# STUDIES OF THE G PROTEIN BETA 3 SUBUNIT IN HUMAN HYPERTENSION

*by*

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# SUMMARY

There have been many reported associations between hypertension-related phenotypes and DNA markers of candidate genes, but none of them have been consistently replicated across diverse populations. Part of the inconsistency may be due to the fact that most DNA markers studied so far do not have a functional impact on the structure or expression of the gene product. Thus, most reported genetic associations have been attributed to linkage disequilibrium with putative functional changes elsewhere at the genetic locus. One strategy to reduce such confounding in association studies may be to select DNA markers that are proven, by various assays, to directly mark a functional change in the gene of interest. In this context the GNB3 gene encoding the human G protein β3 subunit located on chromosome 12p13 has been proposed as a putative candidate gene for hypertension. The 825T allele was associated with the occurrence of a splice variant, which produced a 123-base pair deletion of nucleotides 498 to 620 of exon 9 resulting in the loss of 41 amino acids and 1 tryptophan-aspartic acid WD repeat domain of the Gβ3 subunit (Gβ3-s). It has been hypothesised that the C825T polymorphism of the G protein $\beta_3$ subunit (GNB3) which leads to the formation of a splice variant may be associated with increased signal transduction, and hypertension.

I have studied the GNB3 gene as a candidate gene for hypertension and left ventricular mass in a systematic and comprehensive manner. To be absolutely certain about association, the studies should be done in large population samples, independently replicated, and the association should be observed in both population based and family based studies. In addition it should be demonstrated that the alleles affect the gene product and function in a physiological meaningful way.

I have carried out association studies in three independent populations - A case-control study with well characterised hypertensives and their matched controls; a twin study looking at echocardiographic LV mass; and a large family based study analysing blood pressure and ECG LV mass. In addition I have also looked at three additional candidate genes including ACE, aldosterone synthase and beta-1 adrenoceptor. Though these studies concluded that there was no association between the C825T polymorphism and blood pressure or LV mass, I have demonstrated a significant heritability of various electrocardiographic measures of LV mass and an interaction between GNB3, ACE and SF1 explaining the variability of continuous measures of LV mass in this population.

I studied the functional aspects of the GNB3 polymorphism by measuring platelet aggregation in normal human volunteers, using it as a marker of G-protein related signal transduction. However, there was no association between epinephrine or platelet activating factor induced platelet aggregation and the GNB3 genotype.

Finally, I peformed molecular studies using cells transfected with $\beta_3$ (wild-type) and $\beta_{3\text{-}s}$ (splice-variant) of GNB3 along with a fusion construct of $\alpha_{2A}G_i$, and measuring of GTPase activity and calcium signalling. There was no significant difference between $\beta_3$ or $\beta_{3\text{-}s}$ transfected cells, in terms of epinephrine stimulated GTPase activity, onset-delay of calcium release and time-to-peak of calcium signal. However, cells expressing $\beta_{3\text{-}s}$ showed a significantly lower rate of calcium release compared to cells expressing $\beta_3$. These results indicate that the G protein $\beta_{3\text{-}s}$ subunit has no functional difference or possible slightly reduced effect compared to the wild type.

Thus I have systematically shown that the GNB3 C825T polymorphism does not play a major role in the causation of hypertension using a multi-pronged approach fulfilling all the stringent criteria needed to confirm association. I have shown that the functional difference between the Gβ3-s splice variant and the wild type Gβ3 subunit is small thus may not have a major effect on phenotype independently. It is well known that hypertension and its complications are the consequence of interactions between many genetic and environmental factors. In this scenario, effects conferred by a single gene variant either must be very strong or found at a very high frequency to be statistically detectable, and further studies looking at epistatic interactions with this polymorphism would facilitate the genetic dissection of hypertension. I have shown a statistical interaction in ECG phenotypes suggesting that the net effect of this variation may manifest, only when it's associated afferent or efferent pathways are also affected. However these findings need to be further tested in large association studies especially with ascertainment for high blood pressure, which can identify individuals in the extremes of LV mass distribution and also in population cohorts followed up over a longer time frame.

The approach used in this study offers a paradigm for the study of candidate genes and loci identified in population based studies of complex human disease.

# OUTLINE OF THESIS

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# DECLARATION

The experimental design of the work presented in this thesis was that of the author and his supervisors, Professor JMC Connell, Professor AF Dominiczak and Professor G Milligan. The author with the following exceptions performed all experimental work.

Dr G Inglis performed the genotyping of ACE and Aldosterone synthase. Dr T Stanton performed beta-1 adrenoceptor genotyping for the MIDSPAN population using protocols devised by the author. The author performed platelet aggregation experiments with technical help from Dr S Miller.

Dr L Swan recruited subjects for the twin study and collected echocardiographic and phenotypic data. The author performed the candidate gene assays and analyses.

The MIDSPAN database, which contains the screening phenotypic information on the siblings used in this study, was used with the permission of Prof G Watt.

# PUBLICATIONS ARISING FROM THE THESIS

The review in chapter 1 has been published as follows

- Padmanabhan N, Padmanabhan S, Connell JMC: **Genetics of cardiovascular disease- The renin-angiotensin-aldosterone system as a paradigm.** Journal of the Renin-Angiotensin-Aldosterone system 2000;1:316-24
- Lee WK, Padmanabhan S, Dominiczak AF: **Genetics of Hypertension: From experimental models to clinical applications.** Journal of Human Hypertension 2000; 14: 631-647.

**This has also been published as a web-resource on the genetics of hypertension**

- http://www.gla.ac.uk:443/hypertension/Htngen/default.htm

The work in chapter 5 has been published as follows

- Swan L, Birnie DH, Padmanabhan S, Inglis G, Connell JM, Hillis WS. **The genetic determination of left ventricular mass in healthy adults.** Eur Heart J. 2003 Mar;24(6):577-82.
- L Swan, DH Birnie, S Padmanabhan, G Inglis, JMC Connell, WS Hillis: **The genetic contribution to left ventricular structure – an adult twin study.** [Abstract] Hypertension 2000;36(4):667

  Oral presentation at the International Society of Hypertension Conference - 2000, Satellite Symposium on Genetics of Experimental and Human Hypertension

This work has also led to a related publication

- Stanton T, Inglis GC, Padmanabhan S, Dominiczak AF, Jardine AG, Connell JM.**Variation at the beta-1 adrenoceptor gene locus affects left ventricular mass in renal failure.** J Nephrol 2002 Sep-Oct;15(5):512-8

The work in Chapter 6 has been published as follows

- **S Padmanabhan, Inglis G, MacFarlane P, Watt G, Dominiczak AF, Connell JMC.** Heritability and genetic determinants of electrocardiographic measures of left ventricular mass.(P650) **Journal of Hypertension 2004;22:S180**

  Presented at the 20th Scientific Meeting of the International Society of Hypertension, 2004.

The work in chapters 7 and 8 has been published as follows

- Tomaszewski M, Charchar FJ, Padmanabhan S, Zukowska-Szczechowska E, Grzeszczak W, Dominiczak AF. **Cardiovascular diseases and G-protein beta3 subunit gene (GNB3) in the era of genomewide scans.** J Hum Hypertens. 2003 Jun;17(6):379-80.
- Padmanabhan S, Miller S, Pediani J, Milligan G, Dominiczak AF, Connell JMC. **Is the C825T polymorphism of the G-Protein b-3 subunit functional?** [Abstract] Hypertension 2002; 40(4): 568

  Presented at the European Council for Cardiovascular Research, 2002.

Other publications arising from the study of genetic analysis of complex traits in chapter 1 has led to the following publications

- Charchar FJ, Tomaszewski M, Padmanabhan S, Lacka B, Upton M, Inglis GC, Anderson NH, McConnachie A, Zukowska-Szczechowska E, Grzeszczak W, Connell JMC, Watt GCM, Dominiczak AF: **The Y chromosome effect on blood pressure in two European populations.** Hypertension 2002 Feb;39(2 Pt 2):353-6
- Tomaszewski M, Brain NJ, Charchar FJ, Wang WY, Lacka B, Padmanabhan S, Clark JS, Anderson NH, Edwards HV, Zukowska-Szczechowska E, Grzeszczak W, Dominiczak AF. **Essential hypertension and beta2-adrenergic receptor gene: linkage and association analysis.** Hypertension 2002 Sep;40(3):286-91

# ABBREVIATIONS

| | |
|---|---|
| **ACE** | Angiotensin Converting Enzyme |
| **ADP** | Adenosine Di Phosphate |
| **AGT** | Angiotensinogen |
| **AME** | Apparent Mineralocorticoid Excess |
| **AT1R** | Angiotensin Type I Receptor |
| **ATP** | Adenosine Tri Phosphate |
| **B1AR** | Beta 1 Adrenoceptor |
| **BMI** | Body Mass Index |
| **CAD** | Coronary artery Disease |
| **CVD** | Cardiovascular Disease |
| **CYP11B2** | Aldosterone Synthase Gene |
| **DBP** | Diastolic Blood Pressure |
| **DZ** | Dizygous twins |
| **ECG** | Electrocardiogram |
| **ECHO** | Echocardiogram |
| **eNOS** | Endothelial Nitric Oxide Synthase |
| **FBAT** | Family Based Association Test |
| **Gβ3** | GNB3 wild type |
| **Gβ3-s** | GNB3 splice variant |
| **GDP** | Guanosine 5'-diphosphate |
| **GNB3** | G Protein Beta 3 subunit |
| **GPCR** | G Protein Coupled Receptor |
| **GRA** | Glucocorticoid Remediable Aldosteronism |
| **GTP** | Guanosine 5'-triphosphate |
| **IBD** | Identity by Descent |
| **IC** | Intron Conversion |
| **JNC VII** | VII Joint National Committee of Prevention, Detection, Evaluation, and Treatment of High Blood Pressure |
| **LD** | Linkage Disequilibrium |
| **LVH** | Left Ventricular Hypertrophy |
| **LVM** | LV Mass |
| **LVMI** | LV Mass Index |
| **MAP** | Mean Arterial Pressure |
| **MZ** | Monozygous twins |
| **NO** | Nitric Oxide |
| **PAF** | Platelet Activating Factor |
| **PCR** | Polymerase Chain Reaction |
| **PTX** | Pertussis Toxin |
| **QTDT** | Quantitative TDT |
| **SBP** | Systolic Blood Pressure |
| **SF1** | Aldosterone Synthase C344T Polymorphism |
| **SNP** | Single Nucleotide Polymorphism |
| **SOLAR** | Sequential Oligogenic Linkage Analysis Routines |
| **STDT** | Sibling Disequilibrium Test |
| **TDT** | Transmission Disequilibrium Test |
| **WHR** | Waist - Hip Ratio |

# Chapter 1 - INTRODUCTION

## 1.1 Hypertension

Hypertension is one of the most common diseases afflicting humans throughout the world. Because of the associated morbidity and mortality and the cost to society, hypertension is an important public health challenge. Over the past several decades, extensive research, widespread patient education, and a concerted effort on the part of health care professionals have led to decreased mortality and morbidity rates from the multiple organ damage arising from years of untreated hypertension.

### *1.1.1 Historical Perspective*

The first report of direct BP measurement dates back to 1726, when Stephen Hales cannulated a horse's crural artery and assessed the height of the blood column before and after haemorrhage. He described the importance of blood volume in blood pressure regulation. The contribution of peripheral arterioles in maintaining blood pressure, described as "tone," was first described by Lower in 1669 and subsequently by Sénac in 1783. Claude Bernard, Charles E. Edouard, Charles Brown-Séquard, and Augustus Waller observed the role of vasomotor nerves in the regulation of blood pressure. William Dayliss advanced this concept in a monograph published in 1923. Cannon and Rosenblueth developed the concept of humoral control of blood pressure and investigated pharmacologic effects of epinephrine. Three contributors who advanced the knowledge of humoral mechanisms of blood pressure control are T.R. Elliott, Sir Henry Dale, and Otto Loew.

The monaural stethoscope appeared in the early nineteenth century, well before Riva-Rocci's introduction of the sphygmomanometer cuff in 1896. Firmly shifting attention away from assessing the pulse wave,[1] Korotkoff reported the auscultatory

method of measuring arterial pressure in 1905, permitting measurement of diastolic pressure and systolic pressure for the first time.[2]

As early as 1827, Richard Bright observed the changes of hypertension on the cardiovascular system in patients with chronic renal disease. By 1852, Johnson had described medial hypertrophy of the renal afferent arterioles from high BP, and Traube associated left ventricular hypertrophy (LVH) with increased arterial tension in 1856. Clinical pathologic studies by Sir William Gull and H.G. Sutton (1872) led to further description of the cardiovascular changes of hypertension. Frederick Mahomed was one of the first physicians to systematically incorporate blood pressure measurement as a part of a clinical evaluation. In 1939, Keith, Wagener, and Barker published their landmark study of survival curves for four groups of hypertensive patients classified by eye ground patterns.[3] With these and many subsequent advances, the medical focus shifted from simple measurement of BP to its clinical consequences.

Early efforts to reduce extreme hypertension by adrenalectomy or sympathectomy frequently led to disastrous results leading some authorities to conclude that such hypertension was not merely primary but "essential," because aggressive pressure reductions were deleterious. Serious work on experimental hypertension started with Goldblatt's description of the renal artery clip model in 1934.[4] Thus began a series of discoveries of one intriguing complexity after another: renin, angiotensin, atrial natriuretic factor, cation fluxes, endothelial factors, vascular remodelling, central monoaminergic, and hypothalamic factors, among others.

### *1.1.2 Definition of Hypertension*

Defining abnormally high blood pressure is extremely difficult and arbitrary. Furthermore, the relationship between systemic arterial pressure and morbidity appears to be quantitative rather than qualitative. A level for high blood pressure must be agreed upon in clinical practice for screening patients with hypertension, for instituting diagnostic evaluation and for initiating therapy. Because the risk to an individual patient may correlate with the severity of hypertension, a classification system is essential for making decisions about aggressiveness of treatment or therapeutic interventions.

The classification of hypertension is based on the average of 2 or more properly measured, seated BP readings on each of 2 or more office visits.[5] Table 1.1-1 shows the classification of hypertension for adults aged 18 years or over, based on recommendations of the Seventh Report of the Joint National Committee of Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VII).[6]

| BP Classification | Systolic BP | | Diastolic BP |
|---|---|---|---|
| Normal | <120 | And | <80 |
| Prehypertension | 120-139 | Or | 80-89 |
| Stage 1 hypertension | 140-159 | Or | 90-99 |
| Stage 2 hypertension | ≥160 | Or | ≥100 |

**Table 1.1-1 JNC VII Classification of hypertension**

Because of the new data on lifetime risk of hypertension and the impressive increase in the risk of cardiovascular complications associated with levels of BP previously

considered to be normal, the JNC VII[6] report has introduced a new classification that includes the term "prehypertension" for those with BPs ranging from 120 to 139 mm Hg systolic and/or 80 to 89 mm Hg diastolic blood pressure (DBP). This new designation is intended to identify those individuals in whom early intervention by adoption of healthy lifestyles could reduce BP, decrease the rate of progression of BP to hypertensive levels with age, or prevent hypertension entirely.

This classification does not make different treatment recommendations according to the presence or absence of risk factors or target organ damage. JNC VII[6] suggests that all people with hypertension (Stages 1 and 2) be treated. The treatment goal for individuals with hypertension and no other compelling conditions is 140/90 mm Hg. The goal for individuals with prehypertension and no other risk factors or target organ damage compelling indications is to lower BP to normal using the recommended lifestyle modifications.

### *1.1.3 Epidemiology*

Worldwide, hypertension affects an estimated 690 million persons, primarily adults. Estimates of prevalence of hypertension are variable because the number of measurements made, the method and the circumstances in which measurements are made all vary. Also important here are age, gender, race, and socio-economic status of the population studied. National health surveys in various countries have shown a high prevalence of poorly controlled hypertension. These studies have reported that prevalence of hypertension is 22% in Canada, of which 16% is controlled; 26.3% in Egypt, of which 8% is controlled and 13.6% in China, of which 3% is controlled. Hypertension is a worldwide epidemic; in many countries 50% of the population older than 60 years has hypertension. Overall, approximately 20% of the world's

adults are estimated to have hypertension (defined as blood pressure in excess of 140/90 mm Hg). The prevalence dramatically increases in patients older than 60 years.

### 1.1.3.1 United Kingdom studies

In a representative population sample of 10359 Scottish men and women aged 40 to 59 years between 1984 and 1986, 1262 (25 %) men and 1061 (20 %) women were considered to be hypertensive Hypertension was defined in this study as a blood pressure of 160/95mmHg or higher, or receiving antihypertensive drug treatment and was based on the average of two measurements at a single screening visit in a primary care setting.[7] A more recent survey of 12116 English adults aged 16 years or more in 1994 yielded similar results.[8] Based on the average of the second and third of three readings at a single screening visit in the respondent's home, 19% of men and 20% of women had a blood pressure of 160/95 mmHg or higher or were receiving antihypertensive drug treatment. Both surveys probably overestimate the prevalence of hypertension in the United Kingdom because they were based on the average of readings at a single screening session.

### 1.1.3.2 United States studies

The age-adjusted prevalence of hypertension varies from 18-32%, according to data from the National Health Examination Surveys 1988 to 1991.[9] The proportion of the United States population having a systolic blood pressure of 140 mmHg or higher, or a diastolic blood pressure of 90 mmHg or higher, was 4% for young adults aged 18 to 29 years, increasing to 65% for those over 80 years. This was based on the average of six measurements at two visits, or currently being treated with an antihypertensive drug.[9] In 1999-2000, 28.7% of NHANES participants had hypertension, an increase

of 3.7% (95% confidence interval, 0-8.3) from 1988-1991.[10] Overall, in 1999-2000, 68.9% were aware of their hypertension, 58.4% were treated, and hypertension was controlled in 31.0%.[10]

Recent data from Framingham, United States suggest that the increasing use of antihypertensive medication has resulted in a decline in the prevalence of hypertension. In an analysis of 10333 subjects aged 45 to 74 years, examined over a 40-year period from 1950 to 1989, the prevalence of hypertension decreased from 18.5 to 9.2 per cent for men, and from 28.0 to 7.7 per cent for women. This was using definitions of 160 mmHg systolic or 100 mmHg diastolic on or off treatment, and based on an average of two separate measurements at each visit.[11] This is in contrast to the NHANES study (1999-2000) which suggests that hypertension prevalence is increasing.[10]

#### 1.1.3.3 Age

A progressive rise in blood pressure with increasing age is observed. The third NHANES survey[10] reported that the prevalence of hypertension grows significantly with increasing age in all sex and race groups. The age-specific prevalence was 3.3% in white men (aged 18-29 y); this increased to 13.2% in the group aged 30-39 years; 22% in the group aged 40-49 years; 37.5% in the group aged 50-59 years; and 51% in the group aged 60-74 years. In another study, the incidence of hypertension increased approximately 5% for each 10-year interval of age. The prevalence of hypertension increases with advancing age to the point where more than half of people aged 60 to 69 years old and approximately three-fourths of those aged 70 years and older are affected.[9;10] The age-related rise in SBP is primarily responsible for an increase in both incidence and prevalence of hypertension with increasing age.[12] The systolic

blood pressure rises into the eighth or ninth decade, while the diastolic blood pressure remains constant or declines after age 40 years.[13]

#### 1.1.3.4 Sex

The age-adjusted prevalence of hypertension for men was 34%, 25.4%, and 23.2%, and for women was 31.0%, 21.0%, and 21.6% among African Americans, whites, and Mexican Americans, respectively. In the NHANES III study, the prevalence of hypertension in the age group 18-49 years was 12% for white men compared to 5% for white women. However, the age-related blood pressure rise for women exceeds that for men, thus by 70 years or older the prevalence of hypertension was reported at 50% for white men and 55% for white women.

#### 1.1.3.5 Lifetime Risk of Hypertension

Whereas the short-term absolute risk for hypertension is effectively conveyed by incidence rates, the long-term risk is best summarized by the lifetime risk statistic. This is the probability of developing hypertension during the remaining years of life (either adjusted or unadjusted for competing causes of death). Framingham Heart Study investigators recently reported the lifetime risk of hypertension to be approximately 90% for men and women who were nonhypertensive at 55 or 65 years old and survived to age 80 to 85.[14] Even after adjusting for competing mortality, the remaining lifetime risk of hypertension was 86 to 90% in women and 81 to 83% in men. The impressive increase of BP to hypertensive levels with age is also illustrated by data indicating that the 4-year rate of progression to hypertension is 50% for those 65 years and older with BP 130-139/85-89 mm Hg and 26% for those with BP 120-129/80-84 mm Hg.[15]

### *1.1.4 Hypertension and Risk*

Hypertension plays a key role in the worldwide mortality and morbidity from cardiovascular diseases, which account for 15 million (30% of total) deaths. Hypertension is also the key risk factor in fatal and nonfatal cerebrovascular accidents, which amount to 5 million stroke deaths per year. Another 30 million people live with disabilities caused by stroke. In fact, the Global Burden of Disease Study suggested that in 1990 alone, 2.9 million deaths or 5.8% of total deaths were attributable to hypertension.[16]

#### 1.1.4.1 Blood Pressure and Cardiovascular Risk

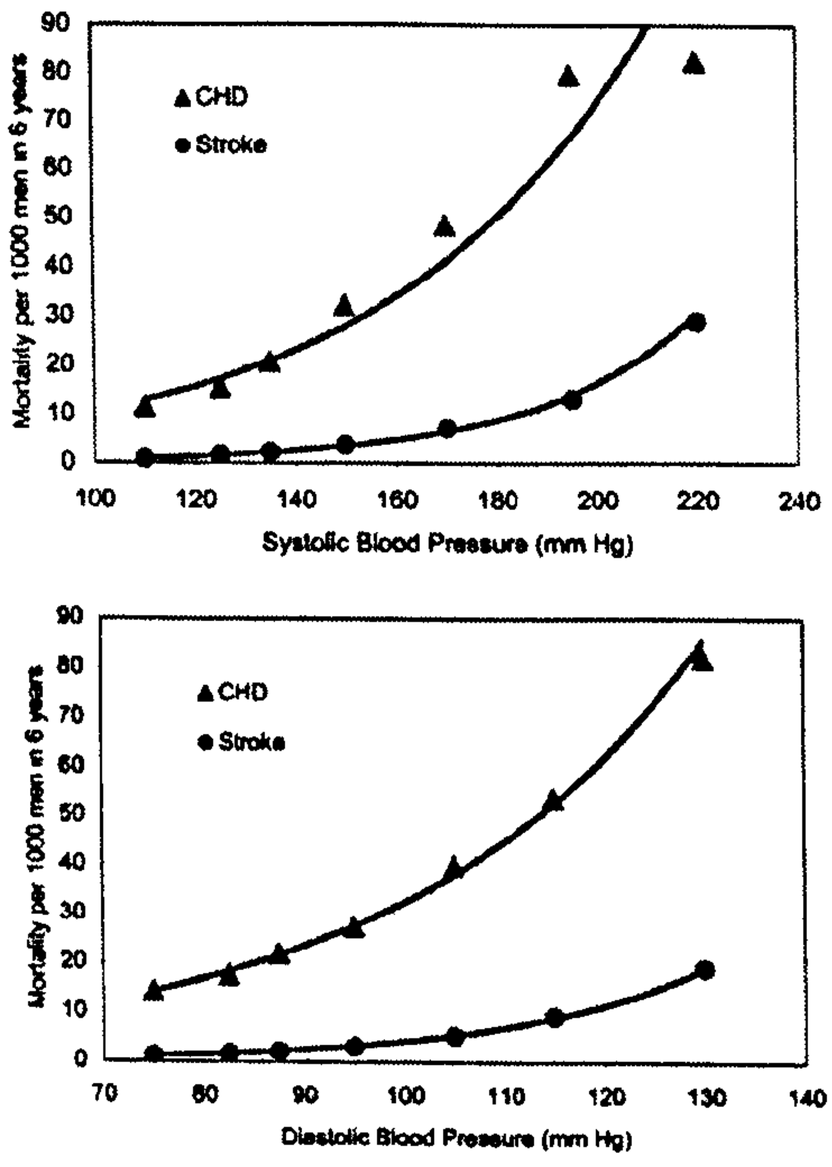


**Figure 1.1-1 Coronary heart disease (CHD) and stroke mortality in relation to systolic and diastolic blood pressure in the MRFIT study.**

**Systolic pressure (upper panel) and diastolic pressure (lower panel) on the X-axis and mortality rate on the Y-axis. Adapted from Neaton et al.[17]**

The relationship between BP and the risk of cardiovascular events is continuous, consistent, and independent of other risk factors. The higher the BP, the greater the chance is of heart attack, heart failure, stroke, and kidney diseases. The risk of both

coronary atherosclerosis and stroke increase exponentially as blood pressure rises (Figure 1.1-1).[17]

Data from observational studies involving more than 1 million individuals have indicated that death from both ischaemic heart disease and stroke increases progressively and linearly from BP levels as low as 115 mm Hg systolic and 75 mm Hg diastolic.[18] The increased risks are present from 40 to 89 years old. For every 20 mm Hg systolic or 10 mm Hg diastolic increase in BP, there is a doubling of mortality from both ischaemic heart disease and stroke.

In addition, longitudinal data obtained from the Framingham Heart Study have indicated that BP in the 130-139/85-89 mm Hg range are associated with a more than 2-fold increase in relative risk from cardiovascular disease (CVD) compared to those with BP levels below 120/80 mm Hg.[19]

At any blood pressure, the absolute risk of coronary disease is considerably greater than for stroke, although the relative risk for stroke increases more rapidly than coronary artery disease. An insight into this finding comes from autopsy studies, which show that the carotid and intracerebral vascular beds are relatively protected from atherosclerosis as compared to the coronary circulation, particularly at lower blood pressures.[20;21]

Probably the major means whereby hypertension accelerates atherosclerosis is through pressure-driven convection of LDL and other atherogenic particles into the arterial intima.[22-25] Indeed, without at least high arterial pressures, atherosclerosis

does not exist in the vascular tree[26] even in patients with homozygous familial hypercholesterolemia.[27] Increased turbulence (a rare occurrence in the human circulatory system) does not increase atherosclerosis; instead higher shear stress is a strong stimulus for the release of nitric oxide, which locally decreases the risk of atherosclerosis. Focal areas of low shear stress are at inherently increased risk of atherosclerotic disease (such as the coronary arteries where flow stops during each systole).[28]

Interestingly, in most studies, stroke risk has been little affected by serum total cholesterol,[29;30] although some recent studies identify clear associated risk.[31] At least some association with all standard cardiovascular risk factors should be expected, not only because thromboembolic stroke may be caused by carotid tree atherosclerosis, but because aortic plaques have been strongly implicated as an embolic source for ischaemic stroke.[32-34]

### 1.1.4.2 Metabolic syndrome and dyslipidaemic hypertension

The frequent coexistence of abnormal plasma lipids and hypertension was termed familial dyslipidaemic hypertension as a descriptive, clinical diagnosis.[35;36] Studies in twins confirmed a genetic predisposition to the coexistence of both hypertension and dyslipidaemia and emphasized the markedly greater risk for CAD when both hypertension and dyslipidaemia were present as compared to either separately.[37] Using data from this study, 46% of CAD risk associated with hypertension can be attributed to the frequently accompanying lipid abnormalities. This may help explain why blood pressure reduction with antihypertensives alone achieves only about half the expected reduction in CAD.[38] Much greater reductions in CAD risk were

achieved in a Swedish primary prevention trial when both blood pressure and lipids were reduced.[39]

Genetic epidemiology strongly points to an interaction between excess accumulation of fat, particularly when centrally distributed, and genetic predisposition that leads to the major features of the metabolic syndrome: hypertension, diabetes, and dyslipidemia.[35;40-43] Figure 1.1-2 illustrates the relationship between metabolic

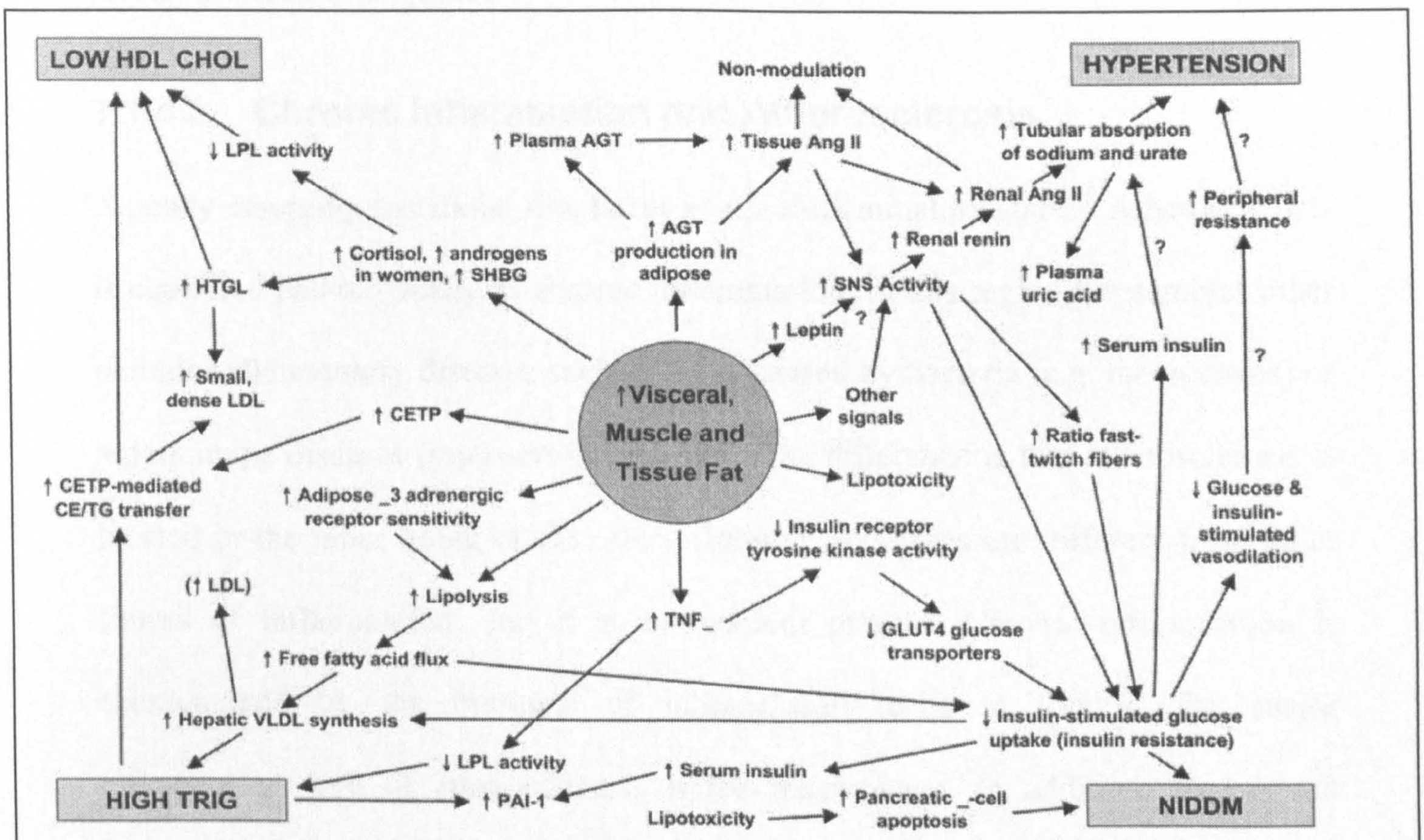


FIGURE 1.1-2 METABOLIC SYNDROME

syndrome and excess fat accumulation (particularly in visceral and in muscle tissues but other tissues as well). Recent advance in adipocyte biology revealed that adipose tissue is a secretory organ. Such fat-derived secretory factors are now called adipocytokines, conceptually.[44] Adipocytokines known to date include leptin, TNF-α, resistin, PAI-1, and adiponectin. Physiological production and secretion of adipocytokines maintains metabolic and vascular homeostasis, by acting on the

adipocyte itself through auto- and para-crine fashions, and on remote organs through blood stream. Dysregulated production of too much or too little adipocytokines, in obesity and lipodystrophy can lead to the development of metabolic syndrome, including insulin resistant diabetes and vascular disease. Restoring the plasma levels of adipocyotkines have been shown to reverse such metabolic syndrome associated with obesity and lipodystrophy. Adipocytokines are now becoming a central target to tackle the metabolic syndrome.

#### 1.1.4.3 Chronic Inflammation and Atherosclerosis

A newly emerging metabolic risk factor is a proinflammatory state.[45] Atherosclerosis is classified pathologically as chronic inflammation. In this regard it resembles other chronic inflammatory diseases such as those caused by bacteria (e.g. tuberculosis) or autoimmune diseases (lupus-erythematosus). The difference is that atherosclerosis is located in the inner lining of the artery (intima), its causes are different from other causes of inflammation, and it is an indolent process. Chronic inflammation is characterized by the presence of inflammatory cells in tissues. The major inflammatory cell of atherosclerosis is the macrophage. In addition, the smooth muscle cells of the arterial wall can acquire inflammatory properties. One unique feature of atherosclerosis is that macrophages and smooth muscle cells acquire large amounts of cholesterol, which eventually kills the cells. The causes of chronic inflammation in the artery include lipoproteins, products of cigarette smoke, and elevated blood glucose. Ultimately, when coronary plaques grow large enough, they can rupture to produce heart attack. Plaque rupture appears to occur in areas where inflammation is most active.

In persons with the metabolic syndrome, chronic inflammation in the arterial wall appears to be enhanced. This is reflected in the blood by " inflammatory markers". One of these is called C-reactive protein, which is released by the liver in the presence of enhanced inflammation. High levels of cytokines, which are proteins that activate macrophages, are another indication of enhanced inflammation. Excess cytokines, which are commonly associated with the metabolic syndrome, may arise from an excess of adipose tissue. All the features of the proinflammatory state have not been defined; however, the relationship between inflammatory processes and the metabolic syndrome is becoming a topic of intense research. If a proinflammatory state is in fact one of the risk factors of the metabolic syndrome, importantly, it could contribute to the development of premature coronary heart disease, and perhaps even to type 2 diabetes.

### 1.1.4.4 Other Risks

Hypertension is not just a risk factor for atherosclerosis. High blood pressure can have direct adverse effects on arteries, arterioles, and the heart, resulting in potentially severe consequences beyond the more common manifestations of myocardial infarction and atherothrombotic stroke. Even modestly elevated blood pressure is a major risk factor for congestive heart failure.[46;47] Hypertension is a major contributor to left ventricular hypertrophy, a major risk factor for sudden death independent of other risk factors.[46;48;49] Hypertension can lead progressively to arterial and arteriolar hypertrophy, arteriosclerosis and arteriolosclerosis, and with very high pressures to fibrinoid change and fibrinoid necrosis in arterioles. These latter changes can result in lumen compromise of arterioles resulting in lacunar stroke, Charcot-Bouchard aneurysms, glomerulosclerosis, nephrosclerosis, and ultimately malignant hypertension in the kidney and retinal ischaemia and blindness.

The risk of intracerebral haemorrhage is increased 33-fold at stage 3 or higher pressures compared to normal blood pressure.[50] Untreated, malignant hypertension is associated with a 5-year mortality rate of 95% with 65% dying from congestive heart failure, 14% from renal failure, 11% from myocardial infarction, and just 5% from cerebral hemorrhage.[51]

### 1.1.4.5 Systolic Blood Pressure

Impressive evidence has accumulated to warrant greater attention to the importance of SBP as a major risk factor for CVD. Changing patterns of BP occur with increasing age. The rise in SBP continues throughout life, in contrast to DBP, which rises until approximately 50 years old, tends to level off over the next decade, and may remain the same or fall later in life.[9;12] Diastolic hypertension predominates before 50 years of age, either alone or in combination with SBP elevation. The prevalence of systolic hypertension increases with age, and above the age of 50 years, systolic hypertension represents the most common form of hypertension. DBP is a more potent cardiovascular risk factor than SBP until age 50; thereafter, SBP is more important.[52] Clinical trials have demonstrated that control of isolated systolic hypertension reduces total mortality, cardiovascular mortality, stroke events.[53-55] Both observational studies and clinical trial data suggest that poor SBP control is largely responsible for the unacceptably low rates of overall BP control.[56;57] In the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) and Controlled Onset Verapamil Investigation of Cardiovascular End Points (CONVINCE) trial, DBP control rates exceeded 90%, but SBP control rates were considerably less (60 to 70%).[58-60] Poor SBP control is at least in part related to physician attitudes. A survey of primary care physicians indicated that three-fourths of them failed to initiate antihypertensive therapy in older individuals with SBP of

140 to 159 mm Hg, and most primary care physicians did not pursue control to less than 140 mm Hg.[61;62] Most physicians have been taught that the diastolic pressure is more important than SBP and thus treat accordingly. Greater emphasis must clearly be placed on managing systolic hypertension. Otherwise, as the population becomes older, the toll of uncontrolled SBP will cause increased rates of cardiovascular and renal diseases.

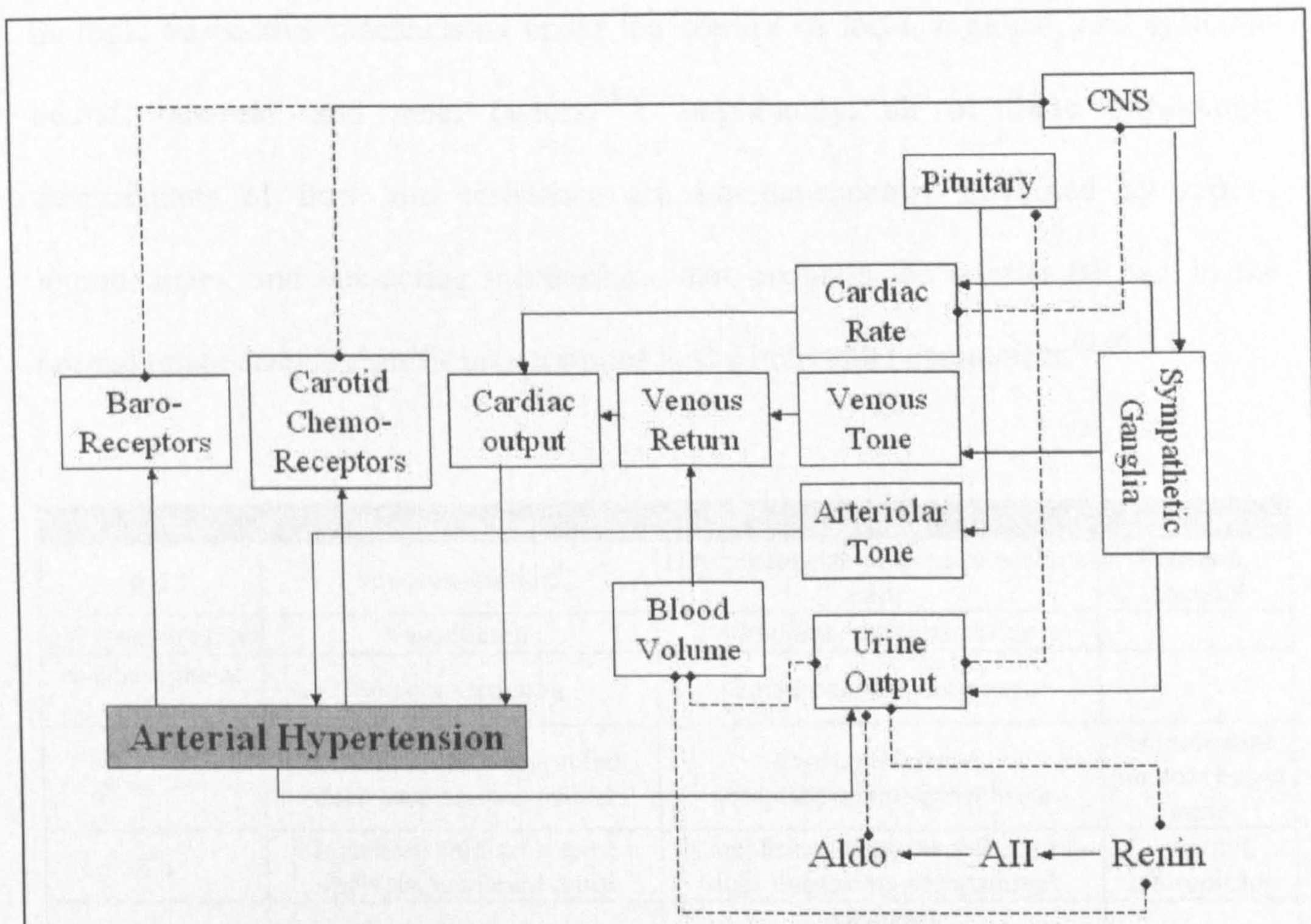


**Figure 1.1-3 Factors that determine arterial blood pressure.**

**SNS=sympathetic nervous system; PNS=parasympathetic nervous system; Ach=acetylcholine; RBC=red blood cells; ADH=antidiuretic hormone; ALDO=aldosterone; →=stimulatory (positive) influence; – –• =inhibitory (negative influence); AI=angiotensin I; AII=angiotensin II; ANP=atrial natriuretic peptide; K=potassium; PGE=prostaglandin E.[63]**

### *1.1.5 The physiology of normal BP control*

The mean arterial BP level is determined by the product of cardiac output (CO) and total peripheral resistance (TPR). These two principal determinants are importantly influenced by many physiologic factors as demonstrated in Figure 1.1-3. Cardiac

output, for example, depends primarily on heart rate and stroke volume. Heart rate is governed by beta-1 and cholinergic receptors under the control of sympathetic and parasympathetic stimulation, respectively. The stroke volume is determined by the ventricular force of contraction (also under autonomic stimulation) and the filling pressure which is in turn determined by the intravascular fluid volume status and the venous capacitance. Similarly, the systemic vascular resistance is influenced by multiple vasoactive mechanisms under the control of local, regional, and systemic neural, humoral, and renal factors.[64;65] Importantly, all of these physiologic determinants of flow and resistance are interdependently governed by rapid-, intermediate-, and late-acting mechanisms that maintain the arterial BP within the normal range despite significant variations in the individual parameters.[63;66]

| Receptors | Agonist actions | Agonists | Antagonists |
|---|---|---|---|
| α-1 | Vasoconstriction[a] | Norepinephrine>dopamine>dobutamine | Prazosin, labetalol |
| α-2, central effect | Vasodilation | Clonidine, norepinephrine | |
| α-2, peripheral effect | Venoconstriction | Clonidine, norepinephrine | |
| α-1, α-2 | Net vasoconstriction; reflex decreased cardiac output | Ergot, midodrine, norepinephrine>epinephrine | Phentolamine, phenoxybenzamine |
| β-1 | Increased cardiac output, lipolysis, increased renin | Epinephrine, norepinephrine, low-dose dopamine, dobutamine[a] | Atenolol, metroprolol |
| β-2 | Vasodilation[b] bronchodilation | Albuterol, epinephrine>>norepinephrine[a] | |
| β-1, β-2 | Net increased cardiac output | Epinephrine, isoproterenol | Propranolol, pindolol |
| α-1, β-1 | Vasoconstriction and increased cardiac output | Epinephrine, high-dose dopamine | Labetalol |
| Parasympathetic | Decreased cardiac output, decreased heart rate | Acetylocholine | Atropine |
| $V_1$ | Increased peripheral resistance | Arginine vasopressin | Selective $V_1$ receptor antagonist (not commercially available) or calcium antagonists |

**Table 1.1-2 Adrenergic receptors in the modulation of arterial blood pressure**

**[a] Predominantly arteriolar. [b] Without chronotropic effects (unlike dopamine and isoproterenol).** [63]

The complex systems and mechanisms that constitute the feedback and reflex control of BP have been extensively studied and characterized. The clinically most relevant ones are depicted in Figure 1.1-4. Rapid responses and reflexes, for example, occurring within seconds after an acute rise in BP include the baroreceptor system, vagus nerve, and the central vasomotor centre of the brainstem.[63] Thus, the rapid-

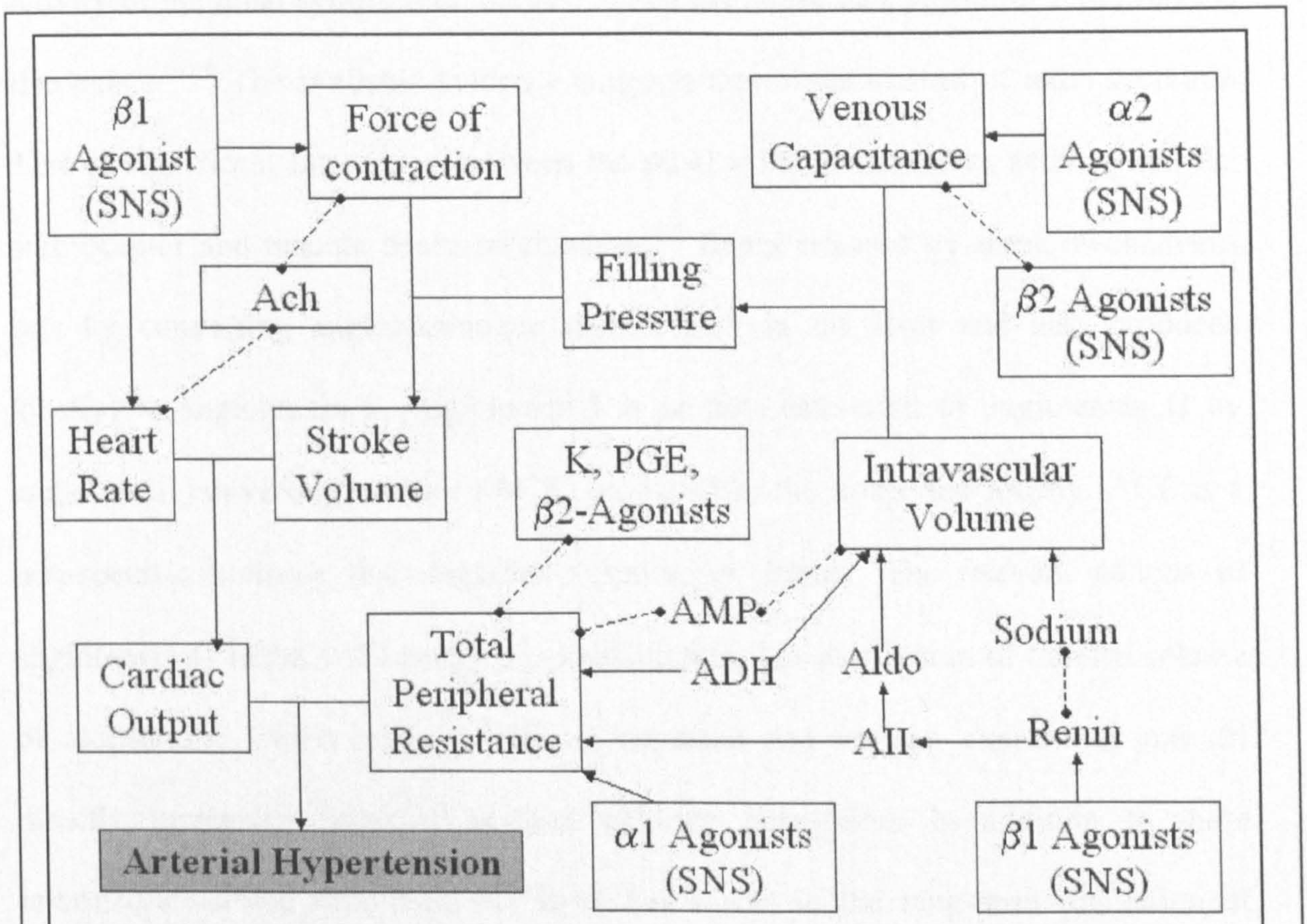


**Figure 1.1-4 Feedback mechanisms in the control of blood pressure.**

**A=angiotensinogen; AI=angiotensin I; AII=angiotensin II; O2=oxygen; ADH=antidiuretic hormone; →=stimulatory (positive) influence; – – •=inhibitory (negative) influence.**

acting reflex and feedback mechanisms in response to acute changes in BP are almost entirely mediated by changes in activity of the autonomic nervous system.[67] The adrenergic receptors, their associated agonists, and their physiologic actions as well as the clinically useful antagonists are shown in Table 1.1-2.

Intermediate-acting responses that occur over minutes and hours involve the renin-angiotensin-aldosterone system, antidiuretic hormone, and the renal juxtaglomerular apparatus (Figure 1.1-5). Both local and circulating renin-angiotensin systems (RAS) play important roles in this feedback mechanism. In the systemic RAS, a drop in BP stimulates renin release via afferent arteriole baroreceptor mechanism and increased activity of the renal sympathetic nerves. A macula densa mechanism of renin release also exists.[68-70] The available evidence suggests that in the control of renin secretion there is significant interaction between the renal sympathetic nerve activity and the baroreceptor and macula densa mechanisms.[69] Renin released by these mechanisms acts by converting angiotensinogen (synthesized in the liver and also produced locally) to angiotensin I. Angiotensin I is in turn converted to angiotensin II by angiotensin converting enzyme (ACE) produced in the lungs and locally. ACE is a non-specific kininase that degrades vasodilatory kinins. The relevant actions of angiotensin II include (1) potent vasoconstriction, (2) stimulation of adrenal release of aldosterone which leads to sodium retention and volume expansion, and (3) directly increasing proximal sodium chloride absorption. In addition to these intermediate-acting responses, the RAS has a role in the long-term regulation of blood pressure.[71]

Antidiuretic hormone (ADH) activity and capillary filtration are two other intermediate-acting mechanisms for BP regulation (Figure 1.1-5). In response to an acute decrease in BP, ADH secretion is increased principally as a result of a decrease in the normal inhibitory tone from the baroreceptors to the hypothalamus. Conversely, increases in BP cause increased baroreceptor activity to the hypothalamus, resulting in increased inhibition of the activity of hypothalamic ADH-

releasing neurons. In the setting of acute, severe hypotension, ADH acts as part of the rapid response and serves as a pressor agent. During periods of prolonged decrease in BP, however, ADH also becomes a part of the late-acting response in continuing to conserve water.

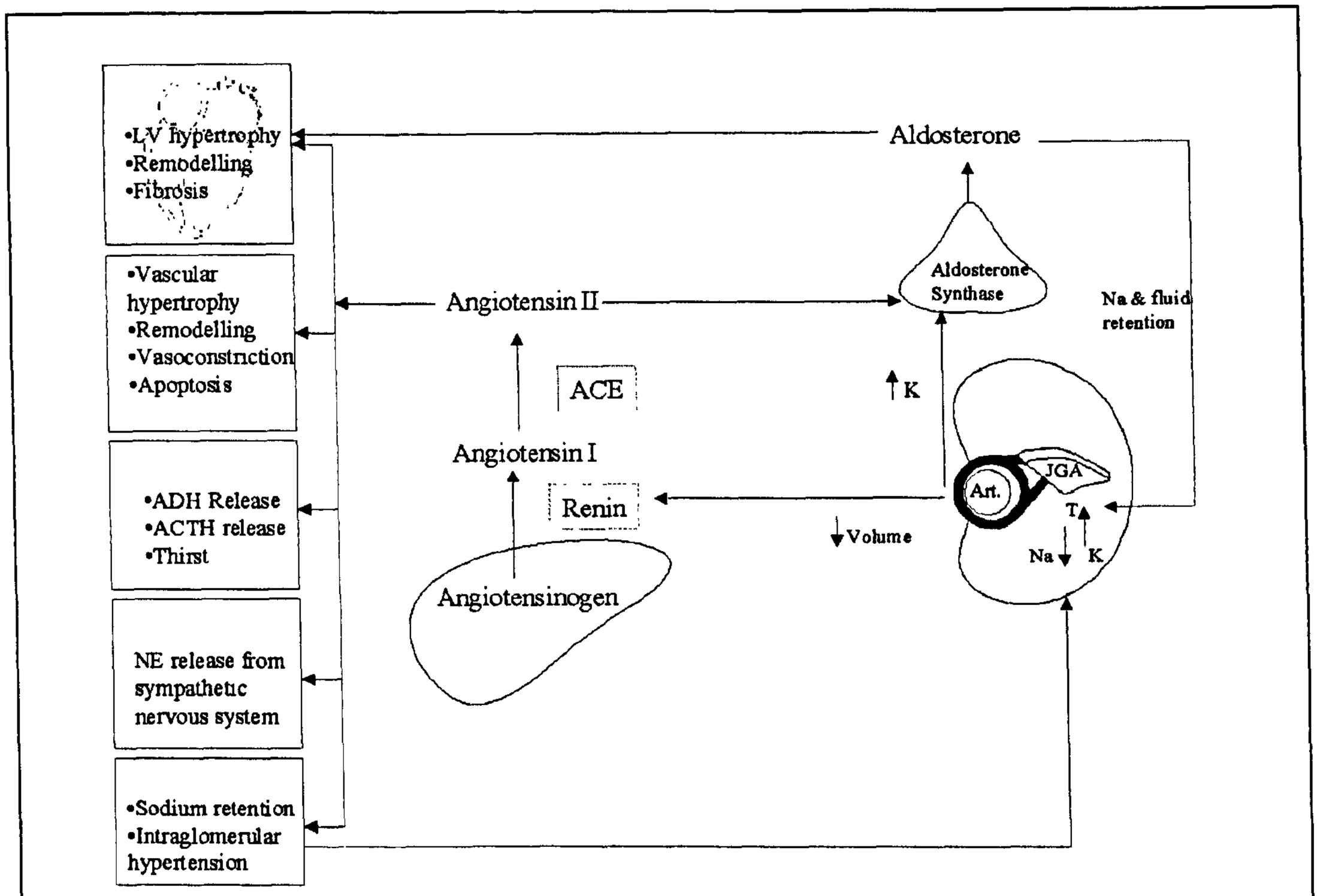


**Figure 1.1-5 The renin–angiotensin system in the control of blood pressure.**

Late-acting mechanisms operate in days to weeks and are very important because of their long-term efficiency in BP regulation.[64] Pressure natriuresis and diuresis are the key mechanisms.[65;72] Very simply stated, an increase in BP level above the normal set point leads directly to an increase in salt and water excretion. In fact, Guyton et al stated that none of the mechanisms discussed above can operate effectively to regulate BP without the participation of this kidney function, a phenomenon they

termed the "overriding dominance of the kidneys" in the long-term regulation of arterial pressure.[64]

Taken together, these complex interrelated mechanisms help regulate BP. An important feature of normal BP control is that significant flexibility is built into the normal level around which BP is regulated. This feature permits significant transient increases in BP level through marked variations in CO and TPR in order to meet physiologic needs. But importantly, counter-regulatory and compensatory mechanisms contained in these feedback loops help to restore BP level to within normal limits again after the end of the acute physiologic demands. The crucial difference between this normal pattern of BP regulation and chronic primary hypertension is an adverse neurohumoral activation, impaired renal handling of sodium, and an increased TPR, the characteristic hallmark of primary hypertension which helps to maintain hypertension and the progression to target organ damage.

#### 1.1.5.1 Pathophysiologic states of chronic BP elevation

The relationships described above suggest that chronic elevations of arterial BP may result from combinations of inappropriate levels of cardiac output (CO) and total peripheral vascular resistance (TPR). The spectrum of these combinations include (1) a high CO and a normal or low TPR, (2) a high TPR and a low or normal CO, and (3) a high CO and a high TPR. Conditions of high CO and relatively normal or low TPR include early phase diabetes mellitus, dialysis patients, and the so-called "hyperdynamic" or "hyperadrenergic" hypertension usually seen in youth. A high TPR associated with a relatively normal or low CO is seen in accelerated or malignant hypertension and hypertension in the elderly. The best example of a balanced increase in both TPR and CO is renovascular hypertension. In all of these

cases, what is missing is an appropriate compensatory mechanism that could normalize the changes in TPR and/or CO in order to return BP level to the normal range.

### *1.1.6 Pathogenesis of Hypertension (Figure 1.3-1,page 114)*

The pathogenesis of essential hypertension is multifactorial and highly complex. Multiple factors modulate blood pressure including humoral mediators, vascular reactivity, circulating blood volume, vascular calibre, blood viscosity, cardiac output, blood vessel elasticity, and neural stimulation. A possible pathogenesis of essential hypertension has been proposed in which multiple factors may interact to produce hypertension, including genetic predisposition, excess dietary salt intake, and adrenergic tone. Although genetics appears to contribute to essential hypertension, the exact mechanism has not been established.

Essential hypertension, or hypertension of unknown cause, accounts for more than 90% of hypertension cases. It tends to cluster in families and represents a collection of genetically based diseases or syndromes with several resultant inherited biochemical abnormalities.[73] The resulting phenotypes can be modulated by various environmental factors, thereby altering the severity and timing of blood pressure elevation.

Many pathophysiologic factors have been implicated in the genesis of essential hypertension:

1. Increased sympathetic nervous system activity, perhaps related to heightened exposure or response to psychosocial stress
2. Overproduction of sodium-retaining hormones and vasoconstrictors

3. Long-term high sodium intake
4. Inadequate dietary intake of potassium and calcium
5. Increased or inappropriate renin secretion with resultant increased production of angiotensin ii and aldosterone
6. Deficiencies of vasodilators, such as prostacyclin, nitric oxide (no), and the natriuretic peptides
7. Alterations in expression of the kallikrein–kinin system that affect vascular tone and renal salt handling
8. Abnormalities of resistance vessels, including selective lesions in the renal microvasculature
9. Diabetes mellitus
10. Insulin resistance
11. Obesity
12. Increased activity of vascular growth factors
13. Alterations in adrenergic receptors that influence heart rate, inotropic properties of the heart, and vascular tone
14. Altered cellular ion transport

The novel concept that structural and functional abnormalities in the vasculature-including endothelial dysfunction, increased oxidative stress, vascular remodelling, and decreased compliance may predate hypertension and contribute to its pathogenesis has gained support in recent years.

Although several factors clearly contribute to the pathogenesis and maintenance of blood pressure elevation, renal mechanisms probably play a primary role, as

hypothesized by Guyton[65] and reinforced by extensive experimental and clinical data. As discussed in this paper, other mechanisms amplify (for example, sympathetic nervous system activity and vascular remodelling) or buffer (for example, increased natriuretic peptide or kallikrein–kinin expression) the pressor effects of renal salt and water retention. These interacting pathways play major roles in both increasing blood pressure and mediating related target organ damage. Understanding these complex mechanisms has important implications for the targeting of antihypertensive therapy to achieve benefits beyond lowering blood pressure.

#### 1.1.6.1 Role of Genes

An estimated 30–60% of the variation in BP between individuals is attributed to genetic factors, after adjustment for age and sex. Depending on the population studied, this estimate may vary from 15–20% to 65–70%. An important genetic component is also suggested by the observation that a child with a history of hypertension in both parents or who has a sibling with hypertension has a 40–60% chance of developing hypertension as an adult, but the risk increases to 80% if the sibling is a monozygotic twin. The inheritance patterns in primary hypertension do not follow classic Mendelian genetics for a single gene locus. Rather, the genetic susceptibility to develop primary hypertension results from the effects of multiple genes. In this regard, an increased risk for hypertension has been noted in a wide spectrum of genetic polymorphisms of angiotensinogen, the endothelial nitric oxide synthase, and the β-2 adrenoceptor. Others have noted an association with α-adducin proteins associated with the actin cytoskeleton and sodium transport. Much attention is also focusing on genetically mediated alterations in the regulation or expression of renal ion channels and transporters.[73] (Detailed discussion in section 1.3)

### 1.1.6.2 Role of the environment

The importance of environmental influences on the blood pressure phenotype has been well described.[74-76] The environmental contribution to the variation in BP between individuals has been estimated to be at least 20%; however, as pointed out by Harrap,[74] environmental effects are most demonstrable between populations rather than between individuals because of presumably larger and more numerous differences. Key environmental factors include geography; dietary intake of sodium, calcium, potassium, and other micro- and macro-nutrients; physical activity; psychosocial stress; socio-economic status; alcohol intake; cigarette smoking; and other lifestyle choices. Among these factors, dietary intake of sodium and salt have been the most studied. Epidemiological and interventional studies have strongly linked salt (NaCl) intake with hypertension- for example populations with a high sodium content in their diet (>4 g/d), such as observed in northern Japan and the Southeastern United States, have a high prevalence of hypertension. Conversely, populations consuming a low sodium diet, such as the Alaskan Eskimo or the Yanamao Indians from the Amazon, have an almost negligible prevalence of hypertension. Similarly, diets low in calcium or potassium have also been associated with a higher prevalence of hypertension.[77] Increasing potassium intake has been found to lower BP in both experimental and human studies. Obesity is perhaps the most salient risk factor for hypertension. The proportion of obese individuals as a function of the total population is continuing to grow in all westernised societies. This parallels the incidence of hypertension in these societies.

Certain metabolic features are associated with the development of high BP. In addition to an elevated glucose (insulin resistance), an elevated haematocrit is also

associated with the development of hypertension. Several studies have also found an elevated serum uric acid to be present in 25% of untreated hypertensive subjects, and also to predict the development of hypertension as well. Controversy exists, however, whether the uric acid is an independent risk factor, or whether it is simply a marker for other conditions associated with hyperuricaemia that may have a role in the development of hypertension, such as renal insufficiency, obesity, insulin resistance, dyslipidaemia, and alcohol use.

There are other important demographic factors for the development of hypertension. Primary hypertension is uncommon in young adults but increases with age, with 65% prevalence in the population at age 65, and 75% at age 75. This age-related increase in prevalence of hypertension has been observed in most Western countries but has not been uniformly observed in all populations. Second, the prevalence of hypertension is greater in men, although the prevalence in women approaches that of men in the postmenopausal years. Certain racial/ethnic groups are also at increased risk based on environmental influences and gene-environment interactions, particularly African-Americans. Obesity, insulin resistance, gout, and sleep apnoea are also associated with an increased risk, as are low socio-economic status and increased stress at work, elevated heart rate and an increased BP response to exercise.[78]

### 1.1.6.3 Inherited Cardiovascular Risk Factors

Cardiovascular risk factors, including hypertension, tend to cosegregate more commonly than would be expected by chance. Approximately 40% of persons with essential hypertension also have hypercholesterolaemia. Genetic studies have established a clear association between hypertension and dyslipidemia.[37]

Hypertension and type-2 diabetes mellitus also tend to coexist. Hypertension is approximately twice as common in diabetics as in non-diabetics, and the association is even stronger in African Americans and Mexican Americans.[79] The leading cause of death in patients with type-2 diabetes is coronary heart disease, and diabetes increases the risk for acute myocardial infarction as much as a previous myocardial infarction in a nondiabetic person.[80] Since 35% to 75% of the cardiovascular complications of diabetes are attributable to hypertension, diabetic patients need aggressive antihypertensive treatment, as well as treatment of dyslipidaemia and glucose control.

Hypertension, insulin resistance, dyslipidaemia, and obesity often occur concomitantly. Other associated abnormalities include microalbuminuria, high uric acid levels, hypercoagulability, and accelerated atherosclerosis. This cosegregation of abnormalities, referred to as the insulin-resistance syndrome or the metabolic syndrome, increases cardiovascular disease (CVD) risk.[80]

#### 1.1.6.4 Sympathetic Nervous System

Increased sympathetic nervous system activity increases blood pressure and contributes to the development and maintenance of hypertension through stimulation of the heart, peripheral vasculature, and kidneys, causing increased cardiac output, increased vascular resistance, and fluid retention.[81] In addition, autonomic imbalance (increased sympathetic tone accompanied by reduced parasympathetic tone) has been associated with many metabolic, haemodynamic, trophic, and rheologic abnormalities that result in increased cardiovascular morbidity and mortality.[82] Several population-based studies, such as the Coronary Artery Risk Development in Young Adults (CARDIA) study,[83] have shown a positive correlation between heart rate and the

development of hypertension (elevated diastolic blood pressure). Most current evidence in humans suggests that sustained increases in heart rate are due mainly to decreased parasympathetic tone, supporting the concept that autonomic imbalance contributes to the pathogenesis of hypertension. Furthermore, increased sympathetic tone may increase diastolic blood pressure by causing vascular smooth-muscle cell proliferation and vascular remodelling as diastolic blood pressure relates more closely to vascular resistance than to cardiac function. Consistent with these population-based observations, norepinephrine spillover studies, which provide an index of norepinephrine release from sympathoeffector nerve terminals, demonstrate that sympathetic cardiac stimulation is greater in young hypertensive patients than in normotensive controls of similar age, supporting the interpretation that increased cardiac sympathetic stimulation may contribute to the development of hypertension.[84]

The mechanisms of increased sympathetic nervous system activity in hypertension are complex and involve alterations in baroreflex and chemoreflex pathways at both peripheral and central levels. Arterial baroreceptors are reset to a higher pressure in hypertensive patients, which reverts to normal when arterial pressure is normalized.[85-87] Resuming normal baroreflex function helps maintain reductions in arterial pressure, a beneficial regulatory mechanism that may have important clinical implications.[88] Furthermore, there is central resetting of the aortic baroreflex in hypertensive patients, resulting in suppression of sympathetic inhibition after activation of aortic baroreceptor nerves.[89] This baroreflex resetting seems to be mediated, at least partly, by a central action of angiotensin II.[90] Angiotensin II also amplifies the response to sympathetic stimulation by a peripheral mechanism, that is, presynaptic facilitatory modulation of norepinephrine release.[91] Additional small-

molecule mediators, which suppress baroreceptor activity and contribute to exaggerated sympathetic drive in hypertension include reactive oxygen species and endothelin.[92;93] Finally, there is evidence of exaggerated chemoreflex function, leading to markedly enhanced sympathetic activation in response to stimuli such as apnoea and hypoxia.[94] A clinical correlate of this phenomenon is the exaggerated increase in sympathetic nervous system activity that is sustained in the awake state and seems to contribute to hypertension in patients with obstructive sleep apnea.[95]

Chronic sympathetic stimulation induces vascular remodelling and left ventricular hypertrophy, presumably by direct and indirect actions of norepinephrine on its own receptors, as well as on release of various trophic factors, including transforming growth factor-β, insulin-like growth factor 1, and fibroblast growth factors.[82] Clinical studies have shown positive correlations between circulating norepinephrine levels, left ventricular mass, and reduced radial artery compliance (an index of vascular hypertrophy).[96;97] Thus, sympathetic mechanisms contribute to the development of target organ damage, as well as to the pathogenesis of hypertension.

