect.

Table 4.3.4: Results of H-FBAT haplotype analyses of a 3 locus haplotype within the FGF1 3' UTR and association to hypertensive status.

	Haplotype alleles					
H	SNP 2	SNP 3	SNP 4	Estimated frequency	z	P
A	C	G	G	53.4%	2.3	**0.020**
B	A	G	G	36.4%	-1.5	0.131
C	A	A	C	7.1%	-1.0	0.297

H = haplotype.

uninformative. However, the particular importance of this SNP supports the findings of the single locus analyses.

4.4: DISCUSSION:

Previous linkage analyses focused our attention on positional and physiological candidate genes in a 7 cM region of human chromosome 5q (Tomaszewski *et al*, 2002). Association studies of two genes were performed in the Silesian Hypertension Study sample, using family-based analysis methods. Work by others had previously implicated the ADRB2 gene encoding the β_2-adrenergic receptor, and this was investigated first. Single locus and haplotype analysis of three non-synonymous, functionally relevant SNPs within the ADRB2 gene did not support its involvement in the development of essential hypertension in our study sample (Tomaszewski *et al*, 2002). This was despite 86% power to detect association ($P<0.05$) with an odd ratio of 1.6 at the two common ADRB2 loci. Attention turned to the FGF1 gene encoding the acidic fibroblast growth factor, a cardiovascular candidate gene which, to our knowledge, has not been studied previously. Single locus and haplotype analyses demonstrated association of polymorphisms within the 3' UTR of the gene. In particular, a single locus, denoted SNP 2 and located 284 bp into the 3' UTR, appears to determine presence or absence of association.

The association analyses of both ADRB2 and FGF1 used family-based approaches. The compact nuclear family structure of the Silesian Hypertension Study sample lends itself to such methodologies. Concerns have been raised by

many regarding the possible effects of population stratification on results of association studies (Ziv & Burchard, 2003) and family-based studies have been developed to address this (Spielman & Ewens, 1996). In increasing robustness to stratification, however, there is generally a loss of power of family-based methods over the case-control method (Risch & Teng, 1998). Simulations have shown that this is particularly acute if parental genotyping is incomplete or absent (Risch & Teng, 1998). Power can be improved, however, by the inclusion of at least one affected parent and additional offspring, particularly if they are also affected (Whittaker & Lewis, 1998). In the Silesian sample, parents were present and genotyped. In addition, owing to the recruitment strategy, prevalence of essential hypertension was high in the group resulting in a high percentage of affected parents and siblings. Thus, whilst controlling for the effects of population stratification, the power of the family-based methods used is potentially maximised. In addition, use of multiple polymorphic loci and consideration of haplotypes results in a more robust analysis (Sharma & Jeunemaitre, 2000). These candidate genes were not studied in the MIDSPAN sample, partly due to unavailability of funding to accomplish this. However, it was also felt that such studies were more usefully pursued in samples with a significant disease burden, such as the Silesian sample, rather than a normal, nominally healthy population sample such as MIDSPAN since allele frequencies of genuine susceptibility loci would be greater.

The ADRB2 gene, encoding the β_2-adrenergic receptor has received much attention from cardiovascular researchers owing to its role in maintenance of vascular tone, heart rate, salt and water excretion and lipolysis (Bray &

Boerwinkle, 2000). Studies of this locus benefit from the existence of relatively well-characterised functional polymorphisms. This knowledge allowed us to select SNPs with known functional significance in line with current thinking (Risch, 2000; Tabor *et al*, 2002). These were Arg16Gly, Gln27Glu and Thr164Ile. Arg16Gly and Gln27Glu affect receptor downregulation. The Gly16 allele promotes agonist-induced receptor downregulation, decreasing numbers of receptors at the cell surface. The Glu27 allele has been shown to result in resistance to agonist-induced downregulation. Where both variant alleles are present, the receptor downregulates at an increased rate comparable to the presence of Gly16 alone (Green *et al*, 1994). The Thr164Ile variant receptor is less common but most functionally altered. The Ile allele causes a slight decease in agonist binding affinity but a substantial decrease in basal and agonist-stimulated adenylyl cyclase activity due to defective G_s protein coupling (Green *et al*, 1993).

Haplotype studies of ADRB2 gene polymorphisms are rare, despite the potential for interactions between the functional polymorphisms described. A case-control study including 707 French hypertensives found no evidence of increased risk of hypertension from haplotypes across four ADRB2 polymorphisms, including Arg16Gly and Gln27Glu (Herrmann *et al*, 2002). This agrees with our findings in the hypertensive Silesian sample. Conversely, two reports of normotensive study groups from Europe reported association of haplotypes of Arg16Gly and Gln27Glu with SBP. A population sample from Italy showed association of SBP with the haplotype Arg16-Glu27 as well as association of the Arg16 allele alone (Castellano *et al*, 2003). A Norwegian case-control study compared

normotensive offspring of hypertensive parents with offspring of normotensive parents. It showed association of a 4 locus haplotype with blood pressure. It was again found that Arg16 was the critical allele (Timmermann *et al*, 1998).

The vast majority of studies of the ADRB2 locus examine polymorphisms as single loci. European, American, Japanese and African Caribbean populations have been investigated. Ethnic differences may explain some of the variability in findings. For example, a case-control study in African Caribbean individuals demonstrated association of the pro-downregulatory Gly16 allele with hypertension (Kotanko *et al*, 1997). Association of the Gln27Glu polymorphism and hypertension was demonstrated in a Japanese case-control study with Gln27 more common in hypertensives (Kato *et al*, 2001). However, in agreement with our findings and those of Herrmann *et al*, studies in white European hypertensive samples from Sweden and the UK show no association of ADRB2 polymorphisms to hypertension (Jia *et al*, 2000; Bengtsson *et al*, 2001). In contrast, positive association of ADRB2 polymorphisms with blood pressure has been reported in normotensive samples from Germany (Busjahn *et al*, 2000), Austria (Gratze *et al*, 1999) and Finland (Bengtsson *et al*, 2001). Association of SBP to ADRB2 has also been reported in white normotensive US sibs (Bray *et al*, 2000b). Studies of intermediate phenotypes in normotensives also suggest association of ADRB2. These include forearm blood flow, response to isoproterenol infusion in individuals from the UK (Cockcroft *et al*, 2000) and salbutamol-induced vasodilatation in Austrian men (Gratze *et al*, 1999). It is notable, then, that association of ADRB2 is negative in hypertensive European populations whereas studies in normotensive groups suggest association.

A possible explanation for the discrepancy in the contribution of ADRB2 to hypertension risk versus normal blood pressure distribution may lie in interaction of other metabolic and cardiovascular phenotypes. ADRB2 influences insulin resistance, obesity and heart failure (Bray & Boerwinkle, 2000). These in turn are known to have very different prevalence in hypertensive and normotensive individuals (Reaven, 2003). They cannot, therefore, be excluded as potential factors confounding the relationship between ADRB2 variants and blood pressure. This possibility is supported by studies of subsets of European hypertensives, for example those with type II diabetes, for whom positive association of ADRB2 to hypertension has been reported (Bengtsson *et al*, 2001). Alternatively, the different contribution of ADRB2 in normotensive and hypertensive individuals may indicate differences in the genetic basis of high and low blood pressure. This remains to be clarified. However, in our hypertensive population there is no evidence to suggest that ADRB2 contributes to hypertension risk across the sample.

The fibroblast growth factor 1 (FGF1) gene, unlike ADRB2, has received little attention from the cardiovascular research community making it an exciting and novel candidate. Studies in animal models have suggested a possible role in blood pressure regulation and, potentially, hypertensive disease. Direct effects of FGF1 on blood pressure have been demonstrated. Infusion into rats causes a dose-dependent hypotensive effect which is blocked by nitric oxide synthase (NOS) inhibitors (Cuevas *et al*, 1991). FGF1 has been shown to stimulate increase in intracellular Ca^{2+} concentrations, and it is possible that FGF1

regulates the calcium-dependent conversion of L-arginine to nitric oxide by NOS (Cuevas *et al*, 1991). Others showed that infusion of FGF1 into rats at physiological concentrations resulted in vasodilatation of resistance arteries and arterioles. Vascular flow also increased and all effects were again blocked by NOS inhibition or endothelial denudation (Wu *et al*, 1996). Systemic infusion into rats has also been demonstrated to induce adrenomedullary secretion, raising plasma adrenaline and noradrenaline via a corticotropin releasing factor-dependent mechanism (Matsumoto *et al*, 1998a). Cerebroventricular or intra-atrial delivery of FGF1 increased plasma corticosterone and adrenocorticotrophic hormone (ACTH) (Matsumoto *et al*, 1998b). This suggests possible roles in metabolic processes and energy expenditure for a molecule which is known to act as an appetite suppressant via glucose-sensitive neurons in the hypothalamus (Matsumoto *et al*, 1998a).

FGF1 is important in development and causes proliferation of fibroblasts and endothelial cells (Powers *et al*, 2000) – hence its designation as a growth factor. However, on binding to FGF receptors, it also triggers expression of a variety of genes. Evidence from studies in cultured endothelial cells suggests that one effect of this may be to upregulate glutathione peroxidase-dependent oxidative stress defence. Studies in cultured human endothelial cells showed that application of FGF1 or the closely related FGF2 increased intracellular reduced glutathione concentrations, and accelerated the removal of hydrogen peroxide applied to the cells (Yang & deBono, 1997).

Although the FGF1 gene has been suggested as a cardiovascular candidate gene previously (Stoll *et al*, 2000), genetic studies have yet to be published. There are presently no known polymorphisms within the coding region of FGF. Consistent with this, we also did not find any coding polymorphisms despite good power to find alleles of 5% frequency or over. In the absence of polymorphic loci in coding regions it has been suggested that 5' and 3' regulatory regions take precedence since they may affect the timing, location or level of gene expression (Tabor *et al*, 2002). Our analysis of three polymorphic loci in the 3' UTR of the FGF1 gene showed association of the locus to hypertension status and blood pressure phenotypes. This is consistent with our linkage studies which were positive for both disease status and quantitative trait in the Silesian group (Tomaszewski *et al*, 2002). Our results implicate SNP 2, primarily, since it was associated both as a single locus and within a haplotype across three loci.

The frequencies of the haplotypes indicate linkage disequilibrium between the three loci. However, LD is not total since the allele at SNP 2 does not definitively predict the allele at SNPs 3 or 4. This may explain why SNPs 3 and 4, as individual loci, are not associated. It is known that LD is not proportional to distance below 60 Kb separation (Wright *et al*, 1999). Complexity in LD over very short distances – in this case, 490 bp – may be expected and has been reported by others in studies of complex diseases such as asthma (Allen *et al*, 2003). Equally, the possibility exists that strong LD may be present between polymorphic loci separated by quite large distances, perhaps as much as 100 Kb (Daly *et al*, 2001). Therefore, association of SNP 2 with hypertension and blood

pressure traits serves to implicate a smaller segment of the original 7 cM linkage region, essentially fine mapping an area of interest.

SNP 2, itself, may be the functional locus but in the absence of functional characterisation it is not possible to conclude this. It may also be in LD with the causative locus. Determination of the strength of LD in the region requires a fine mapping strategy as used by others to describe haplotype block structures (Rioux *et al*, 2001). At present, then, the association at the 3' end of the FGF1 gene may implicate a region of perhaps 200 Kb. Fine mapping within this region will identify the critical haplotype tag associated with the phenotypes of interest. This may result in a number of genetically equivalent loci which cannot be separated based on strength of association, a limitation which has been reported by others (Rioux *et al*, 2001). In this case, study of the functional effects of polymorphisms may be required to determine their relative importance.