Renal sympathetic stimulation is also increased in hypertensive patients compared with normotensive controls. Infusion of the α-adrenergic antagonist phentolamine into the renal artery increases renal blood flow to a greater extent in hypertensive than normotensive patients, consistent with a functional role for increased sympathetic tone in controlling renal vascular resistance.[98;99] In animal models, direct renal nerve stimulation induces renal tubular sodium and water reabsorption and decreases urinary sodium and water excretion, resulting in intravascular volume expansion and increased blood pressure.[100] Furthermore, direct assessments of renal sympathetic

nerve activity have consistently demonstrated increased activation in animal models of genetically mediated and experimentally induced hypertension, and renal denervation prevents or reverses hypertension in these models,[101] supporting a role for increased sympathetic activation of the kidney in the pathogenesis of hypertension.

Of interest, peripheral sympathetic activity is greatly increased in patients with renal failure compared with age-matched, healthy normotensive individuals with normal renal function.[102] This increase is not seen in patients receiving long-term haemodialysis who have undergone bilateral nephrectomy, suggesting that sympathetic overactivity in patients with renal failure is caused by a neurogenic signal originating in the failing kidneys.

#### 1.1.6.5 Vascular Remodelling

Peripheral vascular resistance is characteristically elevated in hypertension because of alterations in structure, mechanical properties, and function of small arteries. Remodelling of these vessels contributes to high blood pressure and its associated target organ damage.[103;104] Peripheral resistance is determined at the level of the precapillary vessels, including the arterioles (arteries containing a single layer of smooth-muscle cells) and the small arteries (lumen diameters < 300 μm). The elevated resistance in hypertensive patients is related to rarefaction (decrease in number of parallel-connected vessels) and narrowing of the lumen of resistance vessels. Examination of gluteal skin biopsy specimens obtained from patients with untreated essential hypertension has uniformly revealed reduced lumen areas and increased media–lumen ratios without an increase in medial area in resistance vessels (inward, eutropic remodelling).[105]

Antihypertensive treatment with several classes of agents, including ACE inhibitors, angiotensin-receptor blockers (ARBs), and calcium-channel blockers, normalizes resistance vessel structure.[106] Of interest, β-blocker therapy does not reverse resistance vessel remodelling even when it effectively lowers blood pressure.[107] To what extent resistance vessel structure plays a direct role in setting the blood pressure and in the pathogenesis of hypertension is a subject of ongoing study and controversy. Furthermore, whether antihypertensive agents that normalize resistance vessel structure are more effective in preventing target organ damage and CVD than agents that lower blood pressure without affecting vascular remodelling remains to be determined.

#### 1.1.6.6 Renal Microvascular Disease

The intriguing hypothesis, proposed by Goldblatt,[4] that primary renal microvascular disease may be responsible for the development of hypertension has recently been revived by Johnson and colleagues[108] and tested in various animal models. These authors have suggested a unified pathway for the development of hypertension whereby the kidney undergoes subclinical injury over time, leading to the development of selective afferent arteriolopathy and tubulointerstitial disease. They hypothesize that the pathway may be initiated by various factors, such as hyperactivity of the sympathetic nervous system[109] or increased activity of the renin–angiotensin–aldosterone system,[110] and that initiation of the pathway may be facilitated by various genetic factors that stimulate sodium reabsorption or limit sodium filtration, as well as by primary microvascular or tubulointerstitial renal disease. These factors result in renal vasoconstriction, which may lead to renal (particularly outer medullary) ischaemia, thus stimulating the influx of leukocytes

and local generation of reactive oxygen species.[111-113] Local generation of angiotensin II at sites of renal injury has been invoked as a stimulus for structural alterations (renal microvascular disease) and haemodynamic effects (increased vascular resistance, low ultrafiltration coefficient, and decreased sodium filtration), which lead to hypertension.[114;115] While this pathway ties in many of the established theories of the pathogenesis of hypertension, it has not yet been confirmed in human disease.[108]

### 1.1.6.7 Arterial Stiffness

Systolic blood pressure and pulse pressure increase with age mainly because of reduced elasticity (increased stiffness) of the large conduit arteries. Arteriosclerosis in these arteries results from collagen deposition and smooth-muscle cell hypertrophy, as well as thinning, fragmenting, and fracture of elastin fibres in the media.[116] In addition to these structural abnormalities, endothelial dysfunction, which develops over time from both aging and hypertension, contributes functionally to increased arterial rigidity in elderly persons with isolated systolic hypertension.[117] Reduced NO synthesis or release in this setting, perhaps related to the loss of endothelial function and reduction in endothelial NO synthase, contributes to increased wall thickness of conduit vessels, such as the common carotid artery. The functional importance of NO deficiency in isolated systolic hypertension is supported by the ability of NO donors, such as nitrates or derivatives, to increase arterial compliance and distensibility and reduce systolic blood pressure without decreasing diastolic blood pressure. Other factors that decrease central arterial compliance include oestrogen deficiency, high dietary salt intake, tobacco use, elevated homocysteine levels, and diabetes. These factors may also damage the endothelium.

The distending pressure of conduit vessels is a major determinant of arterial stiffness. The 2-phase (elastin and collagen) content of load-bearing elements in the media is responsible for the behaviour of these vessels under stress. At low pressures, stress is borne almost entirely by the distensible elastin lamellae, while at higher pressures, less distensible collagenous fibres are recruited and the vessel appears stiffer.[116] Conduit vessels are relatively unaffected by neurohumoral vasodilator mechanisms. Instead, vasodilation is caused by increased distending pressure and associated with increased stiffness. Conversely, conduit vessels do respond to vasoconstrictor stimuli, including neurogenic stimulation during simulated diving, electrical nerve stimulation, and norepinephrine infusion.[118;119]

Increased arterial stiffness also contributes to the wide pulse pressure commonly seen in elderly hypertensive patients by causing the pulse wave velocity to increase. With each ejection of blood from the left ventricle, a pressure (pulse) wave is generated that travels from the heart to the periphery at a finite speed that depends on the elastic properties of the conduit arteries. The pulse wave is reflected at any point of discontinuity in the arterial tree and returns to the aorta and left ventricle. The timing of the wave reflection depends on both the elastic properties and the length of the conduit arteries.

In younger persons, pulse wave velocity is sufficiently slow (approximately 5 m/s) so that the reflected wave reaches the aortic valve after closure, leading to a higher diastolic blood pressure and enhancing coronary perfusion by providing a "boosting" effect. In older persons, particularly if they are hypertensive, pulse wave velocity is greatly increased (approximately 20 m/s) because of central arterial stiffening. At this

speed, the reflective wave reaches the aortic valve before closure, leading to a higher systolic blood pressure, pulse pressure, and afterload and a decreased diastolic blood pressure, in some cases compromising coronary perfusion pressure. This phenomenon explains the increase in systolic blood pressure and pulse pressure and decrease in diastolic blood pressure in the elderly population and is exaggerated in the presence of antecedent hypertension. The increase in systolic blood pressure increases cardiac metabolic requirements and predisposes to left ventricular hypertrophy and heart failure. Pulse pressure is closely related to systolic blood pressure and is clearly linked to advanced atherosclerotic disease and CVD events, such as fatal and nonfatal myocardial infarction and stroke. Pulse pressure is also shown to be a better predictor of CVD risk than systolic blood pressure or diastolic blood pressure.[116]

Most antihypertensive drugs act on peripheral muscular arteries rather than central conduit vessels. They reduce pulse pressure through indirect effects on the amplitude and timing of reflected pulse waves. Nitroglycerine causes marked reductions in wave reflection, central systolic blood pressure, and left ventricular load without altering systolic blood pressure or diastolic blood pressure in the periphery. Vasodilator drugs that decrease the stiffness of peripheral arteries, including ACE inhibitors and calcium-channel blockers, also reduce pulse wave reflection and thus augmentation of the central aortic and left ventricular systolic pressure, independent of a corresponding reduction in systolic blood pressure in the periphery. Antihypertensive drugs from several classes have been shown to reduce systolic blood pressure and CVD morbidity and mortality in patients with isolated systolic hypertension.[120;121]

### 1.1.6.8 Renin–Angiotensin–Aldosterone System

Angiotensin II increases blood pressure by various mechanisms, including constricting resistance vessels, stimulating aldosterone synthesis and release and renal tubular sodium reabsorption (directly and indirectly through aldosterone), stimulating thirst and release of antidiuretic hormone, and enhancing sympathetic outflow from the brain. Of importance, angiotensin II induces cardiac and vascular cell hypertrophy and hyperplasia directly by activating the angiotensin II type 1 (AT1) receptor and indirectly by stimulating release of several growth factors and cytokines.[122] Activation of the AT1 receptor stimulates various tyrosine kinases, which in turn phosphorylate the tyrosine residues in several proteins, leading to vasoconstriction, cell growth, and cell proliferation.[123] Activation of the AT2 receptor stimulates a phosphatase that inactivates mitogen-activated protein kinase, a key enzyme involved in transducing signals from the AT1 receptor. Thus, activation of the AT2 receptor opposes the biological effects of AT1 receptor activation, leading to vasodilation, growth inhibition, and cell differentiation.[124;125] The physiologic role of the AT2 receptor in adult organisms is unclear, but it is thought to function under stress conditions (such as vascular injury and ischaemia reperfusion). Local production of angiotensin II in various tissues, including the blood vessels, heart, adrenals, and brain, is controlled by ACE and other enzymes, including the serine proteinase chymase. The activity of local renin–angiotensin systems and alternative pathways of angiotensin II formation may make an important contribution to remodelling of resistance vessels and the development of target organ damage (including left ventricular hypertrophy, congestive heart failure, atherosclerosis, stroke, end-stage renal disease, myocardial infarction, and arterial aneurysm) in hypertensive persons.[122]

### 1.1.6.8.1 Angiotensin II and Oxidative Stress

Stimulating oxidant production is another mechanism by which angiotensin II increases cardiovascular risk. Hypertension associated with long-term infusion of angiotensin II is linked to the upregulation of vascular p22phox messenger RNA (mRNA), a component of the oxidative enzyme nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase.[126] The angiotensin II receptor–dependent activation of NAD(P)H oxidase is associated with enhanced formation of the oxidant superoxide anion ($\cdot O2^-$). Superoxide readily reacts with NO to form the oxidant peroxynitrite ($ONOO^-$). A reduction in NO bioactivity may thus provide another mechanism to explain the enhanced vasoconstrictor response to angiotensin II in hypertension.[127] The NAD(P)H oxidase may also play an important role in the hypertrophic response to angiotensin II since stable transfection of vascular smooth-muscle cells with antisense to p22phox inhibits angiotensin II–stimulated protein synthesis.[128] Other vasculotoxic responses to angiotensin II that are linked to the activation of NAD(P)H oxidase include the oxidation of low-density lipoprotein cholesterol and increased mRNA expression for monocyte chemoattractant protein-1 and vascular cell adhesion molecule-1.[129;130]

### 1.1.6.8.2 Aldosterone

Aldosterone is synthesized in the zona glomerulosa of the adrenal cortex through the actions of four enzymes. Cholesterol desmolase (CYP11A), 21-hydroxylase (CYP21), and aldosterone synthase (CYP11B2) are cytochromes 450 (CYP), which are membrane-bound haeme-containing enzymes that accept electrons from NADPH through accessory proteins and use molecular oxygen to perform hydroxylations or other oxidative conversions. The other enzyme, 3-beta-hydroxysteroid

dehydrogenase, is a member of the short-chain dehydrogenase family.[131] Aldosterone is crucial for sodium conservation in the kidney, salivary glands, sweat glands and colon. Aldosterone promotes active sodium transport and excretion of potassium in its major target tissues. It exerts its effects via the mineralocorticoid receptor (MR) and the resultant activation of specific amiloride-sensitive sodium channels (ENaC) and the Na-K ATPase pump. Aldosterone and the MR may be involved in the regulation of genes coding for the subunits of the amiloride sensitive sodium channel and the Na-K ATPase pump, as well as of other proteins.[132] Aldosterone indeed increases the number of active sodium channels and augments the action and number of the Na-K ATPase pump units in its target tissues. The heart and blood vessels also express high-affinity mineralocorticoid receptors that can bind both mineralocorticoids and glucocorticoids and contain the enzyme 11β-hydroxysteroid dehydrogenase II, which inactivates glucocorticoids. Activation of these mineralocorticoid receptors is thought to stimulate intra- and perivascular fibrosis and interstitial fibrosis in the heart. The nonselective aldosterone antagonist spironolactone and the novel selective aldosterone receptor antagonist eplerenone are effective in preventing or reversing vascular and cardiac collagen deposition in experimental animals. Spironolactone treatment for patients with heart failure reduced circulating levels of procollagen type III N-terminal aminopeptide, indicating an antifibrotic effect. Spironolactone and the better-tolerated selective aldosterone receptor antagonist eplerenone are being used to treat patients with hypertension, heart failure, and acute myocardial infarction complicated by left ventricular dysfunction or heart failure because of their unique tissue protective effects.[133;134]

Evidence is accumulating that aldosterone excess may be a more common cause of or contributing factor to hypertension than previously thought. Hypokalaemia was thought to be a prerequisite of primary hyperaldosteronism, but it is now recognized that many patients with primary hyperaldosteronism may not manifest low serum potassium levels. Determining the ratio of the plasma aldosterone level to the plasma renin activity in a subject with untreated hypertension may be a more acceptable screening method for distinguishing patients with essential hypertension from those with primary aldosteronism. Accordingly, screening hypertensive patients for hyperaldosteronism has expanded and a higher prevalence of the disorder has been revealed. Prevalence rates between 8% and 32% have been reported on the basis of the patient population being screened (higher in referral practices, where the patient mix tends to be enriched with refractory hypertension and lower in family practices or community databases).[135]

### 1.1.6.9 Vascular Endothelial Dysfunction

Nitric oxide (NO) is a potent vasodilator, inhibitor of platelet adhesion and aggregation, and suppressor of migration and proliferation of vascular smooth-muscle cells. Nitric oxide is released by normal endothelial cells in response to various stimuli, including changes in blood pressure, shear stress, and pulsatile stretch, and plays an important role in blood pressure regulation, thrombosis, and atherosclerosis.[136] The cardiovascular system in healthy persons is exposed to continuous NO-dependent vasodilator tone, but NO-related vascular relaxation is diminished in hypertensive persons. The observation that in vivo delivery of superoxide dismutase (an enzyme that reduces superoxide to hydrogen peroxide) reduces blood pressure and restores NO bioactivity provides further evidence that oxidant stress contributes to the inactivation of NO and the development of

endothelial dysfunction in hypertensive models.[137;138] It has been suggested that angiotensin II enhances formation of the oxidant superoxide at concentrations that affect blood pressure minimally.[126] Increased oxidant stress and endothelial dysfunction may thus predispose to hypertension. This concept is subject to debate and ongoing investigation. It is clear, however, that antihypertensive drugs that interrupt the renin–angiotensin–aldosterone system, including ACE inhibitors, ARBs, and mineralocorticoid receptor antagonists, are effective in reversing endothelial dysfunction. This action may at least partly account for their cardioprotective effects.

### 1.1.6.10 Endothelin

Endothelin is a potent vasoactive peptide produced by endothelial cells that has both vasoconstrictor and vasodilator properties. Circulating endothelin levels are increased in some hypertensive patients, particularly African Americans and persons with transplant hypertension, endothelial tumours, and vasculitis.[139] Endothelin is secreted in an abluminal direction by endothelial cells and acts in a paracrine fashion on underlying smooth-muscle cells to cause vasoconstriction and elevate blood pressure without necessarily reaching increased levels in the systemic circulation. Endothelin receptor antagonists reduce blood pressure and peripheral vascular resistance in both normotensive persons and patients with mild to moderate essential hypertension,[140] supporting the interpretation that endothelin plays a role in the pathogenesis of hypertension. Development of this drug class for the indication of systemic hypertension has been discontinued because of toxicity (teratogenecity, testicular atrophy, and hepatotoxicity). However, endothelin antagonists are indicated for treating pulmonary hypertension[141] and may prove to be clinically useful in the therapy for other forms of vascular disease.

### *1.1.7 Implications for genetics*

The study of hypertension is complicated by the complexity of compensatory mechanisms and the difficulty in determining the initiating cause. For example, manoeuvres that initially cause volume overload and hypertension in the dog eventually lead, through autoregulation of tissue blood flow, to a state of increased peripheral resistance and near normal fluid volume.[65] Indeed, the brilliant work by Guyton and co-workers, identifying the kidney as the dominant, long-term regulator of blood pressure,[64;142] illustrates the need for an appreciation of pathophysiology when considering genetic factors potentially contributing to hypertension. Investigators continue to point out the inadequacies of simplistic Mendelian models in statistical genetics as applied to common disease and the need to consider pathophysiology, biochemistry, and molecular mechanisms in the effort to understand the effects of genetic variants in blood pressure regulation.[143;144]

Recent identification of the genetic basis of most rare, monogenic forms of hypertension and hypotension reiterates the central role of the kidney in long-term control of blood pressure.[73] Several interesting, unsuspected new drug targets have emerged as a result of these studies. These include the chloride channel in the basolateral membrane of epithelial cells of the thick ascending loop of Henle and, possibly, more effective drugs to inhibit the epithelial sodium channel (amiloride is of limited value because it competes with sodium and is hence of little value when it is most needed-during a high salt diet). Importantly, all the monogenic hypertension syndromes identified to date are caused by defects resulting in renal salt retention, whereas all the low blood pressure syndromes share a common mechanism of excess renal sodium loss. The absence of syndromes relating to the many short-term blood

pressure control systems (baroreceptors, α- or β- receptors, etc.) again emphasizes the dominant role of the kidney and its pressure-natriuresis function in long-term blood pressure control.

### *1.1.8 A unifying pathway for the development of hypertension*

A pathway was recently proposed that combines most of the existing hypotheses into one unifying schema.[145] According to this pathway, hypertension is often initiated by a hyperactive sympathetic nervous system (SNS), possibly related to familial, genetic, or environmental influences. Potential factors could include emotional stress, smoking, alcohol, medications, obesity, or baroreceptor dysfunction. Furthermore, activation of the RAS, through either elevations in angiotensinogen (from genetic polymorphisms or oral contraceptives), or increased renin levels (secondary to activation of the renal SNS, renal ischaemia, hypokalaemia, or other mechanisms), could lead to elevated levels of angiotensin II which also feed back to further stimulate the SNS.

In the early phase of hypertension, the BP elevations may be intermittent and the levels may only be in the high-normal range. Unlike normotensive patients, however, the BP variability would likely be greater and with higher and more frequent levels above normal. During this phase, sodium excretion by the kidney is relatively normal. In spite of the fact that norepinephrine and AII have sodium-retaining properties, the increases in BP caused by these agents result in a pressure natriuresis and lead to sodium loss and a normal or low blood volume state.

According to the hypothesis, the intermittent activation of the SNS and/or RAS eventually result in acquired injury to the kidney with an impairment in its ability to

excrete salt. Normally, the kidney responds to elevations in BP by a process of renal autoregulation. In this situation, the afferent arteriole and interlobular artery vasoconstrict secondary to a myogenic reflex (mediated by calcium channels) and by tubuloglomerular (TG) feedback in an attempt to prevent transmission of pressure distally to the sensitive structures in the glomerulus and peritubular capillaries. Presumably, this autoregulatory response explains why arteriolosclerosis of the afferent arteriole is the classic renal biopsy finding in patients with essential hypertension.

Indeed, arteriolosclerosis of the afferent arteriole was interpreted by Goldblatt and others as the primary disease of the renal arterioles that caused essential hypertension. This observation resulted from the fact that arteriosclerosis of this arteriole is both sensitive (observed in >95% of cases) and specific (observed in <30% in other organs) for essential hypertension. According to the above hypothesis, however, this vasoconstriction could be considered a protective, secondary response.

Over time, injury to the kidney could occur via one of two mechanisms. The first mechanism would be some transmission of pressure into the glomerulus caused by an imperfect renal autoregulatory response. Indeed, autoregulation is not immediate, so with sudden changes in systemic BP, some pressure is transmitted into the glomerulus. Certain regions of the kidney, such as the juxtamedullary region, may also have a relative defect in autoregulation. Furthermore, there is evidence for reduced renal autoregulation in conditions associated with a reduction in nephron number, in obesity, and in African Americans. Micropuncture studies have also confirmed that norepinephrine infusion can elevate glomerular and peritubular

capillary pressures in the rat.[146] The consequence is that some increase in pressure can be transmitted, where it could lead to glomerular damage (sclerosis) and tubulointerstitial injury. It is thus of interest that Bohle has described a population of hypertensive patients in which the renal biopsy shows a decompensated nephrosclerosis with glomerulosclerosis and tubulointerstitial damage.[147] Moreover, this pattern is similar to what is observed in hypertensive African Americans, in obesity, and with aging (all conditions associated with salt-sensitive hypertension).

The second mechanism by which injury to the kidney could occur is by ischaemia. As the afferent arteriole thickens in response to the acute elevations in BP, the arteriolar lumen becomes progressively smaller. Eventually, the pressure within the narrowed arteriole cannot support the tension in the afferent arteriole, and the arteriole collapses, leading to distal glomerular and peritubular ischaemia. In addition, the vasoactive properties of both norepinephrine and AII cause prominent vasoconstriction of the arterioles and vasa rectae, resulting in a decrease in blood flow to the peritubular capillaries. Evidence of tubular ischaemia can be found in the majority of biopsies of patients with essential hypertension.[148]

### *1.1.9 Summary*

The complexity of pathophysiologic mechanisms that lead to blood pressure elevation is such that selective, mechanistically based antihypertensive treatment is rarely possible in any hypertensive patient. Hypertension is highly prevalent among middle-aged and elderly persons in our population, and the success rate in controlling blood pressure in these individuals is poor. For example, in the ALLHAT trial,[149] 34% of participants did not achieve the goal blood pressure of less than 140/90 mm Hg despite use of combination therapy, including a β-blocker. Current treatment

guidelines generally recommend a generic approach to treating hypertension, with little emphasis on selecting therapy on the basis of the underlying pathophysiology of the elevated blood pressure.[150-152] With increased recognition of specific causes, it may be possible to develop therapies selective for distinct pathophysiologic mechanisms with fewer adverse effects, resulting in more effective blood pressure reduction.

Use of powerful new techniques of genetics, genomics, and proteomics, integrated with systems physiology and population studies, will make possible more selective and effective approaches to treating and even preventing hypertension in the coming decades.

The use of genetic investigations to identify underlying pathophysiological mechanisms will now be discussed.

## 1.2 Genetics of complex traits

The modern era of the study of genetics was initiated 135 years ago when the Bohemian monk Gregor Mendel published the results of his breeding experiments on the garden pea. In Mendel's time, the abounding theory of heredity postulated a 'blending' of the inherited contributions from the two parents. Mendel's work clearly showed that such blending did not occur, and led to his conclusion of particulate inheritance (the 'gene') and rules of segregation. The relevance of Mendel's work for human traits was first delineated around the turn of the century by Garrod, who reasoned that similar types of transmission rules explained the 'inborn errors of metabolism' typically caused by enzyme deficiencies. At the same time, however, there was another school of thought, primarily emanating from statisticians such as Francis Galton and his student, Karl Pearson. They observed family resemblance for a variety of traits such as anthropometric features and intellectual achievement but they could not discern patterns of inheritance in families that were consistent with mendelian laws. Rather, a 'blending'-type theory seemed more apt, as children's phenotypes tended to be, on average, midway between the parents, with some variability. The resolution of this dilemma did not appear until 1918, when Ronald Fisher published his seminal paper describing 'polygenic' inheritance. Fisher reconciled the two conflicting schools by recognizing that the critical difference lay in the genetic basis for the variation in the trait being studied.

For the traits Mendel studied, the observed variation was due to a simple difference at a single gene (or locus). On the other hand, for the traits studied by the biometrical school, individual differences were not attributable to different alleles at a single locus. Rather, many different genes, each with allelic variations, contributed to the

total observed variability in a trait, with no particular gene having a singly large effect. Thus, an individual phenotype results from the sum total of the effects of all the numerous contributing loci. Furthermore, application of the central limit theorem implicates a continuous normal distribution in the population for such a trait, similar to what is observed. Thus, the lack of mendelian inheritance patterns for numerous human traits did not require the deconstruction of Mendel's theory, but rather an extension of it to a more complex scenario that related genes to phenotype. It is clear that Mendel's success hinged entirely on his selection of single-gene traits, for otherwise the simple rules of inheritance would not have revealed themselves.

### *1.2.1 The genetic basis of human traits and disease*

In human populations, the most common medical disorders with a genetic component are complex traits, which are traits influenced by multiple genetic and environmental factors - including hypertension, coronary artery disease, asthma, schizophrenia. This complexity contrasts with the inheritance pattern of monogenic disorders, in which the presence or absence of disease alleles usually completely predicts the presence or absence of disease (although the severity or age of onset may vary). For genetically complex diseases, risk alleles are less deterministic and more probabilistic—the presence of a high-risk allele may only mildly increase the chance of disease. Furthermore, it has been proposed that these weakly penetrant alleles may be present at high frequency (>1%) in the population.[153;154]

Complex traits are often called quantitative traits to distinguish them from traits that appear in discrete categories. Typically each of the multiple genes underlying a complex trait has the feature that the mean difference in phenotype between

alternative genotypes is relatively small in comparison with the total variance in phenotype in the population.

The normal distribution occupies such a prominent place in the study of complex traits because of a statistical theorem called the central limit theorem, which states that the summation of many random independent quantities conforms to the normal distribution. Since complex phenotypes are determined by multiple genetic and environmental factors, a normal distribution is to be expected if the factors are independent and their effects additive. This is the fundamental precept of biometrical genetics. (*Chapter 10 -Appendix - Quantitative Genetics*)

The widespread presence of high frequency variants in humans was first shown experimentally by Harris among others,[155;156] who found that many proteins have several common, heritable isoforms, thereby demonstrating that common genetic variation could lead to variation in protein structure. The widespread presence of such variation suggested that common variants might be biologically important. Unfortunately, tests of this hypothesis were limited to proteins for which common functional variation could be easily assayed (primarily a few enzymes and determinants of blood group antigens).

The advent of gene cloning and sequencing substantially lowered this technical hurdle. It became possible to easily detect DNA variants in a given gene. The first genetic variants tested were usually restriction fragment length polymorphisms (RFLPs), but with the development of the polymerase chain reaction (PCR) and other improvements in technology, microsatellites, variable number tandem repeats

(VNTRs), insertion/deletion polymorphisms, and single nucleotide polymorphisms (SNPs) could all be analysed.

By determining the genotype of these variants in individuals with disease and in unaffected controls, these polymorphisms could be tested for association with susceptibility to a variety of diseases. The usual conclusion of such studies is that the polymorphism being tested either affects risk of disease directly or is a marker for some nearby genetic variant that affects risk of disease (*Section 1.2.2.2 Population Association Studies, page 77*).

These association studies were further facilitated by the increasingly rapid discovery of common polymorphisms in genes, accomplished by resequencing the same stretch of DNA in multiple individuals. One of the goals of the human genome project has been to identify large numbers of SNPs; indeed, the number of SNPs in public databases is now well over 1,000,000.[157] Association studies have already identified over 600 potential associations between common genetic variants and susceptibility to common disease.

So far, we still have a view that primarily reflects the Mendelist–biometricist dialogue of nearly a century ago. Most human disorders that have been genetically characterized are Mendelian, essentially because the extant molecular tools have enabled the identification of these genes by positional cloning, a procedure now described as 'routine'. By contrast, those disorders or traits for which such approaches have failed are depicted as 'polygenic', multifactorial or 'complex'. Often unwilling to

cede to a notion of 'infinite' genetic complexity, geneticists refer to these cases as 'oligogenic' or 'multigenic', implicating a tractable degree of complexity.

If the genetic variation that contributes to a trait is due to myriad genes, each of modest effect, the task of identifying those individual contributors becomes monumental. However, gene effects typically come in different sizes, even when there are many of them — at least, this has been the lesson from a lengthy history of model systems. There are several measures of gene effects used by geneticists. Many human traits, especially disease outcomes, show family recurrence patterns that are strongly suggestive of interactions between genes or epistasis, implying the existence of multiple, interacting loci. (Appendix - Quantitative Genetics)

### *1.2.2 Strategies to determine the genetic component of disease*

#### 1.2.2.1 Linkage analysis

The situation of gene discovery in humans changed markedly two decades ago when it was recognized that variations in human DNA could be assayed directly and used as genetic markers in linkage studies where a relatively small chromosome region containing the gene of interest is identified through the analysis of co-segregation of genetic markers with disease in families.[158] Before this time, human geneticists performing linkage studies to identify the chromosomal location of disease genes relied on only a handful of blood group and serum protein markers with few successes. The identification of restriction-fragment length polymorphism (RLFP) markers[158] and subsequently abundant highly polymorphic microsatellite (short tandemly repetitive DNA) loci[159;160] has led to the mapping of myriad mendelian disease loci. Development of more efficient molecular tools, especially high-

throughput DNA sequencing, has enabled the identification of disease loci and their mutations by a process characterized as positional cloning. Naturally occurring mutations are identified on the basis of their chromosomal location by taking advantage of the meiotic process of recombination as manifest in families segregating for the disease. Markers closest to the disease gene show the strongest correlation with disease patterns in families, and typically the tracking of recombination events can narrow the region harbouring a disease gene to between 100 and several thousand kilobases.

The remarkable success of positional cloning rests not simply on the advances observed in molecular technology. It also reflects the enormous power of linkage analysis when applied to mendelian phenotypes — that is, those characterized by a (near) one-to-one correspondence between genotypes at a single locus and the observed phenotype. In terms of biometrical genetics, these are loci with very high displacement (Section 10.1.1 Displacement). The observed phenotype corresponds precisely to the underlying genotype with little if any misclassification. The robustness of linkage analysis applied to mendelian traits can be seen by its historic low false-positive rate[161] when the stringent lod-score threshold of 3 suggested by Morton[162] is used (corresponding to a $P$ value of $10^{-3}$ for a sequential test or $10^{-4}$ for a fixed sample-size test[163]).

For common multifactorial disorders, linkage studies are also undertaken, often in nuclear families and sometimes with the inclusion of additional close relatives such as aunts, uncles or cousins. Such families are relatively easy to collect, and they minimise the genetic heterogeneity that might be caused from multiple entries of

susceptibility alleles in more extended pedigrees. Conceptually, the simplest situation arises when studying pairs of affected siblings. The methodology for analysing affected sib-pair data is well established and has been used extensively first starting in the 1970s to investigate linkage between autoimmune disease and HLA antigen.[164-166]

#### 1.2.2.1.1 Affected sib pair analysis

In contrast to traditional lod score analysis, which assumes a genetic model for familial resemblance of a trait, the affected sib-pair method is 'non-parametric' in that no explicit assumptions are made about the mode of inheritance. Rather than assaying for recombination events between disease and marker, the tests of linkage are based on whether affected sib pairs share more than the expected number of marker alleles identical-by-descent (IBD) – two alleles are IBD if they are inherited from the same ancestor. The observed IBD distribution is compared to the expectation under independent segregation of the marker and trait. For independent segregation, each parental allele is transmitted with equal probability, and siblings inherit the same marker alleles 50% of the time independent of affection status. When the data consists of independent affected sib pairs (i.e. each from a different family) with complete information on marker inheritance, the test for linkage can be made by a simple chi square test comparing observed and expected IBD. In practice, a wide variety of approaches has been proposed for affected sib pair tests of linkage.[167] These utilise different methods for taking into account multiple affected siblings within a family, incomplete IBD information, different assumptions about the probabilities of IBD sharing in the presence of linkage, and different statistics for evaluating linkage. Overall one of the best performances for type I and II statistical error is obtained by analysing affected sib pair families as if they were segregating for a rare recessive disease with complete penetrance, while treating unaffected siblings and parents as

having unknown disease phenotype, irrespective of true disease status. In this approach, the test statistic is simply the maximum logarithm of odds ratio (lod) score (MLS) found by estimating a recombination rate between the marker and the 'disease' locus (also called a 'pseudomarker').[168] However, here the recombination rate has no biological significance; it is simply a parameter that adjusts the IBD probabilities for affected siblings. Aside from its robust behaviour, the pseudo-markers approach can be implemented easily in many of the existing programs for classical linkage analysis. Also for this approach, there is no need to decide about weights for sibships with different numbers of affected offspring, as this is accounted for in the linkage analysis.

Whatever method is applied, the evidence for linkage is most often assessed by the MLS statistic, which is analogous to a classical lod score in that its value is the $\log_{10}$ of the odds in favour of linkage. Traditionally, a MLS of 3.0 (odds of 1000:1) in favour of linkage has been assumed at the threshold for declaring significant linkage for monogenetic disorders, in part because this corresponds to a genome-wide significance level, taking into account the testing of multiple markers throughout the whole genome, of about p=0.05.[169] For complex diseases, it has become standard practice to designate regions of potential linkages with less stringent criteria of significance. The idea is that if one is carrying out a genome-wide search, then it is appropriate to use a lower threshold (a MLS of ~2.0) to indicate which areas of the genome should be investigated more thoroughly by typing more markers in the area and/or adding more sib pairs to the original study, or attempting replication in a different study.[170]

A number of practical issues arise when undertaking affected sib-pair analysis. First, it is very important to genotype parents or unaffected siblings if possible. If parental genotypes are completely or partially absent, marker allele frequencies may have a profound effect on the test statistic; for example, as the IBD probability for related individuals that share a single rare allele is high, erroneously assuming that all marker alleles are equally frequent will generally inflate the test statistic in favour of linkage, if one or more of the alleles is more common in the general population. Therefore, marker frequencies should be estimated either from the data or from a matched control population prior to linkage analysis. A second common issue in applying is the verification of putative relationships prior to linkage analysis. In general, any undetected nonpaternity or other pedigree errors reduces the power to detect linkage. There is a high chance of errors being detected if parents or three or more siblings have been genotyped. However, for sib pairs, an erroneous relationship cannot be detected with certainty, unless genotypes on parents or other relatives are available, and a statistical framework must be applied to the total marker data in order to evaluate if the relationship is correct or if the putative siblings are half sibs or unrelated.

It is common practice to apply multilocus and single locus techniques for affected sib pair linkage analysis. Multilocus analysis overcomes to some extent lack of complete knowledge on IBD when parental genotypes are unknown, or when individual markers are not fully informative. Because multilocus methods are sensitive to genotype errors, and different methods have varying power to detect linkage, it is desirable to apply both single marker locus and multiple marker locus to all data sets.[171-174]

#### 1.2.2.1.2 Linkage Disequilibrium in Linkage studies

When marker and disease loci are very close together on a chromosome, genetic crossing over will have occurred at such a low rate that the marker will appear to cosegregate with the gene regardless of the family studied. This is in contrast to the situation of two loci further apart but still linked, in which case repeated crossing over will allow all possible combinations of chromosomal haplotypes to appear with frequencies as predicted by the Hardy-Weinberg equilibrium. Thus linkage disequilibrium can be very useful in defining the ancestral haplotype of a disease gene in relation to several marker loci; it can be used for fine-mapping of the disease gene even when complete linkage is established in families being studied.

There are many measures of linkage disequilibrium. The most commonly used is the disequilibrium coefficient *D*.

$$D=P_{11} - p_1q_1$$

Where $P_{11}$ is the observed frequency of the *1/1* haplotype, $p_1$ is the frequency of the *1* allele at locus *1* in the general population and $q_1$ is the population frequency of the *1* allele at locus *2*. Generally the *1* allele at each locus is defined as the most common of the alleles at that locus. Because *1* is assigned as the most common allele, the coefficient *D* ranges from –0.25 to 0.25. Positive values of *D* indicate that the common alleles at each locus segregate together. Negative values indicate that the common allele at one locus segregates with the rare allele at the other locus. The rate of decay of linkage disequilibrium is dependent on the distance between loci:

$$D_t= D_0(1-\theta)^t$$

Where $t$ is the current generation number, $D_t$ is the current amount of disequilibrium , $D_0$ is the disequilibrium at generation $0$, and $\theta$ is the recombination fraction between loci.

Allelic association due to population admixture, selection or genetic drift between unlinked loci will decay fairly rapidly in comparison to linkage disequilibrium between tightly linked genetic loci and thus is a short-term phenomenon that will be almost impossible to detect in a typical study. However linkage disequilibrium will decay rather slowly, dependent primarily on the recombination distance between markers and the number of generations that has passed since the initial event. The slowness of linkage disequilibrium decay makes this a useful mapping tool.

The general rule of the thumb is that the stronger the disequilibrium, the closer the marker is to the disease locus. This is not always the case however, for several reasons. First the frequencies of the marker alleles have an impact on the power to detect linkage disequilibrium. Mutation rates at the marker locus also affect disequilibrium by increasing the chance that the associated marker allele will change and so seem to be representing a different chromosome.

Population bottlenecks, where the effective population size reduced to a very small number for a period of time before the population size increases again, can create or reinforce an existing association. This is done by the random loss to the genome pool of most chromosomes carrying the susceptibility allele; what remains may only existed in only one individual who survived the bottleneck. Chance loss of susceptibility-allele-bearing chromosome (random genetic drift) can also generate

linkage disequilibrium. Two phenomena that can complicate the analysis of allelic association are selection in favour of a particular phenotype and new mutations arising in the population.

#### 1.2.2.1.3 Non-Mendelian Inheritance

As noted above, linkage analysis and positional cloning have had a remarkable track record in leading to the identification of the genes for many mendelian diseases, all within the time span of the past two decades. Several of these genes account for an uncommon subset of generally more common disorders such as breast cancer (BRCA-1 and -2), colon cancer (familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC)), Alzheimer's disease (β-amyloid precursor protein (APP) and presenilin-1 and -2) and diabetes (maturity-onset diabetes of youth (MODY)-1, -2 and -3). These successes have generated a strong sense of optimism in the genetics community that the same approach holds great promise for identifying genes for a range of common, familial disorders, including those without clear mendelian inheritance patterns. But so far the promise has largely been unfulfilled, as numerous such diseases have proven refractive to positional cloning.

So far, all genes first identified by linkage analysis and subsequently positionally cloned are those with low allele frequency and high displacement (mendelian or near mendelian inheritance) i.e. with large effect and strong genotype–phenotype correlations. By contrast, no genes with moderate or modest displacement, even for rare disorders, have been identified in this way. The literature is now replete with linkage screens for an array of 'complex' disorders such as schizophrenia, manic-depression, autism, asthma, type 1 and type 2 diabetes, multiple sclerosis and lupus, to name but a few. Although many of these studies have reported significant linkage

findings, none has led to convincing replication. Typically, independent studies of the same disorder identify maximal evidence at different chromosomal locations. In effect, linkage analysis, traditionally the most reliable of genetic methods when applied to mendelian traits, has proven to be much less reliable a tool for the study of non-mendelian diseases, with a disappointingly high false-positive rate. The likely explanation is that the biometrical view is closer to reality than the mendelian view for most human traits and diseases.

This does not necessarily mean that no genes underlying non-mendelian traits can be located by linkage analysis. There are several examples of common alleles that have sufficiently large displacement to have been detected by linkage analysis. One example is the role of HLA in type 1 diabetes, where allele sharing by affected sib pairs (ASPs) has been estimated at about 73%.[175] A second example is the role of apolipoprotein E (ApoE) in late-onset Alzheimer's disease, where the ASP allele sharing is estimated at about 60%. Other examples probably exist but have yet to be identified, although the number is likely to be few. Increasing sample sizes may ultimately improve the odds, but there is clearly a limit. In addition, studying more extreme (and less frequent) phenotypes is helpful provided such cases are also genetically more extreme. However, gene effects with displacements of less than 1 standard deviation (s.d.), which are likely to represent most effects, will rarely be identified this way.

Another question relates to whether larger families with many affected individuals would provide better power than smaller families, such as sib pairs. The answer depends on the frequency of the susceptibility allele. For high-frequency alleles,

selection of dense families is likely to increase parental homozygosity at the disease locus and reduce linkage evidence. On the other hand, for rare alleles with large displacement, dense families are usually optimal, because the probability for such a family to be segregating the allele is increased, enhancing the linkage evidence. However, if genome screens of extended pedigrees have been conducted without success, it is reasonable to conclude that rare genes of large effect are unlikely to exist for the trait studied.

### 1.2.2.2 Population Association Studies

An alternative to linkage and allele-sharing methods is association-based analysis. Rather than relying on cosegregation of a susceptibility gene and linked markers within families, association studies looks for significantly increased or decreased frequency of a marker allele with a disease trait and represents deviations from the random occurrence of the alleles with respect to disease phenotype. Allelic association can be due to either direct influence of a DNA variant on phenotype, or from linkage disequilibrium creating combinations of alleles that occur more frequently than would be expected under random association. That is, such studies seek to detect linkage disequilibrium due to linkage between a marker locus and a locus bearing a disease-causing mutation. Until recently, association studies have generally been candidate gene studies. This involves the testing of populations or groups to determine whether a specific allele occurs more frequently in individuals with a disease than in those without a disease. Although these associations must be interpreted with caution, they raise the possibility of a cause/effect relationship between the associated alleles or closely linked loci and the disease. Association studies can play a critical role in the analysis of genetically complex traits, in the evaluation candidate gene loci as well as in the fine-mapping of a region once linkage

studies have indicated a region of interest in the follow-up analysis. The advent of technologies that allow rapid discovery of genetic variants, such as SNPs, has greatly expanded the potential for such investigations. Considerable attention has focussed recently on the scope and feasibility for both genome-wide association and linkage studies using SNPs as a source of markers.[176]

Conceptually, linkage disequilibrium is the same as the standard linkage analysis, except that the recombination distances being measured now are very small (generally < 1cM), and the recombination events can only be inferred based on the level of sharing of the same allele. Although it is easy to find situations in which association will have greater power than linkage to detect susceptibility loci (for example, when the susceptibility is due to a frequent allelic variant that is assayed directly), in practice the relative power depends on assumptions, such as the number of susceptibility variants at a locus, the magnitude of their linkage disequilibrium with other markers, and the comparative frequencies of the marker and the susceptibility variants. Population substructure most often occurs with the recent admixture of populations. In the case of population substructure, alleles may show a statistical association simply by chance due to differences in allele frequencies in the two missing populations. This can occur even when there is no biological association or true genetic linkage. It is more realistic to think of linkage and association as complementary approaches; indeed, association studies are often attempted as an approach to isolate a susceptibility gene in a region that has been identified by linkage.

Association studies are typically conducted using the candidate gene approach. Given the multitude of factors that are involved in the regulation of blood pressure, a seemingly unlimited supply of candidate genes for hypertension can be imagined. When conducting association studies, one must be careful that the subjects with the disease of interest and the control subjects are closely matched with respect to ethnic, geographic and social backgrounds. If not, one might observe differences in allele frequencies between the two groups that have no causal relationships to the disease state.

#### 1.2.2.2.1 Case–control studies

The approach often used for such studies is the case–control design, in which a difference in allele frequency is sought between affected individuals and unrelated unaffected controls. This design does not require the ascertainment of family units and hence has appeal due to the relative ease of implementation.

The control populations should be matched with respect to ethnicity as well as other factors such as age. Spurious associations can result because of population stratification (the existence of multiple population subtypes in what is assumed to be a relatively homogeneous population). Such stratification can represent either recent admixture or the incorrect matching of cases and controls. The existence of these confounding factors can lead to a significant result even in unlinked loci or unassociated loci within stratum.

From an epidemiological perspective, a major limitation in this approach is the potential for confounding (that is, spurious association resulting from correlation with the true risk factor) leading to artefactual as opposed to causal associations. In this

case, the most likely source of confounding is ethnicity, whereby allele frequencies vary by ethnicity and cases and controls are not adequately matched in terms of ethnicity. Although most investigators would at least attempt coarse matching by major demographic groupings (such as race), substratification within racial groups can still lead to bias. This drawback of traditional case–control designs was recognized early on by Lionel Penrose, who recommended the use of unaffected sibs as controls. This paradigm, originally applied to ABO and duodenal ulcer[177], has seen a resurgence in the past few years.[178-181] The disadvantage of this design is that sib controls are over-matched to the index cases, leading to a loss of power compared with a well-designed study involving unrelated controls.[179]

This problem can be addressed by using 'family based controls' as popularised by the transmission disequilibrium test (TDT). [182]

#### 1.2.2.2.2 Family based association study

Family based studies control for the possibility of genetic differences between the case and control populations by comparing the frequencies of alleles transmitted to the affected child to the alleles not transmitted. The only samples needed are those from the affected individual and his or her two parents (the TDT triad). This approach eliminates the concern that population substructure may be the cause of the association. These studies include the transmission disequilibrium test (TDT), the haplotype relative risk test (HRR) and the affected family based control method (AFBAC). The TDT approach tests for linkage in the presence of association. The AFBAC method is designed to detect association in the presence of linkage. Both AFBAC and TDT have little power unless linkage and association coexist. The difference between these two methods is that the TDT can also function as a test of

association in the presence of population admixture and can be used as a valid test of linkage.

#### *1.2.2.2.2.1 Transmission Disequilibrium Test (TDT)*

This test is based on the premise that a parent who is heterozygous with one associated allele and one non-associated allele at the locus under study will transmit the associated allele to the affected offspring more often than expected by chance. The TDT in effect uses the nontransmitted allele as controls, which are well matched for the ancestry of the parent. Stratification is reduced or eliminated as a single individual (the parent) contributes both the control and the disease alleles for this observation.

Transmission Disequilibrium Test (TDT) [182] tests for evidence of both linkage and association (linkage disequilibrium) in triplets of father-mother-affected child – the TDT triad. Here linkage disequilibrium is defined as an increase in frequency of a particular disease-marker haplotype over that expected based on Hardy-Weinberg equilibrium, and it requires both linkage of the disease and marker loci and association between one or more alleles at the locus and the disease allele. This frequency is compared with the Mendelian expectation of 50:50 transmission of the allele. More specifically strong evidence for linkage is detected by examining only the meiosis in heterozygous parents, for which affected offspring exhibited IBD significantly greater than the expectation of 0.5 under the absence of linkage. The disadvantage of this test is that TDT has no power to detect linkage if association is not present. When considering only parent-offspring trios, TDT is similar to Haplotype Relative Risk (HRR). However when applied to affected siblings, TDT provides a test of linkage, not of association. TDT (like other family-based methods) is immune to false-positives from ethnic admixture.[182]

Disadvantages of the TDT are that it is less efficient than a standard case-control study – the primary concerns being power and efficiency.[183] Family-based samples are often difficult to collect and that 50% more genotyping is required than in case-control studies to achieve similar power (the exact loss of power depends on the underlying genetic model). For instance, when parent-child pairs are used for TDT, each informative parent-child pair provides two observed alleles (one transmitted and one non-transmitted), while in a case-control study, each fully informative case control pair provides four alleles for observation. One strategy to balance the concerns of efficiency and potential admixture is to use multiplex sibships and include normal sibs within a TDT study. [183] However, this strategy brings the additional concern that 'normal' sibs may not be through the risk period are subject to censoring.

#### *1.2.2.2.2.2 Likelihood ratio*

In general, it would be desirable to provide an integrated approach to model free analysis that would allow simultaneous evaluation of linkage and association while incorporating data from different types of study designs such as affected sib pairs, parent-offspring and case-control cohorts. Such an approach involves an extension of the pseudomarker linkage test described for the affected sib-pair test.[171-174] The linkage component can be evaluated by calculating a lod score for a disease locus with two alleles, although the test itself is not model dependent. Association is incorporated by allowing for linkage disequilibrium between the marker and the alleles (denoted *D* and *d*) at the putative disease locus. For a marker locus with *n* alleles, under the hypothesis of association and linkage ($H_2$), the likelihood is maximised over the recombination rate, the frequencies of *n*-1 haplotypes associated with *D* and the frequencies of n-1 haplotypes associated with *d* (2*n*-1 parameters). For

the hypothesis of linkage without association ($H_1$), the likelihood is maximised over the recombination rate and the marker allele frequencies, assuming linkage equilibrium with $D$ and $d$ which have fixed allele frequencies (a total of $n$ parameters). Finally, under the hypothesis of absence of linkage ($H_0$), the likelihood is maximised over the marker allele frequencies only, with recombination fixed at 0.5 ($n$-1 parameters).

The likelihood ratio statistics to test for linkage and association are $-2ln(L_1/L_0)$ and $-2ln(L_2/L_1)$ respectively, where $L_i$ is the likelihood under the $i$th hypothesis. The significance of these test statistics can be judged by comparison to chi-square distributions with $n$-1 and 1 degrees of freedom respectively.

#### 1.2.2.2.3 Problems with association studies

Conventional case–control gene-association studies have a long track record of false-positive results. The high false-positive rate has often been attributed to confounding ethnic admixture resulting in population stratification, variable linkage disequilibrium between the polymorphism being studied and the true causal variant, and population-specific gene-gene or gene-environment interactions.[184;185] It is more likely that the high false-positive rate may result from a low prior probability that the few gene polymorphisms examined are in fact causally related to the disease outcomes studied.

One possibility for the irreproducibility that characterizes the vast majority of association studies is that the original observations represent statistical fluctuations (type I error). If this were the case, one would predict that only 5% of subsequent studies would also reach statistical significance with $P < 0.05$, and most associations would never be observed again. However Hirschborn et al[186] found in 166

associations at least 97 were observed again, many of them multiple times. Thus in the absence of a massive publication bias, statistical fluctuation is unlikely to explain all of the initial positive reports.

Another concern is that association studies may be unable to detect disease genes is multiple disease-causing mutations, which arose independently on different haplotype backgrounds, are represented in the sample under study. Studying association in young genetically isolated populations is a strategy to address this. For such populations, linkage disequilibrium is expected to extend over greater distances, and there are likely to be fewer distinct disease-causing alleles present, than in older, outbred populations.

#### *1.2.2.2.3.1 Population stratification*

This occurs when the cases and controls are unintentionally drawn from two or more ethnic groups or subgroups. If one of these subgroups has higher disease prevalence than the others, stratification occurs, because that subgroup will be over represented in the cases and underrepresented in the controls. Any polymorphism that genetically marks the high-risk subgroup (i.e., is found by chance at a higher frequency in that subgroup), therefore, will appear to be associated with and will likely be a false positive. Interestingly, the frequencies of several of the alleles vary substantially between populations, consistent with the possibility of false associations due to ethnic admixture. It should be noted that well-defined subgroups are not necessary to observe stratification; stratification can also occur in a single admixed population where the individuals have varying degrees of genetic contributions from two or more ethnic groups. Even apparently homogeneous, isolated populations (such as Iceland) are in theory susceptible to admixture if there have been multiple distinct waves of

migration from different source populations (e.g., Celtic and Norse, in the case of Iceland).

A few approaches can be used to avoid this problem. First, one can use family-based studies such as the transmission disequilibrium test (TDT).[182] Another possibility is to study multiple case-control populations, each from different ethnic groups, and require that an association be seen in each population. Another approach is by Haplotype Relative Risk (HRR), where a comparison is made between alleles that were or were not transmitted to affected offspring from their parents. HRR ensures that the control and case population are well-matched.[187] Finally, an approach to detect and correct for stratification has been proposed: by typing several dozen random markers, one can empirically determine the degree of stratification in a case control study.[188-190] If significant stratification is detected, one can use these markers to more carefully match cases and controls to remove the effects of stratification.[190] There is some debate as to whether stratification is a significant problem; some authors believe that even minimal ethnic matching of cases and controls is adequate to prevent stratification.[183] However, there are as yet no empirical data that address the degree of stratification found in a typical association study.

#### *1.2.2.2.3.2 Linkage disequilibrium*

Failure of replication can also occur if the polymorphism being tested is not itself the causal variant but is rather in linkage disequilibrium with the causal variant. Linkage disequilibrium, in which nearby variants are correlated with each other more often than expected by chance, depends heavily on population history and on the genetic make-up of the founders of that population. If all examples of a particular stretch of DNA in a population derive from a recent common ancestor, there will have been few

opportunities for recombination events to separate variants within that stretch of DNA and the variants will often be inherited together throughout the population. If, in a different population, the time since a common ancestor is longer, more recombination events will have occurred, disrupting linkage disequilibrium in the region. Furthermore, the particular arrangement of variants in the founders of a population will determine which variants are inherited together. Thus, it is possible that a polymorphism will be in linkage disequilibrium with a nearby disease allele in one population but not in another, leading to variable results of association studies. For example, many of the associations with TNF associations with nearby HLA loci (HLA is a region with strong linkage disequilibrium over large distances).[186] To explore this possibility, positive associations should be followed up by testing adjacent markers (both individually and as multi-marker haplotypes). If linkage disequilibrium is present (and particularly if any of the haplotypes or adjacent markers show stronger association), the possibility exists that the original marker tested is not the causal allele, and further studies of the region are warranted. Although it should be possible to exhaustively test modest sized regions of linkage disequilibrium, special circumstances (e.g., recently admixed populations) may in theory give rise to correlation between markers at much greater distances.

#### *1.2.2.2.3.3 Gene-gene and gene-environment interactions*

Another potential source of variable findings is gene-gene or gene-environment interactions that differ between populations. For example, if the effect of a variant were only manifest in populations with a particular genetic or environmental background, then association would only be seen in populations or subgroups with the appropriate genetic or environmental characteristics. This explanation is commonly invoked to explain differing results of association studies but is less frequently

supported by direct evidence. A further problem arises when considering gene-gene or gene-environment interactions: when combinations of alleles and/or environmental factors are studied, P values are rarely corrected for the number of tests reported (much less the number of tests actually performed). Such "nominally" significant results must be considered to be the product of hypothesis generation rather than hypothesis testing and, therefore, require replication. Perhaps the best possible method of demonstrating that a gene-environment interaction is likely to be correct (and not a statistical fluctuation expected when exploring numerous hypotheses) is to divide the study population randomly into two parts and require that any findings be observed in both parts of the study. Sample sizes need to be increased slightly to maintain power, but the ability to generate and then test hypotheses in the same sample would seem to outweigh this consideration. Otherwise, one requires a replication population that is exactly matched for environmental and genetic background, an extremely unlikely scenario.[186]

#### *1.2.2.2.3.4 Weak genetic effects and lack of power*

Finally, associations can be real but nonetheless not reproducible if the underlying genetic effect is weak. If the subsequent studies are small in size, they will be underpowered to reliably detect weak effects and, therefore, fail to achieve statistical significance. This difficulty is heightened by the "jackpot" effect, in which the first group to publish a significant association involving a weak locus is more likely to have overestimated than underestimated the true effect of the polymorphism. This phenomenon occurs because each study imprecisely estimates the strength of the effect (due to sampling variation). Because a weak effect would in most cases not provide a statistically significant finding in a typically sized study (a few hundred cases and controls), the first published study that does manage to achieve statistical

significance is almost certain to have overestimated the true effect of the variant being tested. Subsequent studies thus need to include much larger numbers of patients to achieve statistical significance. In particular, failure to observe the magnitude of effect seen in the first study should not be taken as a repudiation of the association. This phenomenon is observed for the association of type 2 diabetes and a Pro12Ala polymorphism in the PPARγ gene, where an initial study estimated the effect on diabetes risk to be threefold,[191] but subsequent studies observed very modest risks that usually did not achieve statistical significance.[192;193] Indeed, all of the previous studies, both positive and negative, were consistent with a 1.25-fold effect, and two subsequent large studies confirmed this association.[194;195] Because many alleles may have similarly weak genetic effects, large studies and/or meta-analyses of multiple studies will often be required to determine whether genetic associations between polymorphisms and disease are significant.

Lohmueller et al.[196] propose three general recommendations. First, in light of the seemingly high proportion of false positive reports in the literature, more stringent criteria for interpreting association studies are needed. A single, nominally significant association should be viewed as tentative until it has been independently replicated at least once and preferably twice. A review of 25 associations[196] suggests that two studies with $P < 0.01$ or a single study (other than the first positive) with $P < 0.001$ is strongly predictive of future replication.

Second, large studies should be encouraged, with collaborative efforts probably required to achieve the sample size of many thousands of case–control pairs that is necessary for definitive studies of common variants with modest genetic effects. Even

larger samples will be required to detect gene–gene or gene–environment interactions or associations specific to defined subgroups or to correct for testing association to multiple phenotypes. To help increase the effective sample size, reports of association would ideally include a meta-analysis of all available published data to give a more robust estimate of the genetic effect. To facilitate such meta-analyses and minimize publication bias, all disease association studies that meet minimal quality standards should be published. Such standards could include explicit phenotype definitions, complete listing of all phenotypes analysed, precise localization of the polymorphism(s), low genotyping error rate, analysis that avoids overlap with previous studies and availability of genotype counts for cases and controls (or equivalent data for family-based studies). Publication of non-significant results could be encouraged using forums such as a common association study database or 'brief reports' and 'negative results' sections of specialty journals, with sufficient credit to provide incentives for publication.

Finally, it seems likely that a fraction (perhaps a quarter) of previously published associations represent real associations with common disease. Thus, using large samples to test all previously reported associations, perhaps focusing initially on those associations that have already been replicated at least once, would probably identify a significant number of variants that affect the risk of common disease.[196]

### *1.2.3 Quantitative traits versus Qualitative traits*

Quantitative traits have several advantages in genetic studies compared to a simple classification based on the presence or absence of disease. (Appendix - Quantitative Genetics) Many diseases can be defined in terms of an underlying quantitative liability scale, such as blood pressure for hypertension. Such traits provide a measure

of disease severity, but can also be studied in unaffected family members or in individuals with extreme values of the phenotype, but who are not clinically diagnosed with the disease. For example, young adults who have extreme blood pressure for their age and sex may be useful in linkage analysis even if they may not yet have been diagnosed with hypertension. Another advantage is that a specific quantitative trait may be influenced by a small number of factors, whereas a disease classification often represents a genetically and environmentally more heterogeneous phenotype. In these instances, the quantitative variable will be correlated more strongly with polymorphisms of an underlying gene, and therefore provide a better phenotype both for initial mapping purposes, but also for identifying the causative susceptibility variants at a locus.

A sampling strategy using discordant sib-pairs in the opposite extremes of the distribution of a quantitative trait can in theory reduce dramatically the number of sibling pairs of families that need to be genotyped in order to obtain significant evidence of linkage.[197] Direct linkage analysis of the quantitative variable is an alternative to its transformation into a qualitative phenotype. Selection of families in which some individuals are towards the extremes of the quantitative distribution is still necessary to achieve power. For unselected families, the number of families necessary to detect even loci accounting for a high proportion trait variance, say 10-20% is several thousand.[198]

Several methods have been developed that involve variance component analysis without incorporating a parametric model for the hypothesised major gene.[199;200] Essentially these methods involve first obtaining estimates of IBD statistics for

relative pairs, and then estimating correlations between relatives that are a function of IBD. Although this requires no hypothesis about the number of alleles at the major gene locus, the estimates and test statistics are usually obtained under the assumption of a multivariate normal distribution of the trait within families. Care is needed as multivariate normality can be violated by a number of factors in particular by ascertainment on the trait locus.

A popular model-free framework for sib-pair linkage analysis of quantitative traits is based on the Haseman-Elston approach.[201] This consists of regression of the square of the trait difference on the IBD at the marker locus for sibling pairs; the slope of the regression line is expected to be zero in the absence of linkage, whereas it will be negative in the presence of linkage (because siblings are then more similar in phenotype when they have inherited the same alleles from their parents. A nonparametric approach based on the relationship between IBD and the ranks of sib phenotype differences, rather than the squared difference has been described by Kruglyak and Lander.[202]

A related area that has stimulated extensive methodological investigations recently is linkage disequilibrium mapping with quantitative trait loci. This includes methods that examine the direct relationships between haplotypes or genotype combinations with phenotypes assuming complete identification of the causative variants,[203] and TDT extended to quantitative traits.[204] Techniques are also available that take into account simultaneously linkage and partial linkage disequilibrium with polymorphisms of a gene in either parametric[205] or variance components approaches.[206;207] The variance component method of Fulker et al and Abecasis et

al.[206;207] provides a means of controlling for population stratification that may give rise to apparent association independently of linkage. This is done by partitioning the mean effect for the test locus into two term – one is a nuisance parameter that absorbs the effects of population stratification, while the other depends only on the linkage disequilibrium at the test locus. The variance partition includes a component that enters into the between-relative covariance as a function of IBD at the test locus; this component of variance is nonzero if the linkage disequilibrium between the causative gene variants and the markers that have been tested is incomplete.

#### 1.2.3.1 Association based genome wide studies – use of SNPs

An alternative to genome wide linkage studies and candidate gene studies is the use of genomewide association (linkage disequilibrium) studies. The suggestion of genome-wide searches for gene effects using large-scale testing of single nucleotide polymorphisms (SNPs), or perhaps more appropriately simple nucleotide polymorphisms (which could include short deletions and insertions and multinucleotide changes as well as single nucleotide substitutions), has led to considerable discussion of the efficiency of different approaches.

SNPs have several advantageous properties as DNA markers. They are abundant in the genome: On average, any two copies of a chromosome are expected to differ in approximately one nucleotide position every 1000 bases. SNP markers are biallelic with a maximum heterozygosity of 50%; hence it is expected that a genome scanning set of SNP markers must be denser to be as informative as the highly polymorphic microsatellite marker sets currently in use. Kruglyak performed simulations and showed that a map of 700-900 moderately polymorphic biallelic markers is equivalent to typical 300-400 microsatellite marker maps.[208]

The original suggestion of Risch and Merikangas[209] was to study coding or promoter variants with potential functional significance. Collins et al.[210] subsequently suggested that non-coding or evenly spaced SNPs with high density could be used to track disease loci through linkage disequilibrium. The number of SNPs required for the latter strategy has been the subject of debate, primarily because the extent of linkage disequilibrium in the human genome has not been well studied on a large scale. As opposed to recombination — a biological phenomenon already measured extensively in humans — linkage disequilibrium is a property of populations, and thus depends heavily on their demographic and social histories. Population isolates such as Finns, Ashkenazi Jews and Mennonites have been shown to demonstrate extensive linkage disequilibrium (up to several percent recombination) around rare disease mutations. The degree to which the same will be true for higher-frequency variants is uncertain, although as a general rule the disequilibrium is likely to decline with increasing allele frequency owing to an older coalescence time.

An additional question is whether SNP markers are sufficiently dense in the genome to serve as maps for linkage disequilibrium (LD) and association mapping. Simulation studies[211] to estimate the extent of LD surrounding common gene variants in the general human population on in population isolates have shown that on average, a useful level of LD is unlikely to extend beyond an average distance of roughly 3kb in the general population. This implies that as many as 500,000 evenly spaced SNPs may be required to detect linkage disequilibrium of sufficient magnitude for mapping purposes,[211] even in population isolates, whereas others have argued that founder populations, especially those that have remained small over an extended time period,

such as the Saami of Scandinavia[212] or isolated Sardinian populations,[213] would require far fewer SNPs. Although such populations should improve the chances for detecting rare disease alleles (say less than 5% in frequency), owing to greater linkage disequilibrium per base pair, the same is unlikely to be the case for common alleles (greater than 5% in frequency).[214] Furthermore, the power of association tests diminishes significantly with decrease in linkage disequilibrium, and as a result of discordance between the frequencies of disease and marker alleles.[179;215] Although increasing marker density greatly enhances the chance of including a marker in strong linkage disequilibrium with the disease allele, the same is not true for similarity of allele frequencies because correlations between SNP allele frequencies do not increase inversely with distance between SNPs.[216] Another complication is that, in contrast to linkage analysis, a negative linkage-disequilibrium result in a particular genomic region does not exclude a significant gene effect in that region. It may be that the SNPs used there are in modest or no disequilibrium with the disease allele, and/or the allele frequencies are divergent. Thus, it seems that in a genome-wide random SNP approach, even at high density, many disease-causing genes would be missed.

Several arguments favour using SNPs in coding and promoter regions rather than random SNPs. First, it is these variants, *a priori*, that are most likely to be of functional significance and to influence directly the traits under study. In fact, these are the variants to which random SNP searches are likely to lead. Second, even if not the causative variant in a gene, such SNPs are as likely (or more likely) to be in linkage disequilibrium with the causative allele as are randomly placed SNPs.

### 1.2.4 Multiple hypothesis testing

As the number of SNPs evaluated in association testing increases, a concern arises with false positives. This is true for candidate gene studies, but more so for whole genome random SNP studies where tens to thousands of SNPs will be evaluated. The traditional cut-off for assessing statistical significance ($p<0.05$) by definition can result in 5% false associations occurring simply by chance. The difficulty becomes distinguishing the true associations from these false associations. To account for the multiple hypothesis testing that occurs when many SNPs are evaluated, a simple correction can be applied to the p value called the Bonferroni's inequality. This correction uses the formula

$$P^* = 1-(1-P)^n$$

Where $P^*$ is the overall p-value, taking into account the observed p-value, $P$, and the number of hypotheses tested, $n$.

The major drawback of this method is that Bonferroni's inequality is a conservative correction, especially if some of the hypotheses being tested are not independent. When many SNPs in the same gene are evaluated, for example, and are in LD with each other, the Bonferroni correction would not be appropriate, resulting in the possibility of false negatives or failure to detect a true association. A better approach would be to test the true level of significance through simulations.

Another option would be to not apply a correction at all, but rather, to require any association, whether in the context of a single hypothesis or several thousand, to reach a genome wide significance, similar to what is done in linkage analysis. Genome-wide significance may mean achieving a p-value on the order of $10^{-7}$ or $10^{-8}$ to account for

100,000 to one million multiple comparisons. Regardless of the correction method used, sample sizes will have to be substantially augmented to reach a reasonable level of statistical significance.

### *1.2.5 Genetic heterogeneity in Mendelian disease*

An important issue in the study of Mendelian disease is the phenomenon of genetic heterogeneity, whereby distinct mutations at the same locus (allelic heterogeneity) or different loci (non-allelic heterogeneity) can cause the same, indistinguishable phenotype. Non-allelic genetic heterogeneity is a form of multi-locus model, wherein the predisposing alleles at each locus are typically rare and independently capable of producing disease. By contrast, common predisposing alleles often lead to epistasis or interaction effects among loci. In linkage analysis, allelic heterogeneity does not cause a problem because all families (including those with different mutations) will show linkage to the same chromosomal region. In fact, allelic heterogeneity also provides the strongest evidence for a causal relationship between a cloned gene and disease phenotype. Statistically, it is extraordinarily unlikely to find several different mutations at the same locus in unrelated families with the same disease.

Non-allelic heterogeneity can cause a problem in linkage analysis, depending on its extent. In the extreme situation that any single gene accounts for a small proportion of segregating families, very large families would be required to obtain robust linkage evidence, and positional cloning would still be difficult. But for mendelian disease this has rarely, if ever, been the case. More typically, when non-allelic heterogeneity exists, it involves only a few distinct loci; this degree of heterogeneity usually is not a serious impediment either to linkage analysis or positional cloning, essentially

because the relationship between phenotype and genotype within families remains strong.

Another important issue relating to mutational heterogeneity is the population under study. For mendelian disease, endogamous population isolates with a limited number of founders tend to have less mutational heterogeneity and an increased frequency of founder effects, which makes them particularly useful in studies of positional cloning. When most affected individuals in a population carry a mutation derived from a single ancestor, they effectively create a single large extended pedigree, although most of the distant relationships are missing. Historic recombination events around the disease mutation can still be inferred, however, by examining the extent of DNA shared on present-day disease chromosomes. This approach, referred to as linkage disequilibrium analysis, has been highly effective in leading to the cloning of numerous disease genes.

### *1.2.6 Optimal study designs*

The disappointing results from linkage studies coupled with a biometrical view of the world has led to the suggestion of alternative approaches to tackling the genetics of non-mendelian diseases, namely reversion to the study of candidate genes on a large scale[209] or high-density genome scans that are dependent on linkage disequilibrium.[210] The recent resurgence of association studies using candidate genes has led to much discussion about design issues. However, first it is useful to show directly the greater power of detection of gene effects by direct-association (or linkage-disequilibrium) analysis when the involved variant is in hand as opposed to depending on linkage analysis without linkage disequilibrium. Comparing ASPs (linkage) with case-control pairs (association),[209] showed that for genes with high relative risks ($g \geq 4$) and

intermediate allele frequencies ($p$ = 0.05–0.50) it is realistic to expect linkage analysis to provide statistical evidence for the location of a disease gene. However, for more modest relative risks ($g \leq 2$), linkage analysis will not provide such evidence except in unrealistically large samples. By contrast, case–control association studies, even using a stringent significance level ($5\times10^{-8}$), provide adequate power for genes with relative risks as low as 1.5 (with $p$ = 0.10–0.70).

The simplest design is the epidemiological case–control study, contrasting allele frequencies in cases versus controls. As is true for case–control studies generally, confounding is a problem for inferring a causal relationship between a disease and measured risk factor. One approach to deal with confounding is the matched case–control design, where individual controls are matched to cases on potential confounding factors (for example, age and sex) and the matched pairs are then examined individually for the risk factor to see if it occurs more frequently in the case than in its matched control. If cases and controls are not ethnically comparable, then one can use a matched case–control design, where controls are ethnically matched to cases. This can in theory be accomplished by focusing on homogenous and randomly mating populations, where cases and controls will presumably be ethnically comparable. However, such populations may be more of a theoretical ideal than a reality, as non-random mating patterns exist in nearly all groups. Nonetheless, association studies in Finland are less likely to be subject to confounding problems than in heterogeneous North American populations.

Another solution to this problem involves the use of relatives as controls for cases. The first such described design proposed the use of unaffected sibs as controls,[177] and

this design has recently seen a resurgence of interest.[178-181] Designs involving parents as controls have also been proposed.[187;217-219] Among these, perhaps the test most similar in spirit to the epidemiological matched case–control analysis is the transmission disequilibrium test,[218] in which an allele transmitted by a parent to an affected child is matched to the other allele not transmitted from the same parent; MacNemar's chi-square test of discordance is then applied to the resulting pairs[187]. The two alleles carried by a parent are of necessity ethnically matched, and thus the stratification artefact is eliminated. The same applies to sib controls, whose genotypes are ethnically matched to the cases.

But a significant result from a design using parent or sib controls still does not imply a causal relationship between the tested allele and the disease outcome, because linkage disequilibrium with a linked locus (but not an unlinked locus) will also create a positive result. Nevertheless, it does at least indicate a significant gene effect nearby, if not the tested allele itself. The main drawback of using parents or sibs as controls is either unavailability (for example, with parents for a late-onset disease) and loss of power, especially with sibs.

Whereas the simple case–control design is the mainstay of epidemiology, other family-based approaches are available that are more efficient. In particular, sampling multiplex families, where more than a single individual is affected, can be significantly more efficient than sampling singletons. The increase in efficiency is also a function of the disease allele frequency, and is most pronounced for rarer alleles.

Risch[154] calculated the number of families and total individuals required to detect a gene effect with $g$ = 4.0 (for the homozygote) and $g$ = 2.0 (for the heterozygote), assuming a significance level $\alpha = 5 \times 10^{-8}$ and power $(1 - \beta) = 80\%$. He evaluated two disease allele frequencies, 5% and 20%, and consider designs including one, two or three affected sibs, where the (two) control individuals are either the parents of the sibship, unaffected sibs, or unrelated.

For all designs except sibs, the efficiency is approximately the same when affected and control samples are pooled. For sibs, greater efficiency is possible with individual genotyping,[220] so those cases (pooled versus not pooled) are evaluated separately. The results are provided in Table 1.2-1. Rarer alleles (0.05 versus 0.20) are always more difficult to detect, but the number of subjects required can be reduced substantially by increasing the number affected in the sibship. Using unaffected sibs as controls leads to two to five times the required sample size as using unrelated subjects, depending on the number of affected sibs. Using parents leads to a 40–80% increase, again depending on number of affected sibs. The main conclusion is that if disease-susceptibility alleles are typically low frequency (say ≤20%), multiplex sibships are particularly advantageous; they are also advantageous for more frequent alleles, but the relative advantage is less.[179]

An important remaining question is whether to use parents or sibs as controls and suffer the loss in power (especially with sibs), or use unrelated controls and risk loss of robustness. Population stratification has been invoked numerous times as the cause for an observed high false-positive rate in association studies using candidate genes, yet it has rarely been demonstrated as the culprit.[183] More likely, it is the lack of a

stringent significance level used in such studies that is the problem. If one assumes the prior probability for any particular gene variant to be associated with a disease outcome to be low, most reported significant associations would be false positives.

| Design | | p-0.05 | | p=0.20 | |
|---|---|---|---|---|---|
| No. affected sibs | Two controls | No. Families | No. of Subjects | No. Families | No. of Subjects |
| 1 | Unrelated | 872 | 2616 | 300 | 900 |
| 1 | Parents | 1251 | 3753 | 417 | 1251 |
| 1 | Sibs (NP) | 1715 | 6145 | 604 | 1812 |
| 1 | Sibs (P) | 2032 | 6096 | 655 | 1965 |
| 2 | Unrelated | 265 | 1060 | 102 | 408 |
| 2 | Parents | 448 | 1792 | 173 | 692 |
| 2 | Sibs (NP) | 642 | 2568 | 286 | 1144 |
| 2 | Sibs (P) | 992 | 3968 | 361 | 1444 |
| 3 | Unrelated | 121 | 605 | 52 | 260 |
| 3 | Parents | 218 | 1090 | 101 | 506 |
| 3 | Sibs (NP) | 314 | 1570 | 177 | 885 |
| 3 | Sibs (P) | 605 | 3025 | 258 | 1290 |

**Table 1.2-1 Sample sizes for candidate-gene studies for different designs.**
**p–allele frequency;NP-Not pooled; P- Pooled. Adapted from [179]**

An attractive alternative to using family-based controls is to use random or unlinked genetic markers typed in the same cases and controls to determine the extent of possible confounding by ethnicity.[221] In fact, the same markers can also be used to assess the significance of any putative association,[188] or even used to adjust any candidate gene analysis for potential confounding by stratified analysis. Given the proposals for large-scale genotyping, it seems most likely that this approach will ultimately be most efficient.

### *1.2.7 Population variation and replication*

As discussed above, rare variants (<5% frequency) are most likely to be population specific. In some cases, they may be recent in origin and hence specific to a single

founder population or less recent and generally found in one major ethnic group (for example, haemochromatosis mutation C282Y found only in Caucasians[222]). These are the variants that are most readily detected by a random SNP linkage-disequilibrium approach, but at the same time potentially least replicable by studying distinct populations. In this case it would be worthwhile to examine the same gene in other populations (or even the same population) for other functional variants that are associated with a similar phenotypic endpoint. Discovery of such alleles provides the strongest evidence for a causal link between the gene and the trait, as is the case with family-specific mutations in mendelian diseases.

Common alleles (>10% frequency) are more likely to be found globally. If so, a causal association between a candidate SNP and trait outcome should be reproducible in many ethnically diverse populations. However, whereas pan-ethnic replicability provides support for a causal relationship, its absence does not necessarily negate it. It is well known that the same mutation can cause a major disease phenotype in one strain of mouse but no phenotype in a genetically distinct strain. Thus, background factors (genetic and otherwise) differentiating populations can modify the expression of a gene and lead to different levels of association. For example, this seems to be the case for ApoE and Alzheimer's disease, where the association exists pan-ethnically but is strongest in Caucasians and Asians, and weaker in Hispanics and African Americans.[223]

Another advantage to having an ethnically diverse sample of individuals/families is that patterns of linkage disequilibrium may differ ethnically, helping to resolve causal from non-causal relationships. While populations with high linkage disequilibrium

may be useful for initial detection of SNP associations, several different SNPs may be in strong or complete disequilibrium. Populations with lower levels of disequilibrium can help resolve which SNP effect is primary. Generally, Africans appear to have the lowest levels of linkage disequilibrium and hence are likely to be most useful for such analyses. An example is provided by the association of HLA and narcolepsy. In Caucasian and Asian populations, the alleles *DR2* and *DQ-β-0602* are equally associated with the disease (and in complete disequilibrium with each other), whereas in Africans there is incomplete disequilibrium between them and *DQ-β-0602* shows the primary effect.[224]

In the next section, I shall summarise the various studies that have applied the above concepts in the study of the genetic determination of hypertension.

## 1.3 Genetic determinants of hypertension

Like premature CAD, hypertension is a familial disease and this was recognized as early as 1923 in Germany.[225] Evidence for genetic influence on blood pressure comes from various sources. It is most likely that there are several causal genes, which together contribute to between 30-50% of the variation in blood pressure among individuals. Using historical family data from over 94,000 individuals, Hunt et al.[226] found that the risk of developing hypertension after 1970 in persons under age 50 was approximately doubled for each first degree relative that had developed hypertension before 1970.

### *1.3.1 Heritability of blood pressure*

Adoption, twin and family studies document a significant heritable component to blood pressure levels and hypertension.[227-229] Twin studies document greater concordance of blood pressures in monozygotic than dizygotic twins,[230] and population studies show greater similarity in blood pressure within families than between families. The latter observation is not attributable to only a shared environment since adoption studies demonstrate greater concordance of blood pressure among biological siblings than adoptive siblings living in the same household.[231]

More formal estimates of the heritability of resting systolic and diastolic blood pressures based on family studies, are generally in the range of 15% to 35%. In twin studies, heritability estimates of blood pressure are ~60% for males and ~30-40% for females.[228;232-234] Ideally an estimate of heritability based on the association model would be approximately the same as that derived by multiplying the sib-sib correlation by 2. In previous population studies, sib-sib blood pressure correlations

have been in the range of 0.14 to 0.18.[229] A heritable component to salt sensitivity of blood pressure has been described in blacks.[235] Salt sensitivity is defined as a change in blood pressure in response to changes in salt and water homeostasis; found in 73% of hypertensive and 36% of normotensive blacks, it is generally considered a hallmark of hypertension in blacks. Complex segregation analysis continues to show evidence for hypertension-related "major" genes.[236;237] A large proportion of the phenotypic variation in blood pressure appears to be inherited as a polygenic trait.[238]

Despite very significant recent progress in genomic and statistical tools, the genetic dissection of human essential hypertension still provides a major challenge. Three main lines of investigation have been developed so far. These include linkage analysis in families segregating for rare mendelian forms of hypertension, candidate gene approaches and genome-wide scanning strategies. Of the above approaches, the search for single-gene mendelian forms of hypertension has been the most successful.

Single genes can have major effects on blood pressure, accounting for the rare Mendelian forms of high and low blood pressure.[73] Although identifiable single-gene mutations account for only a small percentage of hypertension cases, study of these rare disorders may elucidate pathophysiologic mechanisms that predispose to more common forms of hypertension and may suggest novel therapeutic approaches.[73] Mutations in 10 genes that cause Mendelian forms of human hypertension and 9 genes that cause hypotension have been described to date, as reviewed by Lifton and colleagues.[73;239] These mutations affect blood pressure by altering renal salt handling, reinforcing the hypothesis of Guyton[65] that the development of hypertension depends on genetically determined renal dysfunction with resultant salt and water retention.

Improved techniques of genetic analysis, especially genome-wide linkage analysis, have enabled a search for genes that contribute to the development of primary hypertension in the population. Application of these techniques has found statistically significant linkage of blood pressure to several chromosomal regions, including regions linked to familial combined hyperlipidemia.[240-243] These findings suggest that there are many genetic loci, each with small effects on blood pressure in the general population. Overall, however, identifiable single-gene causes of hypertension are uncommon, consistent with a multifactorial cause of primary hypertension.

### *1.3.2 Heritability of end-organ damage*

In addition to heritability of blood pressure, predisposition to end organ damage generally attributable to hypertension may be inherited separately from blood pressure. There are "stroke-prone" and "stroke-resistant" spontaneously hypertensive rats (SHR). Cross-breeding the two strains demonstrated independent segregation of the stroke-prone trait.[237] Based on this work in SHR, which found linkage of the stroke trait to an area that included atrial natriuretic peptide, these investigators identified a variant in the atrial natriuretic peptide gene associated with a 2-fold increased risk of stroke in the large, prospective, Physician's Health Study.[244] There are relatively rare Mendelian forms of stroke, both hemorrhagic[245] and lacunar (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy or CADASIL)[246] that are unrelated to hypertension. CADASIL has a prevalence of at least 1 per 100,000 and accounts for about 2% of lacunar strokes under age 65.[247] Leukoariosis is a diffuse lesion of white matter resulting in hyperintensity on MRI scans often seen together with lacunae and thought to be due to a form of arteriolosclerosis or "microatheroma."[248] The extent of leukoariosis was found to be 71% heritable in World War II veteran twins.[249]

Family history appears to influence more common types of end-organ damage, even after adjustment for blood pressure. Thus, family history of atherothrombotic stroke was associated with relative risks of 1.5 to 3 for this most common form of stroke.[250-252] Strong evidence (LOD score 4.40) has been presented for linkage of common forms of stroke to chromosome 5q12 in Icelandic families, apparently independent of known risk factors.[253] Family history of intracerebral haemorrhage was associated with 6-fold increased risk of having an intracerebral haemorrhage independent of blood pressure.[254] For subarachnoid haemorrhage, risk was increased 4-fold by a definite positive family history.[255] Left ventricular mass[256] and other measures of left ventricular size and function are heritable.[257] Pulse pressure, a measure of arterial stiffness or distensibility, was strongly heritable and related to telomere length (which was also highly heritable).[258] Carotid intima-medial thickness (IMT) has been reported to be up to 30% to 64% heritable.[259-262] Presence of carotid plaque was 23% heritable after correction for hypertension.[263] One candidate gene variant (IL6-174G>C CC found in 19% of the population) was associated with much higher plasma interleukin (IL)-6 and increased carotid IMT at high alcohol intakes.[264] A substantial number of gene variants have been associated with carotid IMT; some were also associated with CHD.[265]

### *1.3.3 Gene-environment interaction*

Complex modelling of blood pressure in families suggests inheritance of a gene or genes that lead to a steeper rise in blood pressure with age.[266;267] There are multiple examples of rat or mouse strains that develop high blood pressure only when exposed to a high salt diet, most notable being the Dahl salt-sensitive rat. Chimpanzees living in a natural setting exhibit salt sensitivity in some but not all the individuals.[268]

In humans, environmental stressors include moderate to high salt intake and lack of physical exertion together with an excessively rich diet resulting in a high prevalence of overweight.[269-271] In contrast, when salt intake is very low (below 50 mEq/day), hypertension is rare.[269;272] More recent observations document that even with a relatively high salt intake, blood pressure remains low and hypertension is rare in a rural setting with continued exercise and lean body habitus.[273] Low maternal protein intake and genetic susceptibility can lead to reduced nephron number in several models.[274-278] Recent identification of a substantial reduction in the number of nephrons in kidneys of relatively young primary hypertensive patients who died from accidents provide indirect evidence that such early changes in the structure of kidneys that may favour salt retention may be relevant in human primary hypertension.[279]

### *1.3.4 Genome wide scanning strategy*

Genome-wide linkage studies for complex traits such as essential hypertension are problematic and require careful study design. Phenotypic traits for a polygenic disease aggregate in families but unlike monogenic diseases, they do not segregate in any clear Mendelian fashion. A positive result using a linkage approach that generally tests related individuals, by its nature, would implicate a general region of genomic DNA containing a large number of genes of which the causative gene for a particular disease will still have to be identified. For a complex disorder such as essential hypertension, many inherent problems exist using a linkage approach where on average both parents and children of the affected individual are not available for the study. Therefore the construction of a multi-generational pedigree is difficult and the mode of inheritance required for parametric linkage analysis cannot be calculated. Consequently a non-parametric approach, such as sibpair analysis, that does not

require information on the mode of inheritance or penetrance and has been used in many studies.[280;281] Positive sibpair linkage findings for particular loci obtained to date using microsatellite markers confirm the feasibility of extending this approach to a complete genome scan.[110;282-285]

As a result of there being multiple contributing genes, each with small to moderate effects and environmental factors that have to be taken into account, careful study design in a genome-wide analyses is necessary. At least 22 genome-wide scans have been reported to identify loci for blood pressure using different methodologies. Some used blood pressure as a quantitative phenotype while others used hypertension. Some scans utilized families, others affected or dissimilar sibling pairs. The results of these scans are reviewed in Table 3. Generating much attention is the use of sib pairs with extreme phenotypes, a sampling design recognised as being substantially more powerful than a design that uses randomly ascertained sibs. Though the use of discordant sibs is a powerful tool, as shown by theoretical work and simulation[197;286-290] it is counterbalanced by the large screening effort that is required to identify them. Xu et al[291] carried out a genome scan in search of genes regulating blood pressure and used three sib pair types (discordant, highly concordant and concordant siblings) from a Chinese population, to extract maximal information from their genotyping effort. No chromosomal region was able to achieve 5% genome-wide significance, though analyse on either SBP or DBP revealed several promising regions with maximal lod scores greater than 2.[291] In a similar study, Kruskhal et al using a design that used only discordant sib pairs to localise genes that affect interindividual systolic blood pressure,[292] identified several regions on chromosome 2, 5, 6 and 15 with significant linkage and chromosome 9, 16, 18, 20, 21 with suggestive linkage to differences in

systolic blood pressure levels and to contain genes, the products of which have been suggested to regulate blood pressure.[292] These studies demonstrated how difficult an undertaking genome-wide searches for genes influencing complex traits can be.

Caulfield et al.[293] carried out the BRIGHT study which represents the largest homogenous Caucasian resource that has been published to date (1599 families, comprising 2010 sibling-pairs). They reported a principle locus for hypertension on chromosome 6q (LOD score of 3.21) that attained genome-wide significance using a locus counting method and three further loci with suggestive evidence of linkage on chromosomes 2q, 5q and 9q. [293] In contrast the US National Institute of Health funded Family Blood Pressure Program (FBPP)[294-297] comprising four multi-centre networks (GENOA, GenNet, HyperGEN and SAPPHIRe) which recruited participants with different selection criteria from multiple ethnic groups found no chromosomal region with genome-wide significant evidence of linkage. The highest LOD score, 2.96 was linked to diastolic BP on chromosome 1q in Caucasians using variance components analysis.[297]

Though linked loci with at least suggestive LOD scores were seen on every chromosome, perhaps most striking is the lack of consistently linked loci. This may serve to illustrate the heterogeneity of human hypertension as well as the potential shortcomings of attempting to compare studies using different methodologies. The difficulty in finding reproducible loci for hypertension has been the subject of a number of recent commentaries.[298;299] Currently, identifying susceptibility genes and variants from genome scans remains elusive.(Table 1.3-1)

### 1.3.4.1 Metabolic syndrome and dyslipidaemic hypertension

Genetic insights regarding dyslipidaemic hypertension are beginning to emerge. A locus for blood pressure, fasting insulin, and leptin was found on chromosome 7q.[300] A locus on chromosome 1q21-q23 (near 170 to 180 cM) has been linked in various genome scans to familial combined hyperlipidemia,[301-303] diabetes,[304] and blood pressure.[240;242;292] A similar locus on chromosome 4p may exist.[305] The Lys198Asn variant in the endothelin-1 gene (on 6p24-p23) appears to interact with BMI resulting in mildly greater risk for hypertension in 198Asn carriers with higher BMI.[306-308] The 460Trp variant of α-adducin also appeared to interact with BMI and triglycerides.[309] A variant of the SA gene (SAH) was associated with increased BMI, waist/hip ratio, triglycerides, and blood pressure.[310] The Trp64Arg variant of the β3-adrenergic receptor (ADRB3) has similarly been associated with multiple features of the metabolic syndrome.[311]

## *1.3.5 Linkage analysis*

Major mutations have been identified with the use of this strategy in the following genes: 11β-hydroxylase in glucocorticoid remediable aldosteronism,[312] the β and γ subunits of the epithelial sodium channel in Liddle's syndrome,[313;314] and the 11β-hydroxysteroid dehydrogenase in the syndrome of apparent mineralocorticoid excess.[315;316] Two further mendelian forms of hypertension, Gordon's syndrome (chromosome 1 and 17)[317-319] and hypertension with brachydactyly (chromosome 12)[320] have been mapped to defined chromosomal regions but their respective causal genes still await identification. It should be noted that these rare syndromes with mendelian inheritance account for a very small fraction of the pathological human blood pressure variation. The study of these disorders provide not only novel

diagnostic tools and an opportunity for targeted therapeutic intervention, but also provide an insight into the pathogenesis of the common forms of hypertension.

The most popular candidate gene strategy, which investigates the genes within physiological pathways known to affect blood pressure variation, is a result of lessons learnt while studying these rare syndromes.

| | | | Chromosome and location (cM) of linkage | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ref. | Trait | Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X |
| Krushkal[292] | SBP | W,S | | 58 | | | 190 | 144* | | | | | | | | | 97 | | | | | | | | |
| Xu[291] | SBP | C,S | | | 6 | 73 | | | | | | | 58 | | | | | | 24 | | | 39 | | | |
| | DBP | C,S | | | | | | | | | | 46 | | | | | 10 | 64 | | | | 39 | 25 | | |
| | | | | | | | | | | | | | | | | 5 | | | | | | | | | |
| Levy[240] | SBP | W,F | | | | | | | | | | | | | | | | | 67* | | | | | | |
| | | | | | | | | | | | | | | | | | | | 94 | | | | | | |
| | DBP | W,F | | | | | | | | | | | | | | | | | 74 | 7 | | | | | |
| Rice[321] | SBP | W,F | | 97 | | | 32 | | 135 | 87 | | | | | | | | | | | 4 | | | | |
| | | | | 103 | | | | | | | | | | | | | | | | | | | | | |
| Hsueh[243] | DBP | W,F | | 210* | | | | | | | | | | | | | | | | | | | | | |
| Sharma[322] | HTN | W,S | | | | | | | | | | | | | | | | | | | | | | | |
| Perola[323] | HTN | W,S | | 185 | 166* | | | | | | | | | | | | | | | | | | | 39 | 67 |
| Zhu[324] | HTN | C,S | | 161 | | | | | | | | | | | | | | | | | | | | | |
| Atwood[325] | SBP | W,F | | | | | | | | | | | | | | | | | | 116 | | | 37 | | |
| | DBP | MA,F | | 99* | | | | | | 165 | | | | | | | | | | | | | | | |
| Atwood[326] | PP | MA,F | | | | | | | 114 | 154 | | | | | | | | | | 116 | | | 37 | | |
| Cheng[300] | SBP | W,F | 4 | | | | | | 128 | | | | | | | | | | | | | | | | |
| | MAP | W,F | 4 | | | | | | | | | | | | | | | | | | | | | | |
| Hunt[242] | HTN | W,F | 192 | | | | | | 58 | | | | | 83 | | | 10 | | | | | | | | |
| | | | | | | | | | 127 | | | | | | | | 3 | | | | | | | | |
| | SBP | W,F | | | | | | 89* | | | | | | | | | | | | | | | | | |
| Allayee[305] | SBP | W,F | | | | 90* | | | | | | | | | | | | | | | 10 | | | | |
| | DBP | W,F | | | | | | 89 | | | | | | | | | | | | | | | | | |
| Angius[327] | HTN | W,F | | 12* | | | | | | | 43 | | | | 11 | | 79 | | 15 | | 54* | | | | |
| | | | | | | | | | | | | | | | 5 | | | | | | | | | | |
| Harrap[328] | SBP | W,S | 76 | | | 117 | | | | | | | | | | | | 49 | | | | | | | 42 |
| Rice[329] | SBP | W,F | | | | | | | | | | | 10 | | | | | | | | | | | | |
| | | | | | | | | | | | | | 5 | | | | | | | | | | | | |
| | SBP | B,F | | | | | | | | | | | | | | | | | | | 49 | | | | |
| | DBP | B,F | | | | | | | | | | | | 95 | | | | | | | | | | | |
| Cooper[330] | SBP | B,F | | | | | | | | | | | | | | | | | | | 47 | 78 | | | |
| | DBP | B,F | | 104 | 16 | | | | 81 | | | 76 | | | | | | | | | | | | | |
| | | | | | | | | | 109 | | | | | | | | | | | | | | | | |
| Kristj-ansson[241] | HTN | W,F | | | | | | | | | | | | | | | | | | 89* | | | | | |
| Ranade[295] | HTN | C,S | | | | | | | | | | 30 | | | | 10 | | | | | | | | | |
| | | | | | | | | | | | | | | | | 0 | | | | | | | | | |
| Thiel[297] | DBP | W,F | 170 | | 119 | | | | | | | | | | | | | | | | | | | | |
| Rao[296] | HTN | B,S | | 63 | | | | | | | | | | | | | | | | | | | | | |
| Kardia[294] | HTN | W,S | | | | | | | | | | | | | | | | | | | | | | | |
| | HTN | B,S | | | | | | | | | | | | | | | | | | | | | | | |
| Caulfield[293] | HTN | W,S | | | | | | 190* | | | 145 | | | | | | | | | | | | | | |

**Table 1.3-1 Genome-wide scans reporting suggestive (LOD 1.9) or greater evidence for blood pressure-related traits.**

**HTN, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; W, white; B, Black; C, Chinese; MA, Mexican-American; F,families; S, siblings. *LOD scores 3.0+**

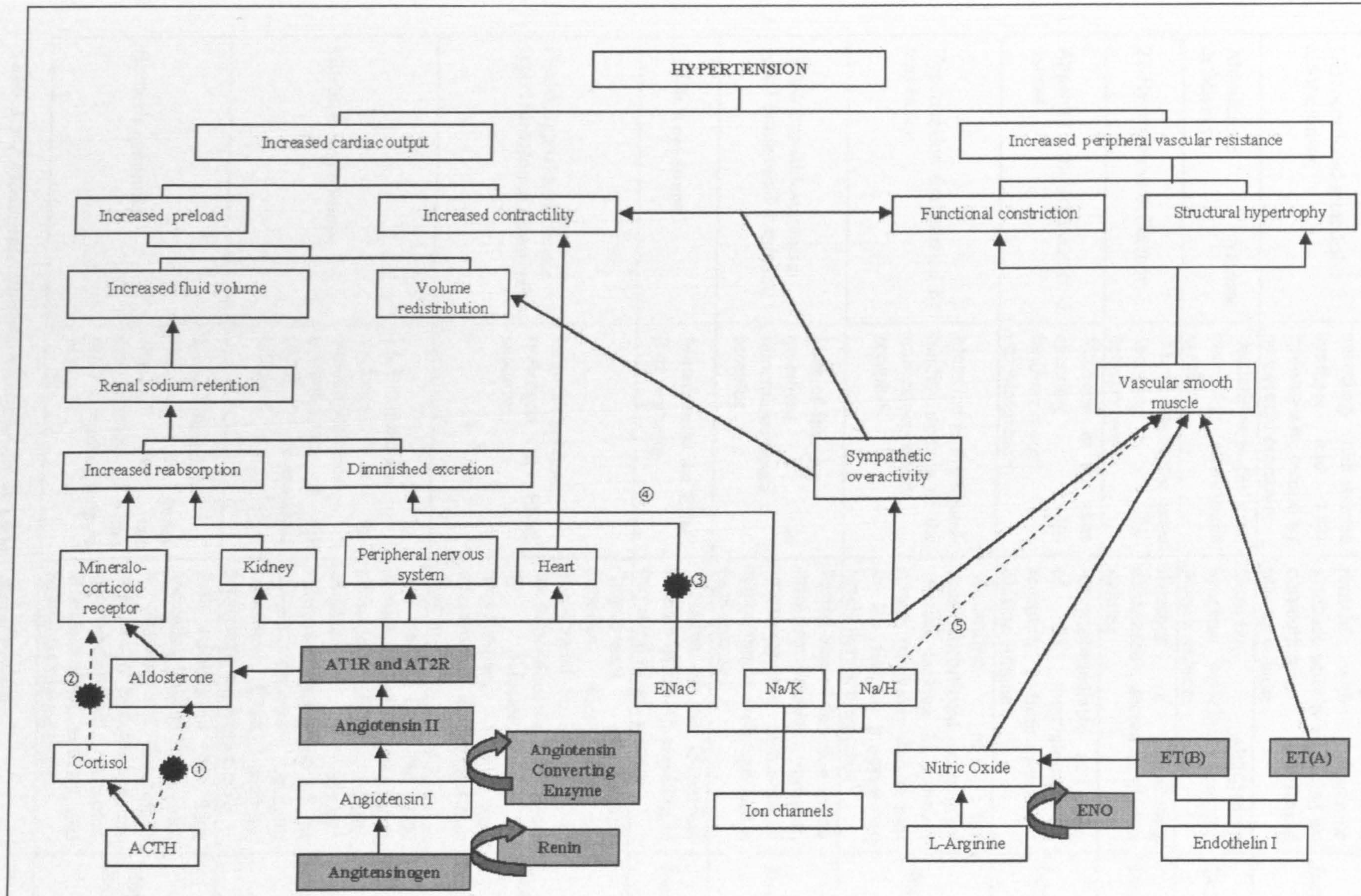


**Figure 1.3-1 Genetic pathways to hypertension**

**The figure shows the location of various candidate genes implicated in the pathogenesis of hypertension. ✱Monogenic Hypertension shown: 1-GRA;2-AME;3-Liddle;4-Adducin;5-GNB3**

| Disease | Mutation | Molecular Mechanism | Effect on Blood Pressure |
|---|---|---|---|
| Glucocorticoid-remediable aldosteronism | Duplication of genes encoding aldosterone synthase and 11β-hydroxylase, caused by an unequal crossover | Ectopic expression of a protein with aldosterone synthase activity regulated by corticotropin; increased plasma volume | Increased |
| Aldosterone synthase deficiency | Mutations in the gene encoding aldosterone synthase | Defective aldosterone synthase activity; decreased plasma volume | Decreased |
| 21-Hydroxylase deficiency | Mutations in the gene encoding 21-hydroxylase | Absence of circulating aldosterone; decreased plasma volume | Decreased |
| Apparent mineralocorticoid excess | Mutation in the gene encoding 11β-hydroxysteroid dehydrogenase | Cortisol-mediated activation of the mineralocorticoid receptor; sodium retention; plasma volume | Increased |
| Hypertension exacerbated by pregnancy | Mutation in the ligand-binding domain of the mineralocorticoid receptor | Activation of the mineralocorticoid receptor by steroids lacking 21-hydroxyl groups (probably due in part to the rise in progesterone levels during pregnancy) | Increased |
| Pseudohypoaldosteronism type I (autosomal dominant) | Loss-of-function mutations in mineralocorticoid receptor | Partial loss of function of the mineralocorticoid receptor, impairing salt reabsorption; improvement with age and a high-salt diet | Decreased |
| Liddle's syndrome | Mutations in the ENaC β or γ subunit | Deletion of the C-terminal domain of ENaC, resulting in increased ENaC activity | Increased |
| Pseudohypoaldosteronism type I (autosomal recessive) | Loss-of-function mutations in ENaC subunits | Impairment of ENaC subunits, which is not ameliorated by activation of the mineralocorticoid receptor by aldosterone; no improvement with age; massive salt supplementation required | Decreased |
| Gitelman's syndrome | Loss-of-function mutations in the sodium–chloride cotransporter of the distal convoluted tubule | Salt wasting from the distal convoluted tubule, leading to activation of the rennin-angiotensin system; subsequent activation of the mineralocorticoid receptor increases ENaC activity, preserving salt homeostasis | Normal or decreased |
| Bartter's syndrome | Loss-of-function mutations in genes required for salt reabsorption in the thick ascending loop of Henle | Salt wasting in the thick ascending loop of Henle leads to activation of the renin–angiotensin system and the mineralocorticoid receptor, increased ENaC activity, and relative salt homeostasis | Normal or decreased |

**Table 1.3-2 Monogenic Diseases That Elevate or Lower Blood Pressure.**

### *1.3.6 Monogenic syndromes*

Table 1.3-2 summarises the various monogenic syndromes associated with blood pressure variation and the molecular mechanism and chromosomal locus.

#### 1.3.6.1 Glucocorticoid Remediable Aldosteronism (GRA)

GRA is an autosomal dominant trait with a clinical phenotype varying from severe early onset hypertension to a much milder blood pressure elevation and mild hypokalaemia. The sine qua non of patients with GRA is that the secretion of

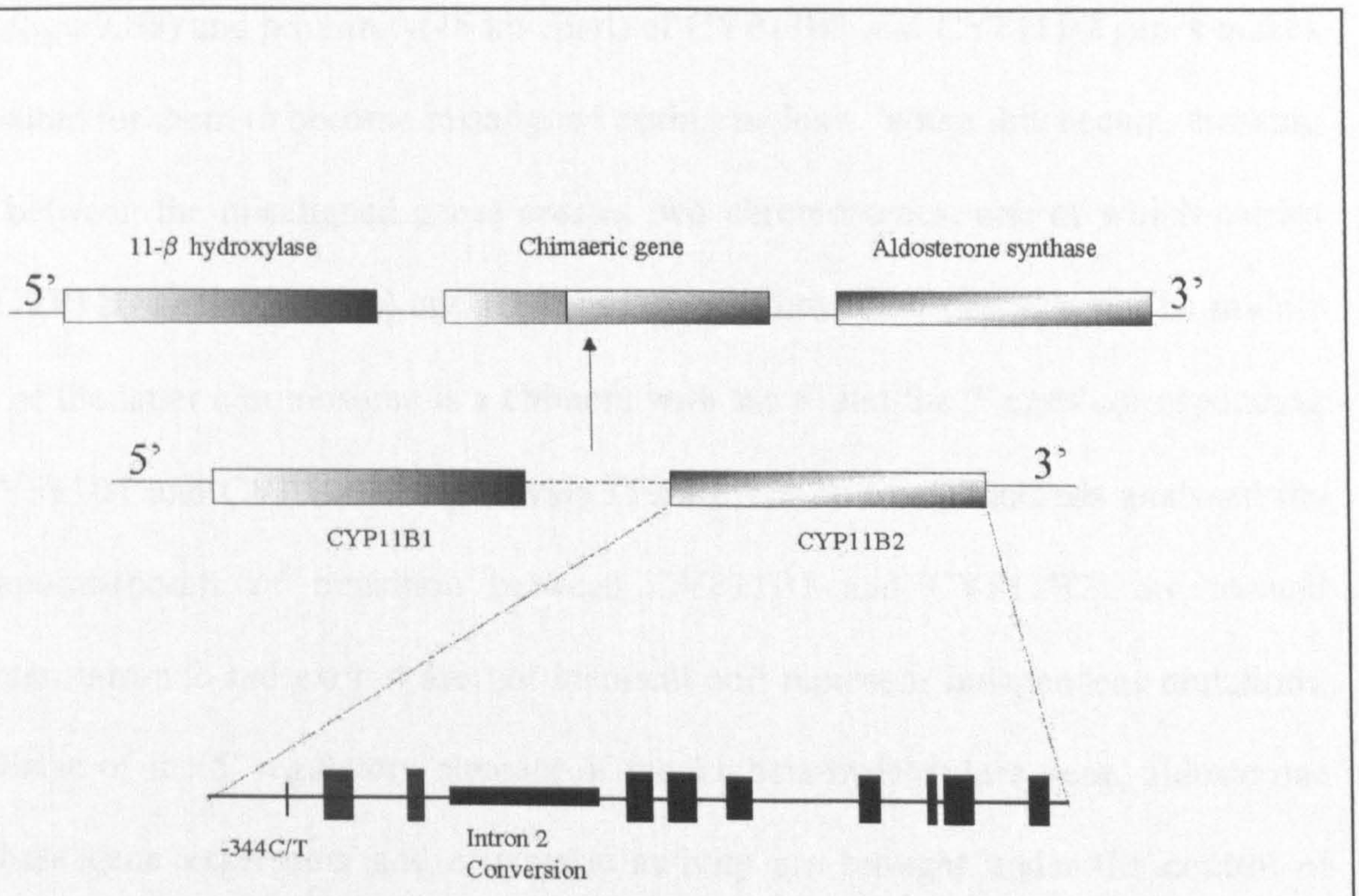


Figure 1.3-2 Genetic variations in the Aldosterone synthase gene.

aldosterone is regulated by adrenocorticotropic hormone (ACTH) rather than its usual secretagogue angiotensin II. It is characterised by moderate hypersecretion of aldosterone, suppressed plasma renin activity and rapid reversal of these abnormalities with glucocorticoids. Elevated 18-oxocortisol is the most consistent

biochemical marker of the condition. The hypertension is of moderate severity and becomes apparent early, with young children having blood pressure greater than the 95th percentile for the age and becoming frankly hypertensive before the age of 20. Associated signs of hypertension are frequent with left ventricular hypertrophy and retinopathy. Some affected kindreds have history of early deaths from strokes in family members before the age of 40. All patients with GRA have a mutation localised to a segement of human chromosome 8, containing aldosterone synthase and steroid 11 beta-hydroxylase genes (CYP11B2 and CYP11B1). The high homology(93%) and proximity(45 kb apart) of CYP11B1 and CYP11B2 genes makes it possible for them to become misaligned during meiosis. When this occurs, crossing over between the misaligned genes creates two chromosomes, one of which carries one CYP11B gene (deletion) and the other carries three CYP11B genes. The middle gene of the latter chromosome is a chimera with the 5' and the 3' ends corresponding to CYP11B1 and CYP11B2 respectively (Figure 1.3-2). In all kindreds analysed the breakpoints(points of transition between CYP11B1 and CYP11B2) are located between intron 2 and exon 4 are not identical and represent independent mutations. By virtue of the 5' regulatory element of the 11 beta-hydroxylase gene, aldosterone synthase gene expression and enzymatic activity are brought under the control of ACTH. This results in ectopic secretion of aldosterone from the adrenal fasciculata and leads to increased salt and water reabsorption and rise in blood pressure.[312] Studies on chimeric cDNA expression in cultured cells showed that chimeric enzymes with amino termini from CYP11B1 and carboxyl termini from CYP11B2 have 18-oxidase activity only if at least the region encoded by exons 5-9 corresponds to CYP11B2. If the sequence of exon 5 instead correspond to CYP11B1, the enzyme has 11 beta-hydroxylase but no 18-oxidase activity,[331] and there is no location of

crossover that yields an enzyme with intermediate levels of 18-oxidase activity. This is entirely consistent with the observation that no breakpoints in GRA alleles occur after exon 4.

Subjects with GRA show a wide range of blood pressure including normotension. Studies to date indicate that this is not related to environmental factors like sodium intake or differences in steroid levels ruling out allelic variations as the cause of variations in clinical severity.

Many kindreds of GRA are of Anglo-Irish extraction[332].[312;333] Subjects with a maternal inheritance of GRA have a significantly higher plasma aldosterone and blood pressure compared to those with paternal inheritance suggesting gene imprinting, but it more likely represents exposure of the foetus to elevated levels of maternal aldosterone which exacerbates the condition.[334] Moreover the chromosomes carrying the chimeric genes tend to occur in association with specific polymorphism of the CYP11B genes. This suggests that one of these polymorphism is or is in linkage disequilibrium with a structural polymorphism that predisposes to unequal crossing over during meiosis. In approximately 40% of alleles in Caucasians, the intron 2 of CYP11B2 has a sequence almost identical to that of CYP11B1. This region could promote misalignment of chromosomal segments during meiosis and thus increase the risk of unequal crossing over. The chimeric genes causing GRA can be readily detected by Southern Blotting or PCR and this gives a test of 100% sensitivity and specificity.[335] The polymorphic variants of CYP11B2 are discussed in section 1.3.7.5 (page 132).

### 1.3.6.2 Liddle's syndrome

Liddle syndrome is an autosomal dominant form of hypertension characterised by hypokalaemia and suppressed renin and aldosterone levels. The high blood pressure responds specifically to amiloride, indicative of an upregulation of the amiloride sensitive epithelial sodium channel (ENaC) activity.

ENaC is composed of three subunits (alpha, beta and gamma), each containing a COOH-terminal PY motif (xPPxY). Mutations causing Liddle syndrome alter or delete the PY motifs of the beta or gamma ENaC which is a conserved motif essential for channel internalisation. Most mutations reported to date result in the elimination of the last 45-76 normal amino acids from the C-terminus of the beta or gamma subunits which lie adjacent on the short arm of chromosome 16[313;314;336] The crucial role played by the PY motif in beta ENaC and gamma ENaC has been highlighted by the identification of the two missense mutations P616L and Y618H in other Liddle syndrome patients.[337;338] No Liddle syndrome mutations have so far been identified in alpha ENaC.

The increased activity of the ENaC is not explained by alteration of single channel conductance but rather by an increased number of channels inserted into the plasma membrane.[339] Since tyrosine from the PPPXY plays a crucial role, it has been proposed that defective endocytosis would lead to an accumulation of ENaC proteins at the apical membrane. The ubiquitin protein ligase Nedd4 binds the PY motif of the EnaC and inhibits the channel. It acts as a negative regulator of the wild-type epithelial channel but is inactive on the Liddle form of the channel. Association between Nedd4 and ENaC might bring the ca-lipid binding and ubiquitin-ligase

domains in close proximity to the channel, initiating its removal from the apical membrane. Loss of the Nedd4 binding sites in ENaC observed in Liddle syndrome might explain the increase in channel number at the surface.[340;341]

To test the hypothesis that minor mutations of the gene may be responsible for attenuated forms of Liddle syndrome, the last exon of the beta ENaC subunit gene was analysed in a series of 525 subjects of Caucasian and Afro-Caribbean origin. Seven amino acid changes were detected. These variants were more common in those of African origin. Functional testing of these variants in Xenopus oocytes did not show any significant functional effects, ruling out a substantial role of the beta ENaC gene in essential hypertension.[342](Polymorphisms in ENaC – page 138)

### 1.3.6.3 Syndrome of apparent mineralocorticoid excess

AME is an autosomal recessive disorder causing pre- and postnatal growth failure, juvenile hypertension, hypokalaemic metabolic alkalosis and hyporeninaemic hypoaldosteronism. A low salt diet or blockade of mineralocorticoid receptors with spironolactone ameliorates the hypertension, whereas ACTH and hydrocortisone exacerbate it. Levels of all known mineralocorticoids are low. Cortisol half-life in plasma is increased along with the ratios of cortisol to cortisone and of 5alpha- to 5beta cortisol metabolites in the serum and urine. An elevated ratio of cortisol to cortisone metabolites in the urine (tetrahydrocortisol plus allotetrahydrocortisol to tetrahydrocortisone [(THF+aTHF)/THE]) is considered pathognomic for this disorder. This suggests that cortisol acts as a stronger mineralocorticoid than is normally the case.

The defect in AME is a deficiency of 11 beta-hydroxysteroid dehydrogenase type 2(HSD11B2) enzyme activity. Both aldosterone and cortisol in vitro are potent activators of the mineralocorticoid receptor in contrast to the situation in vivo where aldosterone is a vastly more potent activator of the renal mineralocorticoid receptor. Cells that respond selectively to mineralocorticoids contain the enzyme 11 beta-hydroxysteroid dehydrogenase that metabolises cortisol to cortisone, a steroid that is incapable of activating the mineralocorticoid receptor. This mechanism protects the mineralocorticoid receptor from cortisol, resulting in selective activation by aldosterone. The active component of liquorice, glycyrrhetinic acid inhibits 11-beta HSD in isolated rat kidney microsomes and the similar abnormalities occurring with liquorice intoxication in humans is a reversible pharmacological counterpart to the syndrome of apparent mineralocorticoid excess.[343]

There are two distinct isoenzymes of the 11 beta-HSD. The liver or type 1 isozyme has a relatively low affinity for steroids and is expressed at high levels in the liver but poorly in the kidney and is not defective in AME. The kidney or the type 2 isozyme has high steroid affinity and is expressed at high levels in the kidney and placenta.

Thus far 18 different mutations in the HSD11B2 gene have been published involving 21 kindreds with AME, resulting in neutralising enzymatic activity. There is significant correlation between the degree of enzymatic impairment and biochemical severity as measured by ratio of cortisol to cortisone metabolites.[344]

While apparent HSD11B2 deficiency causes hypertension, it is reasonable to hypothesise that milder decreases in enzymatic activity might be associated with the

common 'essential' hypertension. In rats placental 11-HSD activity is inversely correlated with placental weight and directly correlated with term foetal weight. Patients with AME are often born with mild to moderate intrauterine growth retardation. It is likely that low levels of 11-HSD in the placenta permits excessive quantities of maternal glucocorticoids to cross the placenta and inhibit foetal growth. In retrospective human population studies, low birth weight and increased placental weight are increased risk factors for subsequent development of hypertension. Although variations in 11-HSD might in principle be responsible for this correlation, studies in humans have not found a relation between 11-HSD activity and birth weight.[345]

### 1.3.6.4 Pseudohypoaldosteronism type II (Gordon's Syndrome)

Pseudohypoaldosteronism type II, also known as familial hyperkalaemia and hypertension or Gordon's syndrome, is characterized by hyperkalaemia despite normal renal glomerular filtration, hypertension and correction of physiologic abnormalities by thiazide diuretics. The blood pressure level is not high , and the biological phenotype is rather attenuated, composed of fluctuating hyperkalaemia with hyperchloraemic acidosis. The response of hyperakalaemia to dietary salt restriction and to mineralocorticoids is heterogeneous, suggesting genetic heterogeneity. Analysis of linkage in eight PHAII families showing autosomal dominant transmission demonstrates locus heterogeneity of this trait, with a multilocus lod score of 8.1 for linkage of PHAII to chromosomes 1q31-q42 and 17p11-q21.[317;318] Disse-Nicodeme et al.[319] analysed a large French pedigree in which 12 affected members over 3 generations confirmed autosomal dominant inheritance. Affected subjects had hypertension together with long-term hyperkalaemia, hyperchloraemia, normal plasma creatinine, and low renin levels. Genetic linkage was

excluded for the 2 previously mapped pseudohypoaldosteronism type II loci, PHA2A on 1q and PHA2B on 17p11-q21, as well as for the thiazide-sensitive sodium-chloride cotransporter gene 16q. Wilson et al.[239] identified two genes causing pseudohypoaldosteronism type II after a genomewide screen using 380 microsatellite markers showed strong linkage to 12p13. Both encode proteins in the WNK family of serine–threonine kinases. Mutations in WNK1 are intronic deletions on chromosome 12p. Missense mutations in hWNK4, on chromosome 17, also cause pseudohypoaldosteronism type II. Immunofluorescence assays have shown that the proteins localize to distal nephrons and may serve to increase transcellular chloride conductance in the collecting ducts, leading to salt reabsorption, increased intravascular volume, and diminished secretion of potassium and hydrogen ions. Analysis of 2 obvious candidate genes, SCNN1A and GNB3, located within the interval showed no deleterious mutation. This is a form of hypertension which most closely mimics essential hypertension and the mild phenotype may leave a large proportion of patients with Gordon's syndrome undiagnosed

### *1.3.6.5* Autosomal Dominant hypertension with brachydactyly

A unique Turkish kindred was described in 1973 with autosomal dominantly inherited brachydactyly and severe hypertension where the two traits cosegregated completely. This syndrome has been mapped to a 4 million base pair segment in chromosome 12p.[320;346] A similar syndrome has been described in a family in Canada and another in the US[347].[348] The importance of this syndrome to primary hypertension is speculative. A putative mechanism in these families is the presence of a vascular anomaly involving the posterior inferior cerebellar artery at the ventrolateral medulla which was present in all 15 affected subjects examined and absent in none of the 11 non-affected siblings.[349] No detailed neurophysiological studies have been done in

subjects with this syndrome and hence one is not sure whether this form of hypertension has a neurovascular basis. Though recently a direct causal relationship has been shown between raised blood pressure and neurovascular compression of the ventrolateral medulla in human beings with surgical decompression resulting in a decline in blood pressure.[350]

### *1.3.7 Candidate genes*

One of the logical and promising strategies is the investigation of candidate genes that encode key components of the physiological mechanisms impaired in (or characteristic of) hypertensive patients. Thus far, the candidate gene approach has provided more examples than the linkage approach of gene variants that appear to affect blood pressure. Reasonable candidate genes to consider include genes related to physiological systems known to be involved in the control of blood pressure and genes known to affect blood pressure in mouse models. Lessons learned from the studies of candidate genes[351] to date include the

- shortcomings that result from limited statistical power of many studies
- expected variation from one population to another
- the need for better phenotyping of study subjects
- the relatively small effect of the genes studied on population prevalence of hypertension
- the lack of sufficient certainty of consequences of any genes studied thus far to make treatment recommendations based on genotype.

There have been many reported associations between hypertension-related phenotypes and DNA markers of candidate genes. However, alleles of very few genes are consistently related to intermediate phenotypes across diverse populations. Part of the

inconsistency may be due to the fact that most DNA markers studied thus far do not have a functional impact on the structure or expression of the gene product. Thus, most reported genetic associations have been attributed to linkage disequilibrium with putative functional changes elsewhere at the genetic locus. Since this may vary between populations, factors such as admixture can result in false conclusions about genetic associations. One strategy to reduce such confounding in association studies may be to select DNA markers that are proven, by various assays, to directly mark a functional change in the gene of interest. Additionally, identifying the genetic determinants of an intermediate quantitative trait such as blood pressure, may help to identify the genetic determinants of a disease, such as hypertension, which is defined by threshold values imposed on the quantitative trait.

#### 1.3.7.1 Angiotensinogen

The renin angiotensin system regulates blood pressure and sodium balance. Angiotensinogen is a key component of this system. The candidacy of angiotensinogen for a role in the genetic basis of hypertension is supported by the observation that plasma angiotensinogen levels track with raised blood pressure through families. Transgenic mice with overexpression of a rat angiotensinogen gene develop hypertension and knockout mice with a disrupted gene and absent angiotensinogen production develop low blood pressure.

The potential role of the AGT gene in human essential hypertension has been tested using a highly polymorphic dinucleotide GT repeat displaying 10 alleles and located in the 3' region of the angiotensinogen gene. When a total of 379 hypertensive sib-pairs were studied for the AGT gene, a 17% excess of allele sharing was found when the sib-pairs were stratified according to the severity of hypertension. Significant

linkage was found in the male pairs while no excess of shared angiotensinogen alleles was observed in female-female comparisons, suggesting the influence of an epistatic hormonal phenomenon. From this study, it was estimated that mutations at the AGT locus might be a predisposing factor in at least 3-6% of hypertensives younger than 60 years of age.[110] Subsequently a large European study involving 630 sib-pairs found no evidence of linkage[352].[353] Also no evidence of linkage was found in sib-pair studies in Chinese and Afro-Caribbeans.[283;354] Conversely in Mexican-Americans, a marginally significant linkage was found in 180 affected individuals belonging to 46 families.[355]

Several missense mutations have been found, mainly located on exon 2 of the AGT gene. Most of them were rare and detected in a few hypertensive probands. Two polymorphisms T174M and M235T were found to be in complete linkage disequilibrium. The M235T polymorphism which showed association with hypertension and plasma angiotensinogen was studied in many case-control studies. The frequency of the AGT T235 allele varies strongly with ethnic groups, being the predominant allele in the African population(0.90-0.95) and more frequent in the Asians(0.75) than in the Caucasians(0.40). In a meta-analysis of all relevant articles published between 1992 and 1996, involving 5493 Caucasian patients, the AGT T235 allele was significantly associated with hypertension in subjects with a positive family history of hypertension and in those with more severe hypertension.[356] A positive association of the T235 allele with hypertension was found in the Japanese, and native adult Canadians.[357;358] The T174M polymorphism did not show any linkage with hypertension in most case control studies. A metaanalysis of 69 association studies with 27,906 subjects concluded that the AGT 235 TT genotype conferred 31% greater risk for hypertension (P = 0.001), whereas those with the MT genotype had 11%

greater risk (P = 0.03).[359] The effect was most frequently found in Caucasian populations and in males. Subsequent studies have continued to find mixed, but generally positive association.[360-365] Blood pressure responses to an angiotensin converting enzyme inhibitor were greater among those with the 235T allele in one study,[366] whereas another group reported negative findings.[367]

A G-A substitution at position 6 upstream of the initial transcription site was found to be in linkage disequilibrium with the T235 allele and haplotypes containing both the G-6A and M235T polymorphism were associated with hypertension.[368] The -6A variant in expression studies, was found to result in an increase in function compared to the -6G allele.[369] In the large Family Blood Pressure Program metaanalysis, AGT -6A was associated with the diagnosis of hypertension but not with higher blood pressure in the normal range.[370] Consistent with the notion that the -6A (or 235T) allele of AGT results in increased tissue expression of angiotensinogen, both normotensive homozygous carriers of the 235T AGT variant (most with a positive family history of hypertension)[371] and hypertensive patients with the -6AA genotype[372] showed significant blunting of renal vascular response to infused angiotensin II. This blunting (less than usual reduction of renal blood flow in response to infused angiotensin II on a high salt diet) is presumably the result of higher intrarenal angiotensin II production resulting in downregulation of vascular angiotensin II receptors. There was also blunting of the normal stimulation of adrenal aldosterone production by infused angiotensin II on a low salt diet in hypertensive patients with the AGT -6AA genotype.[372] In one study of normotensive and hypertensive persons over age 60, the AGT 235T genotype was associated with a lesser diastolic response to changes in salt intake.[373] These results may suggest that

the ability of the renin-angiotensin system to respond to marked changes in salt over a relatively short time remains intact with the relatively modest effects of the -6A variant. Nevertheless, 2 long-term intervention studies including a reduction of salt intake[374;375] and one study utilizing increased fruit and vegetable intake[376] have noted greater responses to the intervention in persons with the -6AA genotype. Those with the -6GG genotype had the least response, whereas heterozygotes were intermediate.

Other phenotypes besides blood pressure may be affected by AGT genotype. Left ventricular mass index was found to be increased in persons with the 235T variant in a Chinese study.[377] Persons with LVH who had AGT 174M or 235T alleles responded to irbesartan with much greater reductions in LV mass than those with other alleles. Response to atenolol was not associated with genotype.[378] These results may explain lower LV mass in (mostly treated) hypertensives with 235 MT and TT but the reverse association in normotensives from the HyperGEN echo study.[379] In a family with a known gene causing hypertrophic cardiomyopathy, higher activity variants for several genes of the renin-angiotensin system (including AGT 235T) were associated with greater LV mass in cardiomyopathy gene carriers.[380] Yet another group reported a greater increase in brain infarctions associated with the 235T variant, although no association with extracranial, carotid atherosclerosis was found.[381] The AGT 235T variant has been associated with more rapid progression of IgA nephropathy.[382;383] One group[384] reported more rapid progression to end-stage renal disease in diabetics with the 235T variant while another did not.[385]

### 1.3.7.2 Renin

The renin gene was the first to be investigated in rats and humans as a candidate gene for hypertension. Early association studies with several restriction fragment length

polymorphism provided negative results.[386;387] An affected sib-pair study performed in French families using a combination of RFLPs grouped in haplotypes found no excess concordance for estimated renin haplotypes in affected hypertensive sibs.[388]

### 1.3.7.3 Angiotensin-1 converting enzyme gene

ACE has a key role in angiotensin II generation and bradykinin degradation, and may thus markedly affect vascular wall reactivity and morphology and kidney function. The ACE gene lies within the genomic region which is linked with hypertension in the SHRSP strain of rat. The human ACE gene has been cloned and localised to chromosome 17q23 and a 287bp insertion/deletion(I/D) polymorphism in intron 16 has been identified for use a genetic marker. The polymorphism of the ACE gene strongly modulates plasma ACE level. Therefore any relationship found with the ACE gene marker would be readily attributable to the biological phenotype associated with the polymorphism.

Two studies have reported a linkage with the ACE locus with diastolic blood pressure or mean blood pressure. In a sample consisting of 3095 participants of the Framingham Heart Study, there was an association of the ACE DD genotype with increased diastolic blood pressure in men but not in women.[389] In a large population based sample of 1488 siblings the ACE locus was found to be linked to diastolic blood pressure and mean blood pressure in adolescents, at a mean age of 15 years.[390]

The distribution of the common alleles of the ACE gene was studied in 170 Caucasian adults with contrasting genetic predisposition to high blood pressure, defined on the basis of personal and parental blood pressures. Young adults with the greatest predisposition who had high blood pressure and two parents with high blood pressure

did not show any significant difference in the distribution of markers of the ACE gene, either as genotype or allele frequencies, when compared with young adults with the least predisposition to high blood pressure.[391] The DD genotype is also associated with a higher risk for cardiovascular disease, particularly acute myocardial infarction, left ventricular and carotid wall hypertrophy, restenosis after transluminal coronary artery angioplasty and reduction in LV mass index in response to enalapril and not associated with stroke.[392-394]

Multiple variants that are in linkage disequilibrium with the I/D polymorphism have been described[395] but it is unknown whether any of these are implicated alone or in combination in the determination of plasma ACE levels. A combined cladistic/measured haplotype analysis of ten polymorphisms spanning 26 kb of the ACE gene excluded upstream sequences including the ACE promoter from harboring the major ACE-linked variant involved in 36% of total trait variability.[205]

More recently, however, evidence for association of the ACE I/D variant have emerged in the context of interaction. Thus, associations of the DD genotype with hypertension are reported for men but not women[389] and in the context of higher risk variants of angiotensinogen,[365;396;397] α-adducin,[398;399] and aldosterone synthase.[399] Other variants of ACE may also affect activity and blood pressure association.[400] ACE DD genotype was associated with greater left ventricular mass index in endurance-trained athletes.[401]

### 1.3.7.4 Angiotensin type I receptor

There is little data concerning the biology of AT1R in hypertension, as it is a cellular component of the RAS and thus less accessible to quantitative measurement.

Though polymorphisms have been found in the coding region and in the 3' untranslated region, only one polymorphism 1166C of the A1166C polymorphism was more frequent in hypertensives.[402] No evidence for linkage was found in a sib-pair study involving 267 pairs from 138 pedigrees, using a dinucleotide microsatellite marker.[403] Either the association found with the A1166C polymorphism is spurious or the sib-pair study is not sensitive enough to detect the linkage, due to its lower power compared to the association study. Another study performed in Australian subjects found a positive association with the A1166C polymorphism, but it was not the case in German hypertensives.[404;405] Analysing the A1166C polymorphism jointly with the ACE I/D polymorphism, showed that the increased risk of MI associated with the DD genotype was restricted to subjects bearing the 1166C allele either in the heterozygous or homozygous state.[406] In a study of Finnish hypertensive twins, two intragenic markers for the type 1 angiotensin II receptor (AT1) showed some evidence for linkage in the total sample. A closer examination of this gene locus was carried out using subgroups of non-obese sibpairs with early onset of hypertension and uniform geographical origin. These stratifications yielded suggestive evidence for linkage of hypertension to the genetic area containing the AT1 gene, with a maximal multipoint logarithm of the odds (LOD) score of 2.9.[407] In the same study, a genetic association study carried out in an independent series of 50 hypertensive cases and 122 normotensive controls showed an increase in the frequency of the A1166-->C allele of the AT1 gene in the hypertensive individuals.[407]

Since no alteration in the coding region of the gene has been detected in hypertensives, the next step would be to look for polymorphisms in the regulatory

regions of the gene resulting in modification of receptor number rather than receptor affinity.