Within the local region around the associated SNP loci, density of known genes is relatively low, compared to the genomic average of approximately 10 per million bases (Cartegni *et al*, 2002). The 100 Kb proximal of SNP 2 contains the FGF1 gene including regulatory regions (Chiu *et al*, 2001). In the distal 100 Kb, no other genes are known except for GRAF (GTPase regulator associated with focal adhesion kinase) which is located 85 Kb distal of the FGF1 gene. GRAF is a GTPase-activating protein which interacts with focal adhesion kinase (FAK). FAK provides a link between integrins and the signalling cascades that are activated by them (Hildebrand *et al*, 1996). Integrin signalling plays a part in regulation of vascular tone, perhaps partly through regulation of ion channels.

Signalling through $\alpha_V\beta3$ integrins results in NO production by endothelial cells and there is evidence to suggest that extracellular matrix deposition and integrin expression in hypertension are altered (Martinez-Lemus *et al*, 2003). The GRAF gene cannot be dismissed out of hand as a candidate, therefore. However, its distance from the locus of association weakens the case in favour whilst strengthening that of FGF1. Given that FGF1 has plausible biological merit and is the only known gene within 180 Kb, FGF1 is a logical focus for further mapping and functional studies.

In summary, haplotype and single locus association analyses, using family-based methods, were applied to the investigation of two candidate genes, ADRB2 and FGF1, in the 5q31.1-q33 linkage region previously described. Analysis of three common, functional polymorphisms probably excludes an effect of these polymorphisms as hypertension susceptibility loci in our Silesian Hypertension Study group. However, such analyses are unlikely to describe all haplotype structure within the gene and must have lower power to exclude other loci, particularly those in low LD. Analysis of polymorphisms in the 3' UTR of FGF1, however, revealed association to both hypertension and blood pressure phenotypes and implicated, particularly, a SNP locus 284 bp into the 3' UTR. No other known genes exist within 85 Kb of the associated loci. Thus, FGF1 has been confirmed as a positional candidate as well as being a physiological candidate. Further mapping and functional studies of this gene are required to confirm its contribution to hypertension risk.

CHAPTER 5

CANDIDATE GENE ANALYSES OF FAMILIAL INTRACRANIAL ANEURYSMS

5.1: INTRODUCTION:

Familial intracranial aneurysms (FIAs) form as a result of complex processes involving genetic and acquired risk factors (Alberts, 1999). Whilst as many as 95% remain asymptomatic (Schievink *et al*, 1994), the minority that rupture do so with devastating effect. Aneurysmal subarachnoid haemorrhage is associated with 50% mortality and about a third of survivors have serious neurological deficits (Schievink, 1997). Prognosis for those patients who undergo elective surgery for intracranial aneurysms (IAs) is considerably better. Mortality of 1% and morbidity of 4% underline the benefit of detecting and treating intracranial aneurysms early (King *et al*, 1994).

At present the aetiology of FIAs is poorly understood. Mechanical factors such as haemodynamic stress are involved and may account for the common siting of aneurysms at arterial bifurcations, particularly around the circle of Willis. Other factors such as inflammatory responses are thought to act on regions of the arterial wall weakened by other processes. However, the underlying weakness may be genetically determined (Zhang *et al*, 2003). Presently, no biochemical markers of aneurysm formation are known and, without an understanding of the genetic basis, it is difficult to effectively identify at-risk individuals in families with a history of FIA (van Gijn & Rinkel, 2001).

Studies of the pattern of inheritance in intracranial aneurysm families have identified autosomal dominant, autosomal recessive and multifactorial inheritance patterns (Bromberg *et al*, 1995) . This indicates the possibility of

major gene effects, but also the probability of genetic heterogeneity (Schievink, 1997). However, in the event that major gene effects exist they may be detectable by linkage analysis or association-based methods. Such studies in FIA patients are presently rare (Zhang *et al*, 2003). Here a candidate gene approach is used to examine the contribution of four genes to the formation of intracranial aneurysms in families from the west of Scotland who have a history of FIA.

A present hypothesis suggests that the underlying defect, which leads to intracranial aneurysm (IA) formation, involves an imbalance in extracellular matrix formation and/ or degradation (Zhang *et al*, 2003). It may be this that amplifies the long-term effects of ageing and haemodynamic stress. Rare Mendelian connective tissue disorders such as autosomal dominant polycystic kidney disease, Marfan syndrome and Ehlers-Danlos syndrome type IV arise from mutations in genes encoding structural proteins. Features of these pathologies include vascular catastrophes such as arterial dissections and ruptures as well as aneurysm formation on both systemic and cerebral arteries (Schievink, 1997). It is reasoned that mutations in genes underlying these Mendelian disorders or other components of matrix formation and decomposition may lead to the formation of IAs seen in families. Selection of the candidate genes studied has been based on this hypothesis. Consequently, genes encoding collagen type III, fibrillin 1, polycystin 1, and serine protease inhibitor A1 have been studied.

Collagen type III is a major component of the arterial wall and is encoded by the COL3A1 gene on chromosome 2q32.2. Three collagen α1(III) chains form the

finished homotrimeric collagen III fibre (Fig. 5.1.1) and provide tensile strength to the arterial wall (van Vlijmen-van Keulen *et al*, 2002).

Mutations in COL3A1 are responsible for Ehlers Danlos syndrome type IV (EDS IV). The majority of deaths in EDS IV patients are due to spontaneous rupture, dissection and aneurysm formation of large to medium-sized arteries (Schievink, 1997). The COL3A1 gene has therefore attracted considerable interest as a candidate for aneurysmal disorders. Studies of collagen type III levels in skin fibroblasts from abdominal aortic aneurysm (AAA) patients suggest that 10% of AAA patients may have reduced collagen III levels (van Keulen *et al*, 1999). Reduced collagen type III production has also been reported in IA patients (van den Berg *et al*, 1997). Some IA patients may have impaired secretion of collagen type III, as low as 50%, in the absence of differences in initial production. This may be due to structural defects affecting thermal stability (Majamaa & Myllyla, 1993). Crosslinking of collagen fibres, essential to the integrity of the tissue, may also be reduced (Gaetani *et al*, 1996).

Despite the biochemical data, sequencing or single strand conformational analyses (SSCP) of the coding regions of COL3A1 have failed to reveal relevant mutations in IA patients (Kuivaniemi *et al*, 1993; van den Berg *et al*, 1999). However, such mutations affecting the triple helical domain of the gene have been reported in AAA families (Tromp *et al*, 1993). Linkage studies have not been performed in IA families. However, a single case-control study of 19 IA patients and 15 controls showed a significant association of an AvaII restriction polymorphism to IA (Brega *et al*, 1996).

FBN1 on chromosome 15q21.1 encodes fibrillin 1, the principal structural component of elastic-fibre-associated microfibrils (Fig. 5.1.1). It is crucial to the elasticity and resilience of blood vessels (Kielty *et al*, 2002). The consequences of disruption of this role are seen in Marfan syndrome where mutations in FBN1 cause aortic ruptures and dissections. Case reports of intracranial aneurysms in Marfan patients also exist (Schievink, 1997). Its significance in at least a subset of AAA families has been suggested by linkage of intragenic markers (Vaughan *et al*, 2001), and mutations have been described in thoracic aortic aneurysm patients (Milewicz *et al*, 1996) and AAA patients (Francke *et al*, 1995). Studies of FBN1 in IA patients have yet to be published. However, some groups are at present studying this gene in families having isolated features of Marfan syndrome including arteriopathies and, specifically, intracranial aneurysms (Zhang *et al*, 2003).

The PKD1 gene encodes polycystin 1 and is located on chromosome 16p13.3. The putative functions of polycystin 1 include cell-cell adhesion and cell signalling, but its function is not precisely known at present (Weston *et al*, 2003). Mutations in the gene result in autosomal dominant polycystic kidney disease (ADPKD). A primary characteristic of this disorder is the formation of renal cysts which affect nephrons and collecting ducts. However, aortic aneurysms and dissections are also common and there is a firm link with increased risk of IAs (Schievink, 1997). As might be expected from the vascular aspect of ADPKD, polycystin 1 is expressed in medial layers of arterial walls (Griffin *et al*, 1997) (Fig. 5.1.1).

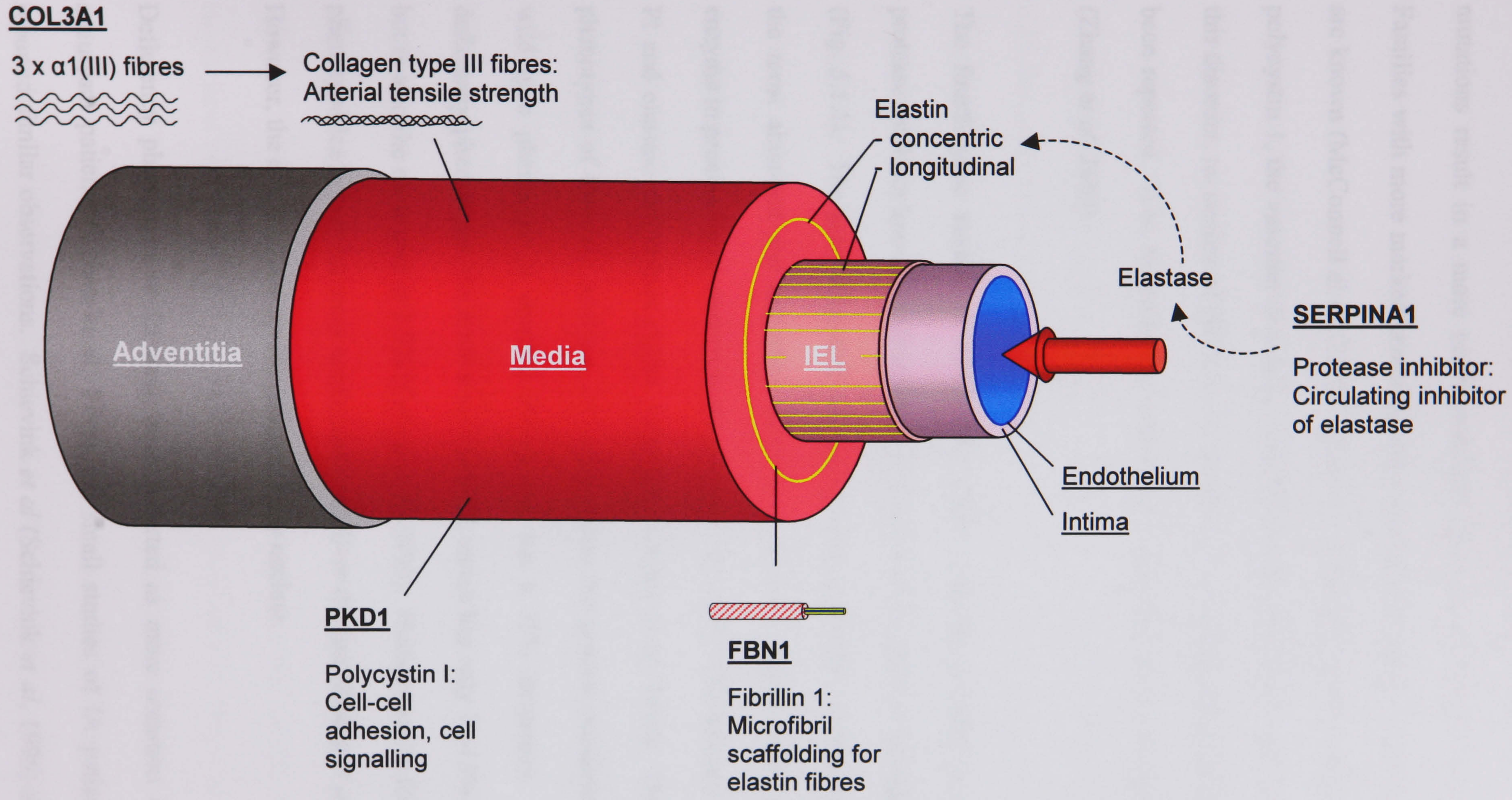


Fig. 5.1.1: Schematic representation of the structure of the cerebral arterial wall, and the location and role of candidate genes investigated.
IEL = internal elastic lamina; PI = protease inhibitor

Studies to correlate site of mutation with phenotype suggest that more 5' PKD1 mutations result in a more cerebrovascular phenotype (Rossetti *et al*, 2003). Families with more marked cerebrovascular but less severe renal manifestations are known (McConnell *et al*, 2001). However, despite the arterial expression of polycystin 1, the vascular defects in ADPKD and the confirmed risk of IA from this disorder, no studies of PKD1 in IA families or aortic aneurysm families have been reported. It is, nevertheless, considered a candidate gene worthy of study (Zhang *et al*, 2003).