### 1.3.7.5 Aldosterone synthase

Two polymorphisms of CYP11B2 have been studied extensively in hypertension. One is a single-nucleotide polymorphism in the 5' promoter region of the gene at –344C/T that alters a putative recognition site for the transcription factor SF1.[408] The

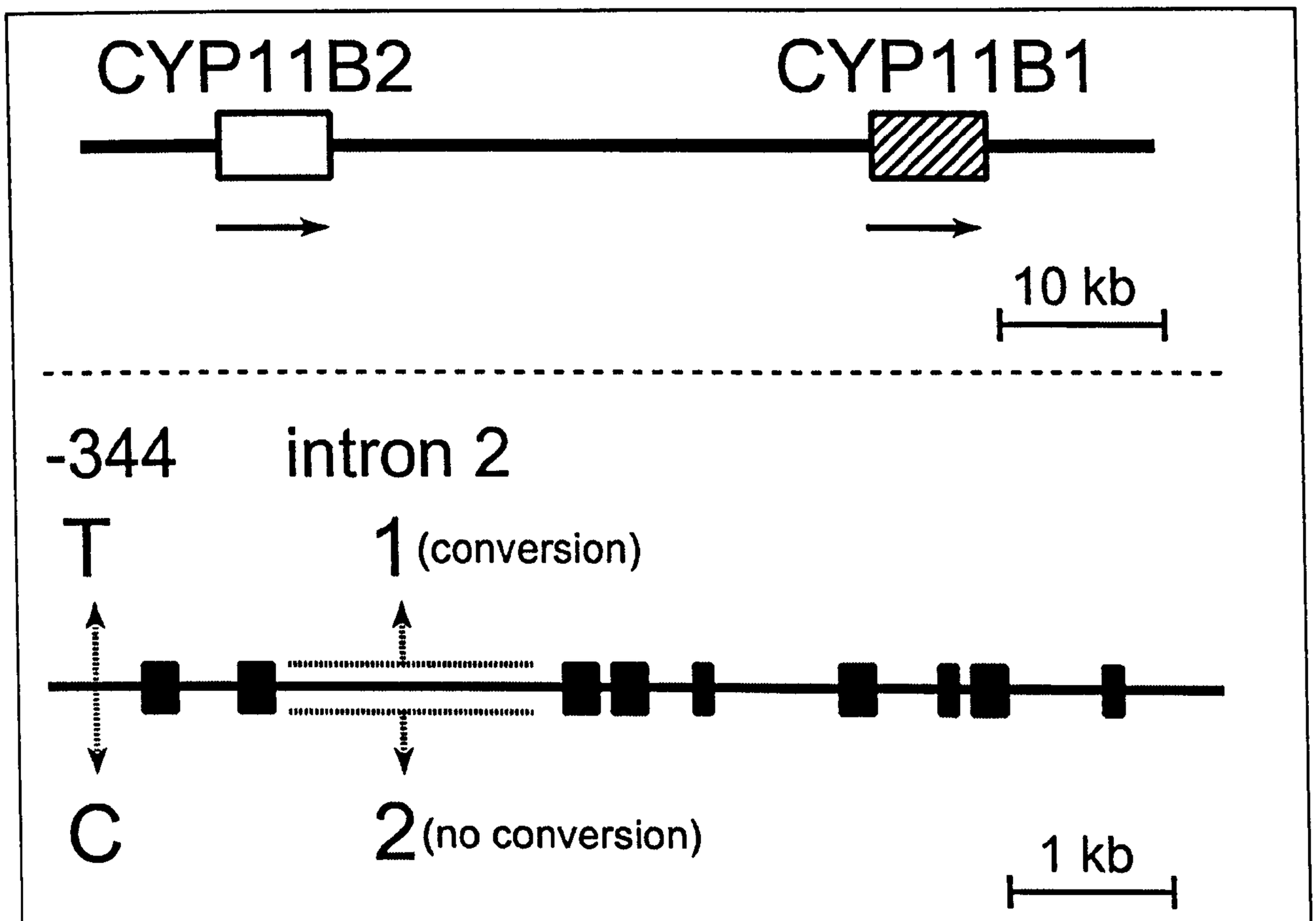


**Figure 1.3-3 Top, Relative orientations of the CYP11B2 (aldosterone synthase) and CYP11B1 (11ß-hydroxylase) genes.**

**Arrows indicate the direction of transcription, and a scale of 10,000 bases (10 kb) is marked. Bottom, Schematic of the CYP11B2 gene showing the locations of polymorphisms at the -344 position in the promoter and in the second intron (an intervening region that is spliced out of mRNA). Protein-encoding regions in the gene are indicated by boxes. A scale of 1000 bases (1 kb) is marked.**

immediate biologic importance of this is unclear, as recent reports suggest that, whereas binding of SF1 is reduced 4-fold by the change from C to T, there is no discernible effect on gene transcription studied in vitro systems.[409] The second involves intron 2 of CYP11B2, which is replaced, in part, by the corresponding intron

from CYP11B1. The 2 polymorphisms are in close linkage disequilibrium, so that the common haplotypes identified are T conversion (38%); C wild-type (45%), and T wild-type (16%).[410] The T allele has been associated with increased urinary excretion of the aldosterone metabolite tetrahydroaldosterone,[410] higher plasma aldosterone levels[411] lower aldosterone levels.[412] Some studies have found the T allele and Intron 2 conversion to be more frequent in patients with essential hypertension by us and several other groups,[410;413] while others have not. More recently, the T allele and intron 2 conversion have been shown to associated with hypertensive patients with a high aldosterone-renin ratio, and allele frequencies were normal in subjects with a normal ratio.[414]

Though there is some evidence to link the CYP11B2 locus and hypertension, particularly that associated with mineralocorticoid excess, Fisher et al [415] showed that the variance in renin status attributable to familial factors was ~35% and the CYP11B2 SF1 polymorphism was not shown to be a major determinant of this. Connell et al[416] suggest that the impairment of β-hydroxylation associated with the T allele leads to a subtle, chronic increase in adrenocorticotrophin drive to the adrenal cortex, with eventual development of hyperplasia. This might be responsible for the long-term development of a resetting of the aldosterone response to angiotensin II, giving rise to the phenotype of hypertension with a raised aldosterone-renin ratio.

### 1.3.7.6 Endothelial nitric oxide synthase gene

Several lines of evidence suggest that blood pressure levels result from an equilibrium between a vasoconstrictor tone (mainly renin-angiotensin and endothelin systems), and a vasodilator tone, due to nitric oxide, prostacyclin and other less characterised factors, such as endothelium derived hyperpolarising factor(EDHF).

The Nitric Oxide Synthases are a family of complex enzymes that catalyse the five-electron oxidation of L-arginine to form NO and L-citrulline. The endothelial isoform of NO synthase (eNOS) represents a major candidate gene since this isoform is responsible for the constitutive regeneration of NO from arginine by the endothelial cells, which in turn activates guanylate cyclase in the vascular smooth muscle cells. The involvement of he eNOS gene was demonstrated by basal blood pressure increase in mice homozygous for the knockout of the eNOS gene, as compared to wild-type mice.[417] No polymorphisms have been detected in the coding region of the of the eNOS gene. Using a highly informative dinucleotide repeat inside intron 13, two sib pair studies showed no significant excess of allele sharing in siblings.[418;419] These two studies do not support a prominent role of the eNOS gene in essential hypertension. There is conflicting evidence for effects of variants of endothelial nitric oxide synthase (NOS3) with both positive[420] and negative[421-423] association studies. Persons with the CC genotype of a -786T>C variant had higher blood pressure and were more than twice as likely to be hypertensive.[424]

### 1.3.7.7 Endothelin system genes

Endothelins (ETs) are 21-amino-acid peptides produced in many cells and tissues. The vascular ET system is represented mainly by ET-1 produced in endothelial cells and is cleaved from a larger precursor, 38 amino acids in length, by the endothelin converting enzyme (ECE). It binds to the $ET_A$ receptor on the vascular smooth muscle and provokes vasoconstriction. Studies with endothelin receptor antagonists have indicated that endothelin-1 probably has complex opposing vascular effects mediated through vascular smooth muscle and endothelial ET(A) and ET(B) receptors. Endogenous generation of endothelin-1 appears to contribute to maintenance of basal

vascular tone and blood pressure through activation of vascular smooth muscle ET(A) receptors. At the same time, endogenous endothelin-1 acts through endothelial ET(B) receptors to stimulate formation of nitric oxide tonically and to oppose vasoconstriction.

Polymorphisms of the ET-1 and ET(A) receptor genes have shown non significant or marginally significant associations with hypertension.[425] The Lys198Asn variant in the endothelin-1 gene (on 6p24-p23) appears to interact with BMI resulting in mildly greater risk for hypertension in 198Asn carriers with higher BMI.[306-308] It is worth noting that the ECE gene is located on chromosome 1p36 on which a locus for systolic BP was found by sib-pair analysis with a protein marker namely 6-phosphogluconate dehydrogenase.[426]

### 1.3.7.8 Adducin

Genetic hypertension in the Milan hypertensive (MHS) rats is due to a renal abnormality in the ability to excrete salt. This alteration is genetically determined, since hypertension can be transplanted with MHS kidney in normotensive Milan normotensive (MNS) rats, even when the kidney derives from a young prehypertensive animal. Na/H countertransport in the brush-border vesicles, Na-K co-transport in the luminal membrane of the thick ascending limb, and the activity and mRNA expression of the basolateral Na-K ATPase both in the proximal and ascending limb tubules are all increased in MHS as compared with MNS. Since these differences in ion transport disappear after removal of cytoskeleton, cross-immunization studies set up to detect possible differences between the cytoskeletal components of the two strains revealed a difference in a protein subsequently identified as adducin.

Adducin is an α/β heterodimeric protein which participates in the assembly of spectrin-actin cytoskeleton, modulates the actin polymerisation, binds calmodulin, is phosphorylated by PKC and tyrosine kinase and regulates cell signal transduction. Point mutations in both the α and β adducin subunits account for around 50% of the difference in blood pressure between MHS and MNS strains.[427] Transfection of hypertensive and normotensive α adducin variants in rat kidney cells showed that the former increases the surface expression and maximal rate of Na-K pump.[428] A direct and specific interaction between adducin and Na-K ATPase suggests the possibility of such an interaction in intact renal membranes.[429]

There is a close homology (94%) for the alpha adducin gene between rat and man. Initial positive association studies in humans[430] were followed by significant linkage in hypertensive siblings[284] and identification of a Gly460Trp variant (with an allele frequency of 0.13-0.16 in controls) associated with hypertension. Subsequent association studies have been reminiscent of findings after the discovery of the AGT association, with mixed but generally positive findings, especially for Caucasian populations.[431-433] Province et al found the association of ADD1 Gly460Trp with hypertension, with an approximately 50% to 70% increase in risk in Caucasians carrying the 460Trp variant, increasing to an odds ratio of 4.2 in older, heavier persons with higher triglycerides; no effects of were seen in African-Americans.[309]

Pathophysiological studies in humans are consistent with a significant increase in salt retention associated with the ADD1 460Trp variant. Thus, greater reductions in blood pressure in response to a diuretic[284] and greater increases in blood pressure after a

saline load[398] were seen in 460Trp carriers. Carriers also showed lower fractional excretion of sodium and a flattened pressure-natriuresis curve.[434] Finally, 460Trp homozygotes more frequently had low-renin hypertension and displayed greater fraction sodium reabsorption.[435;436]

Effects of the ADD1 gene on blood pressure appear to interact strongly with other genes. The increase in blood pressure after a salt load was much greater in carriers of the 460Trp ADD1 variant who also had the ACE DD genotype than in those with the ACE II genotype.[398] ACE genotype had no effect (or possibly reverse effect) in those homozygous for the more common 460Gly variant. A prospective study suggests a similar interaction affecting the risk of incident hypertension.[399] In a well matched case-control study, Clark et al[437] found no evidence to suggest an association between either the alpha-adducin G460W or the ACE I/D polymorphism and hypertension. Furthermore, they found no interaction between alpha-adducin G460W, ACE I/D, and aldosterone synthase SF-1 and IC polymorphisms in the hypertensive population.

Other phenotypes may be affected by ADD1 variants. Presence of ADD1 460Trp was associated with an approximate 2.5-fold increased risk of CAD or peripheral artery disease in a prospective follow-up of hypertensive patients. The risk was not seen in normotensive subjects.[438] In a case-control study, diuretic therapy was associated with a 50% reduction in MI and stroke in 460Trp carriers; noncarriers had no risk reduction associated with diuretic use.[439] Risk of left ventricular hypertrophy was increased 15-fold in 460Trp homozygotes.[440] Interestingly, increased salt intake has also been strongly associated with increased cardiovascular morbidity and mortality[441]

and left ventricle mass[442] independent of blood pressure. Furthermore, salt-sensitivity has also been associated with greater total mortality independent of blood pressure.[443]

### 1.3.7.9 ENaC

Variants of the endothelial sodium channel initially were not associated with essential hypertension,[351] but more recent studies have found some association[444;445] or linkage.[446;447] Hypertensive black Africans with the Thr594Met variant (found in about 5% and associated with hypertension in a previous study) were highly responsive to amiloride.[448] Approximately 7% (10 of 139 tested) of hypertension in blacks was attributable to the Arg563Gln mutation of endothelial sodium channel in one study.[449]

### 1.3.7.10 GNB3

Detailed in Section 1.6.3 GNB3, page 183.

### 1.3.7.11 Dopamine Receptor

An indirect association exists between the renal dopamine system and ion transport. The renal dopamine system may be considered apart from the sympathetic nervous system in that kidney dopamine is synthesized independently of nerve activity. Proximal tubule cells synthesize dopamine from L-DOPA in tubular fluid. Synthesis of dopamine is increased by a high-sodium diet and is also strongly influenced by delivery rate of L-DOPA in filtered fluid in the proximal tubule. Dopamine, by way of the dopamine D1 receptor, downregulates sodium transport by NHE3 in the apical membrane through cAMP-mediated effects and also downregulates Na+-K+ ATPase through diacylglycerol and PKC-mediated effects.[450] The D1 receptor has other intracellular effects and there are other dopamine receptors.[451] Of particular interest

are observations of defective receptor coupling associated with increased D1 receptor serine phosphorylation in both spontaneously hypertensive rats and humans with essential hypertension.[452] This increased receptor phosphorylation (resulting in uncoupling of the D1 receptor from downstream adenylate cyclase activation) was later traced to increased activity of G protein-coupled receptor kinase 4 (GRK4γ).[453] In particular, several variants of the γ subunit of GRK4 showed increased activity by biochemical measures and in transgenic mice. Cursory data in human populations suggested increased risk for hypertension associated with several GRK4γ variants all showing higher than normal activity.[453] Significant interaction between variants of GRK4γ (termed "FJ" in this study) and variants of the renin-angiotensin system was also found[397] with reportedly high prediction of hypertension status using combinations of genotypes.[454] There are reports of variants in the D1 and D2 receptor genes being associated with hypertension.[455;456]

### 1.3.7.12 β-Adrenergic Receptor

Genome-wide scans have now reported linkage to one or more sites on virtually every chromosome. In one of these scans, investigators noted that the β2-adrenergic receptor was a candidate under one of the peaks (a positional candidate gene approach). An Arg16Gly variant was found to be associated with a significant 1.8-fold increase risk of hypertension.[457] Confirmatory results for this variant have been reported in other populations[458;459] as well as a negative study.[460;461] Attenuation of the vasodilatory response in humans with the Gly16 allele provides biological plausibility.[462] Evidence for association of hypertension with variants of the β1[463] and β3-adrenergic receptors[311;464] has also been published.

### 1.3.7.13 Kallikrein

Several studies find reduced urinary kallikrein in association with human hypertension and multiple studies support a role for kallikrein produced in the connecting tubule of the kidney (through local generation of kinin and bradykinin) in promoting diuresis and natriuresis in the face of a high salt intake.[465] Nevertheless, linkage and association studies of human hypertension have generally been negative for tissue kallikrein (KLK1) and other genes of the renal kallikrein system.[421;466] Furthermore, a recently identified variant causing marked decrease in kallikrein activity was not associated with increased blood pressure.[467] Potentially, other factors, such as dietary potassium intake, may interact to modify associations with urinary kallikrein.[468] Whereas gene delivery of human tissue kallikrein reduced blood pressure in spontaneously hypertensive rats,[469] mice made deficient in either the bradykinin receptor-2 or tissue kallikrein were not hypertensive.[470] Further work will be required to provide further clarification of the role, if any, of the renal kallikrein-kinin system in hypertension.

## 1.4 Left Ventricular Mass

### *1.4.1 LV Mass and LV Hypertrophy*

LV mass shows a continuous distribution in the general population,[471] whereas LV hypertrophy is an operational category that defines the upper end of LV mass distribution. [471;472] Left ventricular hypertrophy is often assumed to be little more than a marker for hypertension. In fact, the relation between diastolic or systolic blood pressure and left ventricular mass is not always close.[473;474] Left ventricular hypertrophy is an independent risk factor for myocardial infarction and death in men and women with hypertension[471;472] and in asymptomatic subjects with normal blood.[49;475] In hypertensive patients it is a stronger coronary risk factor than casual blood pressure readings. The Framingham Heart Study showed an apparently continuous relation between LV mass and cardiovascular event rate in the general population.[476] However, the important clinical issues regarding the shape of the relation between LV mass and cardiovascular risk in essential hypertension and the prognostic impact of LV mass values below the commonly agreed-on upper normal limits have not been addressed.

Though left ventricular hypertrophy strongly predicts morbidity and mortality in individuals with or without known cardiovascular disease, the factors associated with the wide variability of LV mass in the general population remain incompletely characterized. A number of constitutional and environmental factors have been identified that influence left ventricular size; these include age, sex, body size, blood pressure, physical activity, salt intake, alcohol consumption, and the presence of heart disease or diabetes.[48;477-479] Other studies have identified additional haemodynamic

associations with LV mass-beyond the well-known association of blood pressure-of the level of haemodynamic volume load and myocardial contractile efficiency.[480-482]

Blood pressure, obesity, and age are important determinants of left ventricular mass[48]; however, they account only for part of the observed variance. Body growth influences cardiac development. The close relation between body size and left ventricular (LV) mass[483] during childhood and adolescence is the hallmark of this influence. However, in infancy virtually the entire variability of LV mass is explained by body size, whereas with increasing age the ability of body size to precisely predict LV mass decreases. In fact, the difference between the value of observed LV mass and that predicted from body size increases with increasing age (heteroscedastic distribution of residuals).[484] This phenomenon might be explained by the progressive haemodynamic load that faces the left ventricle right after birth as the fundamental stimulus for LV muscular development.

Although blood pressure, stroke volume, and decreases in contractile efficiency are important determinants of LVH,[485] this complication is not present in all patients with essential hypertension and can develop before the establishment of hypertension in some individuals.[486] Epidemiological studies have demonstrated that subjects with LVH may have near normal blood pressure,[48] indicating that additional factors besides blood pressure may be important in the development of hypertrophy. Race is known to have a significant effect on left ventricular mass even following correction for other associations.[487] Left ventricular mass is higher in black subjects than in their white counterparts but it is unclear if this represents a greater cumulative exposure to

stimulants of hypertrophy such as hypertension or a familial or genetic pre-disposition to the development of hypertrophy.

Studies in families and twins have shown that left ventricular mass is a familial trait, indicating the influence of both genetic and environmental factors.[488;489] Evidence that left ventricular mass is a familial trait[488;490-492] suggests the influence of genetic factors, while the absence of simple mendelian patterns of inheritance (except in rare syndromes[493]) identifies left ventricular mass as a complex phenotype that is influenced by interacting genetic and environmental factors.

Thus the pathogenic mechanism of LVH may be multifactorial, involving both haemodynamic and non-haemodynamic factors, such as the sympathetic nervous system and the renin-angiotensin system.[494] Recent investigations into the genetic determinants of LVH have focussed mainly on the renin-angiotensin-aldosterone system, with focus on the I/D polymorphism of the ACE gene, but results have been conflicting.[495-498]

### *1.4.2 Measurement of LV Mass*

Definitions of left ventricular hypertrophy have varied and normotensive control subjects may have an incidence from 1.3% to 9.4% according to which one of 13 criteria is applied.[473] For research purposes it is probably better to treat left ventricular mass as a continuous variable without categorising it as either normal or abnormal; in clinical use, thresholds for hypertrophy of 134 g/m2 in men and 110 g/m2 in women seem reasonable. However, even these may vary with population characteristics such as age, race, and physical activity, so that matched contemporaneous control groups

should ideally be used. LV mass can be measured by echocardiography, ECG or MRI. As the major analysis in this thesis is based on Echo and ECG LV mass, these will be detailed more extensively here.

### *1.4.3 Echocardiographic LV Mass*

Echocardiographically measured LV mass has been the gold standard until recently, now it is replaced by MRI. LV measurements are obtained at end diastole. End-diastolic measurement criteria include both the American Society of Echocardiography (ASE) and the Penn conventions. The ASE convention uses a leading edge measurement with end diastole identified at the beginning of the QRS complex of the simultaneously recorded ECG.[499] The Penn convention excludes endocardial or epicardial surfaces in the measurement of wall thickness and includes endocardial surfaces in the LV dimension measurement.[500] End diastole is defined as the peak of the R wave of the QRS complex.

The LV measurements include interventricular septal thickness at end diastole (IVSTd), the posterior wall thickness at end diastole (PWTd), and LV internal dimension at end diastole (LVIDd). From the diastolic measurements, LV wall mass (LVM) is calculated from the Penn convention, according to the Equation of Devereux and Reichek[500] by the formula

$$\textbf{LV Mass (gms)} = 1.04 \times (\textbf{IVSTd} + \textbf{PWTd} + \textbf{LVIDd})^3 - (\textbf{LVIDd})^3 - 13.6$$

LV mass index (LVMI, grams per square meter ($g/m^2$) is calculated by dividing LV mass by body surface area

$$\textbf{BSA } (m^2) = 71.84 \times \textbf{height (cm)} \times 0.725 \times \textbf{weight (kg)} \times 0.425.$$

Another index (LVMI, grams per meter (g/m)) can be calculated dividing LV mass by height.

The diagnostic criteria for LV hypertrophy using LV mass index ($g/m^2$) were >=134 $g/m^2$ for men and >=110 $g/m^2$ for women, representing the sex-specific 97th percentiles of a previously published reference standard in a normal population,[501] both based on Penn convention measurements. LV hypertrophy criteria using LV mass index (g/m) were >=143 g/m for men and >=102 g/m for women, based on similar reference criteria.[502]

M mode echocardiography tends to overestimate left ventricular mass, and two dimensional echocardiographic methods are more accurate, particularly when cavity size or myocardial thickness is non-uniform.[503] Generalisation to the whole left ventricle from M mode dimensions recorded at the base of the heart is inappropriate in the presence of established infarction; this diminishes the importance of some studies.[504]

## *1.4.4 ECG LV Mass*

### 1.4.4.1 ECG Voltage Criteria

Electrocardiographic left ventricular hypertrophy (LVH) has been recognized as a risk factor for cardiac events for some time and electrocardiographic left ventricular hypertrophy (ECG-LVH) was of nearly paramount importance as the primary epidemiological tool and clinical diagnostic method for LVH detection in patients with hypertension, acquired and congenital valvular and other cardiac defects. The clinical significance of ECG-LVH has diminished with the improvement of

echocardiographic imaging technology. The epidemiological significance of ECG-LVH is primarily in the realm of risk evaluation.

Most of the seminal data on ECG LV mass comes from the Framingham study,[505;506] but the assessment of the actual impact of LVH has been confounded by the use of different definitions. The most commonly used electrocardiographic definitions of LVH have been the voltage criteria of Sokolow and Lyon,[507] and that of Casale et al.[508] There have been many attempts at improving the predictive value of the electrocardiogram by relating the 12 lead ECG to echocardiographic estimates of LV mass.[508-510] Casale and colleagues[508] found that augmenting the Cornell criterion (RaVL+SV3) with information from the T wave in V1 improved the performance of the ECG in estimation of LV mass. Okin[510;511] suggested the use of a time-voltage criterion for identification of LVH. However, considering sex, age, body surface area, the duration of the terminal P in V1, and the S voltage in V1 and V4 explains more of the variance than this model,[512] than a linear regression model of Wolf,[513] and significantly more than standard criteria. Okin et al.[514] examined the test accuracy of the criteria for LVH in relation to body mass index in 250 patients and confirmed the need to consider BMI in LVH estimates. This was reinforced by findings from the Framingham study where incorporation of obesity and age into ECG algorithms consistently improved their performance in the detection of hypertrophy.[515;516] The landmark Losartan Intervention For Endpoint reduction in hypertension (LIFE) study used 2 of these ECG criteria to diagnose LVH and confirm reduction of cardiovascular events with regression of LVH.[517] The study initially used the product of QRS duration and Cornell voltage (RaVL+SV3), with an adjustment of 8 mV for females and a partition value of 2440 mV·ms (the Cornell product criterion).[510;518]

More recently, Rautaharju, using data from the third National Health and Nutrition survey, and the Atherosclerosis Research in Communities study demonstrated that Sokolow-Lyon voltages decreased and Cornell voltages increased significantly with increasing breast tissue.[519] However, the overall conclusion was that these effects were small, and that when entered into a multivariate equation, chest size was the dominant variable.

Crow et al.[520] studied the association between eight ECG criteria and echocardiographic LV mass estimates in men and women with mild hypertension. Electrocardiograms and echocardiograms were recorded at baseline, 3 months, and annually for 4 years. The ECGs were computer processed to define 8 different criteria, and the researchers found a poor correlation between ECG and the echocardiogram. However, this result may have been confounded by poorly reproducible echocardiographic measurements.

Rautaharju derived the optimal ECG models for predicting LV mass adjusted for body size from Cornell voltage (CV).[521;522] The equations for white men and women for LV mass in grams are given below.

**Men: LVM = 0.026* CV + 1.25 * W + 34.4**

**Women: LVM = 0.020 * CV + 1.12 * W +36.2**

Okin et al[511] showed that time-voltage-area measurements derived from digital ECGs improved the ECG identification of LVH beyond that available from standard QRS voltage. They found the sensitivity for identification of LVH using 12 lead voltage was 46%, 12 lead voltage-time product 54% and 12 lead voltage area 76%.

A summary of various ECG criteria for LV mass is given in Table 1.4-1.

| ECG criteria | Measurement |
|---|---|
| Sokolow-Lyon voltage | $SV_1 + RV_5$ or $V_6$ |
| Cornell Voltage | $RaVL+SV_3$ |
| Sokolow-Lyon voltage-duration product | $(SV_1 + RV_5$ or $V_6)$*QRS duration |
| Cornell voltage-duration product | $(RaVL+SV_3)$ *QRS duration |
| 12 lead voltage | Sum of QRS voltage in all 12 leads |
| 12 lead voltage -duration product | (Sum of QRS voltage in all 12 leads)*QRS duration |
| LV mass – Rautaharju | Men = 0.026* CV + 1.25 * W + 34.4<br>Women= 0.020 * CV + 1.12 * W +36.2 |

**Table 1.4-1 ECG criteria for LV mass**

### 1.4.4.2 Minnesota Code

The "Minnesota code" early on became the de facto standard for the measurement of ECG abnormalities in epidemiological trials. Computerization has solved many of the problems that the Minnesota code was designed to address.[523] The most commonly used computer coding system in epidemiological trials has been the NOVACODE system.[521] The US pooling project categorized ECG findings into major and minor groupings. Because some authors have found this useful in achieving statistical significance where the individual ECG abnormalities fail to do so, it has intuitive appeal for epidemiology. However, the clinical utility of this simple dichotomization is uncertain and, remarkably, the final report of the Pooling Project [Pooling Project Research Group, 1978] does not make it clear why these particular abnormalities were chosen, or indeed why they opted to categorize at all. Despite this, the categorization proved popular and was adopted in numerous trials.[524-529] The presence of major Q waves on the ECG was an exclusion criterion; thus, studies that used the Pooling Project categorization did not consider major Q waves.

A critical factor in the adoption of any screening test is the prevalence of a positive test in the asymptomatic, apparently healthy population. There were few studies that fully presented ECG data on asymptomatic participants.[525;530-535] In addition, there was some variation in the exclusion criteria for cardiac disease (symptoms, etc.) in those studies that did. However, despite the wide inter-population variation in prevalence, and despite some studies finding no intra-population difference in prevalence of ECG abnormalities between those with a diagnosis of heart disease and those without,[526] findings from 18,403 British men in the Whitehall study suggest caution in the combination of these two groups for analytical purposes.

A summary of the Minnesota codes used is given below.

| | Minnesota codes |
|---|---|
| Major ECG findings | $IV_{1-2}$ or $V_{1-2}$ or $VI_{1-2}$ or $VII_{1-2}$ or $VIII_{1-3}$ |
| Minor ECG findings | $I_3$ or $II_{1-2}$ or $III_{1-2}$ or $IV_3$ or $V_3$ or $IX_1$ |
| Only minor ECG findings | Minor ECG changes but no major ECG changes |
| Ischaemic ECG findings | $I_3$ or $IV_{1-3}$ or $V_{1-3}$ or $VII_1$ |
| ST depression | $IV_{1-3}$ |
| Abnormal T wave | $V_{1-3}$ |
| Arrhythmias | $VIII_{1-6}$ or $VI_{1-2,4}$ |
| Bundle branch blocks | $VII_{1-2,4}$ |
| Left ventricular hypertrophy | $III_1$ and ($IV_{1-3}$ or $V_{1-3}$) |
| Left axis deviation | $II_1$ |

**Table 1.4-2 Minnesota codes and ECG abnormalities**

### *1.4.5 Reliability of ECG LV Mass estimates*

Any test considered as a screening test should be considered in terms of its sensitivity, specificity and predictive value. If we are to assess the prognostic value of a screening ECG, we need to compare the test characteristics to the ultimate endpoint: mortality.

In this way, we can gain some idea for the amount of variance we are able to account for using the ECG. Only one paper in the literature has previously attempted this.[533] These calculations are displayed in Table 1.4-3. As is clearly seen, the sensitivity estimates of individual ECG abnormalities are very low. We know that attributable risk relates to population prevalence and that low prevalence will result in low sensitivity, and this seems to be what is happening here. The data is calculated only from those studies with stringent exclusion criteria, so we could be certain of assessing the true screening qualities of the test. The sensitivity values seem to be highest for LVH and this almost certainly relates to the higher prevalence of this abnormality and (at least when defined by the Framingham investigators using ST depression inclusive criteria) greater risk.

| Study | Q Waves | | ST Depression | | BBB | | LVH with strain | |
|---|---|---|---|---|---|---|---|---|
| | Sens | Spec | Sens | Spec | Sens | Spec | Sens | Spec |
| Framingham | 19 | 98 | 18 | 98 | | | 37 | 94 |
| British Regional | 21 | 96 | | | 5 | 98 | 2 | 99 |

**Table 1.4-3 Sensitivity and specificity estimates of ECG abnormalities to mortality**
**Adapted from Menotti et al.[533]**

All ECG abnormalities increase with age and that some are more prevalent in men (Q waves, RBBB) while others are more prevalent in women (ST depression, LBBB). A striking finding is that ST depression inclusive-LVH (that is, LVH with strain) has a 33% five-year mortality in men and a 21% five-year mortality in women.[505] Also, unrecognised Q wave infarction is associated with the same risk as symptomatic infarction.[536] Studies have shown that ST depression is poorly reproducible, more prevalent in women yet its prevalence is associated with increasing risk.[537]

| Criteria | | White Men | Black Men | White Women | Black Women |
|---|---|---|---|---|---|
| Sokolow-Lyon | Specificity(%) | 90.6 | 86.6 | 96.3 | 94.6 |
| | Sensitivity(%) | 21.3 | 50.0 | 13.2 | 23.1 |
| | Prevalence(%) | 28.6 | 39.7 | 38.6 | 43.0 |
| Cornell Voltage | Specificity(%) | 95.5 | 95.8 | 91.6 | 85.3 |
| | Sensitivity(%) | 15.8 | 46.2 | 27.8 | 53.8 |
| | Prevalence(%) | 39.9 | 63.4 | 42.2 | 59.6 |

**Table 1.4-4 Sensitivity, specificity and correct fraction in the reported prevalence by two sets of ECG-LVH criteria with Echo-LVH as standard.**

**Adapted from Rautharju et al.[538]**

Rautaharju et al[538] showed that the sensitivity of ECG-LVH criteria varies from 15% to at most 55% at varying levels of specificity and that echo-LVH was relatively close to 15% in various gender and racial subgroups. The first conclusion of their study was that it is impossible to obtain ECGLVH prevalence 20% or higher as reported in some studies unless the specificity of the criteria is below 85%. The second conclusion was that it is in fact possible to get estimated LVH prevalence values agreeing within +/ - 10% with the true LVH prevalence with a variety of combinations of sensitivity and specificity when the fraction of true cases in the reported prevalence unacceptably low. A seemingly good prevalence estimate may be based on false information! On the other hand, estimates with a reasonably high correct fraction, such as at least 50%, are in most instances associated with a substantial over- or underestimate of the true prevalence. Actual data are summarized in Table 1.4-4 demonstrating that even with better criteria such as the Cornell voltage, reported prevalence deviated by more than 10% of the expected prevalence of 15% in all subgroups by race and gender, and the correct fraction in the reported

prevalence was only approximately 40% except 66% in black males. It can be concluded that the comparison of LVH prevalence data from contrasting populations with the present LVH criteria is an exercise in futility.

### 1.4.5.1 Reasons for Large Fraction of False Negatives by ECG-LVH Criteria

There is a multitude of reasons for the large fraction of echo-LVH missed by ECG-LVH models. One rational explanation attractive to frustrated electrocardiographers is that these false positives really represent a true diagnostic finding. Pathophysiologically valid and clinically important reasons for false negatives include situations where fibrotic tissue adds to the anatomical LVM and diminishes body surface potentials. CHD, clinical and subclinical, and LVH commonly coexist. Ischaemic injury in CHD and myocardial damage can interfere with normal ventricular conduction and diminish body surface potentials. Left ventricular remodelling in CHD and MI is different from at least initially more physiological adaptation in hypertension. The discrepancy between echo and ECG findings calls for an explanation when causative pathological condition can be suspected in a clinical situation. There are numerous other explanations, however, to account for the large fraction of false negative ECG-LVH findings and suggest that either the " gold standard" or our ECG-LVH model or both have serious deficiencies.

Early repolarisation is benign[539;540] and that the risk of bundle branch block depends on the population in which it appears.[541;542] The prevalence of atrial fibrillation rises exponentially with age and is associated with higher risk than any other ECG abnormality in this age group.[543] Sutherland et al. have confirmed the often-quoted observation that T wave inversion and high voltage QRS are more common in Blacks

than whites, but in general, do not predict coronary heart disease to the same extent.[529] Wannamethee et al. have also noted that elevated heart rates, but not ventricular premature beats, are independent risk factors specific for sudden cardiac death.[544]

### *1.4.6 LV Mass and Risk*

In the Framingham study, increased left ventricular mass was associated with an adverse outcome including cardiovascular death, all cause mortality, and in men, sudden death.[49] Early data from the Framingham study showed that patients with ST-T repolarisation abnormalities ("strain") on the electrocardiogram had a six-fold increase in cardiac deaths over a 20 year follow up period.[545] Echocardiography, however, is 5-10 times more sensitive than the electrocardiogram and detects left ventricular hypertrophy in 25-30% of all hypertensive patients.[473] In such patients, myocardial infarction or death occurs at the rate of 4.6 events per 100 patient years, three times the risk in patients with hypertension and normal left ventricular mass.[471] Even in asymptomatic subjects with normal blood pressure, increased left ventricular mass emerges as a risk factor for coronary disease, cardiac death, and all cause mortality.[49] The relative risk of all cause mortality is 1.5 in men and 2.0 in women for every 50 g/m increment in left ventricular mass indexed to height. The relative risk of sudden death is 1.7 per 50 g/m increment.

In all these studies multivariate analysis shows that, as a risk factor, left ventricular hypertrophy is independent of systolic and diastolic blood pressure, smoking, and cholesterol concentrations.[49;471;472;475] It is not always independent of age.[49;472] The relation between left ventricular mass and established coronary artery disease has also been examined in black subjects: left ventricular hypertrophy with normal coronary anatomy was associated with a five year mortality of 16.1%, which was similar to the

mortality (17.6%) with multivessel disease and normal left ventricular mass. Left ventricular hypertrophy was a more important independent predictor of death than multivessel disease or impaired left ventricular systolic function.[504] Other studies have shown a similar effect on the risk of death and reinfarction after Q wave[546] or non-Q wave infarction.[547]

What explains the association between left ventricular hypertrophy and increased risk? Although left ventricular mass may correlate poorly with casual systolic blood pressure and even less well with diastolic blood pressure, it is better related to 24 hour[548] or 30 year history of blood pressure.[549] Therefore, increased left ventricular mass could still be a marker for end organ effects of long-term hypertension. Left ventricular hypertrophy, however, is also known to be determined by other factors that may possibly increase coronary risk--these include obesity, age, blood viscosity, salt intake, and various genetically determined influences. The DD genotype of the angiotensin converting enzyme gene is linked with left ventricular hypertrophy but not with hypertension and has been shown to carry a higher risk of fatal and non-fatal myocardial infarction than the II genotype in some but not all studies.[550-552] Left ventricular hypertrophy may also occasionally develop as a result of established coronary disease as repeated ischaemia in dogs can induce left ventricular hypertrophy.[553]

Left ventricular mass may itself contribute to coronary risk through increased oxygen demand and reduced coronary reserve, impaired endocardial autoregulation, and possibly small vessel disease.[554] Finally, increased left ventricular mass may result not

only from cell hypertrophy but also from increases in collagen, which may provide a substrate for malignant arrhythmias and sudden death.[555]

The basic pathophysiological mechanisms underlying the association between LV mass and cardiovascular risk remain elusive. LV mass may be considered a time-integrated marker of exposure to high BP values and as a sensitive indicator of cardiac end-organ damage. It is well established that LV mass (1) increases myocardial oxygen consumption while reducing coronary blood flow reserve, (2) is associated with an increase in atherosclerotic lesions at cardiac and extracardiac levels, and (3) is associated with enhanced arrhythmogenesis.

### *1.4.7 Prevalence of ECG Left Ventricular Hypertrophy*

The prevalence of ECG-LVH varies vastly with LVH criteria used, source population, gender, race, age and the survey period. Most population studies have used either Sokolow-Lyon criteria or Minnesota Code that contains Sokolow-Lyon criteria as a subset of code 3.3. LVH prevalence in general high with these criteria but drops to quite low levels if combined with repolarisation abnormalities. All studies showed increases in prevalence of ECG-LVH with increasing age. The high values seen in the young men can be readily explained by physical fitness and muscular hypertrophy associated with testosterone. One can hypothesize that with aging men are less physically active and have correspondingly lower voltage R waves. Then, with further increasing age in both men and women, pathological processes set in, and the size of the R wave increases again. It has previously been demonstrated that gender differences in body size, obesity, and LVM do not completely account for the gender differences in QRS duration and voltage measurements.[556] In fact, recent studies in both humans and animals have emphasized gender differences in the response to

pressure overload. Although degree of hypertrophy seems to be similar,[557;558] male animals exhibit earlier transition to heart failure, with cavity dilatation, loss of concentric remodelling and diastolic dysfunction. This falls into line with human echo studies that show that for obesity and hypertension, relative increase in left ventricular mass is similar[559] among men and women, but that overall, other factors including risk[560] are not.[561]

It is not clear why there are such a wide variety of LVH prevalence estimates from electrocardiograms carried out in different populations using the same criteria. For the 50-59 year old males, the Finnish cohort of the Seven countries study had a mean prevalence of LVH (Minnesota Code 3-1) of 19%; the Finnish Social Insurance study had a mean prevalence of 27.3% and this relative high prevalence even extended to females (mean 13.5%). The figures demonstrate how far these points are outliers. Of all the other countries with predominant Caucasian populations, only Copenhagen (12%) and the Moscow cohort of the European study (18.7%) came close to these estimates. Estimates were also high in the black population, both from the Jamaica study (29.9% in the 40 to 49 age group) and 19.8% in Evans County. The wide variation is demonstrated by studies such as the Whitehall study which found a prevalence of less than 1% in British civil servants aged 50-59, and the age-pooled, white male cohort of the Charleston study. These wide variations demand some explanation. Many studies were rigorous in their training of coders and use of independent assessments. In particular, the Finnish social insurance study used 2 independent coders, and two or three independent medical readers at the University of Minnesota read all ECGs from the Seven Countries participating centres. Some important points can be made. Firstly, it seems likely that at least some of the differences noted are real. It would not seem unreasonable to conclude on the basis of

the above, that black populations and the Finnish population have truly higher mean R wave amplitude than many others. As discussed above, this may not necessarily imply a greater prevalence of echocardiographic LVH, although comparison of the relative weight and skin fold thickness measurements from the Seven Countries study suggests no difference between the Finnish population and the others (Finnish relative weight: 92.5%, others: 92%. Finnish skin fold: 15, others: 17.7). However, Finland did have the highest rates of hypertension and the CHD death rate was higher than all other countries.

A clearer pattern is the lower prevalence of ECG-LVH when the criterion requires ST depression. The Belgian study found the age-pooled prevalence of LVH by this definition to be 0.8% male and 0.5% female. The Honolulu Heart program found the prevalence of high R wave-LVH to be 5.4% compared to the prevalence of 0.6% when both high R waves and ST-T depression is used. Although the Charleston study found a low prevalence of ST depression inclusive LVH (0.9%) in their age-pooled, male-only sample, they in fact found LVH by high R wave criterion to be only 0.3%. This contrasted with the findings in the black population where they found the prevalence of LVH by these criteria to be 7.8%. This was mirrored in the high R wave criteria of the Evans county study that found the prevalence in blacks to be over double that in whites (19.8% compared to 7.4%).

### *1.4.8 LVH - Outcomes and Risk Prediction*

Although using echocardiographic LV mass as a gold standard to refine electrocardiographic estimates is valid, more important is the prognostic value of ECG detected LVH.

Most risk evaluation studies have found a significant association between ECG-LVH and mortality risk only for those criteria that combine high QRS amplitudes with repolarisation abnormalities. As expected, mortality risk is highest in hypertension clinic populations selected for intervention trials. An example is the report from the Italian PIUMA study,[562] which showed that cardiovascular disease (CVD) mortality risk was not significantly increased for the Sokolow-Lyon and Cornell voltage. The risk was not significant also for the Framingham criteria that included left ventricular strain with high QRS amplitudes. CVD mortality risk was over four -fold for the authors' Perugia Score that in essence is a logic combination of three criteria (Cornell voltage >2,400 μV in men and >2,000 μV in women or Romhilt-Estes score > 5 or left ventricular strain). The promising feature of the reported risk for this new criterion was that its prevalence in the study population was high, 17.8%, with concomitant population attributable risk of 37.0%. A high population attributable risk has connotations of etiological implications. Encouraging reports about the feasibility to use ECG-LVH in identification of subgroups at increased mortality risk have raised hopes that monitoring of the reduction of ECG-LVH may open doors for a convenient monitoring of the success of hypertension intervention efforts. An early example comes from MRFIT with the observation that there was a significant decrease in the Cornell voltage in the special intervention group compared with the usual care group of the study. Also regression exceeding the limit of short-range variability of the Sokolow-Lyon voltage was actually associated with independent excess CVD mortality risk, and there was no evidence for reduced risk by other criteria evaluated.[563]

It is now clear from several trials, meta-analyses,[564] and one meta-analysis review[565] that there is a strong relationship between changes in blood pressure and LVH regression. The overall ranking of anti-hypertensives according to Jennings & Wong[565] was: calcium antagonists, angiotensin converting enzyme inhibitors, diuretics, alpha-blockers, beta-blockers, and lifestyle change. Percent reductions in left ventricular mass were typically 12% for ACE inhibitors, 11% for calcium channel blockers, 5% for beta-blockers, and 8% for diuretics. Evidence of LVH regression with anti hypertensive treatment also comes from population data. Mosterd et al.[11] presented data from 10,333 participants who were 45 to 74 years of age at entry. From 1950 to 1989, the rate of use of antihypertensive medications increased from 2.3% to 24.6% among men and from 5.7% to 27.7% among women, while the age-adjusted prevalence of a systolic blood pressure above 160mmHg or diastolic blood pressure above 100mmHg declined from 18.5% to 9.2% among men and from 28.0% to 7.7% among women. They report that this decline was accompanied by reductions in the prevalence of LVH from 4.5% to 2.5% in men and from 3.6 % to 1.1 % in women. In fact, the Framingham investigators removed LVH from the most recent version of their prognostic score (previously the most important factor in the score) since its prevalence has declined probably due to the improved treatment of HBP.[566]

Some important data from Framingham has shown that reduction of electrocardiographic LVH is associated with a decrease in risk. Levy[567] studied 274 men (mean age, 60 years) and 250 women (mean age, 64 years) who were free of overt cardiovascular disease but manifested ECG evidence of left ventricular hypertrophy. Logistic regression analyses of pooled biennial examinations were used to determine risk for cardiovascular disease as a function of baseline voltage (sum of

R wave in aVL plus S wave in V3) and repolarisation abnormality. Subjects with a serial decline in voltage were at lower risk for cardiovascular disease; those with a serial rise were at greater risk. An improvement in ST depression was associated with a marginally significant reduction in cardiovascular risk in men only. Worsening of ST depression was associated with increased risk for cardiovascular disease in both sexes.

While high R wave LVH may simply be a marker of physiological response to hypertension, ST depression inclusive-LVH is associated with an up to 15 fold increase in the risk of cardiac death, making it a more potent risk factor than any other, and suggesting that we take seriously its detection and reversal. Of the cross sectional studies which presented data on individuals with no history of cardiovascular disease, only the Belgian study provided an estimation of ST depression inclusive-LVH prevalence. This study estimated the age-pooled prevalence (25yrs-74yrs) at 0.8%. That is, screening asymptomatic individuals might pick up the one person with unrecognised ST depression inclusive-LVH out of one hundred screened.

The expectation of reduced risk with reduction of ECG-LVH conflicts with recent echocardiographic evidence that has failed to demonstrated improved risk with LV mass reduction with ACE-inhibitors, at least in high-risk hypertensive patients with diabetes.[568] The concept of attributable risk has to be used with caution, and it is unrealistic to assert that ECG-LVH in itself would be a causative factor in mortality. It is plausible to speculate, however, that certain repolarisation wave features (not

necessarily isolated negative T waves) combined with high QRS amplitudes may be a marker for a substrate for adverse cardiac events.

### *1.4.9 Genetics of LV Mass*

Little is known of the genetics of left ventricular mass outwith the narrow confines of a rare group of inherited cardiomyopathies[569] although these familial disorders may provide insight into potential candidate genes for the study of more common forms of hypertrophy. However, before embarking on such investigation it is important to determine the extent to which left ventricular mass is heritable in the population under investigation.

Even before the advent of echocardiography investigators used populations of twins to assess the degree of heritability of left ventricular mass. Cardiac size on CXR was studied by Klisouras in 1971[570] who found no significant genetic contribution. In the 1980s a number of small studies of children and young adults investigated LV mass and its response to training.[491;492;571] The conclusions from these studies were contradictory with some suggesting a specific genetic component and others simply a familial one.[572] Underpowered and highly selected populations go some way to explain these differences. The largest twin study to date was performed on 254 twin pairs living in the Commonwealth of Virginia.[488] The mean age of the subjects, who were identified from school rosters, was 11.2 years. Verharen concluded that a genetic input was an important determinant of left ventricular mass in these children.

Recent attention has concentrated on investigating specific polymorphisms that may be important in the aetiology of left ventricular hypertrophy. These have included polymorphisms of the angiotensin converting enzyme, G protein beta-3 subunit, aldosterone synthase, and beta-1 adrenoceptor genes (Table 1.4-5).

In humans the gene for angiotensin-converting enzyme (ACE) occurs in two allelic forms, distinguished by the presence (insertion, I) or absence (deletion, D) of a 287-base-pair repetitive DNA domain in intron 16. Homozygosity for the D allele was recently reported to be an independent risk factor for electrocardiographically defined left ventricular hypertrophy.[573] In a large cross-sectional sample the DD genotype was associated with an overall relative risk of 1.76 that was attributable to a fourfold increase in risk in a subsample of 172 normotensive men. Some studies have associated the D genotype of the ACE gene to LV hypertrophy, sudden cardiac death, myocardial infarction.[496;574] However other studies have found no relationship.[495]

The ACE D/I polymorphism is known to account for about half the variance in ACE plasma levels, with higher values occurring in persons with two D alleles.[575] On the basis of a number of experimental and clinical observations, the renin–angiotensin system was postulated to be in an activated state in DD homozygotes, leading to speculation that this genotype was associated with cardiac hypertrophy. Angiotensin peptides exert trophic influences on cardiomyocytes in culture,[576] and the expression of genes encoding components of the renin–angiotensin system is up-regulated in hypertrophy and remodeling.[577;578] The ability of ACE inhibitors to induce the regression of hypertensive left ventricular hypertrophy and prevent ventricular remodelling after myocardial infarction provides an intriguing clinical correlate to these observations.

The 825T allele of the G-protein beta3 polymorphism is associated with the generation of a novel splice variant, enhanced intracellular signal transduction, and

arterial hypertension.[579] A recent study showed the T allele related to impaired LV diastolic filling in essential hypertensives.[580] It has also been associated with left ventricular hypertrophy in essential hypertensives.[581]

A polymorphism in the intracellular cytoplasmic tail near the seventh transmembrane-spanning segment of the human beta1AR has recently been identified in a cohort of normal individuals, with the Arg-389 receptors showing higher basal adenylyl cyclase activity and enhanced coupling to Gs.[582] This suggests that this polymorphism may play a role in regulating cardiac function and may be a risk factor in heart failure and hypertension.

Aldosterone controls sodium balance and intravascular volume and thus helps regulate blood pressure.[132] Therefore, genetic variations in the regulation of aldosterone synthesis might influence the structure and function of the left ventricle. Aldosterone secretion is regulated primarily by the renin-angiotensin system. In response to decreased intravascular volume, renin is secreted by the renal juxtaglomerular apparatus and converts angiotensinogen to angiotensin I, which is then converted to angiotensin II by ACE.[132]

Aldosterone has been shown to stimulate the growth of cardiac myocytes and accumulation of cardiac extracellular matrix. Two polymorphisms of the aldosterone synthase gene(Figure 1.3-3) - C344T polymorphism at the SF1 transcription factor binding site and a gene conversion in intron2 have been associated with LV mass and diastolic function in one study.[498] However a larger study found no relationship.[497]

**Table 1.4-5 Summary of genetic association studies of LV mass and function**

| Gene | Date | Ref | Journal | Country | Ethnic Group | Cases | Assoc |
|---|---|---|---|---|---|---|---|
| ACE | 1994 | Schunkert[573] | N Engl J Med | Germany | Caucasian | 290 | Yes |
| ACE | 1994 | Iwai[583] | Circulation | Japan | Japanese | 142 | Yes |
| ACE | 1996 | Lindpaintner[495] | N Engl J Med | USA | - | 2439 | No |
| ACE | 1997 | Montgomery[584] | Circulation | England | Caucasian | - | Yes |
| ACE | 1997 | Beige[585] | J Hypertens | Germany | Caucasian | 343 | No |
| ACE | 1998 | Kauma[586] | Eur Heart J | Finland | Caucasian | 430 | No |
| ACE | 1999 | van Suylen[587] | Am J Respir Crit Care Med | Netherlands | Caucasian | 87 | No |
| Angiotensinogen | 1997 | Ishanov[588] | Am Heart J | Japan | Japanese | 96 | Yes |
| Angiotensinogen | 1997 | Beige[585] | J Hypertens | Germany | Caucasian | 343 | No |
| Angiotensinogen | 1998 | Kauma[586] | Eur Heart J | Finland | Caucasian | 430 | No |
| AT1R | 1998 | Ishanov[589] | Jpn Heart J | Japan | Japanese | - | No |
| CYP11B2 | 1998 | Kupari[498] | Circulation | Finland | Caucasian | 84 | Yes |
| GNB3 | 2000 | Poch[581] | Hypertension | Spain | Caucasian | 60 | Yes |
| GNB3 | 2001 | Zeltner[590] | Hypertension | Germany | Caucasian | 95 | No |
| GNB3 | 2001 | Semplicini[591] | Am J Hypertens | Italy | Caucasian | 207 | Yes |
| GNB3 | 2002 | Sedlacek[592] | Hypertension | USA | Caucasian | 1720 | No |

## 1.5 G proteins

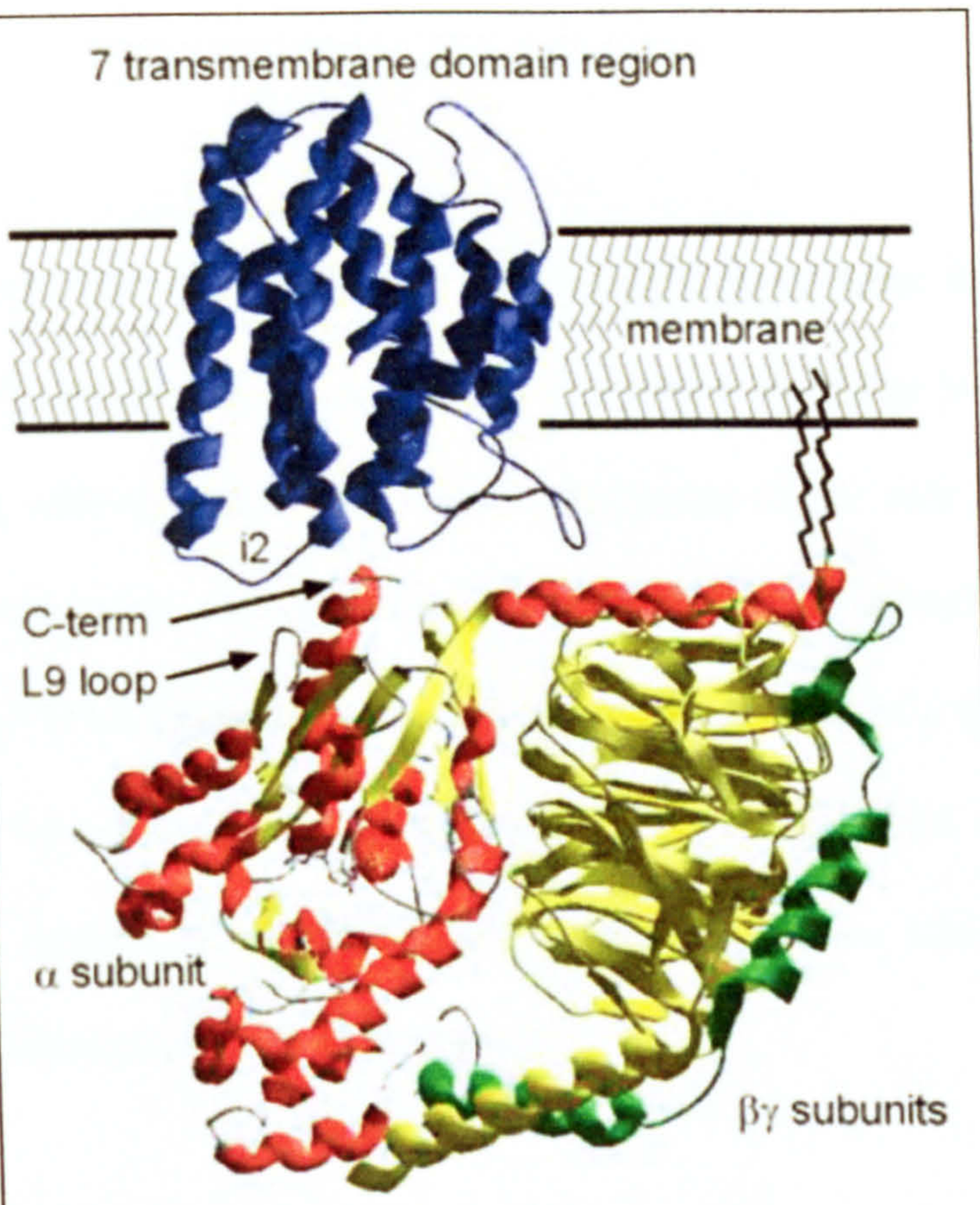


**Figure 1.5-1 Structure of G Proteins.**

**The αβγ trimer is shown in its probable orientation relative to a G protein-coupled receptor and the cell membrane. GDP is cradled between two domains of Gα**

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are signal transducers, attached to the cell surface plasma membrane, that connect receptors to effectors and thus to intracellular signalling pathways.[593] Receptors that couple to G proteins communicate signals from a large number of hormones, neurotransmitters, chemokines, and autocrine and paracrine factors. G proteins play important roles in determining the specificity and temporal characteristics of the cellular responses to signals. G proteins consist of three subunits, α, β, and γ. When signalling, they

function in essence as dimers because the signal is communicated either by the Gα subunit or the Gβγ complex. In most cases, Gβγ subunits cannot be dissociated under nondenaturing conditions. Currently there are 20 known Gα, 6 Gβ, and 11 Gγ subunits.

On the basis of sequence similarity, the Gα subunits have been divided into four families (Figure 1.5-2) and this classification has served to define both receptor and effector coupling, although there are always exceptions to the rule. The $G_s$ and $G_q$ families have very well defined effector pathways, the adenylyl cyclase and phospholipase C-β (PLC-β) pathways, respectively. The $G_i$ and $G_o$ families are more amorphous, and here the signal flows through both the Gα and Gβγ complexes. Perhaps the best understood of the $G_i$ family pathways is the transducin pathway, which mediates detection of light in the eye.

These four broad G protein families transduce signals from a very large number of extracellular agents. The agents listed in Figure 1.5-2 constitute a very small subset of the extracellular signals that can couple to the various G protein pathways. The extracellular signal is routed to specific G proteins through distinct types of receptors. For example, epinephrine's signal is transmitted through the β-adrenergic receptor coupled to $G_s$, the $\alpha_2$-adrenergic receptor to $G_i$, and the $\alpha_1$-adrenergic receptor to $G_q$ and $G_{11}$. The G proteins, in turn, through signalling pathways described in more detail below, regulate important cellular components, such as metabolic enzymes, ion channels, and the transcriptional machinery. The resulting alterations in cellular behaviour and function are manifested in many critical systemic functions, including embryonic development, learning and memory, and homeostasis. This results in the

propagation of regulated activities through increasingly complex layers of organization to serve as the basis of integration at the systemic level.

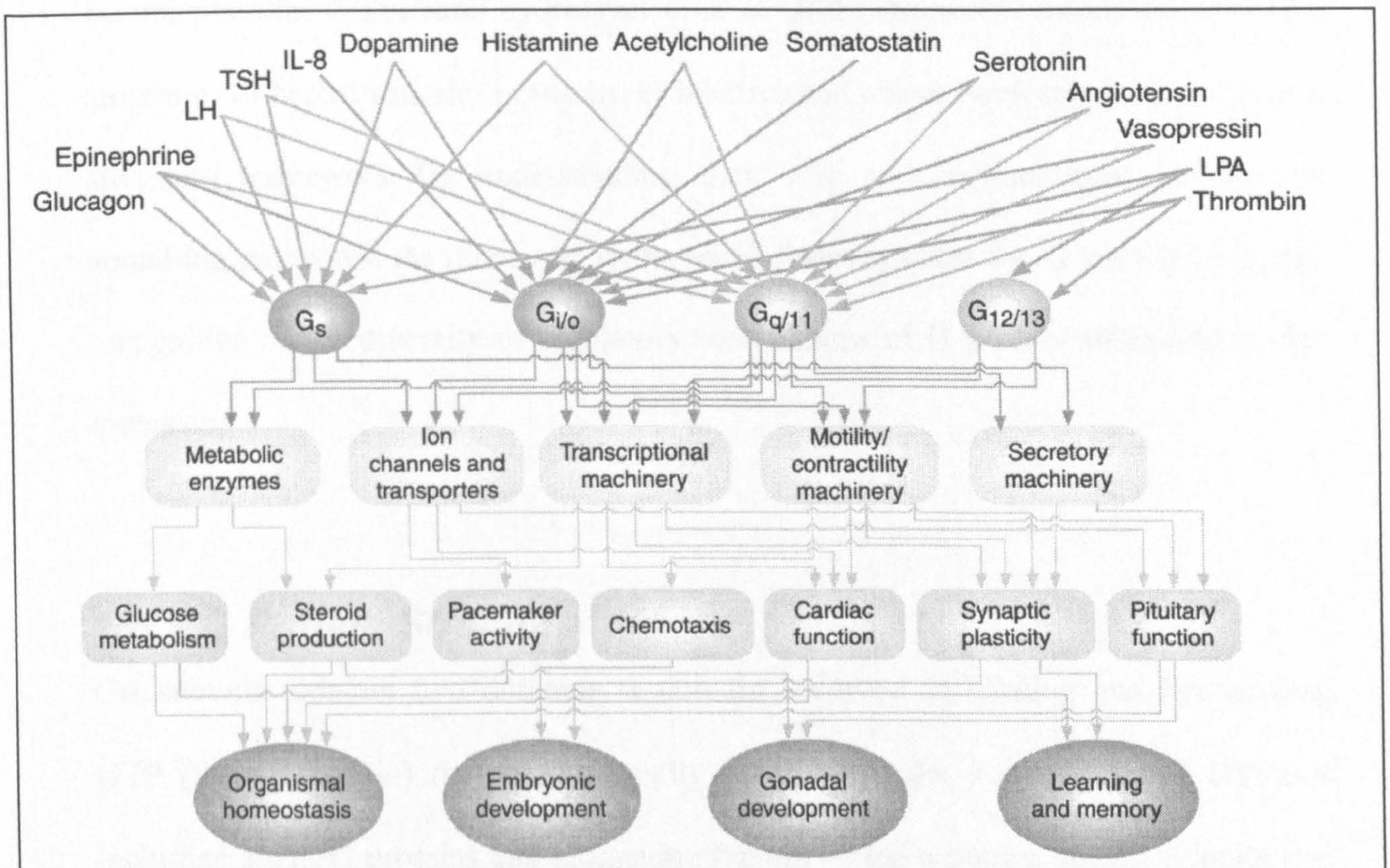


**Figure 1.5-2 Regulation of systemic functions by signalling through G protein pathways.**

**A schematic representation of how signalling through G protein pathways can regulate systemic functions. Many extracellular agents, such as hormones (for example, glucagon, luteinizing hormone, and epinephrine), neurotransmitters (acetylcholine, dopamine, and serotonin), chemokines (IL-8), and local mediators (LPA), signal to the four main G protein families to regulate such cellular machinery as metabolic enzymes, ion channels, and transcriptional regulators. Modulation of the activities of the cellular machines in turn gives rise to altered cellular functions, such as changes in glucose metabolism in liver and muscle or altered activities of pacemaker cells in the heart. These cellular activities contribute to the regulation of large-scale systems such as organismal homeostasis and learning and memory. Thus, G protein pathways can propagate regulatory information through layers of increasing organizational complexity. At all levels, the examples shown here represent only a sample of extracellular agents that couple to the four G proteins, and the functions regulated by these pathways.Adapted from[594]**

G proteins are inactive in the GDP-bound, heterotrimeric state and are activated by receptor-catalysed guanine nucleotide exchange resulting in GTP binding to the $\alpha$ subunit. GTP binding leads to dissociation of $G\alpha{\cdot}GTP$ from $G\beta\gamma$ subunits and activation of downstream effectors by both $G\alpha{\cdot}GTP$ and free $G\beta\gamma$ subunits. (Figure

1.5-3) G protein deactivation is rate-limiting for turnoff of the cellular response and occurs when the Gα subunit hydrolyses GTP to GDP. The recent resolution of crystal structures of heterotrimeric G proteins in inactive and active conformations provides a structural framework for understanding their role as conformational switches in signalling pathways. As more and more novel pathways that use G proteins emerge, recognition of the diversity of regulatory mechanisms of G protein signalling is also increasing.