The fourth gene studied is SERPINA1 which encodes a serine or cysteine protease inhibitor known variously as 'protease inhibitor' (PI) or 'α1-antitrypsin' (Fig. 5.1.1). The gene is located on chromosome 14q32.13. Its product, PI, is the most abundant and important inhibitor of elastase which, itself, is a key enzyme in processing of elastic tissues (Zhang *et al*, 2003). The balance between PI and elastase determines elastin catabolism (Baker *et al*, 1995). Deficiency phenotypes of Protease Inhibitor (PI) exist within the general population. The wild type phenotype is denoted PiMM and has a 95% frequency. Of the deficiency phenotypes, the most severe is PiZZ which has only 10-15% activity, but is also the most rare at 2-3% (Cohen *et al*, 1990). Homozygosity for the 'Z' phenotype leads to pulmonary emphysema and liver disease (Zhang *et al*, 2003). However, the effect of less severe deficiencies is unclear.

Deficiency phenotypes of PI have been reported as more common in aortic aneurysm patients (Cohen *et al*, 1990) and small studies of IA patients have reported similar observations. Schievink *et al* (Schievink *et al*, 1996) showed a

2-fold prevalence of such deficiency phenotypes in 100 consecutive IA patients compared with the general population and others have reported as high as 8-fold over-representation in London IA patients, although this was not significant after multiple testing correction (StJean *et al*, 1996). PI activity may also be reduced in intracranial (Tartara *et al*, 1996) and aortic aneurysm patients (Cohen *et al*, 1987).

Investigation of the candidate genes described was conducted in Scottish families with history of FIA and SAH. Further analysis of PI was performed using DNA samples obtained from FIA families recruited in the south of England. To assess the association and linkage of IA formation to these candidate genes, an intragenic or proximal microsatellite was genotyped for each gene.

5.2: METHODS:

5.2.1: STUDY RECRUITMENT:

Recruitment of Scottish individuals to this pilot study took place during 1998 to 1999 following the strategy described in Section 2.2.1. It was conducted by a single research nurse from the case files of one neurosurgeon, Mr K.W. Lindsay, who has a special interest in FIA. The original study design specified recruitment of affected sibling pairs, a design since used by others in recently published FIA genome-wide scans. However, in the absence of suitable families or due to unwillingness on the part of individuals to participate, focus turned to recruiting with the following priority: 1) any affected family members; 2) parents

of affected persons; 3) any other family members. Affected status was based on history of aneurysmal SAH or angiographic screening for IA by Mr Lindsay's team. Data on IA and SAH risk factors such as age, sex, hypertension and smoking history were recorded. Eighty individuals from 21 families were recruited, including 28 affected by IA.

DNA from Southampton FIA families was the generous gift of Prof. I. N. M. Day at the University of Southampton. DNA was derived from 12 extended pedigrees with history of FIA and/or SAH. A total of 143 individuals was available of which 16 individuals were known to be affected.

5.2.2: CANDIDATE MARKER GENOTYPING:

For each candidate gene, a search of on-line databases and the literature was made to find an intragenic microsatellite marker, if known, or a marker as proximal to the gene as possible. From the Genome Database (GDB, http://gdbwww.gdb.org/), intragenic markers were available within intron 26 of COL3A1 and the 3' UTR of PKD1, and 7 Kb upstream from SERPINA1. Primer sequences for these were taken directly from GDB. A literature search revealed a microsatellite in intron 43 of FBN1 (Miller *et al*, 1996).

5.2.2.1: GENOTYPING OF SCOTTISH FIA SAMPLES:

Fluorescently labelled PCR of the four microsatellite markers was conducted as detailed in Section 2.4.1 using HotStar Taq (Qiagen Ltd, UK). Primer sequences and annealing temperatures are given in Appendix 3. Following PCR, markers

were pooled together and diluted to enable resolution on the ABI 377 semi-automated DNA analyser (Applied Biosystems, UK). PCR products were resolved on the ABI 377 as detailed in Section 2.4.2.1. Data were extracted and analysed with GeneScan v3.1. Genotypes were assigned and checked using Genotyper v2.1. Data were checked by two independent individuals who were blind to phenotype.

5.2.2.2: GENOTYPING OF SOUTHAMPTON FIA SAMPLES:

Only the marker for SERPINA1 was genotyped in the Southampton families. PCR for this marker was performed exactly as for Scottish samples. Resolution and genotyping was accomplished with an ABI 3730 DNA analyser as detailed in Section 2.4.2.2. Data were analysed and genotyped using GeneMapper v3.0. Parity of binning between Genotyper software and GeneMapper software was assured by the use of identical control DNA samples in all runs and genotyping of a subset of Southampton samples on an ABI 377. Genotypic data from 377 and 3730 platforms were compared in this subset using an Excel spreadsheet to ensure agreement.

5.2.3: STATISTICAL ANALYSES:

Genotypic data were held in an anonymised Access database to aid analysis. PEDCHECK (O'Connell & Weeks, 1998) was used to check for and resolve Mendelian errors within families.

Initial analyses of the Glasgow data are described in detail in Section 2.7.3 and were as follows: two-point linkage between each microsatellite marker and the trait was assessed using Affected Pedigree Member (APM) linkage analysis (Weeks & Lange, 1988). This tests for an excess of identity-by-state (IBS) allele sharing between affected relatives, conditional on the degree of relatedness and allele frequencies at the marker locus. Although the IBS method is not as powerful as IBD methods, the small numbers suitable for the APM analysis and lack of parents meant that IBD estimations would have likely been unreliable. This IBS method compromised power for pragmatism. Empirical P values were determined by simulation, over 1000 replicates, using SIM and HIST (Weeks & Lange, 1988).

Linkage was also assessed using the combined Transmission Disequilibrium Test (TDT) (Spielman *et al*, 1993) and sibling TDT (S-TDT) (Spielman & Ewens, 1998) in multiplex families. In this context, TDT/S-TDT is valid as a test for linkage only. Transmission of alleles from heterozygous parents to affected offspring is assessed by TDT. In families having only one parent, the S-TDT uses the unaffected offspring to provide an untransmitted genotype to replace the missing parent. Analyses were performed twice, specifying those with unknown affection status as unknown and then as unaffected.

Linkage in the presence of linkage disequilibrium was tested by use of TDT and S-TDT in simplex families. Two analyses specifying unknown and unaffected status were performed, as for the multiplex family analyses.

The Family-Based Association Test (FBAT) (Horvath *et al*, 2001) tests the null hypothesis of no linkage and no association It allows the analysis of complex pedigree data by decomposing complex structures into individual nuclear families. This was used to test for linkage and association within the complete pedigrees, allowing inclusion of all genotyped individuals. The null hypothesis of no association can also be tested using the empirical variance option within FBAT (EV-FBAT). Markers were therefore analysed for association to the trait with this method.

Finally, a case-control design was used to test for association between markers and trait. Eighteen affected, unrelated individuals were ethnicity, age and sex-matched to 18 controls. Obviously, these are very small groups for the study of a complex trait. Realistically, the power of such small samples is negligible, particularly in the face of probably heterogeneity. It is unlikely that a single gene disorder could be detected, particularly if allelic heterogeneity were present. Such heterogeneity has been reported for Mendelian disorders caused by some of the genes selected. To use the available patients as efficiently as possible, controls were carefully selected from families originating from the same locality as the FIA families, having no known history of cerebrovascular catastrophes, nor connective tissue disorders. The existence of Hardy-Weinberg equilibrium (HWE) was confirmed in controls using Fisher's exact test with 1000 simulations as implemented in GDA v1.0 (Lewis, P. O. and Zaykin, D. 2001; http://lewis.eeb.uconn.edu/lewishome/software.html). Genotype frequencies in case and control groups were compared by an exact χ^2 test using STATXACT-TURBO v2.1 (Cytel Software Corporation, USA).

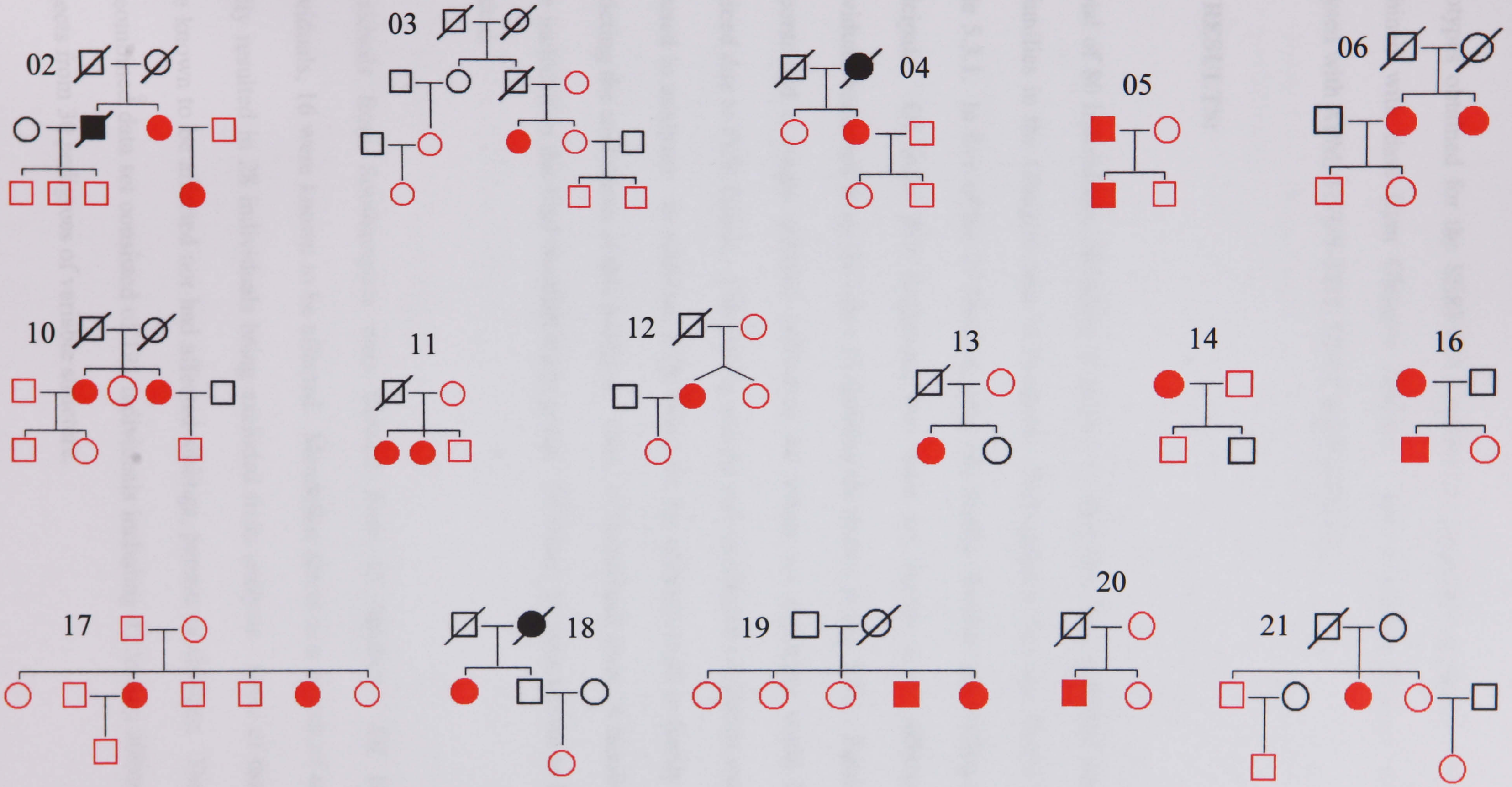


Fig. 5.3.1: Family structures and relationships of Scottish participants in the FIA pilot study. Study participants are red. Shaded individuals were affected. Struck through individuals were deceased. Circles denote females; squares denote males.