## *1.5.1 G Protein Structure*

Gα subunits contain two domains, a domain involved in binding and hydrolysing GTP (the G domain) that is structurally identical to the superfamily of GTPases including small G proteins and elongation factors[595] and a unique helical domain that buries the GTP in the core of the protein(Figure 1.5-1).[596;597] The β subunit of heterotrimeric G proteins has a 7-membered β-propeller structure based on its 7 WD-40 repeats.[598-600] The γ subunit interacts with β through an N-terminal coiled coil and then all along the base of β, making extensive contacts. G protein activation by receptors leads to GTP binding on the Gα subunit. The structural nature of the GTP-mediated switch on the Gα subunit is a change in conformation of three flexible regions designated Switches I, II, and III to a well ordered, GTP-bound activated conformation with lowered affinity for Gβγ.[601] This leads to increased affinity of Gα·GTP for effectors, subunit dissociation, and generation of free Gβγ that can activate a number of effectors. (Figure 1.5-3)

Of the five Gβ subunits cloned thus far, Gβ1–4 share an 80–90% sequence identity and contain ~341 amino acids. The heterotrimeric G protein crystal structure reveals that Gβ subunits contain two distinct structural motifs: the first is an α-helical structure comprising the amino terminus, and the second is a seven-bladed propeller structure. Each of the seven blades comprises a WD repeat (normally bounded by a tryptophan (W) and an aspartate (D) residues) that fold into β-sheets. [602] Gα subunits and several effector proteins share several common interacting regions along the Gβ propeller structure. [598-600]

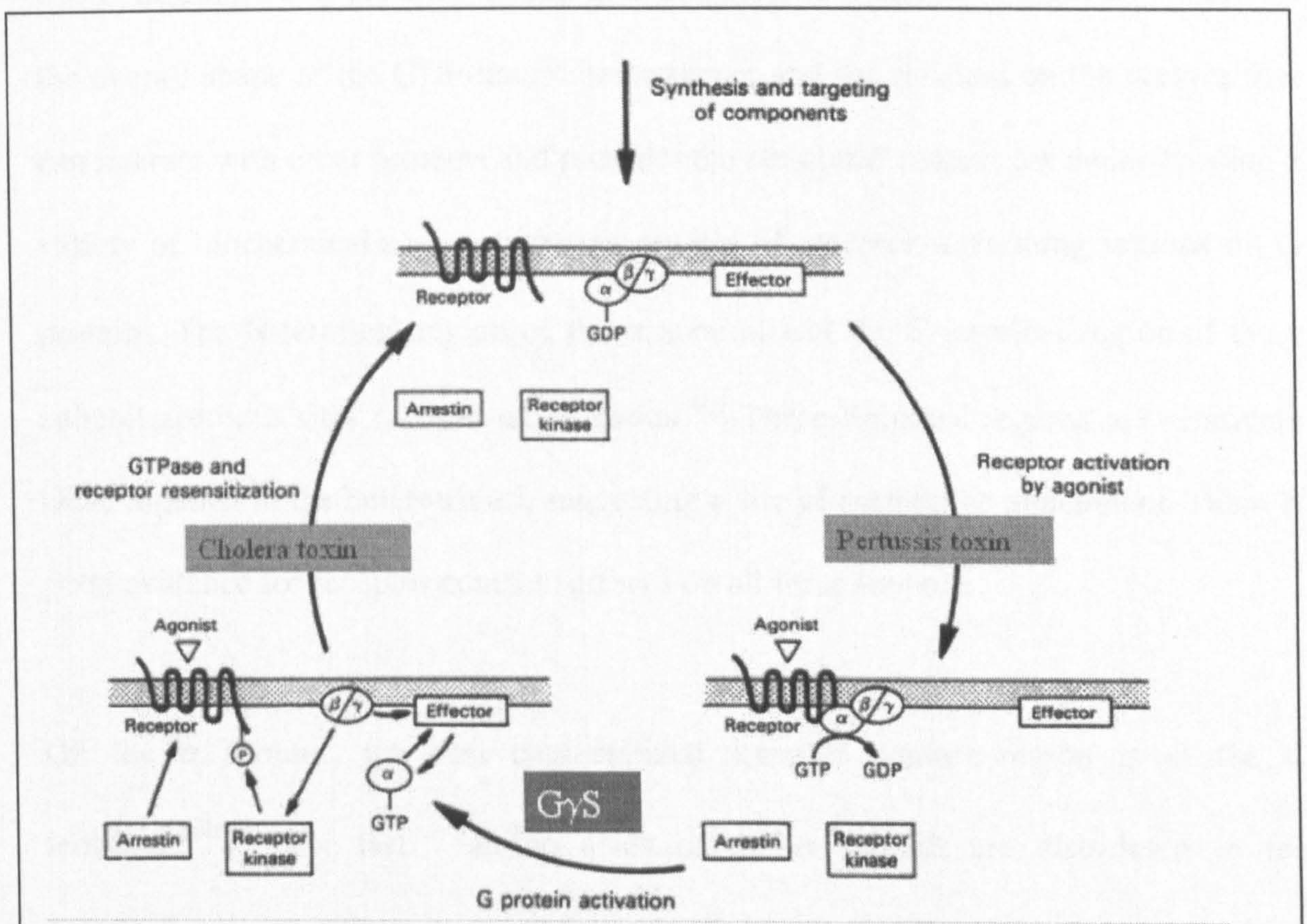


**Figure 1.5-3 G protein signalling cycle**

### *1.5.2 Mechanism of Activation of G Proteins by Receptors*

G protein-coupled receptors have a common body plan with seven transmembrane helices; the intracellular loops that connect these helices form the G protein-binding domain (Figure 1.5-1).[603;604] Both mutagenesis and biochemical experiments with a

variety of G protein-coupled receptors suggest that receptor activation by ligand binding causes changes in the relative orientations of transmembrane helices 3 and 6. These changes then affect the conformation of G protein-interacting intracellular loops of the receptor and thus uncover previously masked G protein-binding sites.[605-607] When an activated receptor interacts with a heterotrimeric G protein, it induces GDP release from the G protein.[608] Upon GDP release and in the absence of GTP a stable complex between the activated receptor and the heterotrimer is formed.

The conformation of the GDP-bound heterotrimeric G proteins $G_t$ and $G_i$[598;600] shows the overall shape of the GDP-bound heterotrimer and the residues on the surface that can interact with other proteins and provides the structural context for understanding a variety of biochemical and mutagenesis studies of receptor-interacting regions on G proteins. The N-terminal region of the α subunit and the C-terminal region of the γ subunit are both sites of lipid modification.[609] These lipidated regions are relatively close together in the heterotrimer, suggesting a site of membrane attachment. There is good evidence for receptor contact surfaces on all three subunits.

On the α subunit, the best characterized receptor contact region is at the C terminus.[608;610] The last 7 amino acids of the α subunit are disordered in the heterotrimer crystal structures, and analysis of receptor-binding peptides selected from a combinatorial peptide library shows that these 7 residues are the most critical.[611] Studies using chimeric Gα subunits confirm that in fact the last 5 residues contribute importantly to specificity of receptor G protein interaction. Elegant mutagenesis studies have shown that the C terminus of the third intracellular loop of receptors binds to this C-terminal region on Gα subunits.

It is clear that the βγ subunits of heterotrimeric G proteins enhance receptor interaction with α subunits.[610] Single Ala mutations in residues of the β subunit that contact the α subunit block receptor-mediated GTP/GDP exchange.[595] This suggests that the β subunit must hold the α subunit rigidly in place for GDP release to occur. Direct binding interactions between receptor and βγ subunit have been reported.[612-614] A cross-linking study demonstrated that the C-terminal 60-amino acid region of Gβ can be cross-linked to an $\alpha_2$-adrenergic receptor peptide corresponding to the intracellular third loop of the receptor.[613] In addition, the C-terminal region of the γ subunit of G proteins has been shown to be involved in receptor coupling and specificity.[612;614]

## *1.5.3 Mechanisms of Effector Activation*

### 1.5.3.1 α Targets

Upon GTP binding to the α subunit, the α·GTP ($a^{*)}$ and βγ subunits dissociate.[598;601] In the GTP-bound, active conformation, a new surface is formed on $G\alpha^*$ subunits,[615] and they interact with effectors with 20-100-fold higher affinity than in their GDP-bound state. The various $G\alpha^*$s interact in a highly specific manner with the well-studied, classical effector enzymes through this surface. $G\alpha^*_s$ activates (and $G\alpha^*_i$ inhibits) adenylyl cyclase, $G\alpha^*_t$ activates photoreceptor cGMP phosphodiesterase, and $G\alpha^*_q$ activates phospholipase C-β. However, this conserved switch surface on Gα subunits does not explain the exquisite specificity of G protein α subunit effector interaction. A chimeric $G\alpha_t/G\alpha_i$ approach identified two other regions that underlie the specific interaction of $G\alpha_t$ with phosphodiesterase γ.[615] Similar regions are

involved in effector interaction of $G\alpha_q$ with PLC[597;616] and $G\alpha_s$ with adenylyl cyclase (AC).[610]

### 1.5.3.1.1 $G_s$ Pathway

The $G_s$ pathway is the original cell signalling pathway to be described, and many key

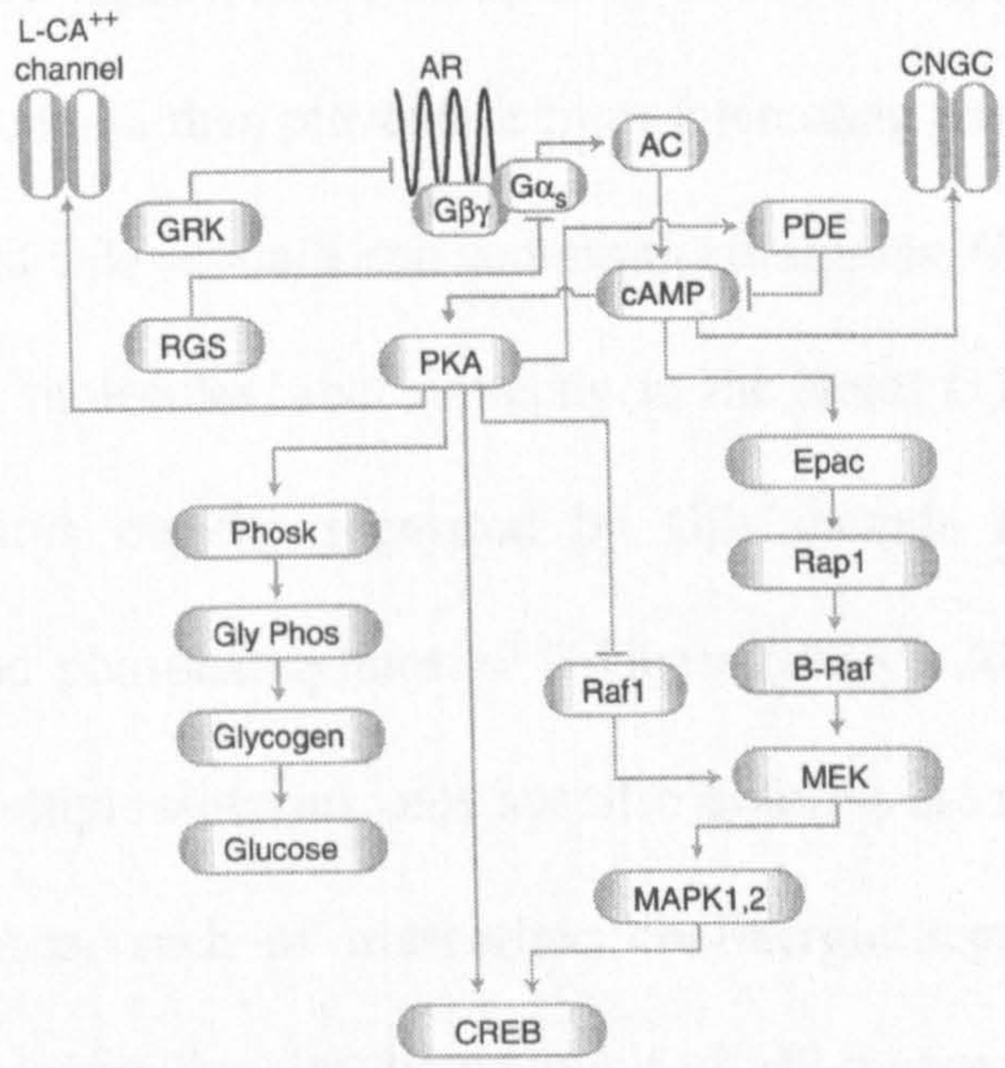


**Figure 1.5-4 The canonical Gs signalling pathway.**

**This schematic diagram demonstrates how the cAMP pathway connects to multiple cellular machines, including ion channels, transcription factors, and metabolic enzymes. AC, adenylyl cyclase; PKA, protein kinase A; PDE, phosphodiesterase; L-$Ca^{++}$ channel, L-type $Ca^{2+}$ channel; CNGC, cyclic nucleotide-gated channel; PhosK, phosphorylase kinase; GlyPhos, glycogen phosphorylase; CREB, cAMP response element-binding protein; EPAC, the cAMP- and AMP-regulated exchange factor for Rap1; Rap1, a small GTPase; MAPK, mitogen-activated protein kinase; Raf1 and B-Raf, MAP kinase kinases; MEK, MAPK/ERK kinase; MEKK, MAPK/ERK kinase; GRK, G protein receptor kinase; RGS, regulators of G protein signalling; βAR, β-adrenergic receptor.adapted from[594]**

concepts, including that of second messengers,[617] protein phosphorylation,[618] and signal transducers,[593;619] have come from the study of this pathway. Most connections in this pathway have been established through biochemical experiments (Figure 1.5-4).

### 1.5.3.1.2 $G_i$ Pathway

This pathway was originally identified by the ability of $G\alpha_i$ to inhibit adenylyl cyclase. Many important hormones and neurotransmitters, including epinephrine, acetylcholine, dopamine, and serotonin, use the $G_i$ and $G_o$ pathway to evoke physiological responses. Signal flow through this pathway is inhibited by pertussis toxin, which adenosine diphosphate (ADP)-ribosylates the G protein α-subunit at its COOH-terminal region and thus prevents it from interacting with the receptor. In this pathway, both $G\alpha$ and $G\beta\gamma$ subunits can communicate signals. $G\beta\gamma$ directly couples to at least four effector molecules, and indirectly to the small GTPase Ras, to activate MAPKs. The effectors directly regulated by $G\beta\gamma$ include PLC-β, $K^+$ channels, adenylyl cyclase, and phosphatidylinositol 3-kinase (PI3K). Although each of these effectors exists as multiple isoforms, only specific isoforms are regulated by $G\beta\gamma$. Key physiological functions, such as muscarinic cholinergic regulation of pacemaker activity in the heart, occur through the coupling of M2-muscarinic receptors to $G_i$ to release a $G\beta\gamma$ subunit that activates $K^+$ channels. $G\alpha_i$ and $G\alpha_o$ can regulate signals from c-Src to signal transducer and activator of transcription 3 (STAT3) and to the Rap pathways, as well as inhibit adenylyl cyclase. The well-studied inhibition of adenylyl cyclase may be physiologically relevant, especially in inhibiting the effects of cAMP to modulate secretion. However, the physiological consequences of $G\alpha_i$ and $G\alpha_o$ regulation of c-Src-STAT3 and Rap pathways remain to be established. Many connections in the $G\alpha_i$ and $G\alpha_o$ pathway have been established by biochemical experiments, although the newer pathways have been studied in transfected cells. It is currently not known how $G\alpha_i$ or $G\alpha_o$ activates c-Src, but some studies indicate possible direct interactions between $G\alpha$ subunits and tyrosine kinases.

### 1.5.3.1.3 $G_q$ Pathway

The $G_q$ pathway is the classical pathway that is activated by calcium-mobilizing hormones and stimulates PLC-β to produce the intracellular messengers inositol trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ triggers the release of calcium from intracellular stores, and DAG recruits protein kinase C (PKC) to the membrane and activates it. These connections have been well-established biochemically. In many cell types, the release of intracellular calcium activates the store-operated calcium channels at the cell surface to allow the inflow of extracellular calcium. $G\alpha_q$, working through PKC and possibly directly, also appears to regulate various isoforms of phospholipase D.[620] $G\alpha_q$ is reported to activate the transcription factor NF-κB through PYK2.[621]

### 1.5.3.1.4 $G_{12}$ and $G_{13}$ Pathways

The $G\alpha_{12}$ and $G\alpha_{13}$ proteins were discovered through sequence similarity to known Gα proteins, and which receptors endogenously couple through $G\alpha_{12}$ and $G\alpha_{13}$ pathways is still unclear. Although from sequence similarity it appears that $G\alpha_{12}$ and $G\alpha_{13}$ belong to the same family, they may produce different signalling outputs but generate a subset of overlapping effects.

$G\alpha_{12}$ has been reported to directly interact with a GTPase-activating protein for Ras, RasGAP, and Bruton's tyrosine kinase (Btk).[622] $G\alpha_{12}$ is thought to stimulate phospholipase D, c-Src, and PKC by as-yet unidentified mechanisms. The endpoint physiological responses of these pathways are not yet fully understood. In many cases it appears that different members of the MAPK family, such as extracellular signal-regulated kinase 5 (ERK5) or c-Jun $NH_2$-terminal kinase (JNK), are activated. This

activation should lead to regulation of gene expression. In fact, $G\alpha_{12}$ was identified as an oncogene in a functional screening assay[623] and hence effects on gene expression patterns are to be expected.

Two receptors that couple to $G\alpha_{13}$ in the native setting are the lysophosphatidic acid (LPA) receptor and the thromboxane A2 receptor. $G\alpha_{13}$ directly interacts with and activates a guanine nucleotide exchange factor for the GTPase Rho, p115RhoGEF, and thus activates Rho, leading to a variety of effects that include regulation of the $Na^+$-$H^+$ exchanger. Through the activation of PYK2, $G\alpha_{13}$ may engage the PI3K pathway to activate the protein kinase Akt and regulate NF-κB.[621] How $G\alpha_{13}$ activates PYK2 is currently not understood.

In $G\alpha_{13}$, knockout mice, lack of $G\alpha_{13}$ led to an impaired angiogenesis of endothelial cells and caused inability to develop an organized vascular system. In addition, $G\alpha_{13}$ (-/-) embryonic fibroblasts showed greatly impaired migratory responses to thrombin, suggesting that chemotaxis was impaired. Interestingly, although $G\alpha_{12}$ shares 67% amino acid identity to $G\alpha_{13}$, it cannot substitute for $G\alpha_{13}$.

The Gs pathway in Figure 1.5-4 illustrates several general patterns that emerge from this complex picture. First, all G proteins engage multiple signalling pathways and consequently different cellular machines. This often helps produce effects with distinct rates of activation and duration of response. In neurons, cAMP can act through PKA to produce short-term effects on channel functions, and through Rap and MAPK to regulate gene expression and produce long-term effects through regulation of the transcriptional machinery. Second, it appears that all G proteins regulate the

activity of GTPases such as Rap and Rho. Third, all G protein pathways either stimulate or inhibit one or more of the MAPK signalling pathways. All of these interconnections result in a complex and likely robust network in which signals from G protein-coupled receptors can be fully integrated with signals from other receptors.

### 1.5.4 $\beta\gamma$ Subunit

Much of the initial focus after the discovery and purification of heterotrimeric G-proteins was directed at the G-protein $\alpha$ subunit because it was a GTPase that acted on both adenylate cyclase and cGMP phosphodiesterase. Even after the identification of the G-protein $\alpha$ and $\beta\gamma$ subunits, their role in signalling was somewhat under appreciated. However, two pieces of suggestive evidence hinted at a broader role for these subunits: (1) the $\beta$ and $\gamma$ subunits seemed heterogeneous in different mammalian tissues, and (2) the tightly bound $\beta\gamma$ complex was an obligatory requirement for receptor activation of a G protein.[624] As the structures of the $\beta$ and $\gamma$ subunits began to be elucidated through molecular-cloning methods, the unexpected finding that a complex could regulate the function of a heart $K^+$-ion-conducting channel pointed to a major function for these subunits as modulators of effector function. In the decade following these initial results, the complex began to be treated as an equal to the $\alpha$ subunit and as a separate arm in the G-protein-signalling pathway.

#### 1.5.4.1 $\beta\gamma$ Targets

Once G$\alpha$·GTP has dissociated from G$\beta\gamma$, free $\beta\gamma$ is an activator of a vast array of proteins, and the list continues to increase.[602] Significantly, the conformation of free G$\beta\gamma$ is identical to G$\beta\gamma$ in the heterotrimer,[599] suggesting that G$\alpha$ inhibits G$\beta\gamma$ interactions with its effectors through the G$\alpha$-binding site on G$\beta$. Evidence for this comes from the laboratory of Iyengar and co-workers,[625] who found a peptide from

ACII (Adenylyl Cyclase 2) that bound to Gβγ and blocked its activation of various effectors, suggesting that part of the effector binding site is shared between ACII, G protein-activated inward rectifier $K^+$ channel (GIRK), and PLCβ. Cross-linking and docking experiments localized the binding site to a part of the Gα-binding region.[626] Besides the Gα-binding region, other regions of Gβγ subunits that have been implicated in effector interaction include the N-terminal coiled coil[627;628] and blades 1 and 7 of the β-propeller of Gβ.[629;630]

Gβγ has well defined effects on some isoforms of the classical second messenger enzymes, PLCβ2 and -β3[631] and AC (Gβγ stimulates $G\alpha_s$-activated ACII, -IV, and -VII whereas it inhibits ACI.[632] It also recruits the β-adrenergic receptor kinase to the membrane where the kinase phosphorylates activated β-adrenergic receptors. It binds to the phosphoprotein phosducin, which is thought to sequester βγ and thereby regulate its availability via a cAMP-dependent protein kinase-regulated mechanism. Phosducin-like proteins have also been shown to bind to Gβγ.[633] Elucidation of the crystal structure of the phosducin-Gβγ complex showed that there is a shared surface on the top of Gβγ for interaction with Gα and phosducin but that a second site of interaction occurs between phosducin's C terminus and β-propeller blades 1 and 7 at the side of Gβγ.[634] Interestingly, the phosphorylation site on phosducin, which regulates its affinity with Gβγ, is far from the protein-protein interface.

$I_{KACh}$ is the inwardly rectifying $K^+$ channel responsible for slowing heart beat in response to the parasympathetic transmitter acetylcholine. It is a homo- or heteromultimer of GIRK[635] monomers found in the heart and brain. Gβγ subunits bind

the N- and C-terminal intracellular domains of GIRKs and directly activate them.[635-637] The Gβγ subunit similarly plays an important modulatory role in certain presynaptic $Ca^{2+}$ channels,[638;639] especially α1A, α1B, and to a lesser extent α1E but not α1C, α1D, or α1S isoforms.[640] It has been shown that Gβγ inhibits $Ca^{2+}$ channel current by directly contacting two regions on $Ca^{2+}$ channel α1 subunits: the intracellular I-II loop[641;642] and the C terminus.[643;644]

Gβγ also directly activates more than one phosphatidylinositol 3-kinase isoform,[645] and a number of kinases as well, for example, the Raf1 protein kinase[646] and Bruton and Tsk tyrosine kinases.[647]

In yeast, Gβγ is the activator of a pheromone-stimulated MAP kinase pathway. It is known to bind to the N-terminal region of the scaffold protein Ste5 in yeast.[648] Recently, Thorner and co-workers[649] showed that Ste5 contains a homodimerisation domain, which is required for β binding. They demonstrated that Gβγ interaction leads to oligomerisation of this domain on Ste5. Most interestingly, dimerisation of this domain by making a glutathione *S*-transferase fusion protein of Ste5 leads to Gβγ-independent activation of the MAP kinase cascade. Recently, yeast Gβγ was also shown to activate Cdc24, the exchange factor for the Rho-type GTPase Cdc42.[650] Gβγ has also been reported to bind to other members of the Rho family of GTPases, Rho and Rac,[651] as well as to the small G protein Arf (ADP-ribosylation factor), which is involved in coat formation and vesicular trafficking.[652]

There are multiple genes for Gβ and Gγ, and most Gβγ pairs can form functional Gβγs.[602] One of the first questions posed was whether different Gβγs regulated

different effectors. The answer from a large number of biochemical experiments was: not much. $G\beta_1\gamma_1$ is better than the others at interacting with rhodopsin and phosducin in photoreceptor cells and somewhat worse than all the other $G\beta\gamma$ pairs at interacting with other effectors. One series of studies that showed selectivity of $G\beta\gamma$ pairs at interacting with receptors and effectors was done using antisense oligonucleotides to suppress the translation of particular proteins, and these studies showed a very high degree of selectivity (see below). Other evidence of specificity, using different techniques, is slowly emerging. $G\beta_5$, a recently discovered $G\beta$ subunit found in the central nervous system,[653] differentially couples to two MAP kinase pathways.[654]

Because $G\alpha$ can inhibit all the actions of $G\beta\gamma$, the $G\alpha$-binding residues are candidate effector activation determinants. Ford et al.[655] tested this idea by singly mutating the 15 $G\alpha$-binding residues of $G\beta$ to alanines, and in all effectors that have been examined, some of the mutants no longer activate the effector. In each effector interaction, however, different residues clustered on the surface of $G\beta$ are critical, suggesting a mechanism whereby a unique contact surface of $G\beta$ can make specific interactions with a number of different effectors. Interestingly, in some cases, removing the side chain increases the potency of the mutant $G\beta\gamma$ to activate an effector.

## 1.6 G protein $\beta_3$ subunit polymorphism (GNB3)

### *1.6.1 G protein and hypertension*

Most transmembrane receptors rely on heterotrimeric GTP-binding proteins (G proteins) to activate or inhibit intracellular signalling cascades. Specifically, a variety of vasoactive or growth-stimulating factors communicate via G proteins in virtually all cardiovascular tissues. Despite this pivotal role in the transmembrane signalling network, only recently an active participation of G proteins was considered in the pathogenesis of hypertension.

A number of endocrine, paracrine, and autocrine factors interact with receptors on vascular smooth muscle cells (and endothelial cells) to functionally regulate vascular smooth muscle tone and hence peripheral resistance. From this functional perspective, peripheral resistance reflects a net balance between vasoconstrictor and vasodilator mechanisms.

| Vasoconstrictors | Vasodilators |
|---|---|
| Angiotensin II (AT) | Adenosine ($A_{2a}/A_{2b}$) |
| Endothelin ($ET_A$) | Glucagon |
| Catecholamine ($\alpha$AR) | Catecholamine ($\beta$AR) |
| Serotonin ($5HT_2$) | Serotonin ($5HT_1$) |
| Substance P ($NK_2$) | Calcitonin Gene-Related Peptide (CGRP) |
| Vasopressin ($V_1$) | Vasopressin ($V_2$) |
| Neuropeptide Y (NPY) | Vasoactive Intestinal Peptide (VIP) |
| Thromboxane $A_2$ ($TXA_2$) | Prostanoids (DP, EP, IP) |
| Leukotrienes ($LTC_4$) | Dopamine ($D_1/D_5$) |
| Histamine ($H_1$) | Histamine ($H_2$) |
| | Bradykinin ($B_1/B_2$) |

**Table 1.6-1 Hormones for G-protein-linked receptors on vascular smooth muscle that modify contractile state**

Impairment of endothelial-mediated vasodilatation has been identified in subsets of hypertensive subjects, particularly in those mechanisms acting via stimulation of NO

synthase and consequent guanylyl cyclase activation. Although these findings have been consistently confirmed in subsets of patients at greatest risk of atherosclerotic complications, findings in essential hypertension has not been universally consistent.[656-658]

In vascular myocytes, activation of receptors linked to adenylyl cyclase and the consequent increase in intracellular camp is an important mechanism mediating vasodilatation. A number of G protein-coupled receptors linked to adenylyl cyclase activation and vasodilatation are expressed in vascular smooth muscle cells namely β adrenergic receptor, $EP_2$, DP, IP prostaglandin receptors, $H_2$ histamine receptor, $V_2$ vasopressin receptor. It has been shown that impaired vascular adenylyl cyclase-mediated vasodilatation is common both to human and animal models of hypertension. In spontaneously hypertensive rats, the reduced relaxation by βAR agonists of femoral arteries is due to functional loss of $G_s$ protein.[659] Also in the renal vasculature of young spontaneously hypertensive rats, vasodilators are ineffective in counteracting the vasoconstrictor effect of angiotensin II, and this defect is related to reduced coupling of the vasodilator receptors to $G_s$ proteins.[660] In Milan hypertensive strain rats, the amount of $G_s\alpha$ protein is reduced in vascular membranes,[661] but paradoxically in the membranes of vascular smooth muscle cells derived from thoracic aortas, there appeared to be increased response to adenylyl cyclase activity reflecting increased βAR coupling to $G_s$ protein.[662] This may reflect a generalised defect in vascular G protein-coupled receptor-mediated adenylyl cyclase activation. Feldman et al[663] showed that in younger white hypertensive subjects fed a normal sodium intake, β-adrenergic-stimulated adenylyl cyclase activation was impaired in lymphocyte membranes. They found that this defect paralleled a reduction in the

ability to form the high-affinity ligand binding state of the β adrenergic receptor, suggesting a functional uncoupling from $G_s$. Mills et al[664] subsequently noted that these defects may be race specific; that is in contrast to the impairment to the impairment in β-adrenergic response in white hypertensive subjects, African American subjects demonstrate enhanced β-adrenergic stimulated adenylyl cyclase activity. The defect in receptor-G protein coupling could represent an alteration at the level of either the receptor of the G-protein. Initially studies focussed on G protein alterations, given the appreciation that altered G protein expression and function were apparent in several animal models of hypertension. But subsequent studies found no alteration in G protein expression, in spite of decreased $G_s$ function.[665] Others have looked at GPCR phosphorylation which could affect the efficiency with which G protein-coupled receptors interact with the G proteins.

### *1.6.2 $Na^+H^+$ Exchanger*

Genes that encode the components of various transport systems that regulate salt and water homeostasis are obvious candidates for influencing blood pressure regulation and hypertension.

The ubiquitously expressed pH-regulating ion transport system, $Na^+/H^+$ exchanger (NHE), swaps extracellular $Na^+$ for intracellular $H^+$. Five isoforms have been isolated in human tissues. Different investigators have demonstrated enhanced NHE isoform-1 (NHE-1) activity in several blood cell types of patients with essential hypertension compared to normotensive control subjects,[666;667] and transgenic mice that overexpress a recombinant NHE protein developed salt sensitive hypertension.[668]

Previous investigations have shown that the enhanced $Na^+/H^+$ exchanger activity in hypertensive individuals is associated with several phenotypes such as left ventricular hypertrophy,[669] insulin resistance,[670] and renal sodium retention.[667] Because the gene encoding the ubiquitous $Na^+/H^+$ exchanger, NHE1 has been excluded as a candidate gene in essential hypertension,[671] research has been focussed on the intracellular and systemic regulations of NHE1.

Siffert et al.[672] immortalized lymphocytes from hypertensive patients with high Na-H exchanger activity and normotensives with low activity. Cells derived from the hypertensives showed persistently greater Na-H exchange, cell proliferation, calcium transients, and inositol phosphate generation in response to agonists such as PAF and somatostatin, all suppressible by pertussis toxin, pointing to a G protein as the source of the enhanced response. No differences in receptor or G-protein number were identified, but increased binding of GTP to G proteins was observed. The finding that immortalized cells of hypertensive subjects displayed enhanced G protein-mediated signalling suggested a genetic alteration rather than a blood pressure effect as the underlying mechanism. Variations in NHE-1 could not be detected,[673] and polymorphisms in the genes that encode the subunits of trimeric G proteins, such as $G\alpha_{i2}$, $G\alpha_{i3}$, $G\beta_1$, $G\beta_2$ have been ruled out.[674]

### *1.6.3 GNB3*

The molecular basis for the altered intracellular signal transduction affecting ion transport in immortalized cells in vitro was found to be a single base change (C→T) at nucleotide 825 in exon 9 of the GNB3 gene on chromosome 12p13, which encodes the $\beta_3$ subunit of the heterotrimeric protein.[579] The 825T allele was associated with the occurrence of a splice variant, which produced a 123-base pair deletion of nucleotides

498 to 620 of exon 9 due to alternative splicing. This resulted in loss of 41 amino acids and 1 tryptophan-aspartic acid WD repeat domain of the $G\beta_3$ subunit. (Figure 1.6-1) By means of RT-PCR experiments, expression of this alternatively spliced mRNA variant was confirmed in B lymphoblasts,[579] neutrophils,[675] and T lymphocytes.[676]

Gβ proteins belong to the superfamily of propeller proteins, and all Gβ proteins identified so far consist of 7 WD repeats that form a regular torus like structure.[602] (Section 1.5.1 page 168) $G\beta_3$ results from alternative splicing of GNB3 and lacks the equivalent of 1 entire WD domain. The C825T polymorphism is located >1700 bp upstream of the alternative splice site, indicating that the effect of the 825T on the splice process is a complex mechanisms. However, such mechanisms have been proposed for other genes.[677] While homozygous 825C allele carriers express only the wild-type gene product, homo- and heterozygous 825T allele carriers apparently express both wild-type $G\beta_3$ as well as $G\beta_{3\text{-}s}$ mRNA. Initially it was unclear how a remote polymorphism located in exon 10 could affect splicing of exon 9, leading to the generation of $G\beta_{3\text{-}s}$.[678] Additional polymorphisms were detected in the intron between exons 9 and 10, in the promoter region, and in the 3'-untranslated region. Interestingly, these were found to be in unusually tight linkage disequilibrium.[679;680] Hence, there exist two complex haplotypes associated with the 825T and the 825C allele which are expected to have an impact on the structure of the $G\beta_3$ pre-mRNA and may, therefore, affect splicing.[679] A second aberrant splicing process was also identified which affects exon 10 (in which the C825T polymorphism is located) giving rise to a splice variant referred to as $G\beta_{3\text{-}s2}$.[681] By means of RT-PCR this splice variant was detected in human heart, T cells, neutrophils, peripheral blood

lymphocytes, and bladder carcinoma predominantly in cells and tissues from 825T allele carriers.[681]

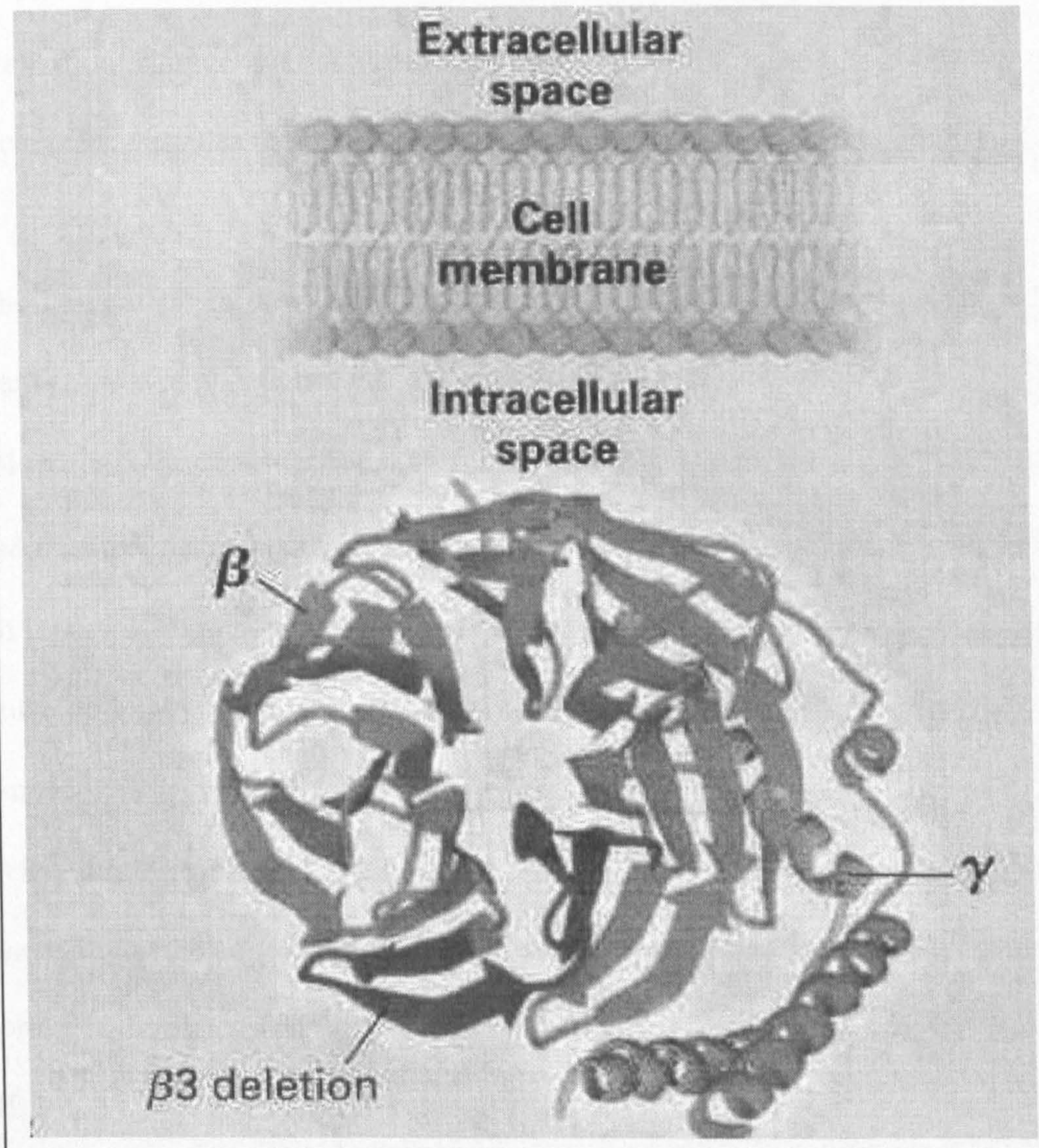


**Figure 1.6-1 G protein β3 subunit – Deletion in the splice variant. Adapted from Farfel[682]**

This splice variant is believed to result in a dominant gain of function.[678] Increased binding of [$^{35}$S]GTPγS in Sf9 insect cells expressing $G\beta_{3\text{-}s}$ suggests that this splice variant results in enhanced activation of pertussis toxin sensitive G proteins.[579]

The C825T polymorphism is a candidate gene for hypertension, because the mutation entails stimulation of the ubiquitously expressed $Na^+/H^+$ exchanger. At the level of the kidney, this may increase $Na^+$ reabsorption and cause chronic volume expansion.[683] Prevention of intracellular acidosis may also lead to proliferation of vascular smooth muscle cells, vascular remodelling and increased peripheral vascular resistance.[683]

In the context of cardiovascular function, one might expect that expression of the variant allele would enhance effects mediated through $G_i$-dependent pathways that are involved in vasoconstriction, such as the $\alpha_2$-adrenoceptor-mediated response. Paradoxically, decreased $\alpha_2$-adrenoceptor-mediated response at a cellular level has been reported in humans with hypertension.[684] Furthermore, the relation between the cellular and the functional responses is unclear and the relation between $alpha_2$-adrenoceptors and the GNB3 splice variant is unknown. Alternatively, increased $G_i$ activity might tonically attenuate $G_s$-mediated responses. Impairment of $beta_2$-adrenergic mediated vasodilatation and other $G_s$-mediated responses have been reported in hypertension.[685] Whether these effects are related to expression of the GNB3 825T splice variant is yet to be determined. Thus these mechanisms remain plausible but as yet unproven hypotheses linking functional abnormalities in G-proteins and hypertension

### 1.6.3.1 Hypertension

There has been variable association between the 825T allele and hypertension both positive[686-690] and nonsignificant[691-693] studies in Caucasian populations where 825T gene frequency is about 25%. Increased risk of hypertension associated with 825T was also reported among blacks[694] who have a much higher gene frequency approaching 80%. A negative study was reported in African Americans.[695] Less

association has been reported in Asian populations, which have intermediate gene frequencies of about 50%,[696-698] although one Japanese study was weakly positive.[699]

Following the initial study by Siffert[579] showing the T allele associated with increased odds of being hypertensive (odds ratio 1.44; 95% CI, 1.09 to 1.88), Schunkert et al [690] reported a mild association between this polymorphism and diastolic blood pressure level, but no association for systolic blood pressure level. They also found an association with lower renin levels and higher aldosterone to renin ratio, features consistent with low renin hypertension. One reason for this may be that some vasoactive factors (norepinephrine via $\alpha_2$ adrenoceptors, angiotensin II and thrombin) are known to transmit some of their effects via pertussis toxin-sensitive $G_i$ proteins.[700;701] Thus, these vasoconstrictors, if activated, may cause enhanced vascular resistance in individuals carrying the mutated GNB3 gene. Alternatively, the higher blood pressure in these individuals may be explained by vascular hypertrophy. In particular, growth factors of vascular smooth muscle cells like platelet derived growth factor are known to activate $G_i$ protein-dependent pathways.[674]

Renin synthesis (reflected by total renin or prorenin plus renin levels) and renin release are strongly augmented by camp, which is under negative control by $G_i$ proteins. Thus, enhanced $G_i$ protein signalling is likely to suppress plasma renin levels. However, this study found no differences with respect to aldosterone levels, despite genotype related differences in renin levels and presumably angiotensin II levels. One explanation of this can be that some stimulatory factors of aldosterone release (e.g. angiotensin II) can act via $G_i$ proteins in a stimulatory fashion, enhancing phospholipase C activity, cytosolic calcium liberation, and the expression of

aldosterone synthase gene.[702] Thus, it may be speculated that in individuals with the TT genotype, lower angiotensin II levels can be compensated for by enhanced postreceptor signalling, and hence explain the inappropriately high aldosterone levels given the suppressed renin levels. This needs to be verified experimentally. Zeltner et al[590] found in young men with normotension or mild incipient essential hypertension (mean age 26 years) that carriers of the 825T allele had faster tubular sodium reabsorption that CC homozygotes. This they attributed to increased activity of $Na^+/H^+$ exchanger.

| Date | Ref | Journal | Country | Ethnic Group | Cases | Assoc |
|---|---|---|---|---|---|---|
| 1998 | Siffert[579] | Nat Genet | Germany | Caucasian | 426 | Yes |
| 1998 | Kato[697] | Hypertension | Japan | Japanese | 718 | No |
| 1998 | Hegele[703] | Hypertension | Canada | Oji-Cree | 447 | Yes |
| 1998 | Schunkert[690] | Hypertension | Germany | Caucasian | 474 | Yes |
| 1998 | Benjafield[687] | Hypertension | Australia | Caucasian | 110 | Yes |
| 1999 | Beige[686] | Hypertension | Germany | Caucasian | 479 | Yes |
| 1999 | Dong[694] | Hypertension | England | Afro-Caribbean | 185 | Yes |
| 1999 | Brand[691] | Hypertension | France | Caucasian | 393 | No |
| 1999 | Brand[691] | Hypertension | Northern Ireland | Caucasian | 171 | No |
| 2000 | Larson[695] | Hypertension | USA | Afro-Caribbean | 472 | No |
| 2001 | Turner[704] | Hypertension | USA | Afro-Caribbean | 197 | No |
| 2001 | Turner[704] | Hypertension | USA | Caucasian | 190 | No |
| 2001 | Zeltner[590] | Hypertension | Germany | Caucasian | 95 | No |

**Table 1.6-2 GNB3 and hypertension**

Hegele et al[703] also reported an association between the T allele and systolic pressure in a sample of Canadian aborigines. The Canadian Oji-Cree are and isolated community with a low prevalence of hypertension. This study found in the young and normotensive population, a very high prevalence of the 825T allele (0.501 compared to 0.25 in a general normotensive German population). They also reported a significantly lower systolic blood pressure in subjects homozygous for the 825T allele, than in subjects with the other two genotypes, and no significant relationship

between a diagnosis of hypertension and the 825T allele. This finding is inconsistent with the expectation based on the functional impact of the mutation in vitro and the association studies in German subjects. One reason for this disparity can be the Oji-Cree population were recruited by community screening and not ascertained on the basis of a diagnosis of hypertension and they were younger with the average age 34 years, which may be too early for the effects of other genetic and/or environmental factors to contribute to the development of hypertension. Also, it can be speculated that the functional impact of the polymorphism could be different when hypertension has become the established phenotype. For example, the tendency of the 825T allele to predispose to elevated blood pressure at the cellular level could be adequately overcome, or perhaps even overcompensated for, by robust counter regulatory mechanisms in the younger subjects. Such possible overcompensation may result in a lower mean blood pressure in subjects with the T allele. With the passage of time, it is possible that the putative counter regulatory mechanisms may become fatigued or fail. Thus, the phenotype associated with the 825T allele in older subjects might be hypertension, which could represent the endpoint resulting from failure of counter regulatory mechanisms. However, there would be few precedents in human pathophysiology for such an explanation. Alternatively, the genomic change at GNB3 position may not have any functional relevance in the Oji-Cree, but may instead be in linkage disequilibrium with another genetic change at this locus, which would be the actual molecular basis for the association with variation in systolic blood pressure. Despite the high frequency of the T allele in this population, only 28 of the 791 subjects were prescribed antihypertensive medications. Though the frequency of the T allele in those taking antihypertensives was 0.571, compared to a frequency of 0.496

in those not on antihypertensives, the small numbers prevented any statistically significant association being detected.

A study of whites with a strong family history of hypertension found an association between hypertension status and GNB3 genotype.[687] In this study, 110 white Australian hypertensives each of whom were the offspring of two hypertensive parents and 189 normotensives whose parents were both normotensive beyond age 50 years, were genotyped for the C825T polymorphism. The T allele tracked with higher pretreatment blood pressure: diastolic=105+/-7, 109+/-16, and 128+/-28 mm Hg (mean+/-SD) for CC, CT, and TT, respectively (P=0.001 by 1-way ANOVA). Blood pressures were higher in female hypertensives with a T allele (P=0.006 for systolic and 0.0003 for diastolic by ANOVA) than they were in male hypertensives.

In African Americans, no association was found between this polymorphism and hypertension status,[695;704] while Dong et al found an association of the GNB3 genotype and hypertension among Afro-Caribbean's in England. Turner et al[704], did not find any association between the C825T polymorphism and blood pressure, but they found the TT genotype to be a significant predictor of greater declines in systolic and diastolic pressure in response to thiazide diuretic therapy. They found in blacks and non-Hispanic whites with essential hypertension, that systolic and diastolic blood pressures declined 5-6 mmHg more in response to diuretic treatment in TT than CC homozygotes with intermediate blood pressure falls in CT heterozygotes. However, no association was seen between 825T and blood pressure response to change in salt intake in young, normotensive men.[705] In the study by Larson et al[695] angiotensinogen A[-6], α-Adducin Gly460Trp and GNB3 polymorphisms were examined for

association with hypertension status in a sample of 904 African Americans. Tests of simple association and multivariate logistic regression analyses revealed no association between hypertension status and any of the studied polymorphisms. The frequency of the 825T allele in this sample of African Americans was considerably higher (0.75 in normotensives and 0.74 in hypertensives) than that reported in whites, or the Canadian Oji-Cree. Turner et al[704] In contrast Dong et al[694] in a study of 428 of black African descent from south London, reported a 3-fold higher risk of hypertension among the 825T allele both as heterozygotes (odds ratio [OR], 3.43 [95% CI, 0.94 to 12.4]) and homozygotes (OR, 3.87 [95% CI, 1. 09 to 13.8]). The estimate of effect and the blood pressure values in the groups carrying the T variant suggested a dominant model for the T allele. This was confirmed by a significant association between the T allele and hypertension (OR, 3.71 [95% CI, 1.05 to 13. 1]), even when adjusted for age, sex, and body mass index (OR, 4.14 [95% CI, 1.11 to 15.4]).

Brand et al[691] looking at 564 Caucasians from France and Northern Ireland and Kato et al[697] studying 718 Japanese hypertensives found no association between the GNB3 gene and hypertension.

### 1.6.3.2 Obesity

Siffert et al[706] reported and association of the C825T allele with obesity in young German men and in Chinese and Black South Africans of either sex and suggested that hypertension associated with the mutation could be mediated via obesity. Reports on the association between measures of obesity and the C825T polymorphism tended to be more consistent that those on cardiovascular traits.[706-708] Siffert et al looking at 197 German hypertensive subjects reported the odds ratio for obesity versus normal

weight was 3.9 [95% confidence interval (CI) 1.1-14.3; P = 0.03] for TT/CC and 1.8 (95% CI 0.7-4.6; P = 0.18) for TC/CC. [708] Similarly the 825T homozygous genotype has been positively associated with higher body mass index, [707;708] body weight,[707] or skin fold thickness among the Inuit,[707] Caucasians,[706] Chinese,[706] or blacks.[706] In a study of African immigrants and African-Americans, Poston et al[709] found individuals with the CC or CT genotype who were physically active had substantially lower BMIs (M+/-SE) (i.e., 25.74+/-2.02) than any of the other groups: sedentary CC + CT (30.58+/-1.03), sedentary TT (30.65+/-1.00) or active TT (29.43+/-1.65). Primiparous homozygous carriers of the 825T allele are at increased risk of obesity and post-pregnancy obesity,[710] however the association was limited to sedentary primiparous women. Hocher et al[711] found that infants whose mothers carried one or two copies of the 825T allele had significantly lower birthweights than did infants whose mothers were homozygous for the 825C allele.

| Date | Ref | Journal | Country | Ethnic Group | Cases | Assoc |
|---|---|---|---|---|---|---|
| 1999 | Hegele[707] | Genome Res | Canada | Inuit | 213 | Yes |
| 1999 | Siffert[706] | J Am Soc Nephrol | Germany | Caucasian | 277 | Yes |
| 1999 | Siffert[706] | J Am Soc Nephrol | China | Chinese | 960 | Yes |
| 1999 | Siffert[706] | J Am Soc Nephrol | South Africa | Afro-Caribbean | 275 | Yes |
| 1999 | Siffert[708] | J Hypertens | Germany | Caucasian | 197 | Yes |
| 2000 | Gutersohn[710] | Lancet | Germany | Caucasian | | Yes |
| 2002 | Poston[709] | Eat Weight Disord | US | Afro-Caribbean | | Yes |

**Table 1.6-3 GNB3 and BMI**

Thus the maternal 825T allele seemingly has a dominant effect on neonatal birthweight, but a recessive effect on postpartum maternal weight. Poch E et al.[712] reported an association between the T allele and insulin sensitivity that was independent of BMI while Wascher TC et al.[713] found GNB3 825T allele was

associated with reduced insulin sensitivity in men with abdominal fat distribution and with more advanced carotid atherosclerosis in middle-aged white men and women. Benjafield et al.[714] found theC825T splice variant of GNB3 makes little if any contribution to obesity in a cross-sectional case-control study of subjects with morbid obesity.

Taken together all these findings suggest that the 825T allele is a marker of adverse metabolic phenotypes. As always in theses types of studies, it is not known whether the associations directly reflect a causative mechanism, or whether they represent linkage disequilibrium with other mutations in GNB3 or other genes. Siffert et al speculate that the 825T allele predisposes to obesity and hypertension via hyperinsulinaemia and increased circulating catecholamines.[708;710] Increased signalling by pertussis toxin-sensitive G-proteins has been shown to stimulate adipogenesis[715] and may be one direct mechanism. Furthermore, increased expression of the $G_{i2}$ subunit in murine embryonic stem cells has been shown to promote terminal differentiation of adipocytes.[716] Alternatively increased $G_i$ activity could tonically attenuate $G_s$-mediated lipolysis. This potential mechanism is notable since impaired $G_s$ function (as occurs in the form of pseudohypoparathyroidism from an early truncation mutant of the alpha subunit of $G_s$) is characterised by impaired adrenergic-mediated lipolysis and obesity.[717]

### 1.6.3.3 Cardiac Phenotypes

| Phenotype | Date | Ref | Journal | Country | Ethnic Group | Cases | Assoc |
|---|---|---|---|---|---|---|---|
| Cardiac function | 1999 | Jacobi[580] | J Hypertens | Germany | Caucasian | 34 | Yes |
| Cardiac function | 2001 | Zeltner[590] | Hypertension | Germany | Caucasian | 95 | No |
| CAD | 2000 | Naber[718] | Hypertension | Germany | Caucasian | 585 | Yes |
| CAD | 2003 | von Beckerath[719] | Atherosclerosis | Germany | Caucasian | 998 | Yes |
| LVH | 2000 | Poch[581] | Hypertension | Spain | Caucasian | 60 | Yes |
| LVH | 2001 | Zeltner[590] | Hypertension | Germany | Caucasian | 95 | No |
| LV mass Index | 2001 | Semplicini[591] | Am J Hypertens | Italy | Caucasian | 207 | Yes |
| Stroke Volume | 2001 | Schafers[720] | Pharmacogenetics | Germany | Caucasian | | Yes |
| LV mass and function | 2002 | Sedlacek[592] | Hypertension | Germany | Caucasian | 2052 | No |

**Table 1.6-4 GNB3 and cardiac phenotypes**

#### 1.6.3.3.1 LV mass and function

Poch et al[581] reported an association between the GNB3 825T allele and LV hypertrophy in 86 patients with essential hypertension and a mean age of 51±1 years. 2-mode echocardiography in these patients showed left ventricular end-diastolic diameter (52.0+/-0.7 versus 48.9+/-0.9 mm, P=0.007), posterior wall thickness (11.3+/-0.2 versus 10.6+/-0.2 mm, P=0.042), and left ventricular mass index (152.7+/-4.4 versus 135.2+/-6.4 g/m2, P=0. 023) were significantly higher in patients with CT and TT genotypes considered together (CT+TT) than in CC patients. The distribution of the genotypes was significantly different when comparing patients with LVH: 20 (0.33) CC and 40 (0.67) CT+TT patients had this complication, and 17 (0.65) CC and 9 (0.35) CT+TT patients did not (P<0.01). The frequency of the T allele was significantly different among patients with (0.40) and without (0.20) LVH (P<0.01). A logistic regression analysis showed that the association between the T allele and LVH was independent of age, mean blood pressure, body mass index, and alcohol consumption. The relative risk of LVH in patients bearing the T allele (CT+TT group) compared with CC hypertensive patients was 3.03 (95% CI 1.14 to 8.05).

Jacobi et al[580] in a study of 34 white patients with established mild to moderate essential hypertension showed that the GNB3 825T allele was not associated with left ventricular mass. However, transmitral flow variables reflecting left ventricular diastolic filling were impaired in patients expressing the TC/TT genotype (ratio of peak late (A) to early (E) velocities: CC versus TC/TT, 0.95 +/- 0.24 versus 1.2 +/- 0.26, P< 0.02; velocity time integrals A/E: CC versus TC/TT, 0.57 +/- 0.16 versus 0.76 +/- 0.23, P< 0.01) while all co-variables such as age, body mass index, ambulatory blood pressure, heart rate and end-diastolic volume were similar between the two groups. This suggested that since alterations in left ventricular filling have been identified as an early marker of hypertensive heart disease, the GNB3 C825T polymorphism might influence cardiac adaptation to increased afterload. An increased heart stroke volume in young, normotensive volunteers was reported.[720]

Zeltner et al[590] in a study of 95 white male students with normal or mildly elevated blood pressure found no association between the GNB3 825T allele and indices of left ventricular mass, left ventricular systolic or diastolic function.

In a study of 2052 individuals from a large population based sample in Germany, Sedlacek K et al.[592] showed GNB3 T allele had no strong and clinically relevant effect on the variability of LVM.

It is hypothesised that GNB3 subunit gene polymorphism may constitute a genetic basis for the enhanced growth phenomena observed in essential hypertension, such as LVH and media hypertrophy of the vessel wall, because it is directly associated with a

functional change and may not merely represent a DNA marker. G proteins mediate part of the actions of vasoactive hormones, such as angiotensin II and norepinephrine.[700] Angiotensin II exerts trophic effects on cardiomyocytes in culture,[721] while growth factors, such as platelet-derived growth factor, can act in part through pertussis-sensitive G proteins in skin fibroblasts, vascular smooth muscle cells, cardiac fibroblasts and may participate in the pathogenesis of LVH.[494]

Indeed, LVH is a complex genetic trait, and a single gene variant, albeit functionally relevant, may carry a minute contribution to the final phenotype. Therefore, it is inherently complicated to demonstrate its influence in even large-scale and well-powered association studies. By contrast, smaller studies have been shown to carry a risk of an -error.[722] Thus, a cautious interpretation of such studies may be advisable.

#### 1.6.3.3.2 Coronary artery disease

Several reported characteristics of the GNB3 C825T polymorphism make it a candidate gene for coronary artery disease. The 825T allele has been shown to be associated with hypertension and obesity which are established risk factors for MI. The 825T allele carriers display an enhanced activation of neutrophils[675] and platelets[723] which could play a role in plaque disruption and subsequent MI. The 825T allele has also been shown to be predictive of enhanced vasoconstriction and myocardial ischaemia after intracoronary $\alpha_2$-adrenoceptor activation.[724]

Naber et al[718] studied 585 patients with angiographically confirmed coronary artery disease (270 with and 315 without previous MI). There was no significant association between GNB3 genotypes and BMI, hypercholesterolaemia, hypertension, diabetes, smoking and previous MI. However the odds ratio for MI associated with the 825T

allele though statistically significant was relatively small at 1.5. Assuming that the complex polygenic nature of ischaemic heart disease implies that interaction of various candidate genes substantial to increase the risk of MI, they followed on to test for interaction of GNB3 with the ACE gene in this population. The D allele of the ACE I/D polymorphism is associated with increased serum ACE concentrations, and thus may become pathophysiologically important in individuals whose genetic makeup comprises an allelic variant that causes an increased cell responsiveness. In this respect the GNB3 825T allele is an attractive candidate to interact with the ACE gene.

| | OR(TT vs. CC) | OR(CT vs. CC) | OR(TT/CT vs. CC) |
|---|---|---|---|
| ACE DD | 7.5(1.5-37.3) p=0.006 | 2.2(1.1-4.3) p=0.03 | 2.4(1.2-4.8) p=0.02 |
| ACE ID | 1.4(0.6-3.4) p>0.05 | 2.0(1.2-3.2) p=0.005 | 1.9(1.2-3.0) p=0.01 |
| ACE II | 0.5(0.1-1.7) p>0.05 | 0.6(0.3-1.1) p>0.05 | 0.5(0.3-1.0) p>0.05 |

Naber et al found a significantly increased OR for MI associated with the 825T allele in individuals with the ACE DD genotype. The increased OR associated with the 825T allele was smaller in individuals with the ACE ID genotype and completely disappeared in conjunction with the ACE II genotype.

Von Beckerath et al.[719] tested whether GNB3 C825T was associated with an increased risk of coronary artery disease (CAD) in 998 angiographically examined, consecutive patients with CAD and 340 angiographically examined, sex- and age-matched controls with no evidence of CAD. The proportion of T allele carriers was significantly higher in the group with CAD compared with the control group (55.6 vs. 48.5; P=0.02). T allele carriage was associated with a 33% increase in the unadjusted risk (OR 1.33 [95% confidence interval, 1.04-1.70]) and a 37% increase in the adjusted risk (OR from the multivariate model 1.37 [95% CI, 1.06-1.76]) for CAD.

Moreover, an increase in T allele carriage was associated with an increase in disease severity (P=0.006; test for trend). The strongest association was observed between T allele carriage and three-vessel disease (unadjusted OR 1.47 [95% CI, 1.10-1.96]).

This observed interaction might result from synergistic but independent effect of each genetic factor. However functional considerations support the hypothesis of a true interaction between both alleles. Hormones of the rennin-angiotensin-aldosterone system, which is affected by the ACE ID polymorphism, activate G protein-coupled receptors, whose signalling properties may be altered in the presence of an 825T allele at GNB3. G protein βγ-subunits play a role in modulating agonist-receptor affinity of the AT-II receptors,[725] and AT-II in particular increases the expression of pertussis toxin-sensitive G proteins,[726] whose signalling properties are in turn enhanced in the presence of an 825T allele at GNB3.[579] In addition, several pathways downstream of the AT-II receptors affect signalling cascades in which G protein βγ-subunits are assumed to be involved, for example activation of phospholipase C[727] and D,[728], regulation of $Ca^{2+}$ channels,[729] and the transactivation of growth factors.[730] For example such an interaction may result in further enhanced platelet aggregation[723;731] with an increased susceptibility for acute coronary thrombosis. Unfortunately, little is known about the specific contribution of GNB3 and its splice variant to these processes. Moreover, besides increased ACE levels, an effect of the ACE D allele on AT-II concentration has not yet been proven. Thus, the molecular nature of the interaction of the GNB3 825T allele and the ACE D allele remains to be elucidated. Also this study was confined only to patients with coronary artery disease, and such a selection may cause a specific bias, this need to be replicated in studies that involve healthy subjects as well.

### 1.6.4 Haplotypes

A number of other variants have been identified that are in linkage disequilibrium with 825T.[679] Six novel single nucleotide polymorphisms in the GNB3 gene termed according to their location as G76A, G1906T, G2906A, A3882C, G5177A, and G5249A and a CACA-insertion-deletion polymorphism position 6496 were found. Genotyping and association studies resulted in the definition of two major GNB3 haplotypes, termed 'C-haplotype' (alleles 825C, 3882A, 5249G, 6496CACA-) and 'T-haplotype' (alleles 825T, 3882C, 5249A, 6496CACA+).[679]

A strong association was seen between 825T and the generation, through an alternate splice site, of yet another short variant of GNB3 ($G\beta_{3\text{-}s2}$), which also showed greater signal transduction activity.[681] $G\beta_{3\text{-}s2}$ is generated by alternative splicing using non-canonical splice sites. Transcripts for $G\beta_{3\text{-}s2}$ are lacking 129 bp of the coding sequence of the wild-type $G\beta_3$ protein. Thus the predicted structure consists of only six propeller blades, which resembles the structure of $G\beta_{3\text{-}s}$. Co-immunoprecipitation analyses indicated that $G\beta_{3\text{-}s2}$ dimerises with different $G\gamma$ subunits, e.g. $G\gamma_5$, $G\gamma_8$ and $G\gamma_{12}$. In Sf9 insect cells, expression of $G\beta_{3\text{-}s2}$ together with $G\gamma_{12}$ enhances receptor-stimulated activation of $G\alpha_{i2}$. Expression of $G\beta_{3\text{-}s2}$ in mammalian cells activated the mitogen-activated protein kinase cascade.[681] The precise mechanism explaining the strong association of 825T and the shorter variants has not yet been elucidated. The fact that only a minority of clones expressed the $G\beta_3$-second variant suggests, perhaps, that there exists another linked site or sites in the gene that control expression.

### 1.6.4.1 Other Associations

Other associations with the 825T variant include enhanced vasoconstriction in response to endothelin-1, norepinephrine, and angiotensin II,[732] but not an α-2 agonist[720] and a substantially increased response to sildenafil.[733] Radial artery hypertrophy was 3 times more frequent ($P < 0.001$) in a healthy population carrying the 825T variant,[734] carotid atherosclerosis,[713] and increased risk of stroke.[735]

Despite the epidemiological evidence, the in-vivo function- at either a cellular or integrative level- of the GNB3 splice variant remains largely unknown. Thus, to determine cause and effect between clinical phenotypes and GNB3 825T, studies are still needed of the relation between this allele and intermediate biochemical and physiological phenotypes. All association studies, taken together highlight the challenges involved when attempts are made to translate genotypic differences into mechanisms that underlie complex phenotypes.

In summary since G protein activation is the key event in intracellular signal transduction, it is logical to assume that the C825T polymorphism may have an impact on a variety of disease processes, signal transduction in human cells and tissues, as well as responses to common drugs. This has been tested in various clinical settings, in some studies, the 825T allele was shown to variably increase the risk for obesity,[706;708;710] hypertension,[689;690] coronary heart disease,[718;719] stroke,[713;735] and depression[736;737] in the white human population of European descent. By contrast, the association of the 825T allele with these disorders in other, non-Caucasian ethnicities remains controversial.[695;697-699;738] Furthermore, the 825T allele serves as a pharmacogenetic marker to predict responses to diuretics,[704] antidepressants,[737]

sildenafil,[733] clonidine,[739] angiotensin II,[732] endothelin-1,[732] and responses to vaccination against hepatitis B.[676] One study showed GNB3 polymorphism was associated to response to diuretics.[704] This has however not been replicated. It is not clear if this is a causal association as it is not clear that the polymorphism is functional. Finally, the molecular and biochemical mechanisms underlying these associations are largely obscure.

# Chapter 2 - OBJECTIVES

Despite the initial molecular work by Siffert et al., subsequent genetic association studies have not confirmed if the GNB3 polymorphism is indeed a candidate gene for hypertension. Many questions remain in relation to the biochemistry associated with the truncated G protein $\beta_3$ subunit ($G\beta_{3\text{-}s}$) and its overall relevance to essential hypertension and other cardiovascular phenotypes. The work presented in this thesis is a detailed study of the C825T G protein $\beta_3$ subunit (GNB3) polymorphism in human essential hypertension

The objective is to investigate if the GNB3 polymorphism is indeed functional and if so, to determine the biochemical, cellular and clinical phenotype associated with it. The plan of the study is to answer the following questions -

- Is the 825T allele associated with blood pressure phenotypes?
    - Case-Control study – To study the association with hypertension
    - Twin study – To study the association with echocardiographic LV Mass
    - Family study – To study the association with blood pressure and ECG LV mass
- Does C825T GNB3 polymorphism increase signalling through GPCRs acting via $G_{i2\alpha}$ ?
    - Platelet aggregation in normal volunteers should show that the presence of 825T allele is associated with increased platelet aggregability

- Does presence of $G\beta_{3\text{-}s}$ lead to increased activation of $G_{i\alpha}$?
    - Use a GPCR-G protein fusion strategy to measure GTPase activity in HEK293 membrane preparations following co-expression with either $G\beta_3$ or $G\beta_{3\text{-}s}$ and $\gamma_5$.

- Is there any difference in effector activation by the two variants of GNB3 subunit?
    - Use a GPCR-G protein fusion strategy to measure single cell calcium signal in EF88 membrane preparations following co-expression with either $G\beta_3$ or $G\beta_{3\text{-}s}$ and $\gamma_5$.

# Chapter 3 - MATERIALS AND METHODS

## 3.1 Genomic DNA Extraction:

Genomic DNA was isolated by phenol-chloroform extraction of EDTA-blood samples.[740]

### *3.1.1 Reagents*

10 ml blood collected into EDTA tubes (Vacutainers – Becton Dickinson)

The following solutions were prepared prior to DNA extraction.

**Erythrocyte lysis mix**

Water – 10 L

Sucrose (0.32 M) – 1090 g

Tris Base (10 mM) – 12.1 g

MgCl2 (5 mM) – 10 g

Adjust to pH 7.5 with conc. HCl

Autoclave at 121°C for 20 minutes

After cooling to room temperature add Triton X-100 – 1%)

**Nucleic Lysis Mix**

Tris Base (10 mM) – 0.6 g

NaCl (0.4 mM) – 11.7 g

EDTA (2 mM) – 0.2 g

Adjust pH to 8.2

Autoclave at 121°C for 20 minutes

**10% Sodium Dodecyl Sulphate (Lauryl Sulphate – SDS)**

SDS – 100 g

Distilled water – 100 ml

**Proteinase K solution**

Proteinase K – 100 mg

10% SDS – 1 ml

Distilled water – 9 ml

**Saturated NaCl**

NaCl – 175 g

Distilled water – 500 ml

**Phenol/Chloroform**

Phenol (equilibrated with 0.1M Tris Rathburn) – 500 ml

Chloroform (equilibrated with 1:24 iso-amyl alcohol)– 500 ml

8-hydroxyquinoline – 2 g

β-mercaptoethanol – 200 µl

### *3.1.2 Apparatus*

- 0.5 ml Eppendorf tubes (Alpha Labs)
- Finnpipettes – 1-10 µl; 5050 µl; 50-200 µl; 200-1000 µl; 1-5 ml
- Universal containers – 30 ml (Sterilin)
- Falcon tubes -50 ml (Becton Dickinson)
- 1.5 ml microtubes (Alpha)

### *3.1.3 Methods*

DNA is extracted from 10 mls of EDTA blood using the following protocol.

In a 50 ml Falcon tube 40 ml of the erythrocyte lysis mix is added to 10 mls of whole blood from EDTA tube collection. They are mixed by inverting five times and then centrifuged at 2800 rpm for ten minutes. The pellet is resuspended in 3 mls of the nucleic acid lysis mix. To this 200 µl of 10% SDS and 100 µl Proteinase K solution are added and incubate at 37°C for 18 hours. After this 1 ml of saturated NaCl is added and the tube shaken vigorously for 15 seconds. 5 mls phenol chloroform

solution is then added to the mix in a taking precautions in a fume cupboard. This in now centrifuged at 2500 rpm for 20 minutes. After centrifugation, the top partitioned layer is transferred to a 30 ml Universal container and 2 volumes 99% ethanol added. The DNA precipitates and it is spooled out carefully and rehydrated in 400 µl distilled water in labelled 1.5 ml microtube. This is left at room temperature for 48 hours and then transferred to –70°C freezer.

### *3.1.4 DNA Quantification*

DNA was quantified using UV spectrophotometry. Initially DNA purity is determined by adding 15 µl of each sample to 735 µl TE, mixing well, and reading the optical density at 260 nm and 280 nm ($OD_{260}$ and $OD_{280}$). The ratio $OD_{260}/OD_{280}$ should be determined in order to assess the purity of the sample. If this ratio is 1.8-2.0, the absorption is probably due to nucleic acids. A ratio less than 1.8 indicates that there may be proteins and/or other UV absorbers in the sample, in which case the DNA is reprecipitated. A ratio higher than 2.0 indicates the samples may be contaminated with chloroform or phenol and should be re-precipitated with ethanol.

After determining purity, the DNA is quantified by measuring $OD_{260}$ and using the following formula.

DNA concentration (µg/µl) = $OD_{260}$ x 50 (dilution factor) x 50/1000 µg/ml

## 3.2 Genotyping C825T G-protein β3 subunit polymorphism

PCR was conducted in a 50 µl volume reaction in a 96 well plate using Perkin-Elmer thermal cycler, using the method of Siffert et al.[579]

### *3.2.1 Instrumentation*

Thermal Cycler: Perkin Elmer

Electrophoresis

Bio-Rad PowerPac 300

Submarine Gel System Bio-Rad Sub-Cell GT

### *3.2.2 Reagents*

The following solutions were prepared for the PCR reaction

**Electrophoresis Buffer (EB)**

a) Prepare 5N NaOH - 200 g NaOH in 1L distilled water

b) In a 10L beaker add 186.12 g EDTA to 1 L distilled water

c) Add 5N NaOH to (b) until the solution clears at pH 7.0

d) Dissolve 2420 g Tris Base in 5 L distilled water and add to (b)

e) Ass approximately 570 ml glacial acetic acid to (b) until pH 8.0

f) Make (b) upto 10 L with distilled water

**Agarose: 2.5% GIBCO-BRL agarose**

a) Add 2.5 g GIBCO-BRL agarose to 100 ml electrophoresis buffer in a 250 ml conical flask

b) Bring to boil in a microwave oven for 60 seconds

c) Transfer the flask to fume cupboard and add I µl Ethidium Bromide (10 mg/ml)

d) Gently swirl liquid to disperse the ethidium bromide and allow to cool for 2 minutes before pouring into gel tray casting assembly

e) Allow the agarose to gel in fume hood at room temperature then transfer to submarine gel tank

**Reaction mixture**

DH20 - 34.8 µL

Buffer - 5 µL -20 mM/L Tris-Hcl (pH 8.4), 50 mMl/L KCl

MgCl2 - 3 μL -1.1 mM/L

(1:10) Primers -1 μL X 2 – 20 pmol each

(1:10) DNTPs -1 μL X4 - 200 μM/L each

Taq Polymerase - 0.2 μL

Genomic DNA – 100 ng

### *3.2.3 PCR components*

Sense primer: 5'-TGACCCACTTGCCACCCGTGC-3'

Antisense primer: 5'-GCAGCAGCCAGGGCTGGC-3'

PCR product with the flanking primers:

TGACCCACT TGCCACCCGT GC cctcagtt cttccccaat ggagaggcca tctgcacggg ctcggatgac gcttcctgcc gcttgtttga cctgcgggca gaccaggagc tgatctgctt ctcccacgag agcatcatct gcggcatcac gtccgtggcc ttctccctca gtggccgcct actattcgct ggctacgacg acttcaactg caatgtctgg gactccatga agtctgagcg tgtgggtaag gGCCAGCCCT GGCTGCTGC

### *3.2.4 PCR protocol*

The PCR reaction is carried out in 96 well Thermowell Plates (MADGEBIO) in Perkin Elmer thermal cycler. Each reaction mix per well is made up with 34.8 μL of distilled water, 5 μL of buffer which contains-20 mM/L Tris-Hcl (pH 8.4) and 50 mMl/L KCl, 20 pmol of each primer made as a 1:10 dilution solution, 200 μM/L of each nucleotide, 0.2 μL of Taq Polymerase and 100 ng of genomic DNA. The stock solution is initially made up using strict hygienic standards to avoid any cross contamination. The DNA was added last. Once all the wells were loaded, the 96 well plate is sealed and transferred to the thermal cycler. The PCR reaction is carried out with the initial denaturation phase for 5 minutes at 94°C followed by 35 cycles

comprising denaturation at 94°C for 1 min, annealing at 60°C for 45 seconds and extension at 72°C for 1 minute followed by a final extension step at 72°C for 7 minutes. After the PCR reaction is completed, the digestion mix is loaded in another 96 well plate. The restriction enzyme placed on ice during loading and taken out of the freezer for the minimum possible time. The digestion mix consists of the restriction enzyme BseD1-0.04 μL (Helena Biosciences), 1.5 μL of Y/TangoBuffer, 8.46 μL of distilled water and 5μL of the PCR product. The 96 well plate is sealed and transferred to the thermal cycler and incubated for 2 hours at 55°C.

#### 3.2.4.1 Electrophoresis and visualization

The digested PCR products are electrophoretically resolved on 2.5% agarose and visualized under UV illumination after ethidium bromide staining. First 10 μl of PCR product is added to 5 μl sucrose dye in a 96 well plate using a 8 channel pipette. This is loaded into 2.5% agarose gel and run for 1 hour at 100 Volts.

The electrophoresis bands are read as follows. The non-digested product (TT genotype) shown a single band of 268 bp, and complete digestion of the PCR product (CC genotype) generates bands of 116 and 152 bp. Heterozygotes (CT genotype) display three bands (Figure 3.2-1).

Figure 3.2-2 and Figure 3.2-3 show the sequencing of the PCR product from two different genotypes to verify that the PCR detected the right genetic polymorphism. Figure 3.2-2 shows the sequence of the CC genotype PCR product after digestion and Figure 3.2-3 the sequence of the CT genotype. At position 140 which corresponds to

the location of the GNB3 C825T polymorphism, Figure 3.2-3 shows a dual peak with both C and T nucleotides present.

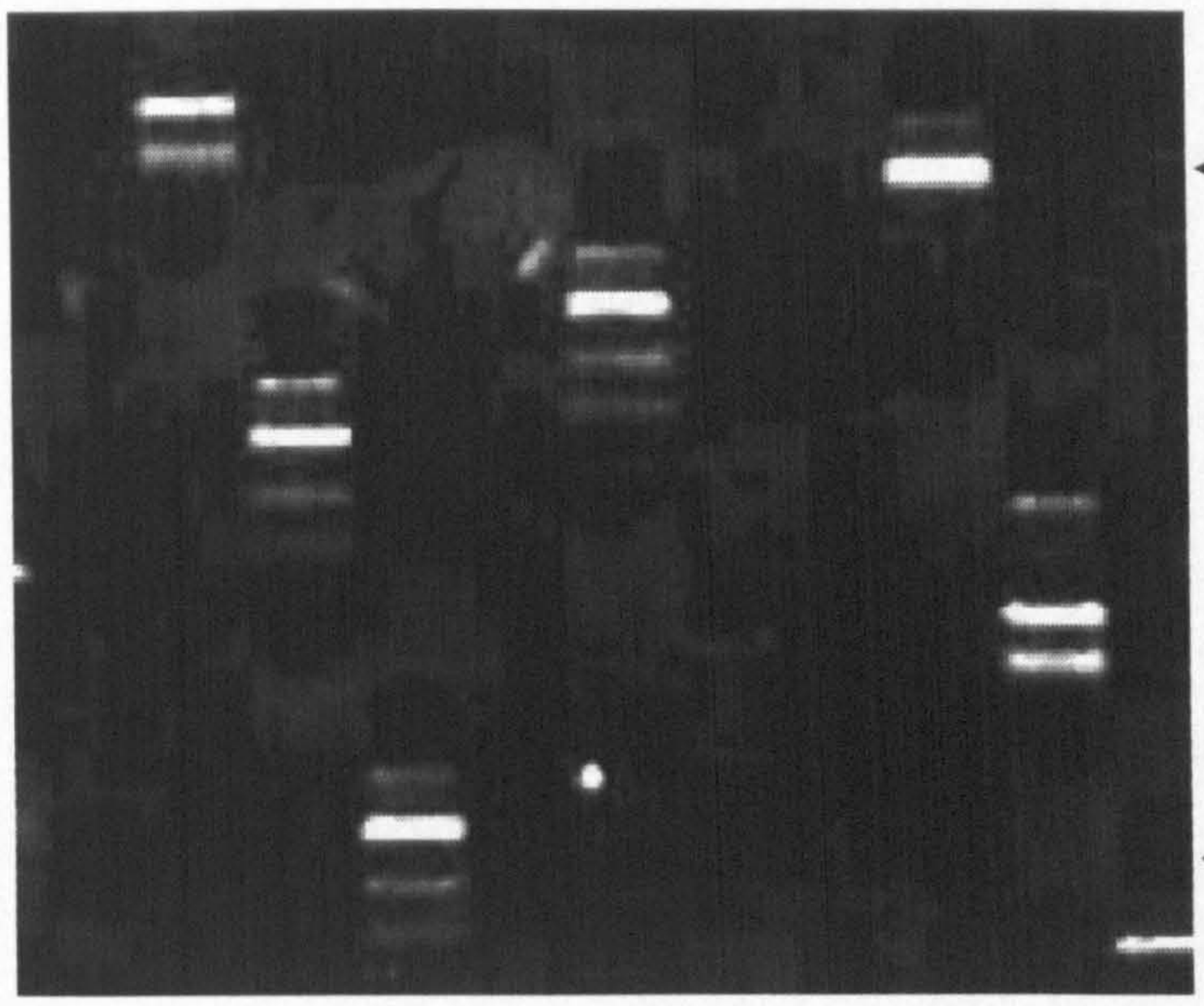


**Figure 3.2-1 PCR gel electrophoresis showing the three GNB3 genotypes**

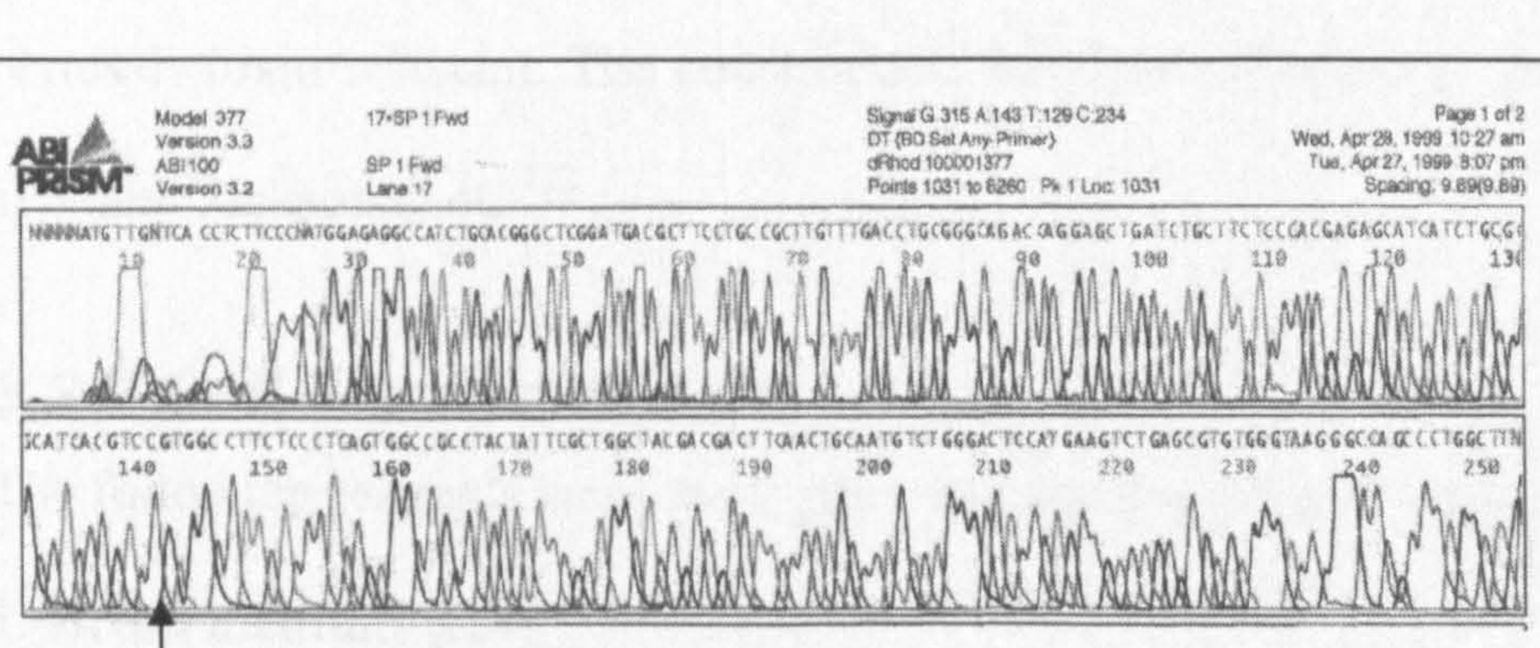


**Figure 3.2-2 Sequence of CC Genotype**

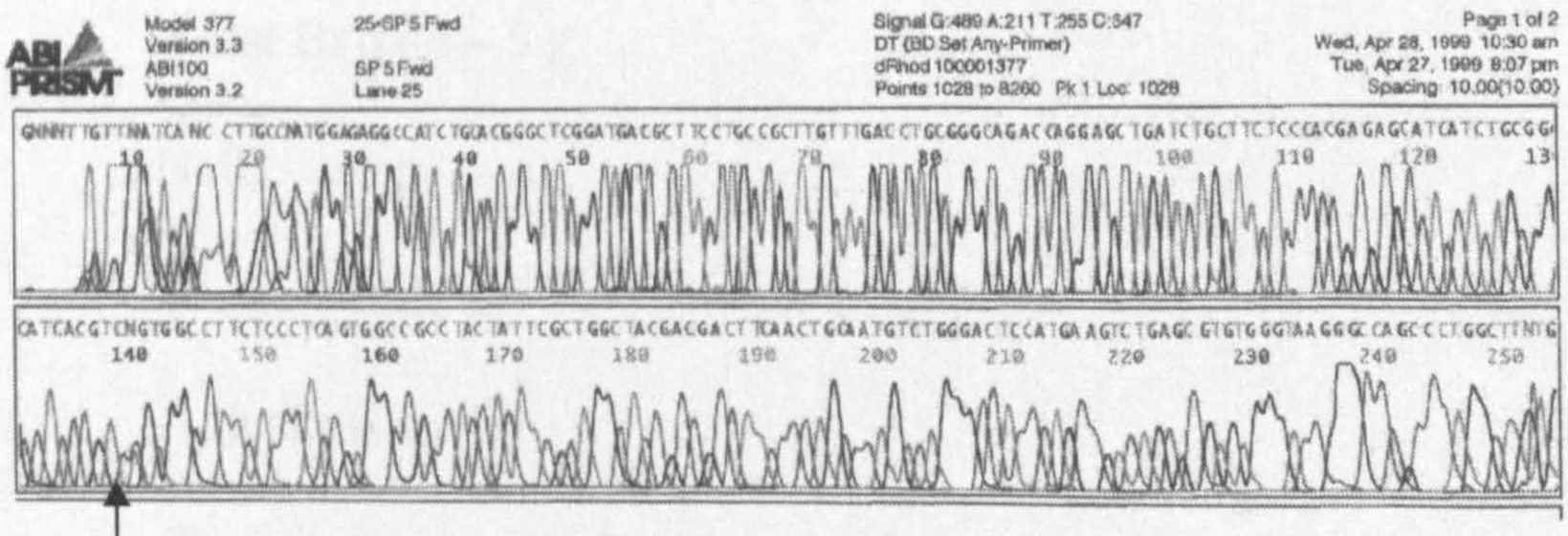


**Figure 3.2-3 Sequence of GNB3 CT Genotype**

## 3.3 Cell Transformation and Amplification of plasmid DNA

All materials for tissue culture were supplied by Life Technologies Inc. (Paisley, Strathclyde, UK). The a2-adrenoreceptor antagonist [3H]RS-79948-197 (90 Ci/mmol) was purchased from Amersham International. [g-32P]GTP (30 Ci/mmol) was obtained from DuPont/NEN. Cholera toxin and pertussis toxin were purchased from Sigma (St. Louis, MO).

### *3.3.1 GPCR-G Protein Fusion construct (Porcine $\alpha_{2A}$ Adrenoceptor-$G_{i1\alpha}Cys^{351} \rightarrow Ile$)*

$\alpha_{2A}$ adrenoceptor-$Cys^{351}G_{i1\alpha}$ is a GPCR-G protein fusion construct obtained from Prof Milligans laboratory and used to study the functional characteristics of the G protein $\beta_3$ subunit and its splice variant. The $Cys^{351}Ile$ mutation renders the fusion construct pertussis toxin resistant. The construction this fusion protein is described by Wise et al[741] and Jackson et al. [742]

### *3.3.2 Cell Transformation*

The following reagents were made prior to commencing cell transformation.

**L-Broth medium (LB)**

Tryptone - 10g

Yeast Extract – 5 g

NaCl – 10 g

Water – 1000 ml

Adjust pH to 7.0

Sterilise in an autoclave

**L- Agar**

Tryptone - 10g

Yeast Extract – 5 g

NaCl – 10 g

Water – 1000 ml

Adjust pH to 7.0

Agar – 15 g

Sterilise in an autoclave

After cooling add stock ampicillin to a concentration of 20-60 µg/ml

**Ampicillin**

Ampicillin 50 mg/ml in distilled water

Working concentration – 20-60 µg/ml

Add stock ampicillin solution to media 1:1000 dilution

First an aliquot of competent bacteria (XL1) is thawed on ice, and 50 µl per transformation is added to a 10 ml sterile tube. Then add 2 µl of selected plasmid DNA (1-5 µl) to the competent bacteria and leave it on ice for 15 minutes. Transformation is done by heat shock at 42°C for 90 seconds in a water bath. After exactly 90 seconds, the tube with the bacteria is returned to ice for 2 minutes. Then 450 µl of L-Broth is added to this and shaken at 37°C for 45 minutes. After this 200 µl of this mix is plated onto ampicillin-agar plate and left inverted at 37°C overnight in an incubator.

The next morning distinct colonies of the transformed bacteria are seen on the agar plate. A single distinct colony from the overnight culture plate is added to a 30 ml sterile universal tube containing 5 ml of L-broth and 5 µl Ampicillin stock using the standard precautions. For control a similar tube is used but without adding the

bacterial colony. The tubes are incubated in a shaker at 37°C for 6 hours, and afterwards the universal container with bacteria is seen to be cloudy while the control container remains clear.

The entire contents of the universal is added into an autoclaved conical flask containing 100 ml of L-broth and 100 µl of ampicillin using standard precautions. The flasks are incubated at 37°C overnight with constant shaking.

The next day Maxiprep of the plasmid DNA is carried out using Qiagen Maxi Kit using instructions provided by the manufacturer.

### *3.3.3 Quantifying plasmid DNA*

The plasmid DNA obtained after maxiprep is diluted to 1:100 solution. Add 1 ml of water to a quartz cuvette as control and add 1 ml of the diluted DNA to another quartz cuvette. Auto zero the photometer with the blank sample at 260 nm and using the cuvette containing the DNA sample, readings are taken at 260 nm and 280 nm.

Knowing that 50 µl/ml of DSDNA has an optical density at 260 nm ($O.D._{260}$) of 1, the concentration of DNA can be calculated as

$O.D._{260}$ X 50 = DNA concentration in µg/ml

| Plasmid DNA | 260 nm (1:100) | 280 nm (1:100) | 260 nm | 280 nm | DNA conc µl/ml |
|---|---|---|---|---|---|
| $\alpha_{2A}$-$G_i$ | 0.12 | 0.07 | 12.0 | 7.0 | 600 |
| $\beta_3$ | 0.121 | 0.062 | 12.1 | 6.2 | 605 |
| $\beta_{3\text{-}s}$ | 0.194 | 0.106 | 19.4 | 10.6 | 970 |
| $\gamma_5$ | 0.151 | 0.076 | 15.1 | 7.6 | 755 |

**Table 3.3-1 Concentrations of the four plasmid DNA used for subsequent experiments.**

## 3.4 Transfections and membrane preparation of HEK293 and COS-7 cells

### *3.4.1 Transfection*

All tissue culture experiments were carried out in the tissue culture laboratory using strict tissue culture protocols. HEK293 and COS-7 cells were cotranfected with GPCR-G protein fusion protein, $G\beta_3$ or $G\beta_{3\text{-}s}$ and $\gamma_5$ DNA. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal-calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 60-mm culture dishes and grown to 60–80% confluency (18–24 h) before transfection with pCDNA3 containing the relevant cDNA species, using lipofectamine reagent (Life Technologies Inc.).[743] Optimem is pre-warmed by placing in a 37°C water bath. DNA is prepared by diluting an appropriate volume of each DNA to 0.1 μg/μl.

The Lipofectamine/Optimem (Lipof/Opt) is prepared for the required number of plates as follows

| | Per plate | 22 plates |
|---|---|---|
| Lipofectamine | 20 μl | 440 μl |
| Optimem | 580 μl | 12760 μl |

| | 1 (μl) | 2 (μl) | 3 (μl) | 4 (μl) | 5 (μl) |
|---|---|---|---|---|---|
| PCDNA3 | 240 | - | - | 120 | 360 |
| $\alpha_{2A}G_i$ | 120 | 120 | 120 | - | - |
| $\beta_3$ | - | 120 | - | 120 | - |
| $\beta_{3\text{-}s}$ | - | - | 120 | - | - |
| $\gamma_5$ | - | 120 | 120 | 120 | - |
| Optimem | 2040 | 2040 | 2040 | 2040 | 2040 |
| Lipof/Opt | 2400 | 2400 | 2400 | 2400 | 2400 |

For each 10 $cm^3$ plate of cells add 9 μg of DNA per plate. Make up the following tubes for transfecting 4 plates with each mixture. The Lipofectamine/Optimem

mixture is added last and drop wise. These tubes are then incubated at room temperature for 30 minutes. Following this add 19200 µl of Optimem to each tube.

For each plate to be transfected, first wash out with 10 ml Optimem, then to each plate add 6 ml of the DNA mixture, which equals 9 µg of DNA per plate. Incubate the plates at 37°C for 5 hours and then add 10 ml of Dulbecco's modified Eagle's medium to each plate and incubate overnight. The next morning, replace the media with 9 mls of fresh Dulbecco's modified Eagle's medium and after 6 hours add Pertussis toxin (25 ng/ml) to each plate as follows in order to bring about ADP-ribosylation of the endogenous $G_i$-family G-proteins,[744] and thus prevent potential interactions between these and the fusion protein.

For 22 plates

Dulbecco's modified Eagle's medium – 22 ml

Pertussis toxin – 27.5µl

Add 1 ml of the mixture to each plate

The plates are incubated overnight at 37°C and the cells harvested the next day for membrane preparation or used for calcium signalling experiments.

### *3.4.2 Membrane preparation*

The cells in each plate are detached mechanically by scraping and suspended in TE buffer containing 0.5 ml of 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5. The cell suspensions in media are centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant discarded. The cells are resuspended in Phosphate Buffered Saline (PBS) by vortexing. The suspension is then centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant discarded. The pellet is frozen at -80°C. After 2 hours, the cell pellets are resuspended in 0.5 ml of 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5 (TE)

and rupture of the cells achieved with 50 strokes of a hand-held Teflon/glass homogeniser followed by passage (10 times) through a 25-gauge needle. The cell lysates were centrifuged at 1000 g for 10 min at 4°C in a Beckman TJ-6 centrifuge to pellet the nuclei and unbroken cells and P2 particulate fractions were then recovered by centrifugation of the supernatant at 200000 g(50000 rpm) for 30 minutes at 4°C in a Beckman TL 100 bench-top ultracentrifuge using a Beckman TLA 100.2 rotor. The membranes are seen as translucent pellets. All the supernatant is removed and the pellet is resuspended in 300 µl of TE buffer. This suspension is added to a tube with TE buffer made upto 4 mls. This is then aliquoted into eppendorf tubes each containing 300 µl and stored at -80°C

### *3.4.3 Membrane protein assay*

Protein concentrations are determined with the bicinchoninic acid (BCA) procedure[745] using BSA as standard. The reaction mix is made up with 1 part of Reagent B (4% copper sulfate) to 49 parts of Reagent A (sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1N sodium hydroxide). Graded concentrations of bovines serum albumin is used for generating the standard protein curve. From this the concentration of the protein is estimated. The reaction is done in triplicate using differing concentration of the protein (1:1, 1:2, 1:3 dilutions). The reaction is carried out in a 96 well plate with 10 µl of the protein preparation and 200 µl of reaction mix added to each well. The plate is covered with an adhesive strip and incubated at 37°C for 20 minutes. The fluorescence from each well is measured at 492 nm in a plate reader. An example of the plate set-up and the calculated protein concentration is given below.

The membrane preparations thus obtained are used for subsequent experiments

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BSA | 0.077 | 0.093 | 0.104 | 0.117 | 0.141 | 0.153 | 0.174 | 0.209 | 0.199 | 0.229 | 0.231 | |
| | 0.079 | 0.087 | 0.099 | 0.120 | 0.135 | 0.153 | 0.175 | 0.176 | 0.192 | 0.229 | 0.230 | |
| Membrane 1:1 10 µl | 0.180 | 0.170 | 0.152 | 0.132 | 0.121 | | | | | | | |
| | 0.185 | 0.142 | 0.151 | 0.119 | 0.108 | | | | | | | |
| Membrane 1:2 10 µl | 0.164 | 0.144 | 0.136 | 0.124 | 0.105 | | | | | | | |
| | 0.158 | 0.133 | 0.132 | 0.11 | 0.1 | | | | | | | |
| Membrane 1:3 10 µl | 0.165 | 0.131 | 0.143 | 0.111 | 0.093 | | | | | | | |
| | 0.141 | 0.122 | 0.122 | 0.112 | 0.098 | | | | | | | |

Final protein concentrations

HEK – 3.286 µg/µl

$\alpha_{2A}$-$G_i$ - 2.351 µg/µl

$\alpha_{2A}$-$G_i\beta_3\gamma_5$ – 2.353 µg/µl

$\beta_3\gamma_5$ – 1.586 µg/µl

PCDNA3 – 1.051 µg/µl

## 3.5 Expression Levels of α2A adrenoceptor

This assay is used to determine the levels of expression of α2A adrenoceptor in the membrane preparations, assessed using $\alpha_2$-adrenoceptor selective antagonist [3H]RS-79948-97. Non-specific binding was determined in the presence of 100 µM idazoxan.

Radioligand binding assays are performed to determine accurately the expression levels of both receptor and G protein, as both signalling molecules are constrained within the same polypeptide and are therefore present in 1:1 molar ratio.

The membrane preparations were made up into a concentration of 0.1 µg/µL and 0.2 µg/µL in TE buffer buffer (10 mM Tris/HCl, 50 mM sucrose, 20 mM MgCl2, pH 7.5) so that assays could be done with both 5 µg and 10 µg of membrane. 500 µL of membrane solution were made up for each concentration.

| | 0.1 µg/µL | | | 0.2 µg/µL | |
|---|---|---|---|---|---|
| | Membrane conc | Membrane µl | TE µl | Membrane µl | TE µl |
| M1 – HEK | 3.286 µg/µl | 15.2 | 484.8 | 30.4 | 469.6 |
| M2 - $\alpha_{2A}$-$G_i$ | 2.351 µg/µl | 21.3 | 478.7 | 42.5 | 457.5 |
| M3 - $\alpha_{2A}G_i$ $\beta_3\gamma_5$ | 2.353 µg/µl | 21.3 | 478.7 | 42.5 | 457.5 |
| M4 - $\beta_3\gamma_5$ | 1.586 µg/µl | 31.5 | 468.5 | 63.1 | 436.9 |
| M5 - PCDNA3 | 1.051 µg/µl | 47.6 | 452.4 | 95.2 | 404.8 |
| RW | 1 | 45 | 450 | | |

**[3H]RS-7**

For each reaction 1.8 nM of [3H]RS-7 is needed. The stock [3H]RS-7 solution is diluted such that adding 20 µL of this to the 180 µL of the reaction mixture will result in a concentration of 1.8 nM of [3H]RS-7. For example the Kd of [3H]RS-7 is 0.3-0.4 nM. The stock [3H]RS-7 solution is 2.41 µM. Thus diluting the stock solution 133.9X will yield a solution of 18nM. When 20 µL of this is added to 180 µL of the reaction mixture, it will yield a final concentration of 1.8nM of [3H]RS-7. For this experiment 3.8 mls of 18nM [3H]RS-7 was made using 28.4 µL of the stock solution and 3771.6 µL of TEM buffer (75 mM Tris/HCl, 5 mM EDTA, 12.5 mM MgCl2, pH 7.4).

The reaction mixture was set up as follows with the membrane being added last and each done in triplicate.

| Tube No. | Membrane µL | [3H]RS-7 µL | Idazoxan µL | TEM µL | Membrane µg |
|---|---|---|---|---|---|
| 1-3 | 50 µL (0) | 20 µL | | 130 µL | 5 µg |
| 4-6 | 50 µL(0) | 20 µL | 20 µL | 110 µL | 5 µg |
| 7-12 | 50 µL(M1) | 20 µL | | 130 µL | 5 µg |
| 13-18 | 50 µL(M2) | 20 µL | 20 µL | 110 µL | 5 µg |
| 19-24 | 50 µL(M3) | 20 µL | | 130 µL | 5 µg |
| 25-30 | 50 µL(M4) | 20 µL | 20 µL | 110 µL | 5 µg |
| 31-36 | 50 µL(0) | 20 µL | | 130 µL | 10 µg |
| 37-42 | 50 µL(M1) | 20 µL | 20 µL | 110 µL | 10 µg |
| 43-48 | 50 µL(M2) | 20 µL | | 130 µL | 10 µg |
| 49-54 | 50 µL(M3) | 20 µL | 20 µL | 110 µL | 10 µg |
| 55-60 | 50 µL(M4) | 20 µL | | 130 µL | 10 µg |
| 61-66 | 50 µL(RW) | 20 µL | 20 µL | 110 µL | 5 µg |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Specific activity** | 184.2 DPM/fmol | | | | | | | | |
| **Reaction Vol** | 200 µL | | | | | | | | |
| | | | | | | | | | |
| | **µgm** | **-Ida** | **+Ida** | | | | | | |
| **Standard** | | 30059 | 30079.9 | 0.8159 nM | | | | | |
| | | 30117.2 | 29869.1 | | | | | | |
| | | 30165.4 | 30060.4 | | | | | | |
| | | | | **Average** | **SE** | **Net-average** | **Net-SE** | **fmol/mg** | **pmol/mg** |
| $\alpha_{2A}G_i$ | 5 | 1116.7 | 79.8 | 1136.29 | 19.59 | 1039.23 | 23.20 | 1128.37 | 1.12 |
| | | | 90.16 | 97.06 | 12.44 | | | | |
| | | 1155.88 | 121.22 | | | | | | |
| | | | | | | | | | |
| $\alpha_{2A}G_i$ $\beta_3\gamma_5$ | 5 | 1977.58 | 53.98 | 1932.07 | 72.12 | 1869.18 | 72.25 | 2029.51 | 2.02 |
| | | 1790.78 | 67.35 | 62.88 | 4.45 | | | | |
| | | 2027.85 | 67.32 | | | | | | |
| | | | | | | | | | |
| $\beta_3\gamma_5$ | 5 | | 89.99 | 83.12 | 0.01 | 9.22 | 7.50 | 10.01 | 0.01 |
| | | 83.14 | 106.36 | 92.35 | 7.50 | | | | |
| | | 83.11 | 80.7 | | | | | | |
| | | | | | | | | | |
| $\alpha_{2A}G_i$ $\beta_3$-s$\gamma_5$ | 5 | 2159.45 | 144.14 | 2188.07 | 59.94 | 2032.55 | 60.49 | 2206.90 | 2.20 |
| | | 2303.21 | 171.21 | 155.51 | 8.10 | | | | |
| | | 2101.55 | 151.19 | | | | | | |
| | | | | | | | | | |
| $\beta_3$-s$\gamma_5$ | 5 | 138.06 | 134.46 | 142.70 | 7.20 | 19.30 | 16.93 | 20.96 | 0.02 |
| | | 156.85 | 93.11 | 123.40 | 15.32 | | | | |
| | | 133.21 | 142.63 | | | | | | |
| | | 184.39 | 101.75 | | | | | | |

**Table 3.5-1 Calculation of levels of expression of α2A adrenoceptor. Ida - Idazoxan**

The tubes were incubated in a water bath at 30°C for 30 minutes. After this, the membranes were filtered out through Whatman GF/C filters using a Brandel vacuum filter with TE buffer. The membranes embedded in filter paper discs were then placed in scintillation tubes. One filter paper disc with membrane immersed in 5 mls of Scintillin in 66 Scintillation tubes. The tubes were vortexed and allowed to stand covered with cap and transferred to a scintillation counter. Radioactivity was determined by liquid scintillation counting. Specific binding is the difference between total and non-specific binding. The following table shows the calculation involved.

## 3.6 High affinity GTPase assays

These were performed essentially as described by Koski et al.[746] with Vmax estimates being obtained following transformation of enzyme velocity vs. substrate (GTP) concentration experiments as described.[747] Non-specific GTPase was assessed by parallel assays containing 100 μM GTP.

Determination of high affinity-GTPase activity is achieved by measuring the agonist-mediated breakdown of substrate γ[$^{32}$P]-GTP to GDP and [$^{32}$Pi], which is counted as an index of enzymatic activity. Although receptor stimulation of GTPase as a measurement of activity of $G_s\alpha$ has been reported, the degree of stimulation is modest. On the other hand, robust GTPase activity measurements mediated through pertussis toxin-sensitive G proteins are common, a reflection of their cellular abundance and higher intrinsic rate of GTP hydrolysis.

To decrease non-specific hydrolysis of GTP, ATP, creatine phosphate and creatine kinase are included in the assay as an ATP regenerating system to prevent the nucleoside diphosphokinase-mediated transfer of [$^{32}$P] to endogenously present ADP,

which can in turn be hydrolysed by specific ATPases. In addition App(NH)P (5'-adenylimidodiphosphate) is added to inhibit the nucleoside triphosphatases and ouabain in included as an inhibitor of $Na^+/K^+$ ATPase. Exogenous $Mg^{2+}$, an important cofactor in G protein activation is also added.

The experiments were carried out in HEK293 cells transfected with α2A Gi along with β3γ5 or β3-sγ5 and the high-affinity GTPase activity measured. High affinity GTPase activity was also measured over a range of concentrations of GTP in the absence and presence of epinephrine. By varying the concentration of the substrate (GTP), it is possible to measure Vmax and Km values for the Gα. In all experiments, cells were exposed to pertussis toxin for 24 hours to eliminate any potential interactions between the receptor element of the fusion protein and endogenously expressed Gi family G proteins.

The membrane preparations were diluted to 0.5μg/μL with TE buffer so that addition of 20μL of the membrane preparation would give 10μg of membrane protein in the reaction mixtures. Thus the membrane preparations were made up as follows

| | Membrane conc | Membrane μL | TE μL |
|---|---|---|---|
| M1 – HEK | 3.286 μg/μl | 33.5 | 186.5 |
| M2 - $\alpha_{2A}$-$G_i$ | 2.351 μg/μl | 46.8 | 173.2 |
| M3 - $\alpha_{2A}G_i\beta_3\gamma_5$ | 2.353 μg/μl | 46.8 | 173.2 |
| M4 - $\beta_3\gamma_5$ | 1.586 μg/μl | 69.4 | 150.6 |
| M5 - PCDNA3 | 1.051 μg/μl | 105 | 115 |
| RW | 1 | 110 | 110 |

[$^{32}$P]GTP is used as substrate and for each reaction mixture 50,000 counts per minute is required. This is calculated as follows

[$^{32}$P]GTP activity(activity) – (activity date-22/07/2000) – 2 μCi/μL

Date of experiment – 26/07/2000

Day difference – 4 days

t½ of [$^{32}$P] – 14.3 days

$$\text{Present activity} = \left( 0.5^{\frac{Days difference}{t^{\frac{1}{2}}}} \right) \times Activity$$

$$\text{Present activity} = \left( 0.5^{\frac{4}{14.3}} \right) \times 2$$

$$= 1.65\ \mu Ci/\mu L$$

To have 50000 counts per minute in each tube and make up a reaction mix for 100 tubes -

$$\text{Total } [^{32}P]GTP \text{ required} = \frac{100 \times 50000}{1.65 \times 1 \times 10^{6}} = 3\ \mu L$$

The reaction mixture is prepared as follows

| | |
|---|---|
| $2x10^{-5}$ Ascorbic acid | 500 μL |
| 2.5 U/ml Creatine Kinase | 200 μL |
| 0.4 M Creatine Phosphate | 250 μL |
| 0.04 M ATP pH | 250 μL |
| 0.04 M APP | 25 μL |
| 0.01 M Ouabain | 1000 μL |
| 4M NaCl | 250 μL |
| 1M $MgCl_2$ | 50 μL |
| 0.1M DTT | 200 μL |
| 0.02M EDTA pH 7.5 | 50 μL |
| 2M Tris HCl pH 7.5 | 200 μL |
| $10^{-4}$ M GTP pH 7.5 | 50 μL |
| [$^{32}$P]GTP | 3 μL |
| Water | 1972 μL |

50 μL of the reaction mixture is aliquoted in each well of a 96 well plate as follows with the membrane being added to each well last. During the whole procedure, the plate is kept on ice.

| Wells | Membrane | Epinephrine | GTP | Water | Reaction mix |
|---|---|---|---|---|---|
| 1-3 | 20 μL [M1] | - | - | 30 μL | 50 μL |
| 4-6 | 20 μL [M1] | - | 10 μL $10^{-3}$ M | 20 μL | 50 μL |
| 7-9 | 20 μL [M1] | 10 μL $10^{-3}$ M | - | 20 μL | 50 μL |
| 10-12 | 20 μL [M2] | - | - | 30 μL | 50 μL |
| 13-15 | 20 μL [M2] | - | 10 μL $10^{-3}$ M | 20 μL | 50 μL |
| 16-18 | 20 μL [M2] | 10 μL $10^{-3}$ M | - | 20 μL | 50 μL |
| 19-21 | 20 μL [M3] | - | - | 30 μL | 50 μL |
| 22-24 | 20 μL [M3] | - | 10 μL $10^{-3}$ M | 20 μL | 50 μL |
| 25-27 | 20 μL [M3] | 10 μL $10^{-3}$ M | - | 20 μL | 50 μL |
| 28-30 | 20 μL [M4] | - | - | 30 μL | 50 μL |
| 31-33 | 20 μL [M4] | - | 10 μL $10^{-3}$ M | 20 μL | 50 μL |
| 34-36 | 20 μL [M4] | 10 μL $10^{-3}$ M | - | 20 μL | 50 μL |
| 37-39 | 20 μL [M5] | - | - | 30 μL | 50 μL |
| 40-42 | 20 μL [M5] | - | 10 μL $10^{-3}$ M | 20 μL | 50 μL |
| 43-45 | 20 μL [M5] | 10 μL $10^{-3}$ M | - | 20 μL | 50 μL |
| 46-48 | 20 μL [RW] | - | - | 30 μL | 50 μL |
| 49-51 | 20 μL RW] | - | 10 μL $10^{-3}$ M | 20 μL | 50 μL |
| 52-54 | 20 μL [RW] | 10 μL $10^{-3}$ M | - | 20 μL | 50 μL |

The plate is covered with an adhesive trip and vortexed. This is centrifuged at 4°C to sediment the contents to the bottom of each well. The reaction in initiated by incubating the plate in a water bath at 37° C for 30 minutes. At the end of 30 minutes, the reaction is terminated by removal of the plate to ice and adding 900 μL of 5% w/v charcoal slurry into each well. 5% w/v activated charcoal in 20mM phosphoric acid is used to separate free [$^{32}$P] from the unhydrolysed [$^{32}$P]GTP. The plate is then centrifuged at 4000 rpm for 15 minutes at 4°C to pellet out the charcoal and the unhydrolysed [$^{32}$P]GTP. The free [$^{32}$P] is present in the supernatant. 300 μL of the supernatant from each tube is pipetted into a microtube and closed with a stopper.

Each tube is placed into a scintillation tube and free [$^{32}$P] is measured by scintillation counting from which the rate of hydrolysis of GTP is calculated. Low affinity hydrolysis of [$^{32}$P]GTP is assessed by incubating parallel tubes in the presence of 100 μM GTP. The basal and stimulated GTPase activity are calculated as follows.

$$\text{GTPase} = \frac{Basal\_hGTP}{\frac{CPM}{pmol}} \times \frac{1000}{\mu g\_membrane} \times \frac{1}{30} \times \frac{1000}{Vol\_Scin\_tube} = \text{pmol/mg/min}$$

$$\text{GTPase} = \frac{Stim\_hGTP}{\frac{CPM}{pmol}} \times \frac{1000}{\mu g\_membrane} \times \frac{1}{30} \times \frac{1000}{Vol\_Scin\_tube} = \text{pmol/mg/min}$$

## 3.7 Single cell (EF88) calcium signalling

EF88 is a fibroblast cell line derived from the embryos of mice in which expression of the α subunits of both $G_q$ and $G_{11}$ had been eliminated by targeted gene disruption.[748-750] The transfection procedure is similar to that described above in section 3.4(page 217). In summary, the cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated foetal bovine serum and L-glutamine (1 mM) in a 95% air and 5% $CO_2$ atmosphere at 37 °C. A portion of the cells harvested during trypsinisation was plated on to glass coverslips, and after a 24-h growth period they were transfected using Lipofectamine (Invitrogen) according to the manufacturers' instructions. After 3 h cells were washed twice with Opti-MEM I and then cultured in DMEM growth medium for a further 24 h, pertussis toxin being added 6 hours into this period as described in section 3.4(page 217). A total of 3 μg of pCDNA3 containing the relevant cDNA species were used to transfect each coverslip.

### *3.7.1 $[Ca^{2+}]_i$ Imaging*

Transfected cells were loaded with the $Ca^{2+}$-sensitive dye Fura-2 by incubation (15-20 min, 37 °C) under reduced light in DMEM growth medium containing the dye's

membrane-permeant acetoxymethyl ester form (1.5 μM). Loaded cells were illuminated with an ultra high point intensity 75-watt xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK) and subsequently imaged using a Nikon Diaphot inverted microscope equipped with a Nikon 40× oil immersion Fluor objective lens (NA = 1.3) and a monochromator (Optoscan, Cairn Research), which was used to alternate the excitation wavelength between 340/380 nm and to control the excitation band pass (340 nm band pass = 10 nm; 380 nm band pass = 8 nm). Fura-2 fluorescence emission at 510 nm was monitored using a high resolution interline-transfer cooled digital CCD camera (Cool Snap-HQ, Roper Scientific/Photometrics, Tucson, AZ).[751] MetaFluor imaging software (version 4.6.8, Universal Imaging Corp., Downing, PA) was used for control of the monochromator, CCD camera, and for processing of the cell image data. Sequential images (2 × 2 binning) were collected every 2 s, exposure to excitation light was 100 ms/image, and all experiments were undertaken in the absence of extracellular $Ca^{2+}$ in saline solution comprising: 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 10 mM D-glucose, 0.01 mM EGTA, pH adjusted to 7.4 using NaOH.

### *3.7.2 $[Ca^{2+}]_i$ Image Analysis*

Ratio images were presented in MetaFluor intensity-modulated display mode,[752] which associates the colour hue with the excitation ratio value and the intensity of each hue with the source image brightness. Briefly background subtracted images acquired at 340 and 380 nm excitation were first used for calculating the 340/380 nm ratio of each pixel. After determination of the upper and lower thresholds, the ratio value of each pixel was associated with one of the 24 hues from blue (low $[Ca^{2+}]_i$) to red (high $[Ca^{2+}]_i$). Pooled average intensity-modulated display ratio intensity values

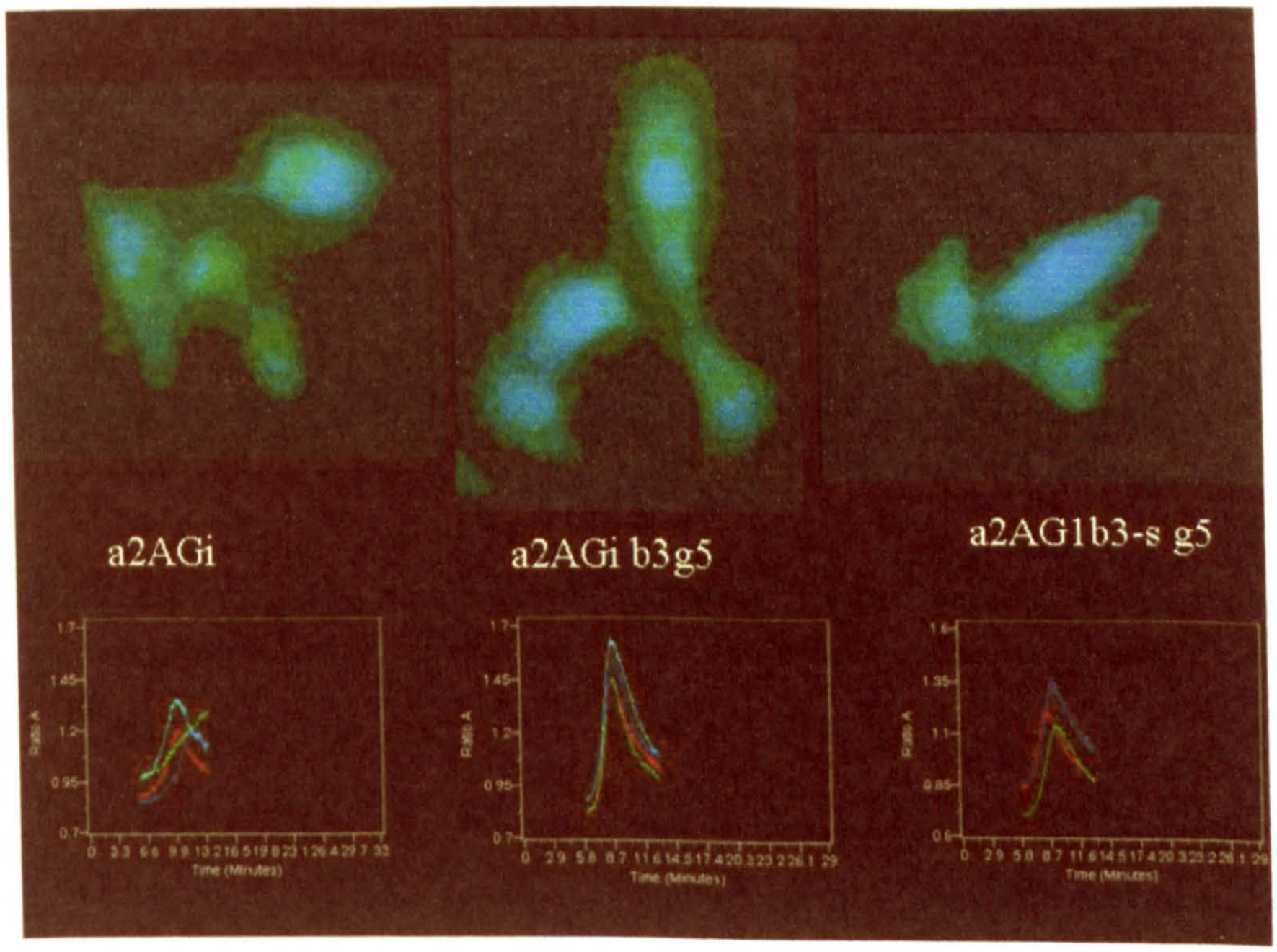


**Figure 3.7-1 Calcium signals in cells transfected with $\alpha_{2A}G_i$, $\alpha_{2A}G_i$ $\beta3\gamma5$ and $\alpha_{2A}G_i$ $\beta3$-s$\gamma5$ showing the difference in calcium signal in cells coexpressing the $\beta\gamma$ subunit. The top panel shows the transfected EF88 cells after stimulation with epinephrine. The bottom panel show the rate of change of Fura-2 fluorescence after stimulation with epinephrine for a single cell selected per experiment.**

# Chapter 4 - GNB3 AND HYPERTENSION – A Case Control Study

## 4.1 Introduction

After the initial report by Siffert et al,[579] there have been numerous association studies in different populations trying to replicate their results. However the association is not clear as summarised in Table 1.6-2. Inconsistency among populations has been noted for a number of other proposed candidate genes for hypertension and the reasons for varying results in case-control studies are detailed in *Section 1.2.2.2* (page 77). It is important, therefore, to identify within specific groups the local relevance of particular genetic markers. I performed a case-control association study for the GNB3 locus in a Scottish group where all the participants were recruited at the same institute with relatively clear classification criteria. The case-control method is a powerful epidemiological method in the study of genetics in complex disease. Cases are defined as those affected with a particular disease of interest. The controls are those who although are at risk of developing the disease are unaffected with the disease. To achieve valid case-control comparison, controls should be selected so that they represent the same source population as the cases. The case and control subjects are then compared with respect to the proportion having the exposure or characteristic of interest. In the candidate gene approach, the frequency of the disease susceptibility gene in the cases is compared with the frequency in a group of controls. As with all case-control studies, selection of an appropriate control group is important to avoid spurious associations that can result from confounding due to recent admixture and selection or drift between unlinked loci.[753]

## 4.2 Recruitment of subjects

### *4.2.1 Cases*

Approval for this study was obtained from the appropriate hospital and community medicine ethics committees, and all subjects gave informed consent. Caucasian

patients with essential hypertension (n=100) were recruited from the Blood Pressure Clinic of the Western Infirmary, Glasgow. All were less than 64 years of age. Secondary hypertension was excluded by physical examination and biochemical and radiological investigations where appropriate. All subjects had a positive family history of hypertension; high blood pressure was diagnosed before the age of 60 years. Subjects with a history of alcohol excess (>21 U per week) and obesity (body mass index [BMI] >33 kg/m2) were excluded. Blood pressure was measured in the clinic by a trained observer with a mercury sphygmomanometer. The diagnosis of hypertension was based on a minimum of 3 blood pressure readings of >160/90 mm Hg before initiation of treatment, although most patients were receiving treatment at the time of the study.

### *4.2.2 Controls*

Control subjects were drawn from the North Glasgow coronary risk survey, which had 200 randomly selected members of the North Glasgow population in each 10-year age/gender band from 25 to 64 years who were part of the World Health Organization Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) project and their inclusion in this study was approved by the West ethics committee. They were normotensive (<140/90 mm Hg), and none were receiving antihypertensive therapy, treatment for heart disease, or hormone replacement therapy. They were individually age- and gender-matched with the cases by random selection from all control subjects who matched the criteria of the cases. Blood pressure was measured on 2 occasions with the Hawksley random-zero sphygmomanometer, with results averaged.

## 4.3 Laboratory methods

The DNA samples from the cases and controls were genotyped for the GNB3 polymorphism as described in *Section 3.2 Genotyping C825T G-protein β3 subunit polymorphism* (page 208).

## 4.4 Statistical methods

Comparisons between cases and control subjects of demographic variables and genotype frequencies were carried out by paired t test and McNemar's test, respectively. Hardy-Weinberg equilibrium was checked by a $\chi^2$ test with 1degree of freedom. Genotype and allele frequencies were compared between groups by a $\chi^2$ test. The details of case-control analysis are summarised in *Section 1.2.2.2.1Case–control studies* (page 79).

When there is no association between genotype and disease, the genotype frequencies will be similar in cases and controls. Although a general test to detect any difference in genotype frequencies can be derived, a trend test that is sensitive to monotonic differences may be the most powerful for genotypic data. The Armitage test for trend has two advantages over other statistical tests of association. First, the test does not require that the genotype frequencies comply with Hardy-Weinberg proportions. Sasieni[754] demonstrated that statistical tests based on the comparison of allele frequencies—rather than genotype frequencies—between unrelated cases and controls can have an increased rate of false-positive conclusions when genotype frequencies do not fit Hardy-Weinberg proportions. Second, the Armitage test for trend provides a flexible analysis method, because different scores can be used to test the dosage of the high-risk allele, depending on whether there is prior knowledge of the disease.

## 4.5 Results

The clinical characteristics of the study group are given in Table 4.5-1. It shows that the age and gender matching of the groups was accurate. The patient blood pressures were those at the time of the study when many were receiving treatment. Despite this, blood pressure in the patient group was significantly higher than in the control group ($P<0.00005$).

The distributions of genotypes and alleles for the two polymorphisms in the case and control populations are shown in Table 4.5-2. The frequencies of the GNB3 C and T alleles were 69% and 31% respectively among controls and 75% and 25% respectively among cases. It can be seen that the frequencies of the GNB3 T allele was not significantly different between the two groups ($p=0.404$).

| | Males (n=46) | | Females (n=54) | |
|---|---|---|---|---|
| | Cases | Controls | Cases | Controls |
| Age (years) | 47.8(11.1) | 47.9(11.1) | 49.5(11.3) | 49.2(11.1) |
| BMI ($Kg/m^2$) | 27.8(2.9) | 25.8(3.5) | 28.1(3.7) | 25.4(3.2) |
| Systolic (mmHg) | 167.8(20.8) [1] | 129.2(13.5) | 170.4(24.9) [1] | 120.6(15.4) |
| Diastolic (mmHg) | 98.4(11.2) | 80.5(8.7) | 101.3(11.8) | 74.4(8.3) |
| Cholesterol (mmol/L) | 6.2(0.4393) | 5.8(1.1) | 6.2(1.1) | 6.1(1.2) |

**Table 4.5-1 Demographic Data**

**Clinical details of the subjects studies. Study included 100 cases and 100 control subjects. Data are mean(SD).**

**[1] P<0.00005 (paired t test, comparing cases with control subjects).**

| | Cases | Controls | McNemar test OR(CI) |
|---|---|---|---|
| Number | 100 | 100 | P= 0.504<br>0.81 (0.46-1.41) |
| CC | 58 | 49 | |
| CT | 34 | 41 | |
| TT | 8 | 10 | |
| | | | |
| FREQ(T) | 0.25 | 0.31 | |
| p | 0.404 | | |

**Table 4.5-2 - Distributions of Genotypes and Alleles for GNB3 C825T Polymorphisms in Case and Control Populations**

**Confidence interval is for difference in proportions between cases and control subjects discordant for T allele expressed as percentage.**

The control and the case groups were in Hardy-Weinberg equilibrium for the polymorphism (cases, P=0.42; control subjects, P=0.81)(Table 4.5-3). A convenient way to view Hardy-Weinberg proportions is the De Finetti diagram. The three sides are the relative frequencies of the three genotypes CC, CT and TT (Figure 4.5-1). Any point within the triangle can be considered a population. If the triangle is drawn with an arbitrarily chosen altitude of 1.0 (since the allele frequencies together will equal 1.0, or 100% of all alleles), the three perpendiculars drawn from any population give the proportions of the three genotypes. Populations in Hardy-Weinberg equilibrium trace a parabola extending from base to base of the triangle. In Figure 4.5-1 both the cases and controls are shown to be in Hardy Weinberg Equilibrium.

| | Controls | Cases |
|---|---|---|
| CC (%) | 49 (48.3) | 58 (56.2) |
| CT (%) | 41 (42.4) | 34 (37.5) |
| TT (%) | 10 (9.30) | 8 (6.3) |
| freq C | 0.69 +/-0.03 | 0.75 +/-0.03 |
| F | 0.033 | 0.093 |
| p (Pearson) | 0.74 | 0.35 |
| p (Exact) | 0.81 | 0.42 |

**Table 4.5-3 Tests for deviation from Hardy-Weinberg equilibrium among cases and controls**

Table 4.5-4 shows the results of the association test carried out using the Armitage test for trend. Whether T or the C allele is taken as the high risk allele, there is no significant association noted in the presence of one or more high risk alleles.

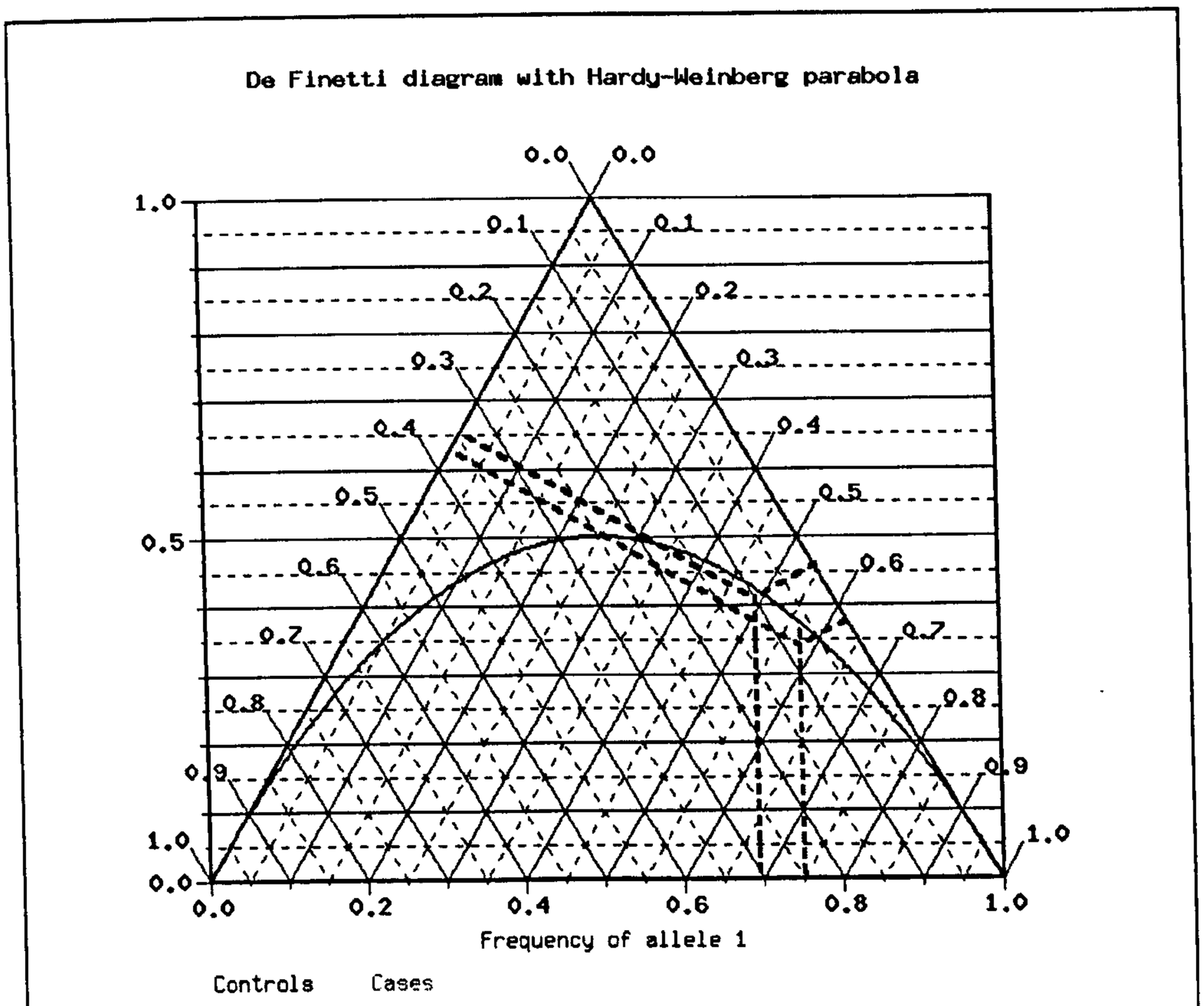


**Figure 4.5-1 De Finetti diagram showing the controls and cases in Hardy Weinberg Equilibrium.**

| Tests for association | | | | | |
|---|---|---|---|---|---|
| | Allele freq. difference | Heterozygous | Homozygous | Allele positivity | Armitage's trend test |
| **Risk allele T** | | | | | |
| | [C] ⇔ [T] | [CC] ⇔ [CT] | [CC+CT]⇔[TT] | [CC]⇔[CT+TT] | common odds ratio |
| Odds Ratio (95%C.I.) | 0.76 (0.49-1.18) | 0.70 (0.39-1.27) | 0.68 (0.25-1.85) | 0.69 (0.39-1.22) | 0.79 |
| $\chi^2$ | 1.51 | 1.09 | 0.59 | 1.63 | 1.42 |
| p | 0.22 | 0.24 | 0.44 | 0.20 | 0.23 |
| **Risk allele C** | | | | | |
| | [T] ⇔ [C] | [TT] ⇔ [CT] | [TT+CT]⇔[CC] | [CC+CT]⇔[TT] | common odds ratio |
| Odds Ratio (95%C.I.) | 1.32 (0.85-2.04) | 1.04 (0.37-2.92) | 1.48 (0.54-4.04) | 1.28 (0.48-3.38) | 1.26 |
| $\chi^2$ | 1.51 | 0.0 | 1.59 | 0.24 | 1.42 |
| p | 0.22 | 0.95 | 0.44 | 0.62 | 0.23 |

**Table 4.5-4 Test for association using the Armitage test for trend in case control population**

| Ethnic Group | Country | Phenotype | Freq(T) Control | Freq(T) Case | No Controls | No Cases | Reference |
|---|---|---|---|---|---|---|---|
| Afro-Caribbean | USA | Essential Hypertension | 0.75 | 0.74 | 432 | 472 | Larson, N. et al.[695] |
| Afro-Caribbean | England | Essential Hypertension | 0.77 | 0.82 | 243 | 185 | Dong, Y. et al.[694] |
| Afro-Caribbean | South Africa | Obesity | 0.83 | 0.9 | | 275 | Siffert, W. et al.[706] |
| Caucasian | Germany | Diastolic Blood Pressure | | 0.33 | | 474 | Schunkert, H. et al.[690] |
| Caucasian | Germany | Essential Hypertension | 0.28 | 0.34 | 1000 | 479 | Beige, J. et al.[686] |
| Caucasian | France | Essential Hypertension | 0.313 | 0.353 | 308 | 394 | Brand, E. et al.[691] |
| Caucasian | Northern Ireland | Essential Hypertension | 0.298 | 0.307 | 163 | 171 | Brand, E. et al.[691] |
| Caucasian | France | Essential Hypertension | 0.306 | 0.33 | 470 | 393 | Brand, E. et al.[691] |
| Caucasian | Germany | Essential Hypertension | 0.25 | 0.31 | 427 | 426 | Siffert, W. et al.[579] |
| Caucasian | Spain | LVH | 0.2 | 0.4 | 26 | 60 | Poch, E. et al.[581] |
| Caucasian | Germany | Obesity | 0.29 | 0.47 | | 277 | Siffert, W. et al.[706] |
| Caucasian | Germany | Renal Transplant | | 0.32 | | 320 | Beige, J. et al.[755] |
| Chinese | China | Obesity | 0.46 | 0.58 | | 960 | Siffert, W. et al.[706] |
| Inuit | Canada | Obesity | | 0.504 | | 213 | Hegele, R. A. et al.[707] |
| Japanese | Japan | Essential Hypertension | 0.496 | 0.492 | 515 | 718 | Kato, N. et al.[697] |
| Oji-Cree | Canada | Systolic Blood Pressure | | 0.501 | | 447 | Hegele, R. A. et al.[703] |
| Caucasian | Scotland | Essential Hypertension | 0.31 | 0.25 | 100 | 100 | |

**Table 4.5-5 Frequencies of T allele in various studies**

## 4.6 Discussion

In the present study, we found no evidence of a significant difference in the frequency of the GNB3 C825T polymorphism between our Scottish hypertensive and normotensive populations. There was no significant difference between the T allele frequency between hypertensives (0.31) and controls (0.25). The odds ratio for hypertension in the presence of the T allele (CT+TT versus CC) was 0.69 (95% C.I., 0.39 - 1.21; p=0.20). Siffert et al[579] demonstrated a significant association of the 825T allele with essential hypertension in a study of 426 hypertensive and 427 normotensive control subjects; the odds ratio for hypertension in the presence of the 825T allele (CT + TT versus CC) was 1.44 (95% C.I., 1.09 -1.88; p=0.025). The CI in the present study and that of Siffert et al[579] partially overlap. If the results of these two studies potentially represent the same value of effect measure, the sample size required to confirm the susceptibility in question must be very large. For example, it is roughly estimated that >1000 individuals are required in each group of cases and controls for detecting an odds ratio ~1.2 with 80% power at a 5% type I error probability.[756] Thus no conclusive claims can be made from this study as the statistical power is insufficient based on this assumption.