Genotypes obtained for the SERPINA1 marker in Southampton families were combined with data from Glasgow families. This combined data set was analysed with APM, TDT/S-TDT, FBAT and EV-FBAT.

5.3: RESULTS:

A total of 80 individuals, including 28 known to have IAs, were recruited from 21 families in the Glasgow area of Scotland. Demographic data are shown in Table 5.3.1. In five of the 21 families, only one family member was willing to participate. Of these five singletons, two were not known to be affected. Individuals recruited from the other 16 families are shown in Fig. 5.3.1. Family 18 contained a single affected individual for whom no genotypes could be obtained due to PCR failure. This family and the two unaffected singletons were not used in analyses. In addition, PCR failed for the affected child in family 5 restricting the usefulness of this pedigree. Thus, 76 individuals from 18 families were included in the final Scottish study group. Of these, 26 were known to be affected.

Individuals from Southampton were derived from 12 families. Of 143 individuals, 16 were known to be affected. Mendelian errors in a branch of one family resulted in 28 individuals being excluded from analysis. None of these were known to be affected nor had affected siblings, parents or children. Thus, the combined data set consisted of 191 individuals including 42 known affected subjects from 30 pedigrees of variable structure.

Table 5.3.1: Demographic and phenotypic data for Scottish FIA study participants.

	Affection status	
	Affected	**Unknown**
N	28	52
Screened for IAs (N)	28	5
% multiple aneurysms	39	-
% SAH	60.7	-
% sex (m:f)	21 : 79	46 : 54
Mean age ± SE / yrs	51.1± 2.0	42.4 ± 2.3
Mean SBP ± SE / mmHg	136.8 ± 4.3	131.0 ± 2.5
Mean DBP ± SE / mmHg	84.5 ± 2.4	79.4 ± 1.7
% on HTN treatment	35.7	11.5
% history of smoking	68	52

Details are shown for patients with a positive affection status for IA and/or SAH versus individuals not known to be affected.

Genotypes for four markers were obtained in Scottish individuals. Locations of these markers relative to candidate genes are shown in Figure 5.3.2. Names, published and observed heterozygosities are shown in Table 5.3.2. Each candidate marker was assessed using linkage and association methods. Results of these analyses are presented in Table 5.3.3.

Linkage of each candidate marker to presence of intracranial aneurysms in the Scottish cohort was assessed by both APM linkage analysis and TDT/S-TDT multiplex-family analysis. No marker was significantly linked to intracranial aneurysm status as assessed by APM. This was the case for the TDT/S-TDT analyses also, whether unknowns were treated as 'unknown' (Analysis I) or 'unaffected' (Analysis II). The lowest P value obtained for each multi-allelic marker is presented. In such small numbers of individuals, trends of significance may be of relevance. Given this, it is noted that the COL3A1 VNTR had the lowest P value in all three linkage analyses. However, it was not affected by the status of unknowns/unaffecteds in the TDT/S-TDT analyses.

P values obtained for TDT/S-TDT in simplex families are also presented. No significant linkage in the presence of association was seen in these analyses but, again, COL3A1 produced the lowest P value. In analyses of both multiplex and simplex families, the specification of status of unknowns had the greatest impact on the P value of the SERPINA1 locus.

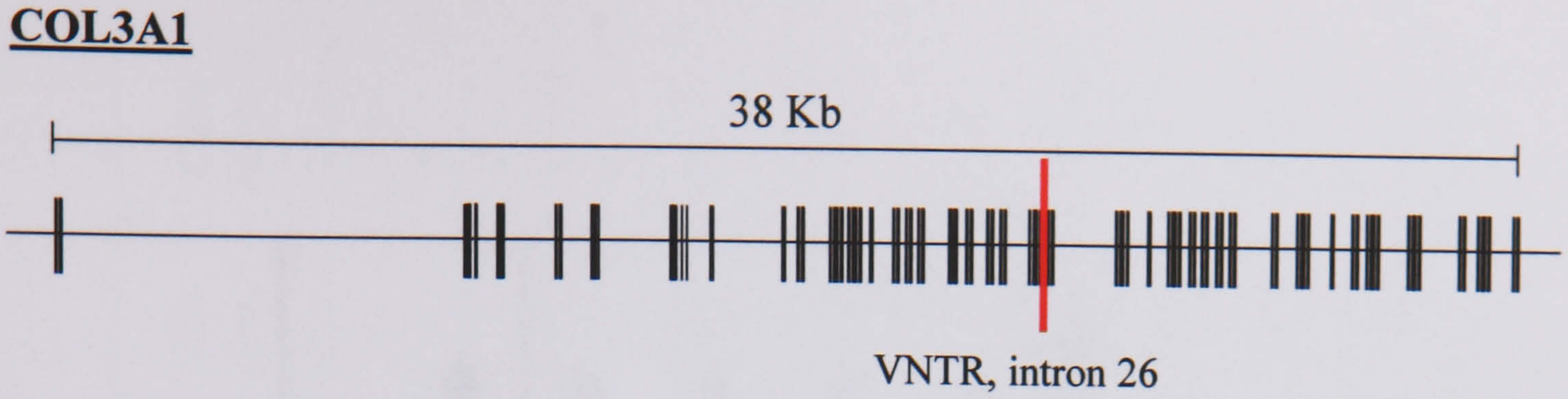


Fig. 5.3.2: A schematic representation of marker positions relative to genes. An intragenic or proximal microsatellite or 'variable number of tandem repeats' (VNTR) marker was genotyped for each candidate gene. Positions of markers relative to gene coding regions are represented schematically. Size of regions spanned by genes are presented.

Table 5.3.2: Names and heterozygosities of candidate markers

Gene	Marker name	Marker heterozygosity	
		Published	Observed ± SD
COL3A1	COL3A1 VNTR	0.70	0.71 ± 0.078
FBN1	MTS-4	0.75	0.59 ± 0.084
PKD1	GDB: 378419	0.56	0.47 ± 0.089
SERPINA1	D14s1142	0.70	0.85 ± 0.060

Published heterozygosities were sourced from the Genome Database or the literature as described in Methods.

Table 5.3.3: Results of linkage and association analyses in selected Scottish and selected combined FIA families.

	APM		TDT/S-TDT (multiplex families)				TDT/S-TDT (simplex)				FBAT	EV-FBAT	Case-control
Locus	P	P_{sim}	z' (I)	P (I)	z' (II)	P (II)	z' (I)	P (I)	z' (II)	P (II)	P	P	P
COL3A1	0.413	0.369	1.500	0.134	1.500	0.134	1.500	0.134	1.500	0.134	0.599	0.692	0.363
FBN1	0.934	0.949	-0.707	0.480	-0.707	0.480	-0.707	0.480	-0.707	0.480	0.948	0.429	0.179
PKD1	0.741	0.757	-0.707	0.480	1.155	0.248	1.155	0.248	1.225	0.220	0.800	0.204	0.217
SERPINA1	0.861	0.873	-0.707	0.480	1.220	0.222	-0.707	0.480	0.894	0.371	0.178	0.062	**0.028**
Combined SERPINA1	0.831	0.834	1.512	0.130	2.250	**0.024**	1.512	0.13	1.768	0.077	0.054	0.071	ND

APM = Affected Pedigree Member linkage analysis; TDT/S-TDT = combined Transmission Disequilibrium Test and Sibling Transmission Disequilibrium Test; FBAT = Family-Based Association Test; EV-FBAT = Empirical variance FBAT; P_{sim}, = empirical P value by simulation; z' = combined TDT/S-TDT z statistic after continuity correction; (I), analyses where 'unknown' status used; (II), analyses where 'unaffected' status used; ND, Not Done. Combined SERPINA1 indicates the analyses in the Glasgow + Southampton dataset.

Results from multi-allelic FBAT, multi-allelic EV-FBAT and χ^2 analyses showed a different trend. In all of these analyses, SERPINA1 produced the lowest P values. The case-control χ^2 test for SERPINA1 gave the strongest indication of association (P=0.028). This was not significant if corrected for multiple testing.

Tests of association are more powerful than linkage analyses, requiring fewer individuals to detect the same magnitude of genotype risk (Risch & Merikangas, 1996). Thus, they may be more useful in studies containing small numbers of individuals as in this case. In this study, use of FBAT also allowed inclusion of a greater number of participants in analysis than IBD linkage or TDT approaches. The χ^2 analysis has, in its favour, the correct specification of affection status of both cases and controls, with little or no ambiguity. Total number of people included in the χ^2 analysis also exceeded that of the APM or TDT/S-TDT analyses. Since the SERPINA1 marker gave the greatest suggestion of significance in these analyses, it was further genotyped in the Southampton family samples and the data combined with the Glasgow genotypes to assess the effect of increased numbers on significance.

Results for analyses of the combined data set are presented in Table 5.3.3. Since matched controls were not available for the Southampton samples, a χ^2 analysis could not be done. Poorly-matched controls may result in type I error in case-control studies due to population stratification (Rabinowitz & Laird, 2000). This is particularly important given the small numbers of individuals studied here. All other analyses were performed using the combined data. A P value of 0.024 was obtained for the 157 bp allele from TDT/S-TDT analysis in multiplex families

when unknowns were set as unaffected. TDT/S-TDT in simplex families resulted in P = 0.077 for the 157 bp allele, again with unaffected status specified. P values bordering on 0.05 were obtained for the multi-allelic FBAT and EV-FBAT analyses (Table 5.3.3). Bi-allelic FBAT analysis was also run on the combined dataset. This gave P = 0.02 from 10 informative families for allele 157. This reflects findings of the TDT tests.

5.4: DISCUSSION:

This study provides case-control study evidence to suggest that association of a marker proximal to the SERPINA1 gene and intracranial aneurysm status may exist in Scottish individuals from FIA families. In a larger cohort consisting of families from Scotland and the south of England, combined TDT and S-TDT analyses suggested that the SERPINA1 marker may be both linked to and associated with intracranial aneurysm status. A case-control design was not possible in this larger data set. However, P values from other family-based analyses support this trend. Linkage and association analyses of markers within the COL3A1, FBN1 and PKD1 genes did not provide any suggestion that these markers are linked to or associated with intracranial aneurysm status.

Clearly, it is not possible to draw firm conclusions from this pilot study including, as it does, small numbers of individuals from families with highly variable structures. P values obtained are not significant if corrected for multiple testing. Any suggestions of linkage or association require investigation in a much larger study. However, it is notable that a number of different analytical

methods, all using the data in different ways, display the same trend towards significance. It is interesting to note that most P values decreased on adding the additional Southampton families. The largest effect was seen for TDT/S-TDT tests when unknowns were specified as unaffected. It is almost inevitable that some of those unknowns will either be harbouring IAs now or will develop them later. This therefore introduces misclassification and must weaken the power of the S-TDT tests. However, the bias in this case would be towards type II error since some affected individuals would be misclassified as unaffected. It would be expected that this should decrease significance as a result. The fact that significance increases is therefore interesting.

No other studies specifically examining linkage or association of the SERPINA1 locus to aneurysmal disease in families have been published. Two recently published genome-wide scans of FIA patients both report linkage on chromosome 14q. Onda *et al* (Onda *et al*, 2001) showed linkage to marker D14s74, 20 cM centromeric of the SERPINA1 locus in Japanese affected sib pairs. Olson *et al* (Olson *et al*, 2002) demonstrated linkage in Finnish affected sib pairs at D14s1426, 12 cM telomeric of SERPINA1. Prevalence of deficiency phenotypes has been reported to be increased in series of IA patients (Schievink *et al*, 1996). However, a causative role has not been confirmed.

Since SERPINA1 expresses protease inhibitor (PI), the most important inhibitor of elastase, the prevailing hypothesis is that a reduction in PI may result in an offset of the balance governing vessel wall turnover. Damaged or absent elastic skeleton is a common feature observed by histological studies of experimental

aneurysms (Yamazoe *et al*, 1990; Kondo *et al*, 1997). Generalised elastic fibre abnormalities have been described in skin biopsies from IA patients, raising the possibility that not only increased degradative proteins but increased susceptibility to their action may exist in some IA patients (Grond-Ginsbach *et al*, 2002). However, the possibility also exists that *increased* levels of SERPINA1 may result in FIA formation. It has been shown that elastase also fulfils a protective role, cleaving other proteases such as plasmin (Moir *et al*, 2002). If elastase is inhibited, the net result will be increased degradation of glycoprotein elements of the extracellular matrix (ECM) by plasmin. This in turn leaves the fibrillar components of the ECM more vulnerable to matrix metalloproteinases (MMPs) (DeClerck & Laug, 1996). Plasmin also activates various members of the MMP family and is expressed at higher levels in aneurysmal tissue compared with normal cerebral arteries, although whether this is cause or effect is not clear (Bruno *et al*, 1998).

This study serves to highlight some of the obstacles which have seen genetic studies of FIA published so rarely. The inability to determine the affection status of anyone not known to be affected is clearly a major difficulty. Linkage analyses solely using affected individuals are one way to minimise misclassification. Such strategies have been used recently in the genome-wide scans mentioned above (Onda *et al*, 2001; Olson *et al*, 2002). However, linkage analyses are inherently less powerful than association strategies (Risch & Merikangas, 1996) and, of course, rely on the availability of affected individuals willing to participate. Herein lies another difficulty in a disorder which affects only 1% to 4% of the population (Rinkel *et al*, 1998) and may carry 50%

mortality and 30% morbidity from SAH (Schievink, 1997). This combined with the difficulty in identifying affected patients by screening (van Gijn & Rinkel, 2001) means recruiting affected individuals is not trivial. This is reflected in the relatively small number of individuals included in the recent GWS studies.

Association studies are more powerful but often rely on the presence of unaffected controls. The case-control paradigm largely avoids the problem of misclassification. Families with a risk for a disorder with consequences as catastrophic as SAH will be aware of this. Thus, controls can be picked with some confidence. However, the problems of admixture and population structure in case-control studies have resulted in their criticism (Tabor *et al*, 2002). Methods such as TDT (Spielman & Ewens, 1996) have been developed to negate these problems by using relatives to derive control genotypes. However, a reliance on the availability of parental data is a drawback in a late onset disorder. This has been partially addressed by S-TDT (Spielman & Ewens, 1998) but one parent is still required and both TDT tests can only use nuclear family data. This presumes that suitable family structures can be recruited and, further, that parents are heterozygous and so informative. This was not the case with the present study in which TDT or S-TDT trios were unusual. FBAT and the general class of family-based association tests allow the use of complex pedigrees. However, they again also use data from unaffected individuals. It is, then, a problem of circular logic: identifying patients is difficult and to recruit and correctly phenotype sufficient for robust genetic studies requires an effective screening protocol; but to develop screening we must have a better understanding of aetiology, which requires genetic studies. Prospective recruitment may solve

some difficulties, but requires long-term follow-up of participants to establish affection status.

This pilot study has provided preliminary evidence that association and linkage of IA status and a marker proximal to SERPINA1 may exist in UK families. Study of this locus in a much larger resource is necessary to determine if SERPINA1 is involved in FIA aetiology. However, as this pilot study has also demonstrated, such a large resource will require a collaborative, multi-centre recruitment program due to the paucity of suitable families at the single centre level and the resulting lack of power. There is also potential for classical linkage studies in extended pedigrees having simple modes of inheritance of IA and/or SAH. However, the high mortality makes it unlikely that such families will be easily obtained. Recruitment for this pilot study is, at present, complete. Considerable funding would be required to expand recruitment to other centres, and to allow a realistic chance of achieving sufficient power given that multiple families must be approached to find one that is suitable.

CHAPTER 6

GENERAL DISCUSSION

In 1990 the World Health Organisation (WHO) stated that coronary heart disease (CHD) and cerebrovascular disease would rank first and fourth, respectively, as causes of global disease by the year 2020 (Murray & Lopez, 1990). Despite significant technological advances in the intervening 14 years, complex disease genetics still represent a considerable challenge.

Essential hypertension and familial intracranial aneurysms are complex disorders sharing common features such as genetic heterogeneity, gene-gene and gene-environment interactions, small individual gene effects, pleiotropy and low penetrance. These features reduce the statistical power of genetic studies of all complex disorders (Glazier *et al*, 2002). Hypertension and intracranial aneurysms also differ in fundamental respects including the quantitative nature of blood pressure versus the dichotomous IA trait; the relative ease of phenotyping for hypertension versus the difficulties of identifying occult, dynamic aneurysmal lesions; and the prevalence in the general population. Genetic study of intracranial aneurysms is at least ten years behind that of essential hypertension and is made more difficult by the lack of confirmed Mendelian disorders, animal models and measurable phenotypes.

6.1: ESSENTIAL HYPERTENSION:

Essential hypertension is a classic example of a complex oligogenic or polygenic disorder, with an estimated 30% to 60% of inter-individual variation due to genetic factors (Ward, 1990). There is wide scope for interaction of multiple physiological systems and environmental factors (Soubrier, 1998). In the realm

of genetics, greatest success has been had in the dissection of Mendelian traits with the application of classical linkage approaches (Lifton *et al*, 2001). However, the application of these strategies in essential hypertension has met with far less success, and has necessitated the evolution and adoption of other complementary approaches (Lander & Schork, 1994).

We examined the distal region of human chromosome 5q by a combination of complementary strategies. The selection of this specific chromosomal segment was based on the presence of good cardiovascular candidate genes and genetic evidence including previous work in humans (Krushkal *et al*, 1998; Krushkal *et al*, 1999), rat models (Jacob *et al*, 1991) and mice (Wright *et al*, 1999). Beginning with a linkage study, we detected a 7 cM blood pressure/ hypertension QTL in a Polish hypertensive family resource (Tomaszewski *et al*, 2002), and confirmed the blood pressure linkage in a normotensive Scottish population sample. The use of independent study groups to confirm linkage has been used before (Zhu *et al*, 2001), but relatively rarely in hypertension linkage studies. Such independent confirmation goes some way toward negating the concerns regarding significance of linkage. The focus on a small chromosomal region also reduces marker number and type I error (Lander & Kruglyak, 1995). The use of both dichotomous trait and quantitative trait analyses again is not unique (Hunt *et al*, 2002), but is not often employed. However, such dual analysis is believed to be more informative (Williams *et al*, 1999).

Evidence of linkage in the 5q31.1-q33 region was followed by candidate gene studies. ADRB2 was selected, again based on the existence of previous evidence

(Bray *et al*, 2000; Bengtsson *et al*, 2001), the plausible physiological role of the β_2-adrenergic receptor in hypertension (Bray & Boerwinkle, 2000) and its positional candidacy within our linkage region. This selection policy was driven by concerns that have been voiced regarding candidate gene selection and, specifically, the lack of reproducibility in candidate gene studies (Tabor *et al*, 2002).

Selection of ADRB2 polymorphisms for study was based on desire to test loci known to affect receptor function. We aimed to directly assess the role of these in hypertension as has been recommended (Risch, 2000). The consideration of haplotypes therefore allowed us to examine the interaction of these functional effects and provided a more meaningful analysis (Sharma & Jeunemaitre, 2000). Further to this, we employed family-based approaches to test for association of gene and phenotype (Tomaszewski *et al*, 2002). This allowed us to side-step some of the pitfalls which are believed to affect the more popular, and genotypically efficient, case-control paradigm (Spielman & Ewens, 1996). By also recruiting carefully selected families with a confirmed history of essential hypertension, we maximised our power to detect association (Whittaker & Lewis, 1998). Exclusion of the ADRB2 locus in our Silesian population does not, of course, imply any exclusion in black or Asian populations. However, taken in context with other studies in white Europeans (Jia *et al*, 2000; Bengtsson *et al*, 2001; Herrmann *et al*, 2002), it does demonstrate that this locus does not have a detectable effect on hypertension risk in such populations (Tomaszewski *et al*, 2002).

Our subsequent selection and analysis of FGF1 was based upon the same criteria of physiological and positional candidacy as ADRB2. Owing to a lack of coding region variants, selection of polymorphic loci for genotyping was necessarily less directed. However, in the absence of coding polymorphisms, those in the untranslated regions have been suggested to assume precedence (Tabor *et al*, 2002). Our demonstration of association of a polymorphism in the 3' UTR of FGF1 represents the first known data suggesting that this locus might have relevance in human hypertension. Further work will concentrate on mapping the association more finely to localise the functional variant. In the absence of coding polymorphisms, 3' UTR and promoter regions are obvious targets. Intronic polymorphisms may also be relevant given their potential for affecting splicing and RNA stability (Cartegni *et al*, 2002). Intronic polymorphisms are known within FGF1 and these must also remain under consideration.

Genetic studies alone will not yield a definitive answer on the importance of FGF1 or any other gene in essential hypertension. For example, the possibility exists that several associated polymorphic loci may be statistically equivalent. Examples of this already exist in a study of Crohn's disease (Rioux *et al*, 2001). Dense mapping with SNPs across a region of 5q31 resulted in strong association of a 250 kb haplotype with Crohn's disease. However, eleven of 301 polymorphic loci were unique to the risk haplotype and their relative importance could not be dissected by genetic methods. Recently, working criteria have been proposed for gene discovery in complex traits, taking into account the need for functional studies (Glazier *et al*, 2002). The need for such rigour has been demonstrated by studies such as that implicating phosphodiesterase 4D (PDE4D)

in ischaemic stroke (Gretarsdottir *et al*, 2003). Detailed genetic and gene expression studies have suggested PDE4D as a putative candidate. However, with no association to either of two coding polymorphisms found, *in vitro* functional testing of regulatory regions has yet to be done to support the association data. This is essential if the role of PDE4D is to be confirmed (Dominiczak & McBride, 2003). Similarly, successful haplotype tagging of the FGF1 gene would need to be followed by expression and, most importantly, functional studies to confirm its role. The lack of coding polymorphisms, which we have already demonstrated, suggests that we too would need to examine regulatory regions.