The lack of association in this study does not appear to be due to ethnic variation. The numbers in other case control studies looking at hypertension are larger. The allele frequencies between cases and controls in this study are comparable with other European studies. There has been variable association between the 825T allele and hypertension both positive[686-690] and nonsignificant[691-693] studies in Caucasian populations where 825T gene frequency is about 25%. Increased risk of hypertension

associated with 825T was also reported among blacks[694] who have a much higher gene frequency approaching 80%. A negative study was reported in African Americans.[695] Less association has been reported in Asian populations, which have intermediate gene frequencies of about 50%,[696-698] although one Japanese study was weakly positive.[699] Although case-control studies can be criticized because of the risk of false-positive findings, particularly where populations are not homogeneous, we were careful to ensure that the matching of cases and control subjects was exact. Furthermore, all subjects were drawn from an ethnically and geographically limited catchment area.

It is also possible that a true mutation as yet unidentified may be in linkage disequilibrium with the GNB3 T allele. Use of genetic linkage analysis, haplotype analysis or intermediate phenotypes would allow for further investigation of this candidate gene in relation to hypertension.

# Chapter 5 - GNB3 AND CARDIAC MASS – Analysis of four candidate genes in a twin study

## 5.1 Introduction

Left ventricular hypertrophy detected on echocardiography is a powerful and independent predictor of cardiovascular complications and death in subjects with uncomplicated essential hypertension. LV mass shows a continuous distribution in the general population,[48] and LV hypertrophy is an operational category that defines the upper end of the LV mass distribution.[471;472] Further details of ventricular mass and risk is detailed in *Section 1.4.6 LV Mass and Risk* (page 153). *Section 1.4.9 Genetics of LV Mass* (page 161) summarises the studies done to elucidate the genetic factors involved in LV hypertrophy. Previous reports have suggested an association between left ventricular indices and ACE, GNB3, aldosterone synthase genes,[495-498;574;580] As shown in Table 1.4-5 most of the candidate genes studied have shown inconsistent results.

In this study I have used the twin model to look at the heritability of LV mass and determine if any component of the heritability could be explained by variations of the GNB3 gene. In addition I have also studied polymorphisms in the ACE and Aldosterone synthase gene, which have previously shown inconsistent associations with LV hypertrophy.

### *5.1.1 Twin study*

The twin approach relies on the fact that monozygotic (MZ) twins are genetically identical whereas dizygotic (DZ) twins share, on average, one-half of their genes. If both twins of a pair are affected, they are said to be concordant. By estimating and comparing concordance rates between MZ and DZ twins, one can get an idea of the role of genetic factors in the aetiology of the disease. If the concordance rate is 100% in MZ twins and between 25%-50% in DZ twins, it may be concluded that the disease

is strictly genetic and probably due to a single recessive (if the concordance rate is ~25%) or dominant (concordance ~50%) gene. For a disorder in which genetic factors are important in aetiology but not the sole factor, the concordance rate in MZ twins will still be greater than for DZ twins, and the concordance rate for MZ twins reared apart will be about the same as for MZ twins reared together. Low concordance in MZ twins or equal concordance between MZ and DZ twins suggests a strong environmental influence on the development of disease. Since most disease have variability in the age of onset, unless the genes involved tightly control the age of onset, twin pairs may become concordant only over time. Thus follow-up of twins is important.

## 5.2 Objective

To determine if any of the echocardiographic measurements of left ventricular mass, left ventricular systolic and diastolic function have a heritable component in a study of 146 twins.

To determine in any of those parameters showing heritability, if polymorphisms of four candidate genes - G protein beta3 subunit, beta-1 adrenoceptor, angiotensin converting enzyme and aldosterone synthase play a role.

## 5.3 Study design

### *5.3.1 Study population*

The population studied were a group of monozygotic and dizygotic twins aged between 30 and 85 years of age (146 adult twins - 69 monozygous and 77 dizygous pairs). Twins were recruited from the general population via the media. To prevent self-selection bias, the purpose of the study was not specified in the advertisement.

The West Ethics Review Committee approved the study. Each twin pair attended the same study morning and completed a health screening and lifestyle questionnaire.

### 5.3.2 Confirmation of zygosity

Zygosity was determined by validated questionnaire and verified by analysing tandem repeat polymorphisms. Four markers were used – AFM238xd10, AFM288vb9, AFM273yfl and AFM199zb6 (Centre d'Etudes du Polymorphisme Humain, Paris, France). Monozygotic twins were homozygous for each marker where dizygotic pairs were heterozygous for at least one marker. The probability of inaccurate assessment of zygosity using this technique was less than $10^{-5}$.

### 5.3.3 Genetic analysis

Genomic DNA was isolated by phenol-chloroform extraction of EDTA-blood samples as described in *Section 3.1Genomic DNA Extraction:* (page 206). The subjects were genotyped for the Insertion/Deletion ACE gene, C825T G-protein β3 , SF1 binding site , intron 2 gene conversion and G1165C beta-1 adrenoceptor polymorphisms by polymerase chain reaction. Genotyping was performed by PCR by the method previously described for C825T G-protein beta-3 subunit polymorphism,[579] (*Section 3.2* page 208) ACE I/D polymorphism[757], SF1 RFLP[498] and Beta-1 adrenoceptor polymorphism. [582] Dr G Inglis performed the genotyping of the ACE and Aldosterone synthase polymorphisms.

### 5.3.4 Echocardiography

The measurement of echocardiographic LV mass is described in section 1.4.3 (page 144). Standard two-dimensional echocardiography was carried out on all the twin subjects by Dr L Swan. Trans-thoracic echocardiography was performed and analysed in line with the American Society of Echocardiography guidelines.[499] Measurements were made over a least 3 cardiac cycles by a single observer blinded to the twins other

results and zygosity determination. Left ventricular mass was calculated using the Penn convention.[500] With the use of these measurements, left ventricular mass was determined by the following equation:

**LV mass (in grams) = 1.04[(LVIDD + VST + PWT)3 - (LVIDD)3] - 13.6**

where LVIDD is end-diastolic left ventricular internal diameter, VST is ventricular septal thickness, and PWT is posterior-wall thickness. Left ventricular mass was used as an unadjusted variable and was also adjusted for height and body surface area (the mass in grams divided by the height in meters).

Other left ventricular dimensions measured were LV end diastolic and endsystolic diameter, posterior wall thickness, interventricular septal thickness and fractional shortening. LV diastolic function was determined by deceleration time, peak E, peak A and E/A ratio on doppler.[758]

### *5.3.5 Anthropometry and BP*

Resting blood pressures and anthropometric parameters were measured at baseline. Ambulatory blood pressure monitoring was performed in a non-hospital environment using a Spacelabs monitor fitted to the non-dominant arm. Tracings were disregarded if less than 85% of the readings were unacceptable.

## 5.4 Statistical analysis

Descriptive data for the cohort are quoted as mean values and standard deviations.

Differences between twin groups were compared using a paired t-test.

Twin analysis was performed using the methodology described by Falconer.[759] In this method the genetic effect is assessed by differences in within-pair variances between monozygotic and dizygotic twins. Intra-class correlation coefficients were calculated for the twin pairs using a statistics software programme (MINITAB 11.2). This test included a test of homogeneity. Heritability, an estimate of the degree to which a variable is genetically determined was calculated for the major phenotypes of left ventricular structure. For each variable in the MZ and DZ groups, intraclass correlations (r) were then compared between the two groups by Fisher's z test. For the variables where r was significantly different in the MZ group than in the DZ group, $H^2$ was calculated ($H^2$ = VA + VD, where VA is the additive variance and VD the dominance variance) by the method of Haseman and Elston. This was calculated using the equation $h^2 = 2(rMZ-rDZ)$ where rMZ is the correlation coefficient for the monozygotic group and rDZ that of the dizygotic group. A p value of less than 0.05 was taken to be statistically significant. For traits with 100% genetic determination, the $H^2$ values would be 1. All those parameters showing significant genetic determination were tested for within pair difference in MZ, DZ concordant and DZ discordant for the four polymorphisms.

## 5.5 Results

### *5.5.1 Demographic data*

The demographic data are shown in Table 5.5-1. The age range of the twins was 31-83 years. The MZ group was slightly older and contained a greater proportion of women. There was no significant difference in BMI, systolic and diastolic blood pressure between MZ and DZ groups.

| | MZ | DZ | p |
|---|---|---|---|
| | | | |
| Zygosity | 69(47.3%) | 77(52.7%) | |
| Sex (M:F) | 16:122 | 47:107 | <0.01 |
| Age | 58.17 ± 0.9 | 53.47 ± 1.06 | <0.01 |
| Pulse | 74.25 ± 0.80 | 74.20 ± 0.82 | 0.96 |
| Weight | 62.25 ± 1.4 | 67.58 ± 1.3 | 0.22 |
| Height | 161.1 ± 0.66 | 165.1 ± 0.79 | <0.01 |
| BMI | 25.05 ± 0.45 | 24.54 ± 0.34 | 0.36 |
| Systolic BP | 132.1 ± 1.4 | 129.8 ± 1.2 | 0.20 |
| Diastolic BP | 81.9 ± 0.81 | 80.6 ± 0.74 | 0.21 |

**Table 5.5-1 Demographic data of twin pairs (Mean ± SD)**

MZ – Monozygous twins; DZ- Dizygous twins

## *5.5.2 LV mass and function*

Table 5.5-2 and Table 5.5-3 show the indices of LV mass and systolic and diastolic function in MZ and DZ twins. There is clearly no significant difference between MZ and DZ groups with regard to any of the indices of LV mass and function.

| | MZ | DZ | p |
|---|---|---|---|
| LV Mass | 176.6 ± 5.83 | 174.6 ± 4.24 | 0.78 |
| LV Mass Index | 104.4 ± 3.39 | 100.6 ± 2.69 | 0.38 |
| IVSd | 1.08 ± 0.02 | 1.05 ± 0.02 | 0.44 |
| IVSs | 1.41 ± 0.02 | 1.40 ± 0.02 | 0.85 |
| LVDd | 4.6 ± 0.05 | 4.75 ± 0.04 | 0.15 |
| LVDs | 2.9 ± 0.05 | 2.97 ± 0.04 | 0.12 |
| PWd | 0.81 ± 0.01 | 0.81 ± 0.01 | 0.71 |
| PWs | 1.24 ± 0.02 | 1.24 ± 0.02 | 0.90 |
| IVS/PW | 1.35 ± 0.03 | 1.31 ± 0.02 | 0.22 |
| Frac. Shortening | 38.55 ± 0.71 | 37.45 ± 0.58 | 0.23 |

**Table 5.5-2 LV mass and systolic function in twin pairs (Mean ± SD)**

MZ – Monozygous twins; DZ- Dizygous twins IVSd – Interventricular septal thickness (Diastole), IVSs- Interventricular septal thickness (Systole), LVDd – LV end-diastolic diameter, LVDs- LV end-systolic diameter, PWd – Posterior wall thickness (Diastole), PWs – Posterior wall thickness (Systole)

| | MZ | DZ | p |
|---|---|---|---|
| Decel Time (ms) | 241.4 ± 3.86 | 242.9 ± 3.14 | 0.75 |
| Peak E (m/sec) | 0.74 ± 0.01 | 0.76 ± 0.01 | 0.18 |
| Peak A (m/sec) | 0.67 ± 0.01 | 0.68 ± 0.01 | 0.64 |
| E/A | 1.12 ± 0.02 | 1.14 ± 0.02 | 0.48 |

**Table 5.5-3 LV Diastolic function in twin pairs (Mean ± SD)**

MZ – Monozygous twins; DZ- Dizygous twins, Decel Time - Mitral Deceleration time, Peak E – Peak mitral E velocity, Peak A – Peak mitral A velocity

### *5.5.3 Heritability*

The intra-class correlation coefficients for the monozygotic and dizygotic groups and their comparison (z) are displayed in Table 5.5-4 and Table 5.5-5. Left ventricular internal dimensions in diastole and left ventricular mass were both under a significant degree of genetic determination (heritability estimates of 0.68 and 0.66 respectively). Two thirds of the variation in LV mass could be explained by a genetic influence. LV mass showed a significant heritability of 0.66 while LV mass indexed to height showed a heritability of 0.99.

Following correction for age, sex, blood pressure and weight (LVMI) there was still a strong genetic component to left ventricular mass (heritability estimate of 0.53). Although there was a trend towards a genetic contribution to diastolic wall posterior wall thickness this did not reach significance.

There was no evidence of genetic determination for the indices of LV systolic and diastolic function namely Fractional shortening, Deceleration time, E/A ratio (Table 5.5-4 and Table 5.5-5).

| Measurement | n-MZ | r-MZ | Z-MZ | n-DZ | r-DZ | Z-DZ | Z | $H^2$ | p |
|---|---|---|---|---|---|---|---|---|---|
| LVMass | 46 | 0.66 | 0.79 | 43 | 0.17 | 0.17 | 2.84 | 0.66 | 0.002 |
| LVMI | 39 | 0.58 | 0.67 | 37 | 0.09 | 0.09 | 2.46 | 0.99 | 0.007 |
| IVSd | 45 | 0.32 | 0.32 | 43 | 0.11 | 0.11 | 0.99 | 0.31 | 0.160 |
| IVSs | 45 | 0.31 | 0.31 | 43 | 0.34 | 0.36 | -0.22 | -0.08 | 0.588 |
| LVDd | 45 | 0.71 | 0.88 | 43 | 0.36 | 0.38 | 2.29 | 0.68 | 0.011 |
| LVDs | 44 | 0.59 | 0.67 | 43 | 0.45 | 0.48 | 0.83 | 0.26 | 0.202 |
| PWd | 45 | 0.42 | 0.44 | 43 | 0.09 | 0.09 | 1.61 | 0.65 | 0.053 |
| PWs | 44 | 0.46 | 0.50 | 43 | 0.34 | 0.36 | 0.62 | 0.22 | 0.267 |
| IVS/PW | 45 | 0.16 | 0.16 | 43 | 0.05 | 0.05 | 0.51 | 0.22 | 0.305 |
| FracS | 44 | 0.16 | 0.16 | 43 | 0.27 | 0.27 | -0.50 | -0.21 | 0.693 |

**Table 5.5-4 LV mass and systolic function - Intraclass correlation coefficient for MZ(r-MZ) and DZ(r-DZ), Z level, broad heritability estimates(H-sq) and p values**

MZ- Monozygous; DZ- Dizygous, r- Intraclass correlation, $H^2$ – Broad sense heritability, IVSd – Interventricular septal thickness (Diastole), IVSs- Interventricular septal thickness (Systole), LVDd – LV end-diastolic diameter, LVDs- LV end-systolic diameter, PWd – Posterior wall thickness (Diastole), PWs – Posterior wall thickness (Systole), FracS- Fractional Shortening

| Measurement | n-MZ | r-MZ | Z-MZ | n-DZ | r-DZ | Z-DZ | Z | H-sq | P |
|---|---|---|---|---|---|---|---|---|---|
| Decel Time | 55 | 0.04 | 0.04 | 64 | 0.03 | 0.03 | 0.089 | 0.03 | 0.46 |
| Peak E | 56 | -0.004 | -0.004 | 65 | 0.11 | 0.11 | -0.64 | -0.23 | 0.74 |
| Peak A | 56 | 0.37 | 0.39 | 65 | 0.23 | 0.23 | 0.86 | 0.29 | 0.19 |
| E/A | 56 | 0.36 | 0.38 | 65 | 0.48 | 0.53 | -0.77 | -0.23 | 0.78 |

**Table 5.5-5 LV Diastolic function - Intraclass correlation coefficient for MZ(r-MZ) and DZ(r-DZ), Z level, broad heritability estimates(H-sq) and p values**

MZ- Monozygous; DZ- Dizygous, r- Intraclass correlation, $H^2$ – Broad sense heritability, Decel Time - Mitral Deceleration time, Peak E – Peak mitral E velocity, Peak A – Peak mitral A velocity

### *5.5.4 Association Study*

As LV mass and LV diastolic diameter showed evidence of genetic determination in the twin study. The next step is to determine if variation in any of the four candidate genes selected plays a role in the genetic determination. This was tested using two approaches.

Firstly one member of each twin pair was selected. Selecting either twin 1 or twin 2 did not influence the results. This was a simple association study using one-way ANOVA to check for an association between genotype and phenotype. Table 5.5-6 shows the genotype frequency of the four genes studies – ACE, GNB3, B1AR and Aldosterone synthase polymorphism. There is no significant association between any polymorphism and LV mass. After correcting for age, sex, blood pressure and weight, by regression and testing the residuals also showed no significant association. Figure 5.5-1 shows LV mass index plotted by genotype for the four genes studies. The results show no association. Though there is a trend to lower LVM index with the G allele of the B1AR polymorphism, it did not reach statistical significance.

Secondly the intrapair differences were compared between MZ concordant (MZ) and concordant and discordant DZ (DZc and DZd) twins for each of the genes studied (ACE, GNB3, B1AR, SF1) separately. Were a second gene locus exerting an effect,

the within pair difference of concordant DZ twins would be expected to lie between those of MZ and discordant DZ twins.

No significant within-pair difference is seen between MZ and DZ concordant twins for each of the genes studied with respect to LV mass, LVDd and PWd (Table 5.5-7). Also no significant within-pair difference is observed between DZ concordant and DZ discordant twins for each of these three echocardiographic indices. With respect to the B1AR gene, the within-pair difference is small in MZ twins and similarly great in DZ concordant and discordant twins, though there is an indication of a trend towards greater within-pair difference in DZ discordant twins.

| | | | | | |
|---|---|---|---|---|---|
| **ACE** | | **II** | **ID** | **DD** | **p** |
| | Genotype frequency | 0.18 | 0.47 | 0.35 | |
| | LV mass | 174.9(53.4) | 178.1(71.3) | 182.2(59.4) | 0.92 |
| | LVDd | 4.75(0.45) | 4.68(0.59) | 4.74(0.57) | 0.84 |
| | PWd | 0.80(0.13) | 0.81(0.14) | 0.80(0.13) | 0.99 |
| | | | | | |
| **GNB3** | | **CC** | **CT** | **TT** | **p** |
| | Genotype frequency | 0.51 | 0.41 | 0.08 | |
| | LV mass | 174.0(56.8) | 180.5(76.1) | 191.5(65.9) | 0.90 |
| | LVDd | 4.74(0.60) | 4.64(0.52) | 4.75(0.63) | 0.83 |
| | PWd | 0.80(0.11) | 0.81(0.14) | 0.82(0.22) | 0.98 |
| | | | | | |
| **Beta 1AR** | | **CC** | **CG** | **GG** | **p** |
| | Genotype frequency | 0.47 | 0.49 | 0.04 | |
| | LV mass | 184.8(63.3) | 171.9(49.1) | 146.7(22.5) | 0.43 |
| | LVDd | 4.81(0.52) | 4.66(0.61) | 4.5(0.34) | 0.49 |
| | PWd | 0.82(0.15) | 0.79(0.12) | 0.77(0.11) | 0.84 |
| | | | | | |
| **SF1** | | **TT** | **TC** | **CC** | **p** |
| | Genotype frequency | 0.24 | 0.55 | 0.21 | |
| | LV mass | 162.4(44.5) | 187.3(75.0) | 178.1(48.0) | 0.29 |
| | LVDd | 4.64(0.37) | 4.78(0.55) | 4.60(0.60) | 0.33 |
| | PWd | 0.75(0.10) | 0.82(0.14) | 0.81(0.13) | 0.12 |
| | | | | | |

**Table 5.5-6 LV mass indices in groups with different genotypes. Association study using one member from each twin pair.**

ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism, LVDd – LV end-diastolic diameter, PWd – Posterior wall thickness (Diastole). Data given as mean (SD).

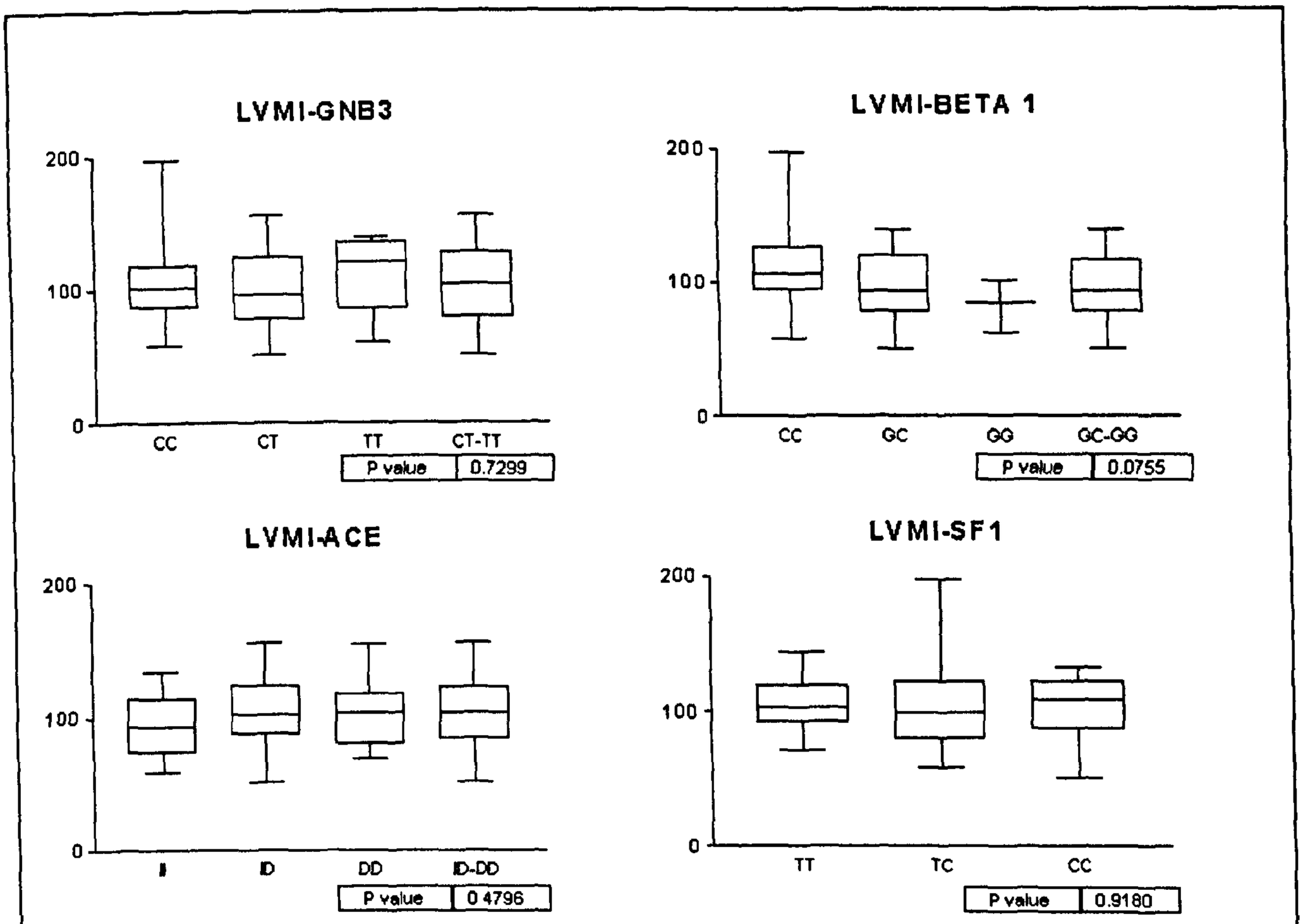


**Figure 5.5-1 LV mass index displayed by genotype.**

**Box plots represent means and SD. ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism**

| | | MZ | DZc | DZd | p (MZ vs DZc) | P (MZ vs DZd) |
|---|---|---|---|---|---|---|
| ACE | LV mass | 37.9 (29.4-46.5) | 46.9 (32.3-61.6) | 40.2 (22.3-58.0) | 0.24 | 0.79 |
| | LVDd | 0.34 (0.28-0.41) | 0.39 (0.29-0.50) | 0.43 (0.25-0.61) | 0.42 | 0.24 |
| | PWd | 0.12 (0.09-0.15) | 0.12 (0.07-0.17) | 0.11 (0.06-0.17) | 0.96 | 0.89 |
| | | | | | | |
| GNB3 | LV mass | 35.8 (26.6-44.9) | 44.0 (27.6-60.5) | 41.9 (20.2-63.6) | 0.33 | 0.52 |
| | LVDd | 0.34 (0.27-0.43) | 0.39 (0.27-0.52) | 0.42 (0.23-0.54) | 0.51 | 0.26 |
| | PWd | 0.11 (0.08-0.14) | 0.12 (0.07-0.17) | 0.12 (0.06-0.18) | 0.71 | 0.72 |
| | | | | | | |
| B1AR | LV mass | 37.7 (28.4-46.9) | 40.2 (26.5-53.8) | 49.9 (23.3-76.5) | 0.75 | 0.25 |
| | LVDd | 0.33 (0.28-0.41) | 0.37 (0.25-0.50) | 0.38 (0.29-0.57) | 0.50 | 0.51 |
| | PWd | 0.12 (0.09-0.15) | 0.11 (0.06-0.15) | 0.12 (0.05-0.19) | 0.76 | 0.83 |
| | | | | | | |
| SF1 | LV mass | 37.9 (29.3-46.5) | 44.0 (26.0-62.1) | 44.7 (30.2-59.2) | 0.48 | 0.38 |
| | LVDd | 0.34 (0.28-0.41) | 0.33 (0.19-0.46) | 0.47 (0.35-0.59) | 0.82 | 0.15 |
| | PWd | 0.12 (0.090.15) | 0.14 (0.08-0.19) | 0.10 (0.06-0.14) | 0.51 | 0.46 |
| | | | | | | |

**Table 5.5-7 Within pair differences in MZ twins, DZ twins concordant (DZc) and DZ twins discordant (DZd) for genotypes.**

ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism, LVDd – LV end-diastolic diameter, PWd – Posterior wall thickness (Diastole). Data given as mean difference (95% C.I.).

## 5.6 Discussion

This study confirms a genetic influence over many aspects of left ventricular structure persisting into adulthood. Left ventricular mass, uncorrected, demonstrated a significant degree of heritability with approximately 70% of LV mass being genetically determined. A familial effect and a trend towards a genetic effect are noted in the measurements of septal and posterior wall thickness. It is, however, left ventricular internal dimensions, rather than wall thickness that were influenced by

genetic factors. Mass remained significant even following correction for basic anthropometric and blood pressure measurements with approximately half of the variation being due to other genetic influences.

Previous twin studies in children and young adults have given conflicting results regarding the heritability of left ventricular structure and function. In the largest twin study to date left ventricular mass was under genetic control in a group of 11 year old children. However, the degree of genetic influence over a given phenotype varies with age and different genes are important at various stages in life. In adult life when many of these disease processes are clinically relevant, the effects of genes may be diminished or "watered-down" by the cumulative effect of environmental and lifestyle factors.[760]

Functional assessments of the left ventricle such as fractional shortening and fractional thickening did not reveal a significant genetic component, environmental factors being more important. Mitral valve E wave forward flow, reflecting the early diastolic filling of the left ventricle, did have a degree of heritability a finding consistent with previous studies.[490] The determinants of the E/A ratio in this population were age and systolic blood pressure but not LV mass.

Twin estimates of heritability are often higher than those of sibling and family cohorts and indeed the estimates from this study were higher than those obtained from the Framingham family study that estimated adjusted LV mass heritability at between 0.24-0.32.[489] In the largest twin echo study in children the estimates of heritability varied from between 63-77%. This study reveals that this genetic influence persists

into adulthood and identifies LV mass in adults as a target for further genetic study and in particular the further study of potential candidate genes. In addition it would be of interest, once the data is available, to compare the proportion of genetic control over LV mass in this "high risk" West of Scottish population to other adult cohorts using a similar twin methodology.

Though previous reports have suggested an association between left ventricular indices and ACE, GNB3, aldosterone synthase genes,[495-498;574;580] in this twin group we found no association between any of the echocardiographic parameters of LV mass and function and the four candidate genes ACE, GNB3, Beta1 adrenoceptor and Aldosterone synthase. In a twin study, Busjahn et al[574] showed a correlation between serum ACE activity and any parameter of cardiac size. They also showed LV posterior wall thickness was greater in subjects with DD genotype. However their twin analysis showed that posterior wall thickness was not merely a function of the ACE genotype but rather a polygenic trait. Additionally, they did not find any association between the ACE genotype and septal thickness. Their study is similar to this twin study as it involved normotensive twins. In previous studies, the relation between GNB3, Aldosterone synthase and LV mass were variable, and this study shows no clear association. The single gene effects on LV mass are probably small and thus genetic dissection of this trait is difficult. Another disadvantage inherent in twin studies is that because they tend to be small studies, any estimates derived from them have necessarily large confidence limits or standard errors.

Poch et al[581] recently provided data from 86 previously untreated hypertensive patients, suggesting an association of GNB3 polymorphism with LVH. In particular,

carriers of the T allele displayed a 3-fold increased risk of presenting with LVH. By contrast, Sedlacek et al[592] evaluated a substantially larger and epidemiologically phenotyped population sample and failed to show any association between the GNB3 T allele and cardiac structure. Though the results of our study is similar to that of Sedlacek et al.[592] it must be pointed out that the phenotypes under investigation may be different in the 2 studies. In this respect, Poch et al[581] focused their work on untreated hypertensive patients. Such selected patients may be more informative to detect minor effects of a given candidate gene with respect to LVH compared with the normal population sample. However, Sedlacek et al[592] analysed multiple subgroups potentially related to the effects of the GNB3 C825T polymorphism, including hypertensive subjects without antihypertensive medication and found no differences according to the genotype status. Furthermore, other phenotypes related to LVH independent of echocardiographic measurements, such as the serum levels of atrial and brain natriuretic peptide, did not display an association with the GNB3 polymorphism. In the present study, normal twins were analysed who were not selected for any cardiovascular phenotype and thus similar to the study of Sedlacek et al.[592]

Another explanation for the differences between the Spanish and the present study may be that the clinically based ascertainment strategy in Spain favoured a higher prevalence of the T allele. In fact, previous studies documented an association of T allele particularly with severe hypertension, including combination antihypertensive therapy[689;690] and end-organ damage.[687] Thus, a positive association of the GNB3 T allele may not reflect only a causative nature of this polymorphism for LVH, but also

differences in ascertainment strategies, resulting in differences in underlying mechanisms such as more severe hypertension in some selected patient groups.

Diastolic left ventricle dysfunction is yet another finding related to hypertension and LVH. Jacobi et al[580] reported in 34 mild to moderate hypertensive patients an association of the GNB3 T allele with impaired diastolic filling (CC versus TC/TT) and velocity time integral A/E (CC versus TC/TT) and concluded that this polymorphism contributes to the development of hypertensive heart disease. In the present study, however, 2 parameters of diastolic left ventricle function (E/A and Deceleration time) and indices of systolic left ventricle function failed to show any association with the T allele status, even in subgroups of untreated hypertensive subjects. Sedlacek et al[592] also failed to show any association with the T allele status and diastolic left ventricle function (E/A and IVRT) and indices of systolic left ventricle function, even in subgroups of untreated hypertensive subjects.

The most likely explanation of the present negative study is that the GNB3 T allele has no strong and clinically relevant effect on the variability of LV mass. Indeed, LVH is a complex genetic trait, and a single gene variant, albeit functionally relevant, may carry a minute contribution to the final phenotype. Therefore, it is inherently complicated to demonstrate its influence in even large-scale and well-powered association studies. By contrast, smaller studies have been shown to carry a risk of an $\alpha$-error.[722] Thus, a cautious interpretation of such studies may be advisable.

# Chapter 6 - GNB3 AND BLOOD PRESSURE AND LV MASS IN THE RENFREW/PAISLEY POPULATION

## 6.1 Introduction

Complex traits are typically divided into two major categories – qualitative or dichotomous traits and quantitative or continuous traits. Most association analyses use categorical definitions of disease. But a complex trait like blood pressure or LV mass is inherently a non-mendelian trait (*Section 1.2.2.1.3 Non-Mendelian Inheritance,* page 75), and thus may provide more effective descriptions of disease. For many of these conditions, all or nothing definitions of disease are arbitrary and unsatisfactory. In quantitative trait analysis, the strength of the genetic component is measured by the heritability ($h^2$) (Heritability is explained in *section 10.4 Broad sense heritability,* page 322 and *section 10.5 Artificial selection and narrow-sense heritability*). The most commonly used method for examining linkage between a quantitative trait and a marker locus is the one proposed by Haseman and Elston.[201] Details of quantitative trait analyses are explained in *section 1.2.3 Quantitative traits versus Qualitative traits*, page 89. As described in *Section 1.2.2.2.3 Problems with association studies,* page 83, part of the drawbacks in association mapping can be overcome by using family members in TDTs (Transmission Disequilibrium Tests). An accurate analysis should use all the information in a pedigree to construct powerful tests of association that are robust in the presence of stratification (*Section 1.2.2.2.2 Family based association study*, page 80). In this study, I study the association of GNB3 polymorphism to blood pressure and ECG measures of LV mass as quantitative traits, in a population of middle-aged siblings, who were not ascertained for any cardiovascular risk factor. I shall also look at three other genes namely ACE, Beta-1 adrenoceptor and aldosterone synthase polymorphism which have been previously associated with LV mass, and with the exception of beta1-adrenoceptor with blood pressure.

## 6.2 Aims

To estimate the heritability of blood pressure and LV mass as determined by various ECG indices in a large population of siblings.

To perform candidate gene studies to see if variations of four candidate genes (including GNB3) could explain the variance in the population of those phenotypes that show significant heritability.

## 6.3 Study design

This study is a family based study of the Renfrew/Paisley offspring population recruited between 1993-94. The details of the population are given below. The data available included blood pressure, anthropometry, digital ECG and DNA samples. The objective of the analysis is to test for association between the four candidate genes and its association with blood pressure and ECG indices of LV mass. As they comprised only siblings with no parental data, the sibling-TDT test is used in the analysis. The population was not selected for any phenotype and hence the analysis is conducted using the data as quantitative traits. The West Ethics committees approved the study protocol.

## 6.4 ECG Indices

The ECG indices of LV mass studied include the Rautaharju equation which gives the LV mass in grams adjusted for weight (Men: LVM = 0.026* CV + 1.25 * W + 34.4; Women: LVM = 0.020 * CV + 1.12 * W +36.2 ).[521;522] Also other indices of LV mass studied include the Sokolow–Lyon voltage (SV1+RV5 or RV6), Cornell voltage (RaVL+SV3), Cornell product [(RaVL+SV3)*QRS duration], 12 lead voltage, the time product of these indices and the voltage time area because as previously

described, they have increased sensitivity and specificity for identification of LVH.[507-511] A summary of these indices are given in Table 1.4-1.

## 6.5 Recruitment of subjects

### *6.5.1 Family Ascertainment*

The subjects upon whom this research is based were offspring of 4064 married couples who were screened in the Renfrew-Paisley (Midspan) Study in 1972–76.[761;762]

In 1993–4, attempts were made to trace offspring either through direct contact with the original study couple or, where both husband and wife were dead, through contacts with informants mentioned on their death certificate. Altogether 4829 offspring aged 30–59 were identified from 2365 couples with children; 3202 of these offspring lived within 45 minutes of Paisley and Renfrew. Of these, 1040 men and 1298 women from 1477 families completed a questionnaire and examination in 1996—a response rate of 73%.

### *6.5.2 Phenotyping*

Information on subjects' personal and family medical history, socio-demographic information, and smoking habits was obtained from detailed self-completed questionnaires. Examinations were carried out in clinics staffed by six research nurses who rotated between measurement stations. Measurements included: 12 lead ECG; blood pressure—recorded using a Dinamapp 8100, three readings were taken from the left arm with the subject seated and allowed to rest for five minutes—the mean of the last two reading for systolic and diastolic blood pressure was used; non-fasting venous blood samples were analysed for total and HDL cholesterol, fibrinogen, white blood count and glucose; FEV1 and FVC were measured using a Vitalograph Spirotrac III. Standing height and sitting height were measured without shoes in the Frankfort plane

to the nearest millimetre using a Holtain stadiometer. Weight was measured to the nearest 100 g with Seca digital scales in stockinged feet wearing indoor clothes.

## 6.6 Genetic analysis

Genomic DNA was isolated by phenol-chloroform extraction of EDTA-blood samples (*Section 3.1* page 206). The subjects were genotyped for the Insertion/Deletion ACE I/D, C825T G-protein β3, Aldosterone synthase SF1 binding site, G1165C beta-1 adrenoceptor polymorphisms by polymerase chain reaction. Genotyping was performed by PCR by the method previously described for C825T G-protein beta-3 subunit polymorphism,[579] (*Section 3.2*, page 208) ACE I/D polymorphism[757], SF1 RFLP[498] and Beta-1 adrenoceptor polymorphism. [582] Dr G Inglis performed the ACE and Aldosterone synthase polymorphism PCR and Dr T Stanton performed the beta-1 adrenoceptor PCR.

## 6.7 Statistical methods

The analytic strategies used in this study were designed to consider all the traits as continuous variables namely ECG indices, blood pressure and anthropometric measurements. This avoids the inherent limitations of choosing arbitrary cut-off points for categorisation. Family based studies control for the possibility of genetic differences between the case and control populations by comparing the frequencies of alleles transmitted to the affected child to the allele not transmitted. The only samples necessary are those from the affected individual and his or her two parents – the TDT triad (*Section 1.2.2.2.2.1 Transmission Disequilibrium Test (TDT)*, page 81). In cases where the parental DNA is unavailable, the traditional TDT approach is impossible. A novel approach that circumvents this difficulty is to use unaffected siblings as controls rather than relying on parental controls. This sib-TDT (S-TDT) approach compares marker allele frequencies in affected and unaffected siblings. The test requires only a

simple affected/unaffected sibling pair, although power can be increased if additional siblings are available. It provides a test for linkage and association and is immune to the effects of sampling bias. (*Section 1.2.6 Optimal study designs*, page 97)

### 6.7.1 Heritability

Heritability quantifies the strength of family resemblance and represents the percentage of variance in a trait that is due to all additive familial effects including additive genetic effects and those of the familial environment. Estimates of heritability for each phenotype was calculated using the variance component method implemented in the programme SOLAR.[199] The procedure involved the comparison of a sporadic model (assuming no familial resemblance) with a polygenic model (including familial correlations) to determine the best fit for the data.

### 6.7.2 Association analysis

A two-stage approach was used in the investigation of association between the polymorphisms of the four genes and blood pressure and ECG measures of heart size. Firstly, the eldest individual from each family was investigated by a mixed effects ANOVA to identify associations between genotype and phenotype. In the mixed effects ANOVA, the data was analysed in a general linear model in SPSS, with the trait as the dependent variable, genotypes as fixed effect and age, systolic blood pressure, weight and height as covariates for the ECG phenotypes, and age, sex BMI as covariates for blood pressure.

In the second stage of the analysis, family based association analysis was carried out using the variance components models of association implemented in the QTDT program and FBAT programs.

### 6.7.3 QTDT

QTDT[206] allows variance component models to be constructed which include the effect of polygenes, shared family environment and any effect of linkage at the locus under study. Association can be tested allowing for linkage and background polygenes. The effects of genotypes on phenotypic means are partitioned into between-family and within-family components, by comparing the association of alleles and trait values across siblings from different families to the association of alleles and trait values across siblings within the same family. Sibling pairs are by definition ethnically and racially homogeneous and any difference in trait scores between siblings of different genotypes at a candidate marker, therefore, reflect true genetic association. QTDT also allows for the partitioning of any observed association into between-family and within-family components, thus providing a check on whether the result could have arisen due to unsuspected population stratification amongst the families studied. This method is designed for families of any size and uses all available genotypic information.[206;207]

Simple linear models do not provide valid tests of linkage disequilibrium when multiple offspring per family are considered. When evaluating if a candidate polymorphism is the disease mutation, it is important to evaluate not only the evidence for association, but also estimate the significance of individual components of variance. QTDT uses variance components to model the phenotypic similarities that are common in family data. A typical model for the variances might include environmental (e), polygenic (g) and additive (a) components of variance. To evaluate the significance of one variance component, two alternative variance models are specified. For example, to estimate the heritability of the trait in the sibling data, a

model with only an environmental variance is compared with a model with polygenic and environmental variances.

To test for association, a model is defined including not only a linear model for the means with the covariates but also a model for the variances. Means and variances are fitted by maximum likelihood using a numeric minimiser. The analysis was carried out using BMI, age and sex as covariates.

NULL MODEL

Means = Mu + Covariate + B

Variances = Ve + Vg + Va

FULL MODEL

Means = Mu + Covariate + B + W

Variances = Ve + Vg + Va

This model includes a mean term (mu), a between family component of association (B) and, in the full model used under the alternative hypothesis, a within family component of association (W).

Variance components models can be sensitive to the phenotypic distribution, especially in small or selected samples. In small samples, in selected samples or when the variance model is incomplete, estimates of the variance components may be biased. Empirical p-values were calculated using a Monte-Carlo permutation framework for the within family component of association. These permutations condition on the trait distribution, linkage and familiality, and provide a test for

linkage disequilibrium. Another attractive feature of using Monte-Carlo permutation to estimate p-values, is that they can provide a global p-value with a built-in adjustment for multiple testing. To do this transmission scores for all markers are permuted simultaneously and the most significant association observed in each replicate is noted. The most significant association in the actual data is then compared to this reference distribution.

### 6.7.4 FBAT

FBAT[763] provides a 2 test statistic that tests the composite null hypothesis of no linkage or no disequilibrium without concerns about confounding because of admixture. FBAT has the advantage over the conventional transmission disequilibrium test (TDT) of being able to use data from all family members, not just case-parent trios.

The FBAT method[763] defines a statistical test that reflects association between a phenotype (T) and a marker value (X). The tests uses the generic form $S = \Sigma T \cdot X$ as a test statistic, where summation is over all offspring in all families in the dataset. The distribution of S under H0 is calculated using the distribution of offspring genotype, conditional on the trait T and on the parental genotype, or the sufficient statistics when parental genotype is unobserved. The value $S - E(S)/\sqrt{Var(S)}$ follows a Normal (0,1). As this is in a region of linkage, with several individuals in each family, calculating an empirical variance provides a valid test of association.

## 6.8 Results

### 6.8.1 Clinical characteristics of the subjects

The Renfrew/Paisley offspring population comprised 2338 individuals from 1477 families. There were a variable number of offspring from each family.

Electrocardiograms were available from 2308 individuals (1035 men and 1273 women) out of 1466 families. The summary of the eligible families is given in Table 6.8-1. The average age of the male subjects was 48 years and the female subjects 44 years. 593 families had more than one sibling. 873 families had only one offspring and thus were not included in the sibling based association study. In order not to lose this group, the analysis was carried out in two strands. First the eldest member of each family was analysed for gene association. This did not include the family data and is thus a simple cross-sectional association study. Secondly all the families with more than one offspring were underwent the family based association study.

| Number of sibs per family | Number of families | Number of individuals |
|---|---|---|
| 1 | 873 | 873 |
| 2 | 424 | 848 |
| 3 | 112 | 336 |
| 4 | 41 | 164 |
| 5 | 12 | 60 |
| 6 | 2 | 12 |
| 7 | 1 | 7 |
| 8 | 1 | 8 |
| Total | 1466 | 2308 |

**Table 6.8-1 - Structure of the 1477 families analysed**

Table 6.8-2 shows the demographics of the population using only the eldest offspring so as to include single offspring families into the study. Males had significantly higher BMI, body surface area, waist/hip ratio compared to women. The various indices of ECG LV mass were also significantly higher in men. There was however no significant difference in BP between men and women.

Table 6.8-2 shows the baseline characteristics and ECG phenotypes of the population. All the phenotypes differed significantly by gender. The mean value of ECG LV mass was 170 gms for men and 130 gms for women. Table 6.8-3 outlines the proportion of variance of the ECG phenotypes that was explained by the covariates in the

population that included just the eldest member from each family. The covariates retained in the final model were age, systolic blood pressure at recruitment, weight and height. The inclusion of BMI, body surface area, diastolic blood pressure, waist-hip ratio did not significantly improve the fit of the models. Age, systolic blood pressure, weight and height accounted for 5-12% of the variation in different ECG phenotypes while Sex, age, systolic blood pressure, weight and height accounted for 18-28% of the variation (except for ECG LV mass which is a compound phenotype that includes weight in its calculation). All the adjusted phenotype values (standardized residuals) had an approximately Normal distribution (Kolmogorov–Smirnov Z value <1·30, P>0·05 for all the traits).

| | Male | Female | p |
|---|---|---|---|
| N | 1035 | 1273 | |
| Age | 44.9(6.3) | 45.4(6.0) | 0.055 |
| SBP mmHg | 131.1(15.2) | 123.5(15.7) | <0.001 |
| DBP mmHg | 79.2(10.9) | 70.9(10.0) | <0.001 |
| BMI | 26.5(4.0) | 25.9(5.4) | 0.003 |
| Creatinine | 107.1(23.1) | 89.7(13.2) | <0.001 |
| Cholesterol | 5.4(1.0) | 5.2(0.9) | <0.001 |
| Diabetes (%) | 0.8 | 2.0 | |
| LVM (Rautaharju) gms | 170.1(23.2) | 130.2(18.6) | <0.001 |
| Cornell Voltage μV | 1295.5(528.1) | 925.8(428.5) | <0.001 |
| Sokolow Lyon μV | 2407.8(704.2) | 2082.5(579.9) | <0.001 |
| 12Lead μV | 14999.4(2941.2) | 12296.1(2495.7) | <0.001 |
| Sokolow Lyon product μV.s | 229.2(73.7) | 182.2(55.1) | <0.001 |
| Cornell Voltage product μV.s | 123.7(53.9) | 81.7(41.3) | <0.001 |
| 12 Lead product μV.s | 1431.8(356.6) | 1080.2(268.5) | <0.001 |

**Table 6.8-2 – Demographics and ECG phenotypes of the eligible study population. (mean and standard deviation)**

| | Phenotype | Men | Women | Sex adjusted |
|---|---|---|---|---|
| age, SBP, height, weight | LVM (Rautaharju) | 0.84 | 0.82 | 0.87 |
| | Cornell Voltage μV | 0.085 | 0.12 | 0.22 |
| | Sokolow Lyon μV | 0.086 | 0.088 | 0.14 |
| | 12Lead μV | 0.088 | 0.069 | 0.26 |
| | Sokolow Lyon product μV.s | 0.08 | 0.081 | 0.18 |
| | Cornell Voltage product μV.s | 0.078 | 0.109 | 0.24 |
| | 12 Lead product μV.s | 0.068 | 0.054 | 0.28 |

**Table 6.8-3 - $R^2$ for models predicting adjusted ECG phenotypes (p<0.001 for all models)**

## 6.8.2 Heritability

| Phenotype | Heritability |
|---|---|
| BMI | 0.55±0.06* |
| Waist/Hip ratio | 0.39±0.07* |
| Systolic BP | 0.35±0.07 |
| Diastolic BP | 0.53±0.06* |
| LVM (Rautaharju) gms | 0.55 ± 0.07* |
| Cornell Voltage μV | 0.32 ± 0.06* |
| Sokolow Lyon μV | 0.32 ± 0.06* |
| 12Lead μV | 0.44 ± 0.06* |
| Sokolow Lyon product μV.s | 0.28 ± 0.06* |
| Cornell Voltage product μV.s | 0.28 ± 0.06* |
| 12 Lead product μV.s | 0.36 ± 0.07* |

**Table 6.8-4 – Heritability estimates (± standard error) for ECG indices of LV mass, blood pressure, BMI and waist/hip ratio.**

* P<0.00001 for testing sporadic vs polygenic models in SOLAR.

Heritability was used to summarise the overall extent to which phenotypic variation is due to additive genetic effects was calculated using individuals from families with more than one offspring. In Table 6.8-4 the heritability estimates are shown from the analysis of these phenotypes performed in SOLAR. The heritability estimates for systolic blood pressure was 35% and diastolic blood pressure 52%. BMI showed a heritability of 55%. The heritability of the electrocardiographic phenotypes that are based on the Cornell voltage (RaVL+SV3) were 48-62% for LV mass, 26-38% for Cornell Voltage, Sokolow-Lyon voltage and Sokolow-Lyon product had a heritability of 22-38%, and the electrocardiographic phenotypes based on 12 lead voltage (12 Lead Voltage and 12 Lead product) had a heritability of 29-50%. These findings provide a strong rationale for genetic studies focused on quantitative assessment of electrocardiographic variables related to LV hypertrophy to identify genes that contribute to these phenotypes and cardiovascular risk. These estimates of heritability for Cornell Voltage is in agreement with previous reports in Caucasian families which range from 26-32%,[256;489] but are lower than an estimate of heritability of 65-72%

obtained in siblings of African ancestry.{Kotchen, 2000 13847 /id} The ECG criteria for LVH that are based on Cornell Voltage was developed using echocardiographic validation,[508;522] therefore it is not surprising that the heritability estimate for this is similar to echocardiographic LV mass.[256;489]

For the ECG measures of LV mass, the maximum heritability was shown by LVM-Rautaharju equation and 12 lead voltage, so further family based association analysis will be done on these indices.

### 6.8.3 Association study

| | Male | Female | p |
|---|---|---|---|
| N | 654 | 812 | |
| Age | 46.5(5.8) | 46.9(5.7) | 0.18 |
| SBP mmHg | 132(15.4) | 125.1(16.4) | <0.001 |
| DBP mmHg | 80.1(10.8) | 71.4(10.1) | <0.001 |
| Height | 1750.2(63.9) | 1611.4(80.4) | <0.001 |
| Weight | 81.5(13.4) | 67.5(13.1) | <0.001 |
| Cholesterol | 5.5(1) | 5.2(0.9) | <0.001 |
| LVM (Rautaharju) | 170.7(23.2) | 130.9(18.8) | <0.001 |
| Cornell Voltage mV | 1315.8(534.7) | 942.5(443) | <0.001 |
| Sokolow Lyon mV | 2387.1(692) | 2078.2(597.2) | <0.001 |
| 12Lead mV | 14941.5(2822.2) | 12305.7(2596.1) | <0.001 |
| Sokolow Lyon product mV.s | 227(70.7) | 182.2(56.7) | <0.001 |
| 12 Lead product mV.s | 1425.3(341.8) | 1083.2(278.6) | <0.001 |
| Cornell Voltage product mV.s | 125.6(54.4) | 83.4(43.3) | <0.001 |

**Table 6.8-5 - Demographics and ECG phenotypes of the eldest member of each of eligible families in the study population. (mean and standard deviation)**

The summary of the demographic and ECG phenotypes of the subset comprising the eldest member from each family is shown in Table 6.8-5. None of the four polymorphisms showed any association with blood pressure or BMI. The results are summarised in Table 6.8-6, which also shows that all four genes are in Hardy-Weinberg Equilibrium. In the mixed effects ANOVA model shown in Table 6.8-7, B1AR was associated with the variability in the Cornell-Voltage based ECG phenotypes. An interaction between GNB3 and SF1 was found to have a significant

association with variability in LV mass based on Cornell Voltage, while an interaction between GNB3, ACE and SF1 was associated the LV mass indices based on 12 lead voltage. GNB3 alone did not have any major effect on LV mass or blood pressure. The main effect of the association with the B1AR polymorphism persisted after Bonferroni correction was applied.

| GNB3 | | | | | | |
|---|---|---|---|---|---|---|
| | CC | CT | TT | HW[c] | ANOVA[d] | |
| N | 496 | 481 | 97 | 0.23 | F | p |
| SBP[a] | 127.5(0.8) | 127.4(0.8) | 126.5(1.6) | | 0.19 | 0.82 |
| DBP[a] | 74.8(0.6) | 74.8(0.6) | 73.9(1.1) | | 0.33 | 0.72 |
| BMI[b] | 26.5(0.3) | 26.5(0.4) | 26.2(0.9) | | 0.87 | 0.42 |
| B1AR | | | | | | |
| | CC | CG | GG | HW[c] | ANOVA[d] | |
| N | 587 | 415 | 72 | 0.93 | F | p |
| SBP[a] | 127.2(0.7) | 127(0.8) | 127.3(1.8) | | 0.02 | 0.98 |
| DBP[a] | 74.9(0.5) | 74.3(0.6) | 74.4(1.2) | | 0.46 | 0.63 |
| BMI[b] | 25.9(0.3) | 26.6(0.4) | 26.7(0.9) | | 0.15 | 0.86 |
| ACE | | | | | | |
| | II | ID | DD | HW[c] | ANOVA[d] | |
| N | 239 | 501 | 334 | 0.05 | F | p |
| SBP[a] | 127.2(1.1) | 126.2(0.9) | 128(1) | | 1.57 | 0.21 |
| DBP[a] | 74.7(0.8) | 73.6(0.6) | 75.3(0.7) | | 2.98 | 0.05 |
| BMI[b] | 26.4(0.6) | 26.3(0.4) | 26.5(0.6) | | 0.12 | 0.89 |
| SF1 | | | | | | |
| | CC | CT | TT | HW[c] | ANOVA[d] | |
| N | 240 | 528 | 306 | 0.67 | F | p |
| SBP[a] | 126.2(1.1) | 128(0.9) | 127.2(1.1) | | 1.17 | 0.31 |
| DBP[a] | 74(0.8) | 75.2(0.6) | 74.2(0.7) | | 1.55 | 0.21 |
| BMI[b] | 26.5(0.5) | 26(0.4) | 26.6(0.6) | | 0.26 | 0.77 |

**Table 6.8-6 - Mixed effects ANOVA of blood pressure and BMI using the eldest member of each family, showing the main effects of the four genes.**

[a] Covariates – age, sex, BMI; [b]Covariates – age, sex; [c]Hardy Weinberg Equilibrium – Exact p value; [d]Mixed effects ANOVA performed using blood pressure and BMI as continuous variables adjusted for covariates.

**HW – Hardy Weinberg Equilibrium; ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism.**

| | LVM | | Cornell Voltage µV | | 12Lead µV | | Cornell Voltage product µV.s | | 12 Lead product µV.s | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F | p | F | p | F | p | F | p | F | p |
| GNB3 | 2.87 | 0.06 | 2.88 | 0.06 | 1.23 | 0.29 | 2.84 | 0.06 | 0.68 | 0.51 |
| B1AR | 4.05 | **0.02** | 3.83 | **0.02** | 0.27 | 0.76 | 3.29 | **0.04** | 0.21 | 0.81 |
| ACE | 0.02 | 0.98 | 0.07 | 0.93 | 0.77 | 0.47 | 0.14 | 0.87 | 1.53 | 0.22 |
| SF1 | 0.82 | 0.44 | 1.48 | 0.23 | 0.02 | 0.98 | 1.28 | 0.28 | 0.3 | 0.74 |
| GNB3 * B1AR | 1.17 | 0.32 | 1.58 | 0.18 | 0.61 | 0.65 | 1.75 | 0.14 | 0.32 | 0.86 |
| GNB3 * ACE | 1.31 | 0.26 | 1.32 | 0.26 | 0.19 | 0.94 | 1.2 | 0.31 | 0.65 | 0.63 |
| GNB3 * SF1 | 2.72 | **0.03** | 2.9 | **0.02** | 1.94 | 0.1 | 2.92 | **0.02** | 3.11 | **0.01** |
| B1AR * ACE | 1.03 | 0.39 | 0.79 | 0.53 | 0.32 | 0.86 | 0.66 | 0.62 | 0.51 | 0.73 |
| B1AR * SF1 | 0.15 | 0.96 | 0.22 | 0.93 | 0.84 | 0.5 | 0.16 | 0.96 | 1.74 | 0.14 |
| ACE * SF1 | 1.45 | 0.21 | 1.21 | 0.31 | 0.29 | 0.89 | 0.87 | 0.48 | 0.39 | 0.82 |
| GNB3 * B1AR * ACE | 1.38 | 0.2 | 1.31 | 0.24 | 1.49 | 0.15 | 1.52 | 0.15 | 1.14 | 0.33 |
| GNB3 * B1AR * SF1 | 2.18 | 0.03* | 1.29 | 0.25 | 0.92 | 0.49 | 1.59 | 0.13 | 1.46 | 0.18 |
| GNB3 * ACE * SF1 | 1.31 | 0.23 | 1.29 | 0.24 | 3.11 | **<0.01** | 1.29 | 0.24 | 2.17 | **0.03** |
| B1AR * ACE * SF1 | 1.3 | 0.24 | 0.78 | 0.62 | 1.2 | 0.29 | 0.62 | 0.76 | 1.25 | 0.26 |
| GNB3 * B1AR * ACE * SF1 | 1.38 | 0.02* | 1.26 | 0.07 | 1.22 | 0.1 | 1.26 | 0.07 | 1.24 | 0.09 |

**Table 6.8-7 - Mixed effects ANOVA of the adjusted electrocardiographic phenotypes using the eldest member of each family, showing the main effects of the four genes and their interactions.**

**ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism.**

### *6.8.4 Family Study*

In the family study, the entire pedigree is analysed for all families with more than one offspring. The phenotypes analysed were systolic BP (SBP), diastolic BP (DBP), LV Mass by Rautaharju equation, 12 lead voltage (12Lead_V). Association was tested in QTDT using the orthogonal model and allowing for linkage and background polygenes. Empirical p-values were calculated using a Monte-Carlo permutation with built-in adjustment for multiple testing. As seen in Table 6.8-8, the strongest association was for B1AR gene polymorphism and 12 Lead Voltage.

Unsuspected population stratification amongst the families studied can lead to spurious results. Population stratification was tested by partitioning the observed

association into between-family and within-family components using the QTDT program. This showed no evidence of population stratification in the study group.

As there is no stratification except for 12 Lead Voltage and the B1AR polymorphism, the next step is to test the total evidence for association (i.e., not a TDT). The results are summarised in Table 6.8-9, which shows no association by variance component testing for all the polymorphisms and the phenotypes tested.

| | GNB3 | | B1AR | | ACE | | SF1 | |
|---|---|---|---|---|---|---|---|---|
| **PROBANDS** | 610 | | 645 | | 703 | | 610 | |
| | $\chi^2$ | p | $\chi^2$ | p | $\chi^2$ | p | $\chi^2$ | p |
| **SBP** | 1.96 | 0.16 | 0.0 | 1 | 0.15 | 0.69 | 2.01 | 0.16 |
| **DBP** | 1.0 | 0.32 | 0.45 | 0.50 | 0.49 | 0.48 | 1.17 | 0.28 |
| **MAP** | 0.0 | 1 | 0.06 | 0.81 | 0.05 | 0.82 | 0.01 | 0.92 |
| **LV mass** | 1.01 | 0.3 | 0.38 | 0.5 | 0.06 | 0.8 | 0.96 | 0.3 |
| **12Lead_V** | 0.12 | 0.7 | 4.07 | 0.04 | 1.18 | 0.3 | 0.13 | 0.7 |

**Table 6.8-8 – Variance component analysis of association between candidate genes and cardiovascular phenotypes in the Renfrew Paisley offspring population.**

**ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism. Systolic BP (SBP), diastolic BP (DBP), mean arterial pressure (MAP), LV Mass by Rautaharju equation, 12 lead voltage (12Lead_V).**

| | GNB3 | | B1AR | | ACE | | SF1 | |
|---|---|---|---|---|---|---|---|---|
| **PROBANDS** | 1992 | | 2161 | | 2122 | | 1992 | |
| | $\chi^2$ | p | $\chi^2$ | p | $\chi^2$ | p | $\chi^2$ | p |
| **SBP** | 1.13 | 0.28 | 0.0 | 1 | 0.40 | 0.53 | 1.23 | 0.27 |
| **DBP** | 1.07 | 0.30 | 2.21 | 0.14 | 1.04 | 0.31 | 1.14 | 0.29 |
| **LV mass** | 1.48 | 0.2 | 0.02 | 0.87 | 0.36 | 0.6 | 1.43 | 0.20 |
| **12Lead_V** | 0.38 | 0.50 | 0.61 | 0.4 | 0.52 | 0.45 | 0.38 | 0.51 |

**Table 6.8-9 – Total association in the whole family using age, BMI and sex as covariates.**

**ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism. Systolic BP (SBP), diastolic BP (DBP), mean arterial pressure (MAP), LV Mass by Rautaharju equation, 12 lead voltage (12Lead_V).**

Finally the whole analysis was carried out after excluding all those ECG's which showed any abnormality namely ischaemia, conduction defects as defined by their Minnesota codes (the Minnesota codes used are shown in Table 1.4-2). Table 6.8-10 shows the results of the analysis that included tests for Association, Linkage, Linkage

in the presence of association, population stratification, and total association. The results are similar with no association detected.

| | n | Association | p | Linkage | p | Linkage + Assoc | p | Stratific-ation | p | Total association | p |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LV MASS | | | | | | | | | | | |
| **GNB3** | 540 | 0.17 | 0.70 | 0 | 1.00 | 0 | 1.00 | 0.14 | 0.70 | 0.03 | 0.90 |
| **B1AR** | 579 | 1.47 | 0.20 | 0 | 1.00 | 0 | 1.00 | 2.17 | 0.10 | 0.07 | 0.80 |
| **ACE** | 648 | 1.46 | 0.20 | 0 | 1.00 | 0 | 1.00 | 2.01 | 0.20 | 0.02 | 0.90 |
| **SF1** | 540 | 0.18 | 0.70 | 0 | 1.00 | 0 | 1.00 | 0.15 | 0.70 | 0.02 | 0.90 |
| 12L –V | | | | | | | | | | | |
| **GNB3** | 540 | 0.99 | 0.30 | 0.04 | 0.80 | 0.18 | 0.70 | 0.67 | 0.40 | 0.32 | 0.60 |
| **B1AR** | 579 | 3.88 | 0.05 | 0.02 | 0.90 | 0.04 | 0.80 | 3.48 | 0.06 | 0.52 | 0.50 |
| **ACE** | 648 | 0.32 | 0.60 | 0 | 1.00 | 0 | 1.00 | 0.06 | 0.80 | 0.48 | 0.50 |
| **SF1** | 540 | 1 | 0.30 | 0.03 | 0.90 | 0.27 | 0.60 | 0.68 | 0.40 | 0.32 | 0.60 |

**Table 6.8-10 Analysis of the ECG indices excluding abnormal ECG's using the Minnesota Code. Age, sex and BMI used as covariates.**

**ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism. LV Mass by Rautaharju equation, 12 lead voltage (12Lead_V).**

## *6.8.5 Family based Association*

The next analysis was carried out using the same phenotypes but using the FBAT program. The results are presented in Table 6.8-11. Similar to the results of the variance component analysis, B1AR shows a significant association with respect to 12 lead voltage measures. The other genes show no association.

| | GNB3 | | B1AR | | ACE | | SF1 | |
|---|---|---|---|---|---|---|---|---|
| N / families[a] | 610/244 | | 645/245 | | 703/283 | | 610/244 | |
| | Z | p | Z | p | Z | p | Z | p |
| Systolic BP | -0.61 | 0.54 | -1.75 | 0.079 | -1.03 | 0.30 | -0.61 | 0.54 |
| Diastolic BP | -0.62 | 0.53 | -1.17 | 0.24 | -0.67 | 0.49 | -0.62 | 0.53 |
| BMI | 0.36 | 0.72 | -1.23 | 0.22 | 0.07 | 0.94 | 0.36 | 0.71 |
| LV mass | 1.3 | 0.19 | -0.72 | 0.47 | -0.09 | 0.93 | 1.3 | 0.19 |
| 12Lead_V | -0.25 | 0.80 | 2.07 | 0.03 | 1.06 | 0.28 | -0.25 | 0.80 |

**Table 6.8-11- FBAT analysis of the Renfrew Paisley Offspring phenotypes and candidate genes.**

**[a] – Number of informative families for the polymorphism**

Systolic BP, Diastolic BP and BMI used as continuous variables adjusted for age and sex. LV mass and 12 lead voltage adjusted for age, sex, height and weight.