Several pathways could mediate a contribution by FGF1 to development of hypertension. These could involve the endothelium and nitric oxide, oxidative stress, the sympathetic nervous system or the hypothalamic-pituitary adrenal axis, based on observations in animal models. Thus, there are many potential gene-gene interactions. There is an increasing acknowledgement that interactions between genes may need to be incorporated into analysis before complex disease genes can be detected (Cordell, 2002). Statistical methods are under development to model QTL interactions in genome-wide linkage (Cordell *et al*, 2000; Cordell, 2002) and linkage disequilibrium analysis (Ott & Hoh, 2001). Such approaches have been explored, for example, in type II diabetes (Cox *et al*, 1999) and asthma (Zandi *et al*, 2001). At the level of candidate genes, analysis of interactions has suggested that gene-gene synergy is detectable. Staessen *et al* (Staessen *et al*, 2001) studied hypertension gene-gene interactions by genotyping polymorphisms in the α-adducin (ADD1,

Gly460Trp), angiotensin I converting enzyme (ACE, I/D) and aldosterone synthase (AS, -344C/T) genes. Prospective analysis of 678 initially normotensive Caucasian individuals, who were followed up for a median 9.1 years, showed that the ACE/DD genotype was associated with 31% increased incidence of hypertension. Incidence increased by 59% if in combination with the ADD1/Trp allele, and 122% in AS/CC subjects. Among subjects having ADD/Trp, AS/CC and ACE/DD there was 252% higher incidence of hypertension. AS/CC genotype was associated with decreased aldosterone secretion in the same population. It was suggested that this could result in over-compensation, by the renin-angiotensin system, in patients already more prone to sodium retention due to ACE and ADD1 variants (Staessen *et al*, 2001). The synergistic effect between the ACE/DD genotype and ADD/Trp allele had been shown previously (Barlassina *et al*, 2000). Hypertensive individuals having both DD and Trp were most responsive to sodium loading and both alleles were associated with reduced plasma renin activity.

Interaction of the M235T locus in angiotensinogen (AGT) and I/D in ACE has been examined in a Caucasian case-control study (Vasku *et al*, 1998). Of interest is the fact that neither AGT nor ACE, alone, was associated with hypertension. However, in 202 normotensives compared with 163 hypertensives, consideration of double homozygotes showed an increased frequency of DD/MM subjects in the hypertensive group (Vasku *et al*, 1998). The implication of the M235 allele is contrary to meta-analyses of AGT (Staessen *et al*, 1999). This suggests that data from gene-gene interaction studies may be as difficult to interpret as single gene candidate studies. However, many lessons have already been learnt with

regard to this. For example, the potential effects of population stratification are now appreciated although a recent review suggests that there are, in fact, few clear examples where population structure has compromised case-control studies; lack of power is perhaps a far greater problem (Cardon & Palmer, 2003). Power will be as important an issue with gene-gene interaction studies as single gene studies.

Available strategies in hypertension research have, to date, failed to consistently identify susceptibility genes for hypertension. Candidate gene studies have been confusing, whilst linkage studies have not lived up to expectations. In the context of the popular genome-wide linkage paradigm, the recent BRIGHT Study genome-wide scan represents the cutting-edge of study design (Caulfield *et al*, 2003). Despite this, it has not eased the concerns of many that GWS studies have failed to provide a consensus on hypertension QTL (Harrap, 2003). However, the recent description of haplotype blocks and the potential for linkage disequilibrium mapping of complex disease genes is now offering new hope for gene mapping in complex disorders such as essential hypertension (Kruglyak, 1999). Already, some mapping success using LD approaches has been reported, for example, with Crohn's disease (Rioux *et al*, 2001), asthma (Allen *et al*, 2003) and ischaemic stroke (Gretarsdottir *et al*, 2003). The approach has yet to be extended to a genome-wide study, and already debate rages as to the number and nature of SNPs to use (Daly *et al*, 2001; Maniatis *et al*, 2002; Dawson *et al*, 2002). However, characterisation of LD across the genome is underway and may provide some answers (Gibbs *et al*, 2003). In addition, there is unprecedented availability of data following immense advances in sequencing, genotyping

(Syvanen, 2001), and microarray technology (Lander, 1999). The availability of the human (Lander *et al*, 2001), mouse (Waterston *et al*, 2002) and ongoing rat genome sequences (http://www.hgsc.bcm.tmc.edu /projects/rat/) is an invaluable asset. In combination, novel strategies, wealth of data and application of technology provide the best opportunity for future dissection of the genetic basis of essential hypertension.

6.2: FAMILIAL INTRACRANIAL ANEURYSM:

The work on candidate genes of familial intracranial aneurysms considers a disorder which has yet to see the sort of detailed investigation directed at hypertension. The aetiology of FIA formation, like hypertension, is complex, most likely involving genetic heterogeneity and certainly affected by environmental factors (Zhang *et al*, 2003). Therefore, the study of genetics of familial intracranial aneurysms is similarly challenging. However, our work here highlights a specific set of obstacles for this disorder. Primary among these is the difficulty experienced in trying to recruit sufficient numbers for study due to high mortality and morbidity. This is reflected in recent genome-wide scans of FIA (Onda *et al*, 2001; Olson *et al*, 2002) which are small in comparison with hypertension scans. To compound this problem, in the absence of biological markers and intermediate phenotypes, analysis can only consider a dichotomous affection status. The expected benefit of quantitative trait analyses is not an option (Duggirala *et al*, 1997). Finally, the occult nature of the lesions means that classification relies on complex screening, the efficiency of which is a matter of debate (Kirkpatrick & McConnell, 1999). In many instances, screening has

not been performed as there is an associated risk and apparently healthy individuals are understandably reticent.

Choice of the FIA candidate genes studied relied solely on plausible physiology, particularly derived from Mendelian disorders (Schievink, 1997). In the absence of genetic animal models (Dobrin, 1999) and, until recently, human genome-wide studies, comparative and positional mapping data were unavailable. With the small size of this study, mapping of large numbers of polymorphisms across the genes would have increased chances of false positives. In light of this, genotyping of a single microsatellite was a sensible option, and one that has been used in many studies before (Caulfield *et al*, 1994). Microsatellite markers may also be more informative than single SNPs owing to their multiallelic nature (Xiong & Jin, 1999).

The preliminary results, suggesting association of a marker near the SERPINA1 locus and intracranial aneurysm, suggest the value of further analysis of this locus. The most pressing requirement is for increased numbers of families to allow a more detailed investigation. Non-synonymous polymorphisms resulting in deficiency phenotypes are known (Crystal, 1989) and these would provide a starting point for haplotype analyses, were sufficient individuals available. It is also possible that the microsatellite tested may be functional. Located in the upstream region, it may affect promoter elements of the SERPINA1 gene. Others have demonstrated such functional effects of microsatellites. For example, a microsatellite in the promoter region of MMP9 was shown to be associated with intracranial aneurysm. Reporter gene assays also showed that it

affected expression, lying between two transcription factor binding sites and perhaps modifying their interaction (Peters *et al*, 1999).

SERPINA1 is of interest in terms of the potential interactions with other components of extracellular matrix remodelling. As has been discussed, recent genome-wide scans have been followed by candidate gene studies which implicated elastin and collagen type I α2 (Onda *et al*, 2001; Yoneyama *et al*, 2004). Some *in vitro* functional studies have been performed in the latter case. However, further work is required for both genes, including detailed functional studies and confirmation in other populations, if presently accepted standards are to be adhered to (Dominiczak & McBride, 2003). Regardless, SERPINA1 fits neatly into the pathology suggested by these genes. With its inhibition of elastase (Zhang *et al*, 2003) there is potential for interaction with variants of elastin. Elastase also protects some components of the extracellular matrix by degrading MMPs (Moir *et al*, 2002). Thus, plausible interactions may be hypothesised with unstable collagen variants or high activity MMPs, both of which have been implicated (Peters *et al*, 1999; Yoneyama *et al*, 2004) as mentioned above.

Studies of inheritance pattern of FIA have shown that some families exhibit an autosomal dominant mode. Others are recessive whilst still others are more complex (Bromberg *et al*, 1995). One conclusion from this may be that there are Mendelian IA disorders yet to be discovered which might shed light on the more complex forms of IA formation. These should be accessible to linkage studies in large pedigrees if enough survivors can be found, and families phenotyped

precisely. Description of Mendelian forms could prove enlightening with regard to potential aetiology of more complex forms. It would also offer further confirmation of the role of genes, as has been the case with the report of NOTCH3 mutations in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) in ischaemic stroke (Joutel *et al*, 1997; Dominiczak & McBride, 2003). There may also be IA subtypes which are highly complex and offer as great a challenge to researchers as essential hypertension, or some that are oligogenic and tractable to genome-wide linkage study. However, no animal models are available to give a perspective on this. The recent genome-wide scans may be the first of many as this approach is tested empirically, although the lack of consensus between them is a familiar story (Harrap, 2003). Alternatively, LD mapping methods, as used recently in ischaemic stroke (Gretarsdottir *et al*, 2003), may prove of worth in aneurysmal haemorrhagic stroke. At a genome-wide level, this will require a major undertaking to recruit enough individuals to provide power. Alternatively, QTL suggested by GWS linkage studies could provide more focused targets for LD mapping. However, a far clearer picture of pathophysiology, subtypes of IA and potential mechanisms is needed before full advantage can be taken of developments in genetic strategies.

APPENDICES

APPENDIX 1:

Suppliers of chemicals and reagents used:

Reagent	Supplier
Agarose-1000	Life Technologies
Ammonium persulphate	BDH
BigDye Terminator Sequencing kit	Applied Biosystems
Bromophenol blue	Sigma
BsrDI restriction enzyme	New England Biolabs
Chloroform	Fisher Scientific
Decon 75	Decon Laboratories Ltd.
Dextran-EDTA loading buffer	Applied Biosystems
dNTPs/deoxynucleoside triphosphates	Promega
EDTA	Fisher Scientific
Ethanol	Sigma
Ethidium bromide	Sigma
Formamide	Amresco
GENESCAN-500 Rox	Applied Biosystems
GENESCAN-500 Tamra	Applied Biosystems
Hi-Di formamide	Applied Biosystems
HotStarTaq DNA polymerase	Qiagen
ItaI restriction enzyme	Roche
Long Ranger 5% acrylamide solution	FMC
MasturePure DNA Purification kt	Epicentre Technologies

MgCl2	Fisher Scientific
MnlI restriction enzyme	New England Biolabs
Nucleofast-96 PCR purification plates	Macherey-Nagal
PCR buffer (10X)	Qiagen
Phenol	Fisher Scientific
Picogreen dsDNA Quantitation kit	Molecular Probes
Proteinase K	Sigma
SDS (sodium dodecyl suphate)	BioRad
Sodium Acetate	Sigma
Sodium chloride	Fisher Scientific
Sucrose	Fisher Scientific
TBE (10X)	National Diagnostics
TEMED	Amresco
Tris	Fisher Scientific
Triton X-100	Sigma
Ultra Pure agarose	Life Technologies

APPENDIX 2:

List of formulations for preparation of solutions.

ABI 377 sample loading cocktail for genotyping (for 96 samples):

106 µl formamide combined with 40 µl ROX or TAMRA standard, and 40 µl of Dextran-EDTA buffer (Applied Biosystems).

ABI 377 genotyping sample:

2 µl of ABI 377 loading cocktail and 1.5 µl of pooled PCR products.

ABI 377 sequencing loading cocktail (96 samples):

435 µl of formamide combined with 115 µl of Dextran-EDTA buffer.

ABI 377 sequencing sample:

Precipitated sequencing products plus 5 µl of ABI 377 sequencing loading cocktail.

ABI 3730 loading cocktail for genotyping (96 samples):

1000 µl of Hi-Di formamide and 1.2 µl of ROX or TAMRA standard.

ABI 3730 genotyping sample:

10 µl of 3730 loading cocktail and 2 µl pooled PCR products.

ABI 3730 sequencing sample:

Precipitated sequencing products resuspended in 15 µl Hi-Di formamide.

10% ammonium persulphate (10 ml):

1g APS in 10 ml sterile deionised water. Store at 4°C.

BigDye Terminator sequencing mastermixes:

For the ABI 377 (1:4 reaction, 20 µl):

2 µl of Ready Reaction Mix (Applied Biosystems), 3 µl 5X sequencing buffer (Applied Biosystems), 3.2 µl sequencing primer (1 µM), 2 µl template DNA, 9.8 µl autoclaved deionised water.

For the ABI 3730 (1:16 reaction, 20 µl):

0.5 µl of Ready Reaction Mix (Applied Biosystems), 3.75 µl 5X sequencing buffer (Applied Biosystems), 3.2 µl sequencing primer (1 µM), 2 µl template DNA, 10.55 µl autoclaved deionised water.

6X DNA loading buffer (50 ml):

25 ml glycerol, 25 ml deionised water, 0.05 g xylene cyanol FF, 0.05 g bromophenol blue.

dNTPs (40 ml, 1 mM):

400 µl each of dGTP, dATP, dCTP and dTTP (provided as 100 mM stocks) added to 38.4 ml autoclaved deionised water.

70% ethanol (100 ml):

70 ml of analar 100% ethanol combined with 30 ml of deionised water.

95% ethanol (100 ml):

95 ml of analar 100% ethanol combined with 5 ml of deionised water.

PCR master mix for Qiagen HotStarTaq (20 µl):

2 µl Qiagen 10X PCR buffer, 4 µl dNTPs (1 mM), 1 µl primers (20 µM), 5 µl genomic DNA template (5 ng/µl), 7.96 µl autoclaved deionised water, 0.04 µl Qiagen HotStarTaq (5 U/µl).

Primer stocks:

Diluted to 100 µM with appropriate volume of autoclaved deionised water.

PCR primer working solutions (20 µM, 100 µl):

10 µl of forward and 10 µl of reverse primer stocks with 80 µl of autoclaved deionised water.

10% SDS (500 ml):

500 ml 20% SDS with 500 ml autoclaved deionised water.

Sequencing primer working solutions (1 µM, 200 µl):

2 µl of primer working solution and 198 µl of autoclaved deionised water.

3M sodium acetate, pH 6.0 (1 l):

408.1 g sodium acetate dissolved in 1 l of sterile deionised water. pH adjusted with glacial acetic acid.

1% sodium chloride (1 l):

10 g sodium chloride dissolved in 1 l of autoclaved deionised water.

1X TBE buffer (1 l):

100 ml of 10X TBE combined with 900 ml deionised water.

1M Tris, pH 8.0 (1 l):

121.1 g Tris base dissolved in 1 l deionised water. pH adjusted with concentrated hydrochloric acid.

APPENDIX 3:

Primer and probe sequences:

1, forward; 2, reverse primer.

# indicates presence of a 5' GTT TCTT tail to aid microsatellite genotyping.

Primer sequence	Fluor Label	T_m (°C)	Mg^{2+} (mM)
Chr 5q linkage			
D5s494			
1-GCT TTC ACG AAG GTA GAT ATT GCT	FAM	53.9	1.5
2-#CCA GGC TAG GCA GAT TAC AGA T		54.8	1.5
D5s642			
1-AGC TCT TTA CTT CTG GAC TTA CAA A	HEX	52.7	1.5
2-#CTA GAC CAT AGA TAA CCC TGT GAT		53.9	1.5
D5s500			
1-ACC TAT TCG ACC TAA TGA CTA AAG A	FAM	52.7	1.5
2-#ATC GGT GAA ATG CAA CTA CTT		48.5	1.5
D5s1480			
1-TTG GGA AGA ATA GCT TTC CC	FAM	49.7	1.5
2-#TTC TAG CTT CCC CCT ATG CT		51.7	1.5
D5s636			
1-AAG GCA TAT GGG AAA TAT CTG T	HEX	49.2	1.5
2-#CCA CAC CAT TAT GAC ATT TTC T		49.2	1.5
D5s820			
1-ATT GCA TGG CAA CTC TTC TC	NED	49.7	1.5
2-#GTT CTT CAG GGA AAC AGA ACC		52.4	1.5
D5s2093			
1-TTG CAG TGA GTC AAG TTC G	FAM	48.9	1.5
2-#CTC GGC ATC ATA TAG AGG C		51.0	1.5
D5s1471			
1-TAT ACA TGT GAA ATT AGA AAG CAC C	FAM	51.1	1.5
2-#TGA GCT ATG TTT GTG GCA GA		49.7	1.5

D5s1456			
1-TAT CGA ATT GTA ACC CCG TT	HEX	47.6	1.5
2-#TCT GGA AAA CCC TAA TTC TCC		50.4	1.5
D5s462			
1-TTC ATT CAT CCA CTC AAA CA	NED	45.6	1.5
2-#AGC TAA TAC AGT CTT TCA AGC AG		51.7	1.5
D5s211			
1-ACT TTG AAA ACC ACT GGC CT	FAM	49.7	1.5
2-#ATG TAT CTA GCC ATG GTA GC		49.7	1.5

ADRB2 SNP
genotyping

Arg16Gly		
1-CTT CTT GCT GGC ACG CAA T	51.0	1.5
2-CCA GTG AAG TGA TGA AGT AGT TGG	55.6	1.5
Gln27Glu		
1-GGC CCA TGA CCA GAT CAG CA	55.8	1.5
2-GAA TGA GGC TTC CAG GCG TC	55.8	1.5
Thr164Ile		
1-GGA CTT TTG GCA ACT TCT GG	51.7	1.5
2-ACG AAG ACC ATG ATC ACC AG	51.7	1.5

FGF1
PCR and sequencing

FGF1 exon 1		
1-GGT CCA AAG CTG TGT CCA AT	51.7	1.5
2-ACC TTC CTC CCA CCT TGA CT	53.8	1.5
FGF1 exon 2		
1-CCG TGA AAT GAA TGA GCA GA	49.7	1.5
2-CTG GAA ACC TCA AAC CTT GG	51.7	1.5
FGF1 exon 3		
1-ACC ATG ACC CCA ATT TTC CT	49.7	1.5
2-ATT TTT GGG TCA ACC AGG TG	49.7	1.5
FGF1 3' UTR		
1-AAC AGA GGG ACC AAA TTG CTT	50.4	1.5
2-CTC AGC CAG TTT CCC TTT CTT	52.4	1.5

FGF1 SNP genotyping

SNP 2 primers			
1-GAT CTC CAA TTG CCT CTT GCA		52.4	1.5
2-GAC CCC TTA ACA CAC TTC ATT TAG C		56.0	1.5
SNP 2 probes			
1-CCC TCC ATG CAA AA	VIC	37.3	
2-CCC TCA ATG CAA AAG	FAM	39.2	
SNP 3 primers			
1-GAT CTC CAA TTG CCT CTT GCA		52.4	1.5
2-CTC AGT AGA GGG AAA TAG TGT GCA GTT		58.2	1.5
SNP 3 probes			
1-CAC TTA GCT GAC CCC	VIC	44.6	
ACT TAG CCG ACC CC	FAM	43.2	
SNP 4 primers			
1-GAA GTG GTT TCC TGA TAA CAA GCA A		54.4	1.5
2-TCT CTG CAC TCC CAC ACT CAG T		56.7	1.5
SNP 4 probes			
1-TTC ATG TGC ATC TGG	VIC	39.2	
2-CAT GTG GAT CTG GGG	FAM	44.6	

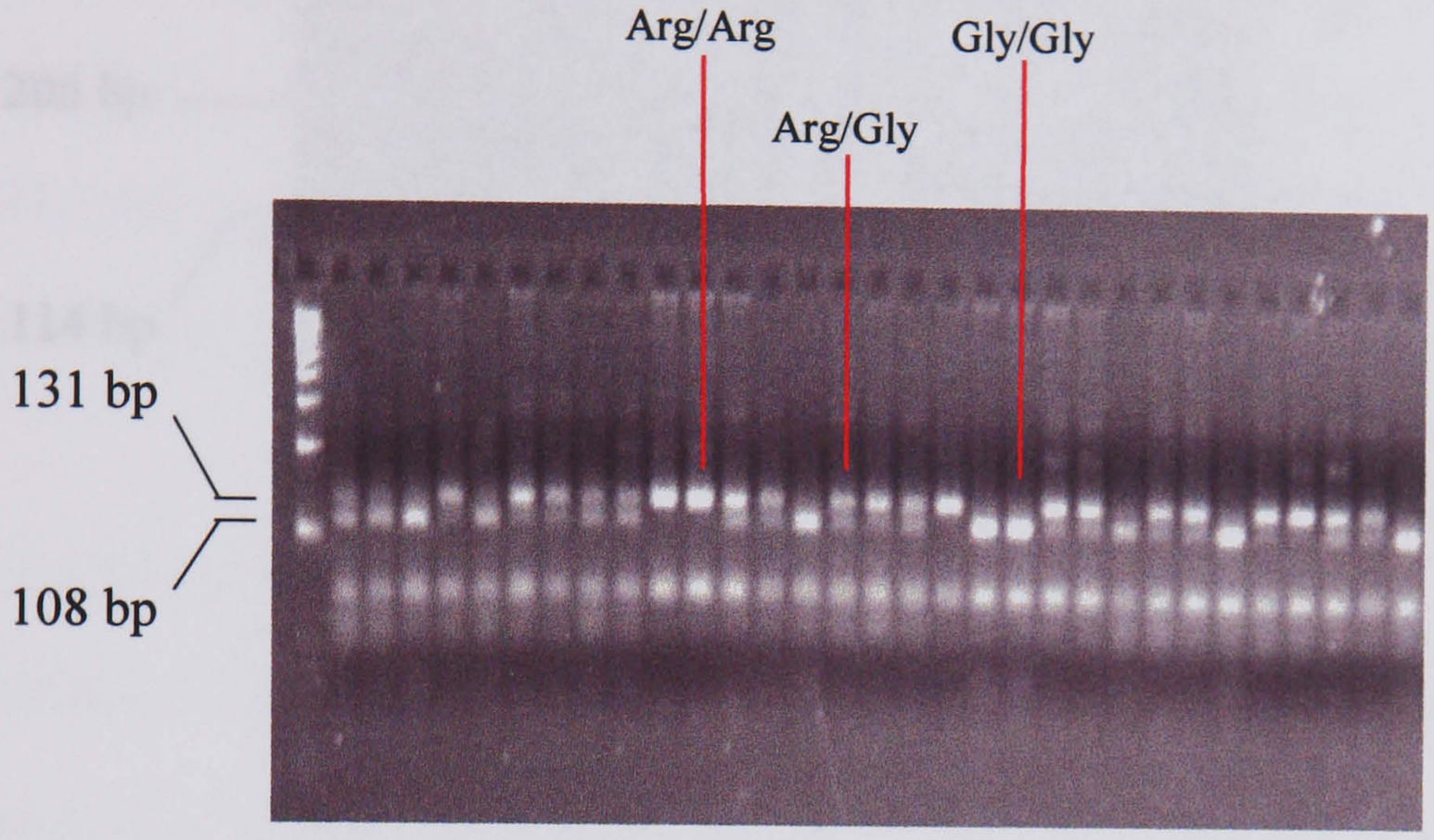
FIA genes

COL3A1 VNTR			
1-CCA TTC TTA CCA GGA GAC CCC TAA AG	TET	59.5	1.5
2-#GGG GGG CAG AGG CTA CAG TGA G		62.2	1.5
FBN1 MTS-4			
1-GAT GTC CCT ATT GCC ATC ACC AC	HEX	57.0	1.5
2-#CCT GTG CAG GGT AAG ACA AG		53.8	1.5
PKD1 BP1			
1-CAC AGC CAG CTC CGA GGG	TET	57.1	1.5
2-#TCC TCC TGG GGG CTG GCT C		59.7	1.5
SERPINA1 D14s1142			
1-TTG CAG GGA GTC AGG TGT ATG	FAM	54.3	1.5
2-#GCA TCA CAC AGA GAC ACG GAT		54.3	1.5

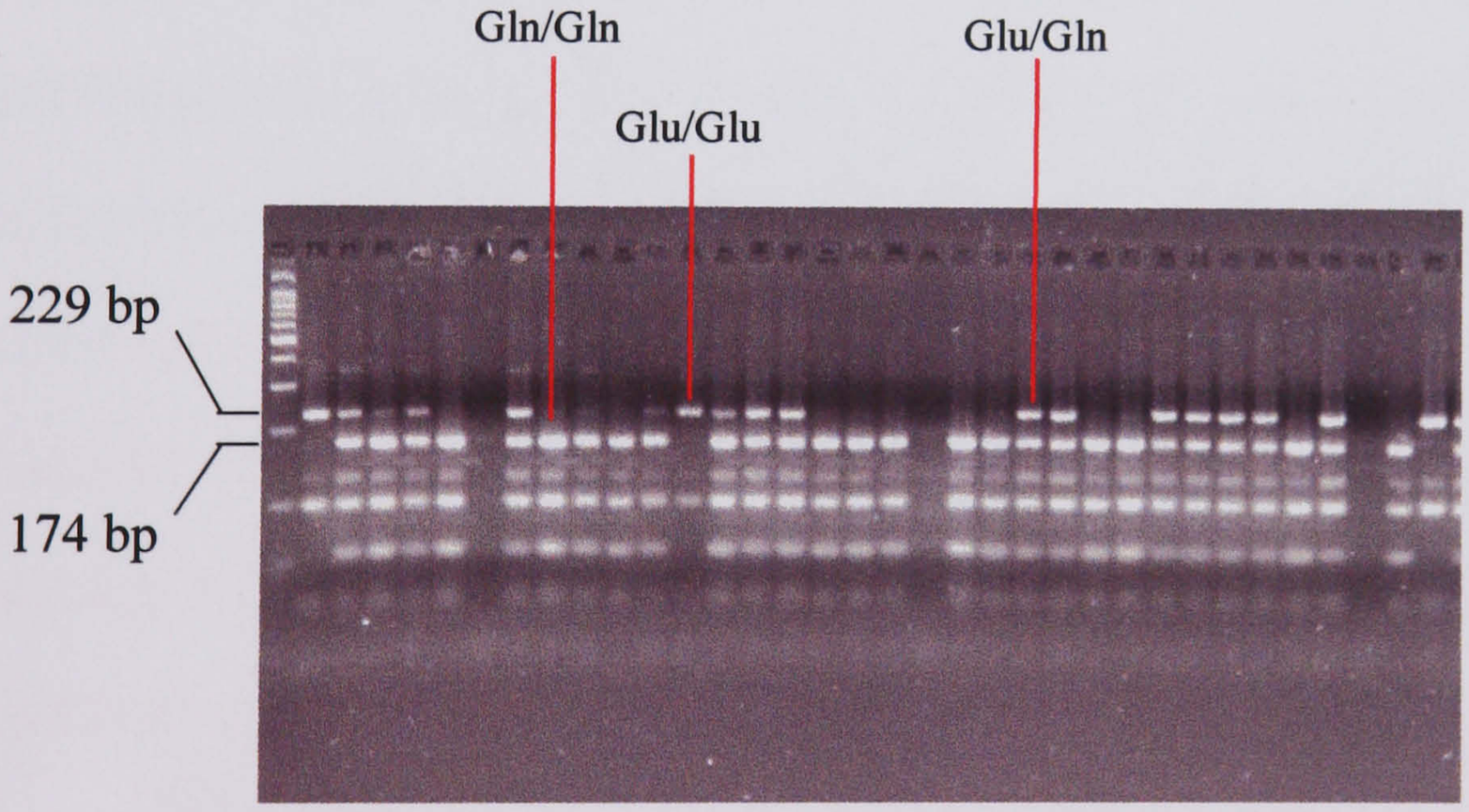
APPENDIX 4

Examples of resolved restriction digest products for ADRB2 SNP genotyping.

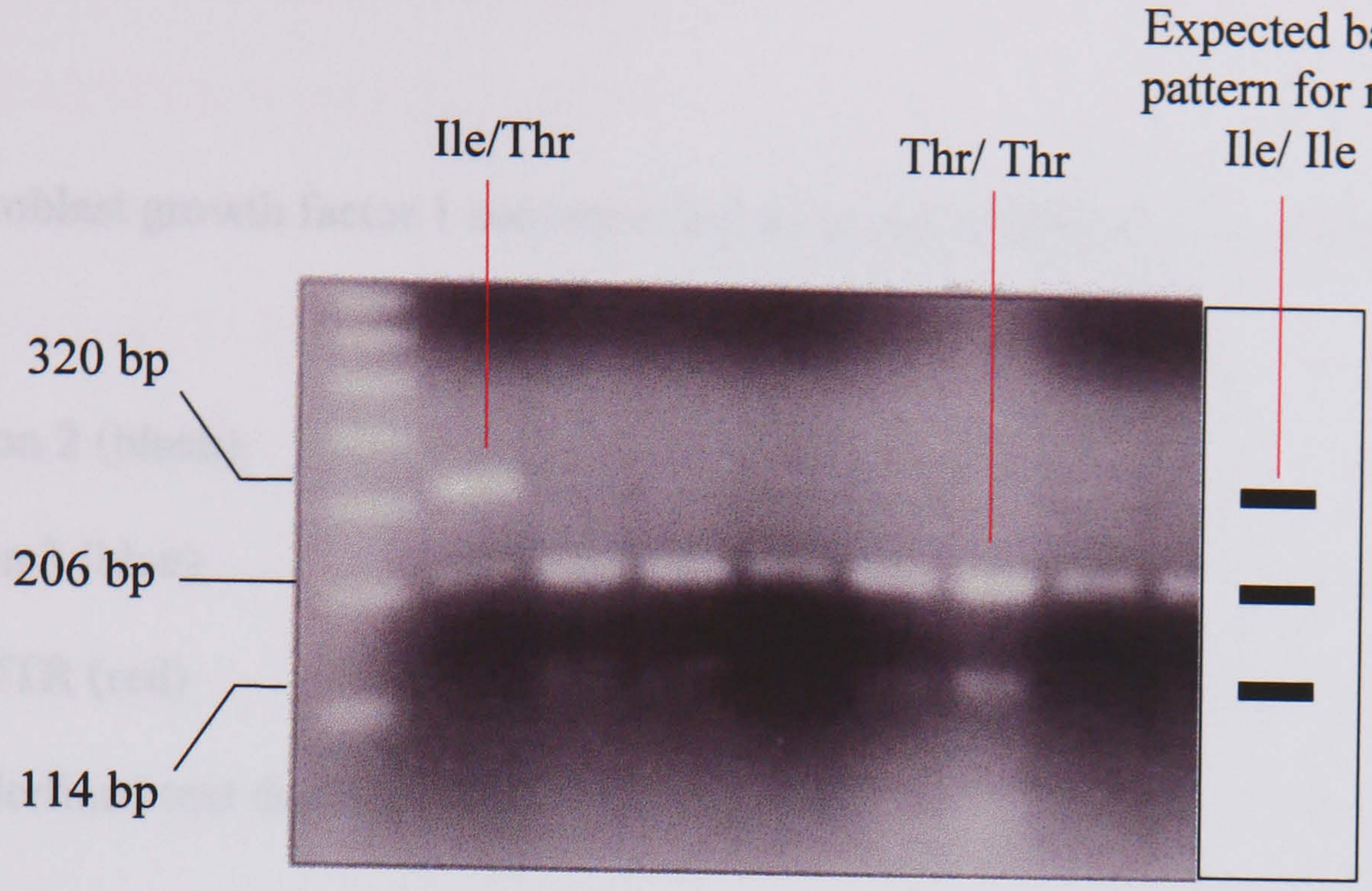
Arg16Gly



Gln27Glu



Thr164Ile


Ile/Thr
Thr/ Thr
Expected band pattern for rare Ile/ Ile
320 bp
206 bp
114 bp

APPENDIX 5

Fibroblast growth factor 1 sequence and positions of SNPs 1, 2, 3, and 4 found.

Intron 2 (black),

Exon 3 (blue)

3' UTR (red)

Underlined text denotes primer annealing sites.

.....CCATGACCCCAATTTTCCTGAAAAGGAGACTGAGGCATGGAGAGC

TTTAGTATTTTGCCCAATGTCACACAGCTAGTAAATGGGGACCCCCAT

GTGAAACTACTCACTGATTGTCCTACTCTCTTGTGGTTTTATCTTTTTA

GCAGACACCAAATGAGGAATGTTTGTTCCTGGAAAGGCTGGAGGAGA

ACCATTACAACACCTATATATCCAAGAAGCATGCAGAGAAGAATTGG

TTTGTTGGCCTCAAGAAGAATGGGAGCTGCAAACGCGGTCCTCGGAC

TCACTATGGCCAGAAAGCAATCTTGTTTCTCCCCCTGCCAGTCTCTTC

TGATTAAAGAGATCTGTTCTGGGTGTTGACCACTCCAGAGAAGTTTCG

AGGGGTCCTCACCTGGTTGACCCAAAAATGTTCCCTTGACCATTGGCT

GCGCTAACCCCCAGCCCACAGAGCCTGAATTTGTAAGCAACTTGCTTC

TAAATGCCCAGTTCACTTCTTTGCAGAGCCTTTTACCCCTGCACAGTT

TAGAACAGAGGGACCAAATTGCTTCTAGGAGTCAACTGGCTGGCCAG

TCTGGGTCTGGGTTTGGATCTCCAATTGCCTCTTGCAGGCTGAGTCCC

TCAATGCAAAAGTGGGGCTAAATGAAGTGTGTTAAGGGGTCAGCTAA

GTGGGACATTAGTAACTGCACACTATTTCCCTCTACTGAGTAAACCCT

ATCTGTGATTCCCCCAAACATCTGGCATGGCTCCCTTTTGTCCTTCCTG

TGCCCTGCAAATATTAGCAAAGAAGCTTCATGCCAGGTTAGGAAGGC

AGCATTCCATGACCAGAAACAGGGACAAAGAAATCCCCCCTTCAGAA

CAGAGGCATTTAAAATGGAAAAGAGAGATTGGATTTTGGTGGGTAAC

TTAGAAGGATGGCATCTCCATGTAGAATAAATGAAGAAAGGGAGGCC

CAGCCGCAGGAAGGCAGAATAAATCCTTGGGAGTCATTACCACGCCT

TGACCTTCCCAAGGTTACTCAGCAGCAGAGAGCCCTGGGTGACTTCA

GGTGGAGAGCACTAGAAGTGGTTTCCTGATAACAAGCAAGGATATCA

GAGCTGGGAAATTCATGTGCATCTGGGGACTGAGTGTGGGAGTGCAG

AGAAAGAAAGGGAAACTGGCTGAG…

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