**ACE – ACE I/D polymorphism, GNB3- GNB3 C825T polymorphism, B1AR – B1AR G1165C polymorphism, SF1- CYP11B2 C344T polymorphism.**

## 6.9 Discussion

This study is a cross-sectional study of an unselected population of siblings from the Renfrew/Paisley area of Scotland. Both a simple association study and a sibling-TDT analysis including family structure were used to look for an association between GNB3 and blood pressure and ECG indices of LV mass. The phenotypes were analysed as quantitative traits. Three other genetic polymorphism of ACE, β1 adrenergic receptor and aldosterone synthase were also analysed. All these genes have been variably associated with blood pressure and LV mass.

The advantage of this study is that it uses a family based approach to detect association with candidate genes. Transmission disequilibrium testing is more powerful in assessing genetic linkage and association and is immune to the effects of sampling bias, thus avoid the misleading results that occur when the study population is not genetically homogeneous. I also used quantitative traits in the analysis, as hypertension and LV mass are inherently non-mendelian traits and quantitative traits may provide more effective descriptions of these complex phenotypes.

### *6.9.1 Blood Pressure*

I found no association between systolic and diastolic blood pressure in the association study using the eldest offspring in each family. Analysing separately for males and females or together using sex, age and BMI as covariates consistently showed no association. I also found no association using a family based analysis. There was no evidence of population stratification and the lack of association was evident with quantitative transmission disequilibrium test (QTDT) and this was verified by the less conservative EV-FBAT.

I studied the West of Scotland population known to have a high cardiovascular risk, and found no relation between GNB3 polymorphism and blood pressure levels at recruitment when the average age of the population was 38-50 years. The association studies between GNB3 and hypertension have been inconsistent and are detailed in *Section 1.6.3.1* page 186. Two longitudinal studies from Europe show contrasting results for association between GNB3 polymorphism and risk of hypertension in a non-hypertensive population.[693;764] In a population based prospective cohort study from Finland of 903 men, aged 42-61 years at baseline and followed up for an average time of 4.2 years, Snapir et al[693] found no statistically significant difference between the GNB3 genotype groups in respect to baseline and end-of-follow-up risk for hypertension or obesity, systolic or diastolic blood pressure, or body mass index. Sartori et al[764] looked at 461 participants of the Hypertension and Ambulatory Recording Venetia Study (HARVEST) study (age, 18 to 45 years) at low cardiovascular risk, according to 1999 ISH/WHO criteria. The study end point was eligibility for antihypertensive medication, that is, progression to grade II hypertension during the first year of observation or office systolic blood pressure 150 mm Hg and/or office diastolic blood pressure 95 mm Hg in two later consecutive visits during follow-up. At baseline, there was no statistically significant difference among genotypes with respect to body mass index, blood pressure, and heart rate. According to survival analysis, the patients carrying the 825T allele had an increased risk of reaching the blood pressure end point (CI, 1.108 to 1.843; P=0.006). They reasoned that in these young hypertensive patients with low cardiovascular risk profile, the genetic influences on blood pressure could be more pronounced than in obese, older patients with long-lasting hypertension, in whom the effects of

environmental factors may prevail. Though at baseline they found no difference in genotype frequencies, they suggest 825T allele of GNB3 gene is associated with earlier blood pressure increase and a more frequent need of antihypertensive therapy during a mean follow-up of 4.7 years. In this study the phenotype studied is slightly different in that, I used the age and sex adjusted blood pressure values as a continuous variable and performed variance component analysis. While most of the other association studies have been case-control studies. One of the drawbacks of variance component analysis is that observations on subjects with incomplete data are assumed to be missing at random and hence ignorable. In this population for blood pressure and BMI, only those who had all the phenotypes recorded were used in the analysis. This large study is a negative association study that supports the results of the case control study described in *Chapter 4* -.

### *6.9.2 ECG LV mass*

The primary aim of this study was to identify measures of cardiac hypertrophy determined by ECG that are most likely to have a genetic basis and determine if variations in the GNB3 genotype had any role in relation to hypertensive LVH as previously reported.[581] I also looked at other candidate genes implicated in LVH namely aldosterone synthase, ACE and beta1 adrenoceptor.

The pathogenic mechanism of LVH is multifactorial, involving both haemodynamic and non-haemodynamic factors, such as the sympathetic nervous system and the renin-angiotensin system. In addition, epidemiological studies have demonstrated that subjects with LVH may have near-normal blood pressure,[48] indicating that additional factors besides blood pressure may be important in the development of hypertrophy. Studies in families and twins have shown that left ventricular mass is a familial trait,

indicating the influence of both genetic and environmental factors. Recent investigation into the genetic factors for LVH have focused mainly on the renin-angiotensin system, with focus on the insertion/deletion polymorphism of the angiotensin-converting enzyme gene associated with LVH,[573] but results have been conflicting.[495]

This study quantifies the significant heritability of ECG indices of LV mass namely LV mass as measured by the Rautaharju equation, Voltage indices namely Sokolow-Lyon, Cornell, and 12 lead voltages. I have also analysed voltage time product and found them to be significantly heritable. The estimates for heritability for these ECG indices were lower than the heritability determined for the twin population (Table 5.5-4). One reason is that it is known that twin estimates of heritability are often higher than those of sibling and family cohorts. It has also been suggested that anatomical and ECG indices of LVH may reflect different phenomena, and they have been found to have independent effects on morbidity and mortality.[506;581;765] The ECG voltage provides a complex measure of cardiac hypertrophy derived from the magnitude of electrical potential generated by the left ventricle and the electromotive forces generated by the heart. In addition to the biological differences between the electrical and anatomic phenotypes, the accuracy and reliability with which a phenotype can be measured can influence familial correlations and heritability estimates. Our heritability estimates are comparable to those reported by Mayosi et al who found Sokolow-Lyon voltage, electrocardiographic left ventricular mass, Cornell voltage, and Cornell product had heritability estimates of 39-41%, 12-18%, 19-25%, and 28-32%, respectively.[766]

Sedlacek et al[592] evaluated a large and epidemiologically phenotyped population sample and failed to show any association between the GNB3 T allele and echocardiographic cardiac structure and diastolic function. This study population is comparable with the of Sedlacek et al[592] in that the population was not selected for any cardiac phenotype, but unlike Sedlacek et al[592] the present study is a family based study using ECG indices of LV mass. The results of my study is similar to that of Sedlacek et al.[592] it must be pointed out that the phenotypes under investigation are different, though the ECG indices of LV mass correlate with ECHO LV mass. Poch et al[581] focused their work on untreated hypertensive patients and such selected patients may be more informative to detect minor effects of a given candidate gene with respect to LVH compared with the normal population sample. However, Sedlacek et al[592] analysed multiple subgroups potentially related to the effects of the GNB3 C825T polymorphism, including hypertensive subjects without antihypertensive medication and found no differences according to the genotype status. Furthermore, other phenotypes related to LVH, independent from echocardiographic measurements such as serum levels of atrial and brain natriuretic peptide, did not display an association with the GNB3 polymorphism. In the present study, normal siblings, who were not selected for any cardiovascular phenotype were analysed and did not show any association with the GNB3 polymorphism.

This study shows an association between B1AR and ECG LV mass in the association study, which used only one individual per family. The effect is also seen in the variance component based analysis of the entire population and on family based association testing. It must be pointed out that the phenotype values were not entirely normally distributed when the entire population was considered, though it showed a

normal distribution when only one member of each family was included. However GNB3 consistently showed a negative single gene association with the ECG phenotypes.

This study also shows a significant statistical interaction between GNB3, ACE and SF1 polymorphisms. It is well known that complex traits like LV mass are the consequence of interactions between many genetic and environmental factors. In this scenario, effects conferred by a single gene variant either must be very strong or found at a very high frequency to be statistically detectable, even if the sample of the association study is large. The candidate genes I have looked at have previously been inconsistently associated with LV mass. The statistical interactions we have shown may explain some of the previous inconsistent results if they can be replicated in other studies. Biologically, it is reasonable to speculate that as G proteins are signal transduction molecules through which the actions of the renin-angiotensin-aldosterone system and the adrenergic system are mediated, they may exert a major effect only in association with variation in these genes. G proteins mediate part of the actions of vasoactive hormones, such as angiotensin II and norepinephrine. Angiotensin II exerts trophic effects on cardiomyocytes in culture and is known to stimulate growth in other cell systems.[721] On the other hand, growth factors, such as platelet-derived growth factor, can act in part through pertussis-sensitive G proteins in skin fibroblasts, vascular smooth muscle cells, and cardiac fibroblasts and may participate in the pathogenesis of LVH.[494] G-protein β3 subunit gene polymorphism may constitute a genetic basis for the enhanced growth phenomena observed in essential hypertension, such as LVH and media hypertrophy of the vessel wall, if it is directly associated with a functional change and does not merely represent a DNA marker.

Though the functional effect of the GNB3 splice variant is in dispute, whether its function is enhanced as hypothesised by Siffert et al.[672] or whether the splice variant is associated with loss of function as described by Ruiz-Velasco and Ikeda[767], the net effect of this variation could be manifest only when its associated afferent or efferent pathways are also affected. However these findings need to be further tested in large association studies especially with ascertainment for high blood pressure that can identify individuals in the extremes of LV mass distribution. In this setting, the practicality of measuring electrocardiographic phenotypes are much greater than that of measuring echocardiographic left ventricular mass in many thousands of individuals. Such ascertainment is also necessary to give power at the stage of defining genotype–phenotype association.[169]

# Chapter 7 - FUNCTIONAL STUDY OF GNB3 – Platelet Aggregation

Platelet aggregation plays a central role in the pathogenesis of acute thrombosis in coronary heart disease.[768] Endothelial injury, as well as atherosclerotic lesions, enhances the adhesion of platelets to the subendothelial matrix through interactions of the platelet glycoprotein (GPIb/IX) complex with von Willebrand factor.[769] Subsequent platelet activation is triggered by many agonists, most of them, including thrombin, ADP, epinephrine, and thromboxane A2, bind to heptahelical receptors coupled to heterotrimeric G proteins.

Some specific endogenous and other environmental factors have been described to affect platelet aggregability such as age,[770] stress,[771-773] hypertension,[774] cigarette smoking and alcohol consumption,[775] but these factors only partially explain the interindividual variability in aggregation responses.

The activation of platelets results in platelet aggregation and release of several vasoactive substances such as catecholamines, 5-hydroxytryptamine, thromboxane A2, and platelet-derived growth factor.[776] These vasoactive substances in turn can promote vasoconstriction, vascular hypertrophy, and atherosclerosis, as well as thrombus formation.[776] In essential hypertension, platelets are believed to be preactivated or hyperresponsive to vasoactive agents. This hypothesis is supported by the evidences demonstrating that platelet release product -thromboglobulin, intraplatelet calcium, intracellular pH, density of platelet 2-adrenoceptors, or platelet aggregatory responses to vasoactive substances are increased in patients with essential hypertension compared with normotensive subjects.[777-783] Especially in essential hypertension, platelet might be overactivated by mental stress or exercise because of the exaggerated responses of systemic haemodynamics and sympathoadrenal system

to stressors.[784] The repeated enhancement of platelet activity during daily stress may accelerate vascular injury and hypertensive organ damage, thereby exposing hypertensive patients to risks of cardiovascular complications.[785;786] Accordingly, it is suggested that platelets play an important role in the genesis of cardiovascular complications in hypertension. In fact, the recent clinical study, called Hypertension Optimal Treatment (HOT) study, has demonstrated that adding 75 mg/day of aspirin to antihypertensive treatment reduced major cardiovascular events by 15% and all myocardial infarctions by 36% in hypertensive patients.[787] Similarly, another study demonstrated that aspirin had a 44% reduction of myocardial infarction in primary prevention.[788] Therefore, it is desirable that antihypertensive agents would have favourable effects on platelet function as well as pressure-lowering effect. The effects of antihypertensive drug on platelet function have been extensively studied in the clinical setting and in in-vitro experiments. Most clinical data suggest that angiotensin converting enzyme inhibitors, calcium antagonists, and β-blocking agents inhibit platelet function in essential hypertension.[778;789;790] However, the results of the previous reports are not consistent in detail regarding antiplatelet effect of these drugs, possibly as a result of differences in study design, subjects selected, or methodology used.

## 7.1 G Protein and Platelet Aggregation

The physiology of G protein function is described in section 1.5 (page 165). The G protein subunits which are activated in human platelets by common agonists have been described: vasopressin (VP) induces platelet aggregation via the V1 receptor involving the pertussis toxin (PTX)-insensitive Gq.[791] Using a knockout mouse model, Offermanns et al.[792] documented that Gq is essential for aggregation in response to TXA2, thrombin, and ADP, whereas G12/13 proteins mediate the platelet

shape change reaction.[793] Activation of human platelets with thrombin receptor-activating peptide (TRAP) results in the activation of Gi, Gq, and G12/G13,[794] the thromboxane A2 (TXA2) receptor agonist U46619 activates Gq, G12 and G13, but not Gi.[794;795] Therefore, platelet aggregation induced by thromboxane A2 requires the secretion of ADP to inhibit the adenylyl cyclase.[796] Epinephrine-induced platelet aggregation is considered to be exclusively mediated by Gi2 after activation of the α2A adrenoceptor,[797] whereas ADP mediates platelet activation through two G-protein-coupled receptor subtypes Gq and Gi.[798] Signalling via G protein-coupled receptors leads to the binding of fibrinogen and von Willebrand factor to the glycoprotein (GP) IIb/IIIa receptor of platelets as a final common pathway by a process described as inside-out signalling.[799] The following are the agonists and their G protein targets which will be studied in this experiment.

- Weak Agonists
    - ADP - Gq, Gi
    - Epinephrine - Gi2
- Strong agonists
    - PAF - Gi

## 7.2 Hypothesis

Given the activation of PTX-sensitive G proteins by epinephrine together with the observation that the α2A adrenoceptor activates G protein heterotrimers containing Gβ3,[800] epinephrine-mediated platelet aggregation should be enhanced in GNB3 825T allele carriers. In contrast, platelet activation evoked by thrombin, ADP, or U46619, TRAP and VP may not be influenced by the allele status at the 825 positions of GNB3.

## 7.3 Methods

### *7.3.1 Subject recruitment:*

Normal volunteers were recruited by advertisement. Any individual above 18 years of age, non-smoker, non-diabetic, non-hypertensive, not on any medications including NSAIDs was invited to take part in the study. They were advised to attend the Clinical Investigation Research unit at 9AM fasting from midnight. On arrival, they were asked to lie on a bed in the research unit and after 5-10 minutes rest, they filled in a questionnaire, signed a consent form and their blood pressure and vital signs recorded. The research protocol was approved by the West Ethics Committee.

### *7.3.2 Collection of Blood:*

Venous blood was collected from the subjects after overnight fast. All subjects confirmed on the day of study that they were non-smokers and they were not on any medications including aspirin or other over-the-counter medications. Blood was drawn from a large vein, usually the antecubital vein without stasis using an 18G butterfly needle into a 50ml plastic syringe. The 50 ml sample of blood for platelet aggregation was anticoagulated with 3.15% Sodium Citrate (9:1 vol/vol), pH 7.4. Also blood samples for routine haematology, biochemistry and a sample of blood for DNA extraction were obtained.

### *7.3.3 Preparation of Platelet-Rich Plasma (PRP) and Platelet-Poor Plasma (PPP)*

Platelet-rich plasma was obtained by a 20 minute centrifugation at 100g at room temperature. The two-thirds of the supernatant PRP was transferred to a polystyrene tube and allowed to sit at room temperature for 20 minutes. Platelet-poor plasma was prepared by further centrifugation of the remaining blood specimen at 2000g for 20 minutes at room temperature.

### *7.3.4 Reagents:*

ADP (Sigma) was prepared as a stock solution of 1000 μM in 1 ml distilled water. Platelet activating factor (PAF) (Sigma) was prepared as a solution of 1000 nM in 0.35% Bovine Serum Albumin (BSA) and 0.15M NaCl. Epinephrine (Sigma) was prepared as a stock solution of 100 μM in 1 ml distilled water. All the stock solutions were frozen at -20°C. Before the start of the experiments, the aggregating agents were made up in the following concentrations for the dose response curves. ADP : 200, 100, 50, 25,12.5, 6.25, 3.125, 1.56 μM in distilled water; Epinephrine: 100, 50, 25, 12.5, 6.25, 3.125, 1.56 μM in distilled water; PAF: 500, 250, 125, 62.5, 31.25, 15.625, 7.8 nM in BSA/saline. These concentrations would provide the necessary final concentrations when it would be diluted 1:10 with PRP during the experiment. Further concentrations were prepared by dilutions during the experiment if needed. Thus the final concentrations of ADP ranged from 0.1 to 20 μM, PAF from 0.7 to 50 nM and epinephrine from0.1 to 10 μM.

### *7.3.5 Platelet Aggregation:*

Platelet aggregation was carried out according to Born's method.[801] using a Chronolog Aggregometer. Measurement was performed on aliquots of 450 μL of platelet-rich plasma with stirring at 1200 rpm at room temperature. 450 μL of platelet-poor plasma served as the reference. 50 μL of agonist was added to the platelet-rich plasma and the changes in light transmission recorded. The aggregation studies were conducted 60 minutes after venepuncture and were completed within 2 hours.

In aggregometric studies, the parameter considered is light absorption, which is evaluated on a scale of 0-100%. Because platelets are randomly dispersed in platelet-rich plasma and virtually absent in platelet-poor plasma, light absorption is very high

with platelet-rich plasma and very low with platelet-poor plasma. At the beginning of each aggregometric experiment, light absorption of the platelet-rich plasma examined is conventionally set at 90% and light absorption of platelet-poor plasma at 10%. When platelets aggregate, light absorption is reduced and the reduction correlates with the extent of platelet aggregation. The extent of platelet aggregation can be determined by measuring the maximum amplitude of increase in light transmission, directly from the aggregometer tracings in light transmission units. A very reliable parameter to quantify platelet aggregation is the agonist concentration able to induce a value of maximal aggregation of 50% ($EC_{50}$). For this purpose, at least four doses of each agonist are used. With the maximal aggregation values corresponding to different agonist concentrations, it is possible to obtain a dose-response curve. This curve is subsequently transferred to a semilogarithmic scale by plotting the logarithms of agonist concentrations on the x-axis and the corresponding maximal aggregation values on the y-axis. A sigmoidal dose-response curve is obtained and using the software Prism (Graphpad), the $EC_{50}$ is determined using nonlinear regression. Reproducibility testing showed an intra-individual variance in response to agonist of 10-15%.

## 7.4 Results

Thirty-six normal volunteers were included in the study. Those volunteers whose platelet aggregation measurements were not possible and those who could not be genotyped were not included. Table 7.4-1 summarises the demographics of this group. There were 13 (36%) with CC genotype, 15 (42%) with CT genotype and 8 (22%) with TT genotype. There were more females in the CC and CT groups but the difference was not significant. The BMI, systolic blood pressure were not

significantly different between the three groups. The TT group had a higher diastolic pressure compared to the other two groups.

**Table 7.4-1 Summary of the normal volunteers and stratified by their GNB3 genotype**

| | CC | CT | TT | P |
|---|---|---|---|---|
| N | 13 | 15 | 8 | |
| Age | 27.6 (7.2) | 28.7 (9.7) | 29.3 (6.5) | 0.9 |
| Male:Female | 5:8 | 10:5 | 4:4 | 0.3 |
| BMI | 24.2 (2.9) | 23.6 (2.1) | 25.8 (6.9) | 0.5 |
| SBP | 117.7 (11.5) | 121.0 (6.4) | 123.5 (20.6) | 0.7 |
| DBP | 70.1 (7.0) | 62.9 (4.4) | 74.3 (13.5) | 0.06 |
| Smoker % | 15.3 | 40 | 25 | 0.34 |
| Epinephrine $EC_{50}$ | 13.25 (10.7) | 19.37 (13.8) | 16.06 (5.7) | 0.43 |
| Epinephrine $EC_{50}$- Males | 22.1 (12.1) | 20.9 (15.4) | 14.2 (6.9) | 0.79 |
| Epinephrine $EC_{50}$- Females | 7.4 (3.9) | 16.4 (11.7) | 17.3 (5.9) | 0.12 |
| PAF $EC_{50}$ | 226 (58.8) | 231.1 (72.0) | 267.4 (96.4) | 0.65 |
| PAF $EC_{50}$ – Males | 243.8 (51.6) | 319.3 (223.6) | 340.4 (38.4) | 0.76 |
| PAF $EC_{50}$ – Females | 214.2 (64.8) | 201.6 (44.6) | 219.0 (92.3) | 0.93 |
| ADP $EC_{50}$ | 1.59 (1.27) | 0.95 (0.84) | 0.86 (0.43) | 0.26 |
| ADP $EC_{50}$ – Males | 1.33 (1.35) | 1.08 (0.99) | 1.07 (0.46) | 0.92 |
| ADP $EC_{50}$ – Females | 1.76 (1.32) | 0.71 (0.46) | 0.73 (0.45) | 0.22 |

Table 7.4-1 shows the dose response curve relating ADP concentration to platelet aggregation. For each individual, aggregation was carried out with the three agonists and dose response curve plotted and the $EC_{50}$ derived using GraphPad Prism. The results were analysed using one-way ANOVA to check if GNB3 genotype influenced platelet aggregation. There is a lack of association between platelet aggregation and GNB3 genotype for all three agonists when the analysis was pooled or stratified by sex.

Figure 7.4-2,Figure 7.4-3 and Figure 7.4-4 demonstrates the lack of association between GNB3 genotype and platelet aggregation. Though the numbers repeating the analysis for each sex separately also showed no association (Table 7.4-1). Using the general linear model to adjust for age, sex blood pressure and GNB3 genotype, only age and blood pressure were found to be significant predictors of epinephrine induced

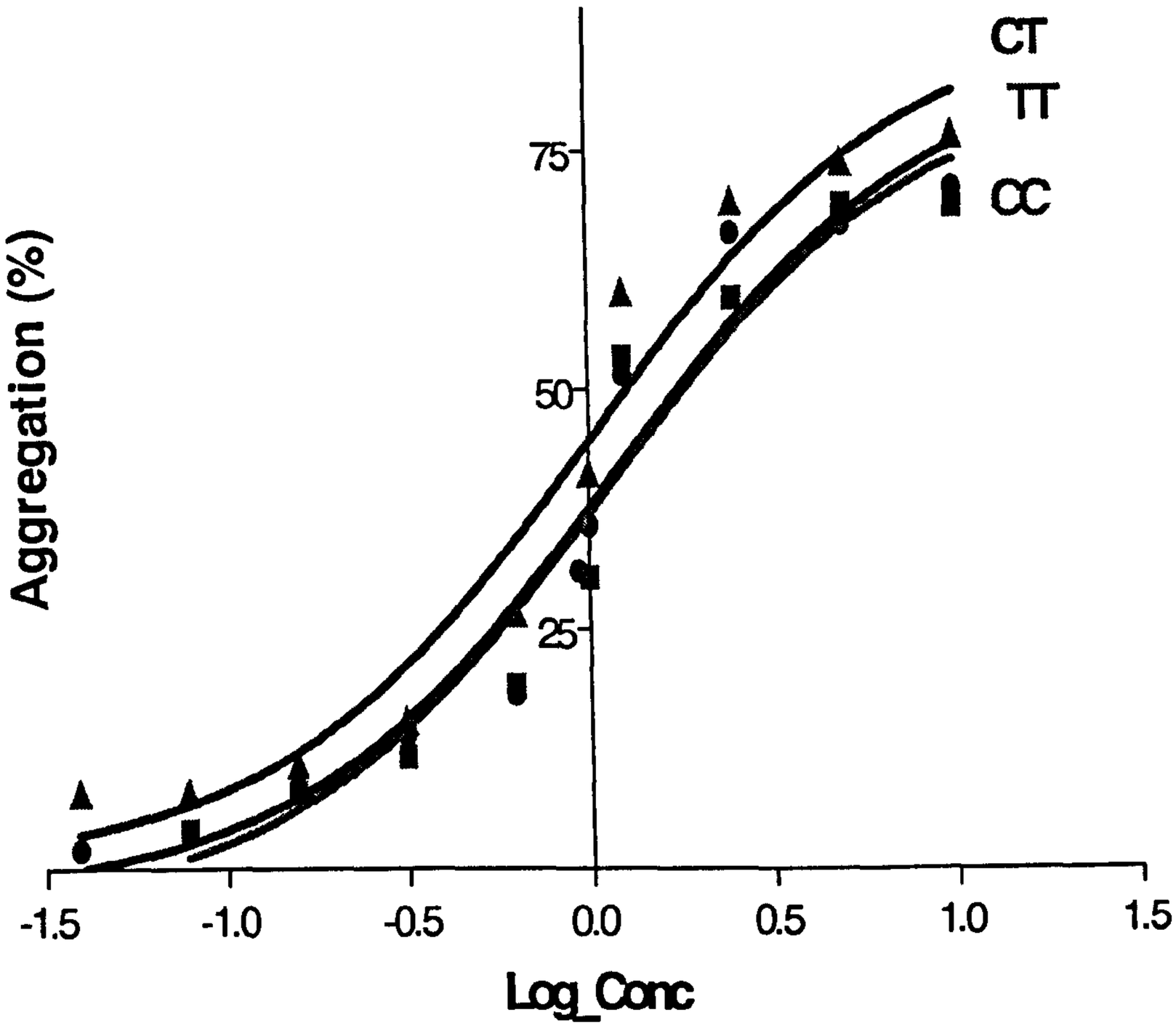


**Figure 7.4-1 An example of the dose response curve with log concentration of ADP plotted on the X-axis and percentage platelet aggregation on the Y axis.**

platelet aggregation (Table 7.4-2). There was no significant effect of any of these parameters on ADP or PAF induced platelet aggregation. GNB3 genotype did not affect any of the three agonist induced platelet aggregation.

| | F | p |
|---|---|---|
| Age | 10.19 | 0.011 |
| Sex | 0.24 | 0.648 |
| Systolic BP | 9.64 | 0.013 |
| Diastolic BP | 6.63 | 0.030 |
| GNB3 Genotype | 0.11 | 0.901 |

**Table 7.4-2 Epinephrine induced platelet aggregation used as the dependent variable and age, sex, blood pressure and GNB3 genotype used as predictors in a general linear model.**

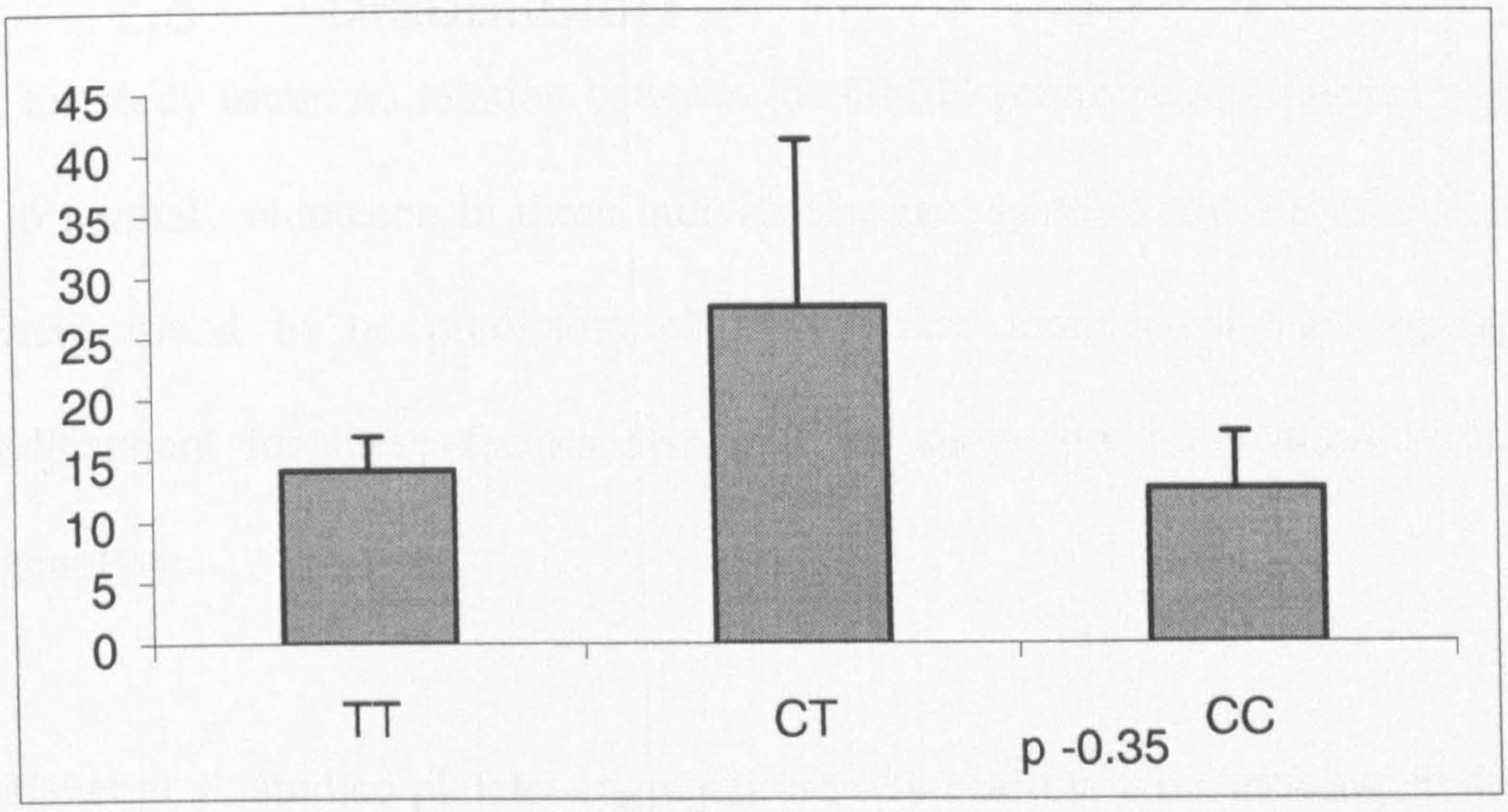


**Figure 7.4-2 EC 50 of epinephrine induced platelet aggregation stratified by GNB3 genotype**

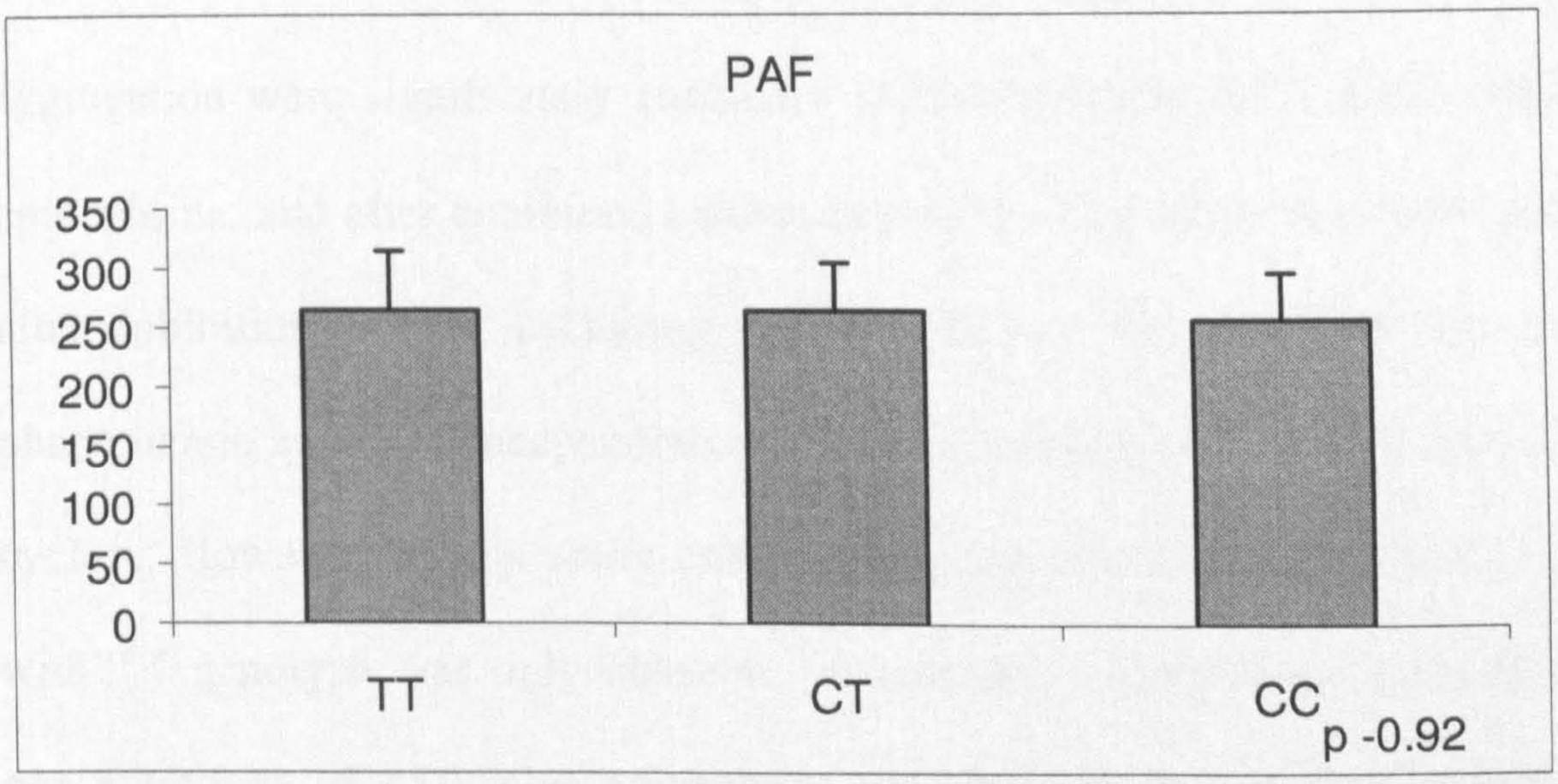


**Figure 7.4-3 EC 50 of PAF induced platelet aggregation stratified by GNB3 genotype.**

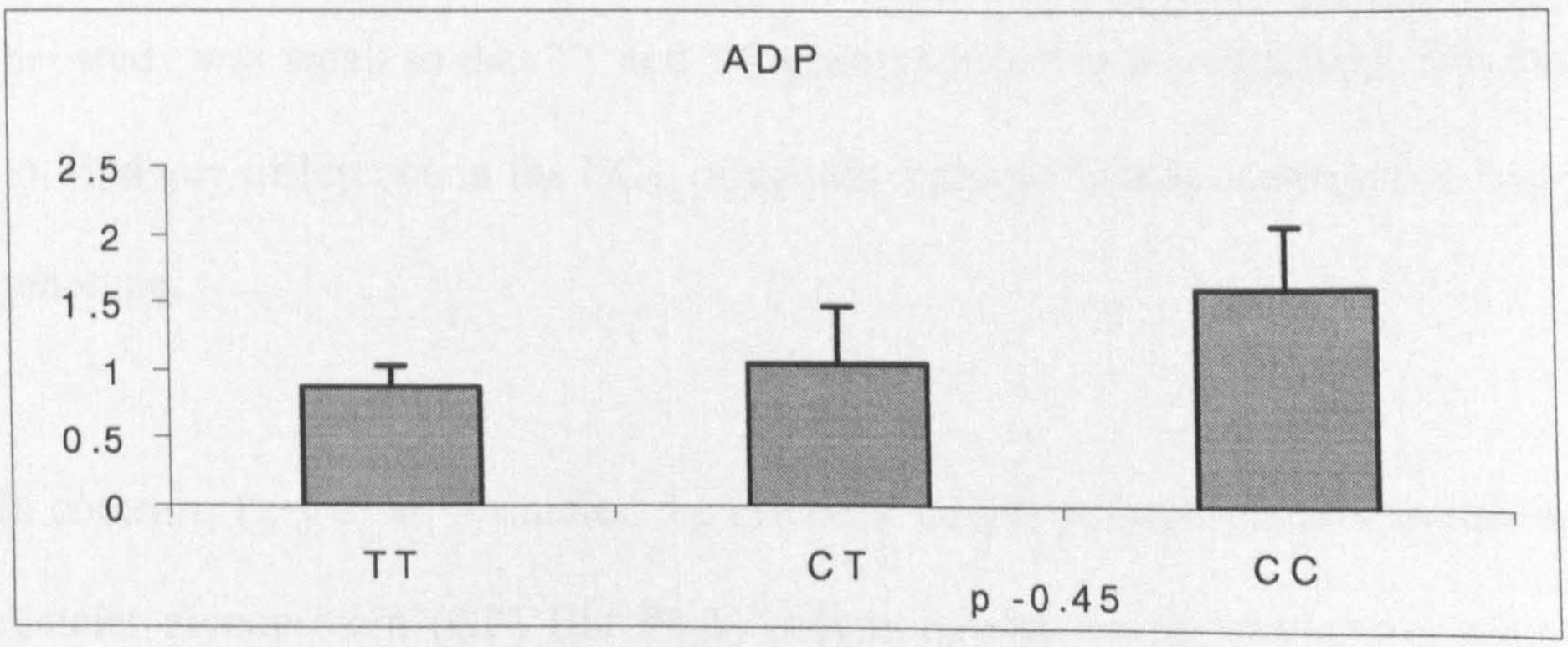


**Figure 7.4-4 EC 50 of ADP induced platelet aggregation stratified by GNB3 genotype.**

## 7.5 Discussion

This study found no relation between the GNB3 genotype and platelet aggregation in 36 normal volunteers. In these individuals, age, systolic and diastolic blood pressure were found to be predictors of epinephrine induced platelet aggregation. But adjustment for these factors also did not show any association with the GNB3 genotype.

Naber et al. studied platelet aggregation in 34 healthy, non-smoking, male individuals between 18 and 40 years of age with no medication known to interfere with platelet function for at least 15 days.[723] They found slopes and maximum of the secondary aggregation were significantly enhanced in platelets from 825T allele carriers after epinephrine, and after combined epinephrine/ADP. This effect was more pronounced after inhibition of the cyclooxygenase-2 pathway by acetylsalicylic acid. This phenomenon appeared independent of platelet secretion, or inhibition of the adenylyl cyclase. However, in this study enhanced aggregation of platelets from individuals with TT genotype was only observed on combined stimulation of platelets by low concentrations of ADP plus epinephrine and more marked during inhibition of the cyclooxygenase pathway by aspirin. Moreover, the number of subjects included in that study was small so that TT and TC genotypes had to be combined. This study did not find any difference in the $EC_{50}$ of agonist induced platelet aggregation and GNB3 genotype.

In contrast, Frey et al.[802] studied the effect of GNB3 polymorphism together with the platelet glycoprotein (GP) IIIa Pl(A) polymorphism on platelet aggregation in 150 healthy, non-smoking individuals aged 18–40. They found that individuals showing

the GNB3 CC-genotype display an enhanced platelet aggregation in response to low concentrations of agonists which act via G protein-coupled receptors. Moreover, non-responders to low concentrations of ADP were more likely to be T-allele carriers. This observation was particularly pronounced with agonists acting via Gi-coupled receptors. This is not surprising since the β3 subunit couples to Gi and altered cellular signalling via Gi coupled receptors has been shown to be associated with the GNB3 genotypes.[579;803]

Increased platelet aggregability is a major cardiovascular risk factor. Though some studies have shown individuals with GNB3 TT genotype have an increased risk for infarction and stroke, the relation between GNB3 and platelet aggregation is tenuous. Our experiment and the two other studies quoted above show no robust association. One can only speculate that other processes increase the risk for myocardial infarction in 825T allele carriers, e.g. increased vasoconstriction.[724;732;804] Also infarction is considered a complex process initiated by rupture of vulnerable plaques. It is sensible to assume that many different mechanisms contribute to this process like vasoconstriction, impaired endothelium function, and coagulation.

# Chapter 8 - MOLECULAR GENETIC STUDY OF GNB3

## 8.1 Introduction

The ability to bind and hydrolyse guanine nucleotides as integral parts of their activation-deactivation cycle is perhaps the most prominent functional characteristic of signal-transducing heterotrimeric G proteins. Measurement of the effects of receptor activation on high-affinity GTPase activity provides the basis of a variety of methods used to probe G-protein function.

The G protein signalling cycle is shown in Figure 1.5-3. Activation of receptor by the agonist initiates the release of guanosine 5'-diphosphate (GDP) from the $\alpha$-subunit of the heterotrimer, followed by the binding of guanosine 5'-triphosphate (GTP). This leads to the dissociation of the $\alpha\beta\gamma$ heterotrimer into the GTP-liganded $\alpha$ subunit and the free $\beta\gamma$ dimer, both of which can interact with a variety of effectors to modulate intracellular second messenger concentrations. Deactivation occurs from hydrolysis of GTP by the intrinsic GTPase activity of the $\alpha$ subunit, and subsequent reassociation of the heterotrimer. (Section 1.5.2, page 169)

As described in Section 1.5.4.1($\beta\gamma$ Targets, page 176), G$\beta\gamma$ serves as the direct activator of certain G protein-responsive $K^+$, $Ca^{2+}$, and perhaps also $Na^+$ channels. Changes in the cytoplasmic free $Ca^{2+}$ concentration constitute one of the main pathways by which information is transferred from extracellular signals received by animal cells to intracellular sites. The intracellular $Ca^{2+}$ signal is conveyed by the magnitude, location and duration of the changes in $Ca^{2+}$. Increases in $Ca^{2+}$ in a given region of the cytoplasmic space are usually initiated by the binding of an extracellular signalling molecule (agonist) to its plasma-membrane receptors. The sarcoplasmic reticulum in excitable cells and the endoplasmic reticulum in non-excitable cells are

the major intracellular $Ca^{2+}$ stores. $Ca^{2+}$ ions flow from within these stores into the cytoplasm through two types of $Ca^{2+}$ channels: IP3Rs and ryanodine receptors (RyRs). IP3, released into the cytoplasm when cells are activated by external stimuli, activates the IP3R. Three isoforms of IP3Rs (IP3R1, IP3R2 and IP3R3) are known. Released $Ca^{2+}$ then exerts a positive feedback on its own release. Subsequent inhibition of the $Ca^{2+}$ release is then caused by the decreasing luminal $Ca^{2+}$ concentration and by the increase in the concentration of the cytoplasmic $Ca^{2+}$/calmodulin-complex.

The functional analysis of the GNB3 is performed by measuring GTPase activity and intracellular calcium signals in cells transfected with $G\beta_3\gamma_5$ along with a fusion construct of $\alpha_{2A}$-adrenoceptor with $G\alpha_i$ subunit. The $\alpha_2$-adrenoceptor subtypes represent the prototypic examples of G-protein-coupled receptors (GPCRs) which mediate inhibition of adenylate cyclase activity via interaction with members of the $G_i$-subfamily of heterotrimeric G-proteins.[805] With the exception of $G_z$,[806;807] the $\alpha$-subunits of the $G_i$-like G-proteins all possess a conserved cysteine residue four amino acids away from the C-terminus which can act as the acceptor for ADP-ribosylation catalysed by pertussis toxin.[808] Thus Senogles[809] and by Hunt et al.[810] have described a scheme to replace the cysteine residue which acts as the target for pertussis toxin-catalysed ADP-ribosylation. In this way, treatment of a cell expressing this pertussis toxin-resistant form of a $G_i$-like G-protein with pertussis toxin results in attenuation of the coupling of a GPCR to the endogenous wild-type $G_i$-like G-proteins, thus allowing examination of the interaction of the GPCR with the mutationally modified G-protein in isolation.

The fusion constructs of $\alpha_2$-adrenoceptor and pertussis toxin resistant $G\alpha_i$ allowed 1:1 stoichiometry between the GPCR and G protein thus enabling specific functional assessment. GTPase activity is used to measure the capacity of a ligand to induce a functional response and allows comparison with various $\beta_3$ subunits. The single cell calcium signalling experiment is used to study the downstream effect of different $\beta_3$ subunits. In these experiments as mentioned above, the $\alpha_{2A}$ G protein and $\gamma$ subunits remained constant. The experiments were also performed using $\alpha_{2A}G_i\beta_1\gamma_2$.

## 8.2 Laboratory methods

The high affinity GTPase assays were performed in HEK293 cells cotransfected with $\alpha_{2A}$ adrenoceptor-$Cys^{351}G_{i1\alpha}$ fusion construct along with $\beta_1\gamma_2$ or $\beta_3\gamma_5$ or $\beta_{3\text{-}s}\gamma_5$. The plasmid DNA incorporating these cDNAs were initially amplified and for use in subsequent experiments. *Section 3.3 Cell Transformation and Amplification of plasmid DNA* (page 214) describes the procedure. After this cell lines were transfected with the cDNA as described in *section 3.4 Transfections and membrane preparation of HEK293 and COS-7* (page 217). The formal protocol for the GTPase assay and calculations involved are described in *section 3.6 High affinity GTPase assays* (page 223). The calcium signalling study was performed in EF88 cells cotransfected with the $\alpha_{2A}$ adrenoceptor-$Cys^{351}G_{i1\alpha}$ fusion construct along with $\beta_3\gamma_5$ or $\beta_{3\text{-}s}\gamma_5$. The protocol for calcium signalling experiment is given in *section 3.7 Single cell (EF88) calcium* (page 227).

## 8.3 Results

### *8.3.1 GTPase Assay $\alpha_{2A}G_i$ cotransfected with $\beta_1$ and $\gamma_2$*

Figure 8.3-1 shows GTPase measured in HEK293 cells cotransfected with $\alpha_{2A}G_i$ and $\beta_1\gamma_2$. The experiment was carried out in three different transfected cell lines - Cells

transfected with $\alpha_{2A}G_i$ alone, $\alpha_{2A}G_i$ with $\beta_1\gamma_2$, $\beta_1\gamma_2$ alone, and PCDNA3 alone. High affinity GTPAse was measured in each case, after 24-hour incubation with pertussis toxin. In Hek293 cells transfected with $\alpha_{2A}G_i$ alone, the basal GTPase activity was 8.19 ± 0.12 pmol/mg/min and after stimulation with 100μM epinephrine the GTPase activity was 36.46 ± 13.07 pmol/mg/min. In HEK293 cells after transfection with $\alpha_{2A}G_i$ and $\beta_1\gamma_2$ subunits, the basal and stimulated GTPase activity were 11.31 ± 0.24 pmol/mg/min and 47.47 ± 18.67 pmol/mg/min respectively. The basal and stimulated GTPase activity when measured in cells transfected $\beta_1\gamma_2$ alone (9.35 ± 16.15 and 0.32 ± 7.60 pmol/mg/min respectively) and PCDNA3 alone (10.24 ± 0.64 and 16.64 ± 7.68 pmol/mg/min respectively) without $\alpha_{2A}G_i$ were small indicating that the measured high affinity GTPase was indicative of the functional response mediated by the agonist epinephrine on the $\alpha_{2A}G_i$ construct. There was no difference in the expression level of the fusion construct in the different experiments. The expression of $\alpha_{2A}G_i$ in cells expressing this alone was 2.41 pmol/mg and in cells cotransfected with $\alpha_{2A}G_i$ and $\beta_1\gamma_2$ was 2.27 pmol/mg.

Figure 8.3-2 shows the result of high affinity GTPase measured over a range of concentration of GTP. Using Michaelis-Menten kinetics, the Vmax and Km is calculated for each experiment. The increase in Vmax due to epinephrine in cells expressing $\alpha_{2A}G_i$ alone was 43.63 pmol/mg/min and in cells expressing $\alpha_{2A}G_i$ with β1γ2 it was 47.77 pmol/mg/min. Given the expression levels above and since the receptor and G protein within the fusion are present in 1:1 molar ratio, the agonist induced turnover number was calculated as 18.06 $min^{-1}$ and 21.02 $min^{-1}$ respectively.

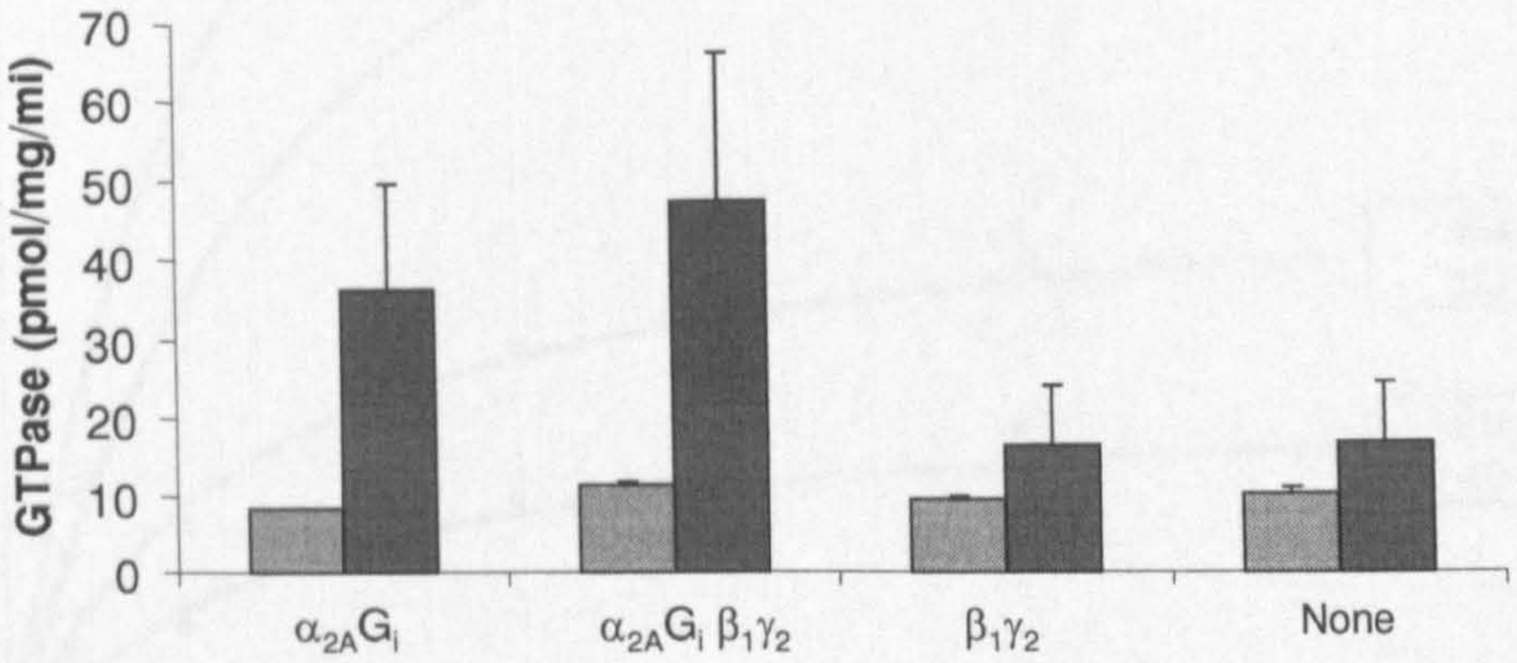


**Figure 8.3-1 Agonist stimulation of high affinity GTPase activity of $\alpha_{2A}G_i$ fusion protein. Membrane fractions from pertussis toxin treated HEK293 cells cotransfected with $\alpha_{2A}G_i$ fusion protein, $G\beta_1$ and $G\gamma_2$ subunits were used to measure GTPase activity.**

**- Basal GTPase activity - GTPase activity in the presence of $10^{-4}$M Epinephrine**

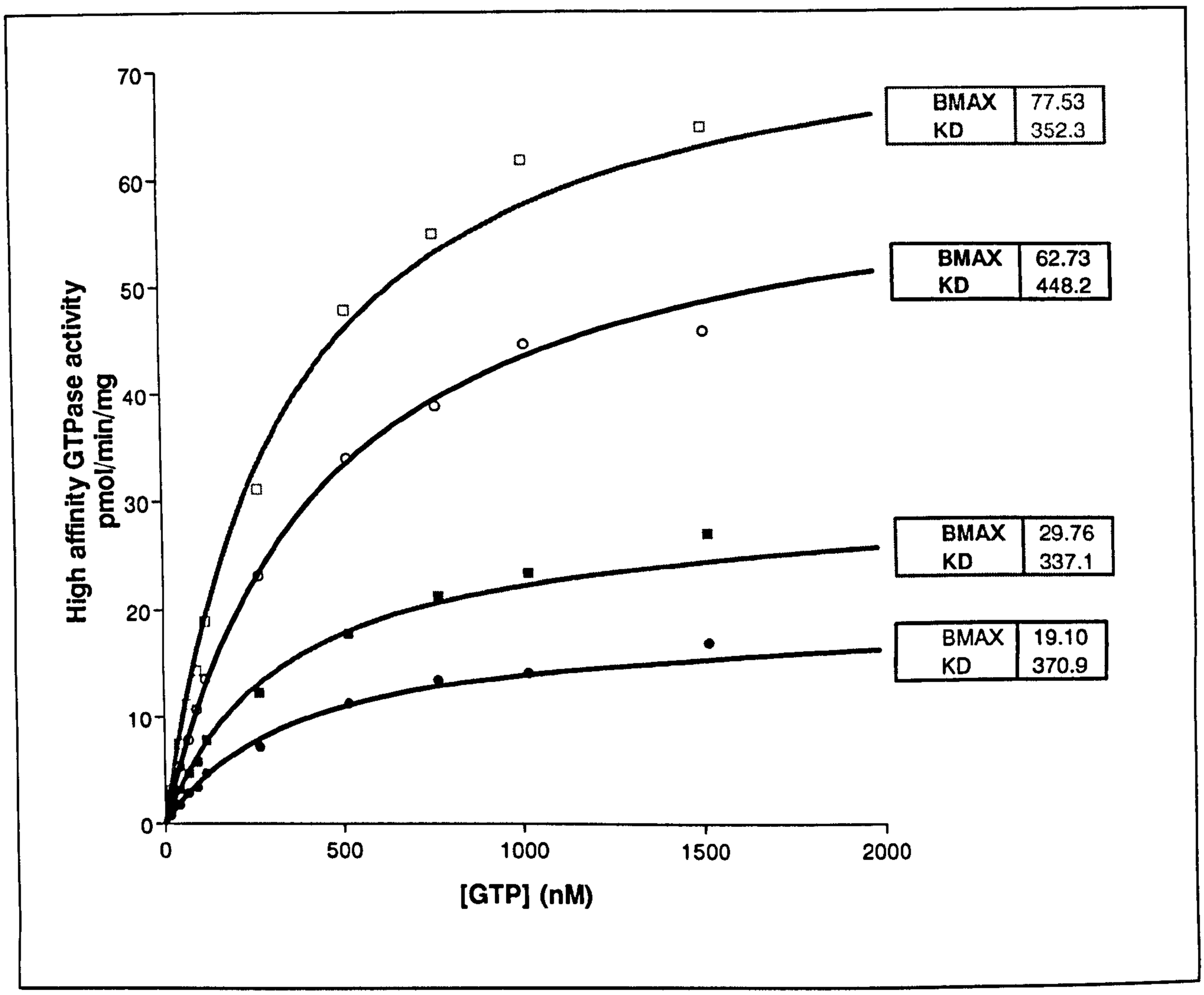


**Figure 8.3-2 GTPase assay $\alpha_{2A}G_i$ cotransfected with β1 and γ2**

Agonist stimulation of high affinity GTPase activity of $a_{2A}G_i$ fusion protein. Membrane fractions from pertussis toxin treated HEK293 cells cotransfected with a2AGi fusion protein, $G\beta_1$ and $G\gamma_2$ subunits were used to measure GTPase activity. High affinity GTPase activity measured for varying concentrations of GTP in membranes of pertussis toxin treated HEK293 cells transfected to express $\alpha_{2A}G_i$ with and without $\beta_1\gamma_2$. In membranes expressing $\alpha_{2A}G_i$: ●- Basal GTPase activity and ○- GTPase activity in the presence of $1x10^{-4}$M epinephrine. In membranes expressing $\alpha_{2A}G_i$ $\beta_1\gamma_2$: ■- basal GTPase activity, □- GTPase activity in the presence of $1x10^{-4}$M epinephrine.

### 8.3.2 GTPase Assay $\alpha_{2A}G_i$ cotransfected with $\beta_3/\beta_{3-s}$ and $\gamma_5$

To compare the functional response between the wild type $\beta_3$ subunit and the splice variant $\beta_{3s}$ subunit, the experiment was carried out in HEK cells cotransfected with $\alpha_{2A}G_i$ $\beta_3\gamma_5$ and $\alpha_{2A}G_i\beta_{3-s}\gamma_5$. The GTPase activity was measured in each case in response to 100μM Epinephrine and Vmax and Km measured by using graded concentrations of the substrate GTP. The results are shown in Figure 8.3-3 and Figure 8.3-4.

Figure 8.3-3 shows GTPase measured in HEK293 cells cotransfected with $\alpha_{2A}G_i$ and $\beta_3\gamma_5$ or $\beta_{3-s}\gamma_5$. The experiment was carried out in cells transfected with $\alpha_{2A}G_i$ alone, $\alpha_{2A}G_i$ with $\beta_3\gamma_5$, $\alpha_{2A}G_i$ with $\beta_{3-s}\gamma_5$, $\beta_3\gamma_5$ alone, $\beta_{3-s}\gamma_5$ alone and PCDNA3 alone and high affinity GTPAse was measured in each case, after 24 hour incubation with pertussis toxin. In Hek293 cells transfected with $\alpha_{2A}G_i$ alone and, the basal GTPase activity was 7.53 ± 2.15 pmol/mg/min and after stimulation with 100μM epinephrine the GTPase activity was 58.69 ± 1.46 pmol/mg/min. In HEK293 cells after transfection with $\alpha_{2A}G_i$ and $\beta_3\gamma_5$ subunits, the basal and stimulated GTPase activity were 9.60 ± 0.56 pmol/mg/min and 76.75 ± 2.75 pmol/mg/min respectively. The basal and stimulated GTPase activity when measured in cells transfected with $\alpha_{2A}G_i$ and $\beta_{3-s}\gamma_5$ subunits were 6.62 ± 0.26 and 57.20 ± 1.59 pmol/mg/min respectively.

Figure 8.3-4 shows the result of high affinity GTPase measured over a range of concentration of GTP. Using Michaelis-Menten kinetics, the Vmax and Km is calculated for each experiment. The increase in Vmax due to epinephrine in cells

expressing $\alpha_{2A}G_i$ alone was 98.94 pmol/mg/min, in cells expressing $\alpha_{2A}G_i$ with $\beta_3\gamma_5$ it was 110.22 pmol/mg/min and in cells expressing $\alpha_{2A}G_i$ with $\beta_{3\text{-}s}\gamma_5$ it was 115.09 pmol/mg/min. The expression of the fusion protein in cells transfected with $\alpha_{2A}G_i$ alone was 1.34 pmol/mg and thus the agonist induced turnover in these cells was 73.75 $min^{-1}$. In cells expressing $\alpha_{2A}G_i$ with $\beta_3\gamma_5$, the expression of the fusion protein was 2.20 pmol/mg and the agonist induced turnover 49.9 $min^{-1}$, and in cells expressing $\alpha_{2A}G_i$ with $\beta_{3\text{-}s}\gamma_5$, the expression of the fusion protein was 2.20 and the agonist induced turnover 52.2 $min^{-1}$.

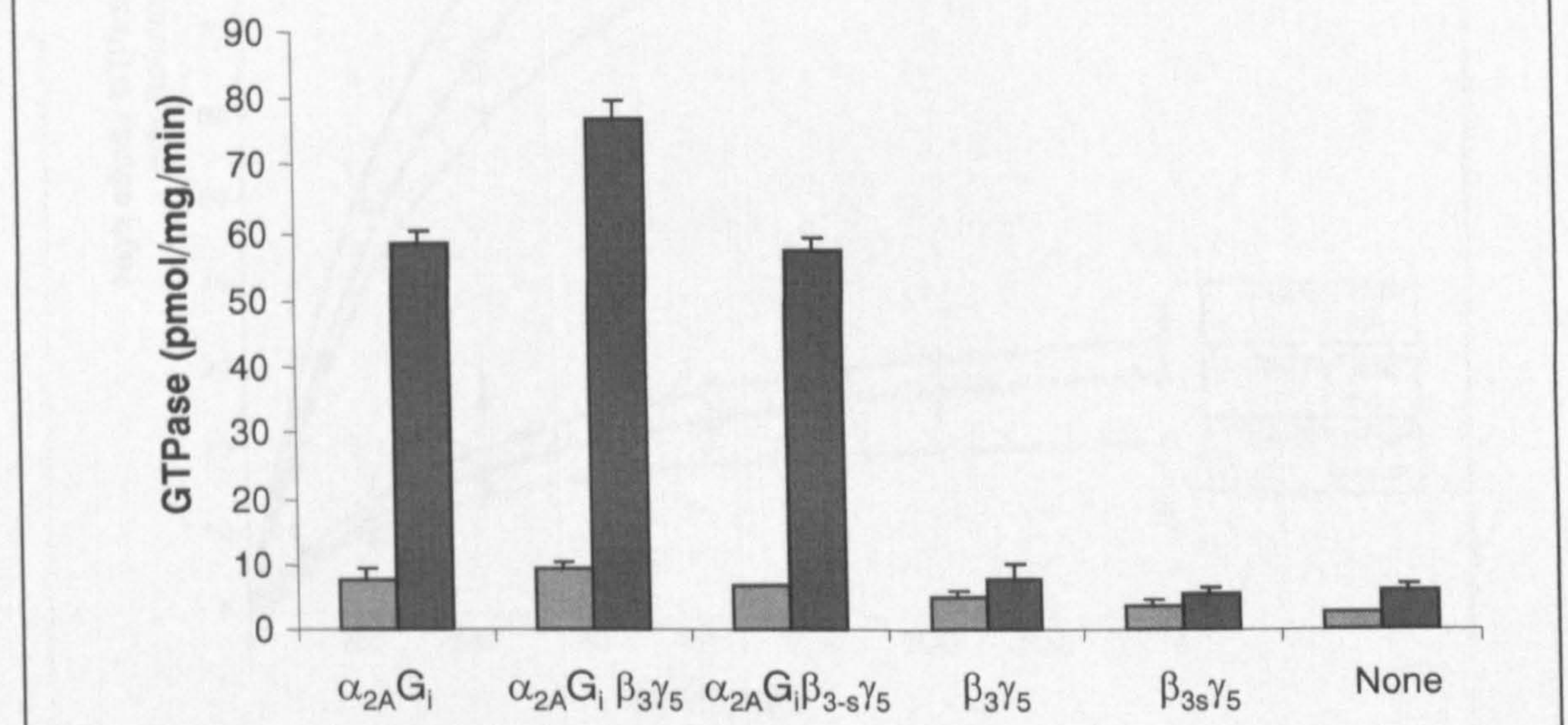


**Figure 8.3-3 Agonist stimulation of high affinity GTPase activity of $\alpha_{2A}G_i$ fusion protein. Membrane fractions from pertussis toxin treated HEK293 cells cotransfected with $\alpha_{2A}G_i$ fusion protein, $G\beta_3$ or $G\beta_{3\text{-}s}$ and $\gamma_5$subunits were used to measure GTPase activity.**

**- Basal GTPase activity GTPase activity in the presence of $10^{-4}$M Epinephrine**

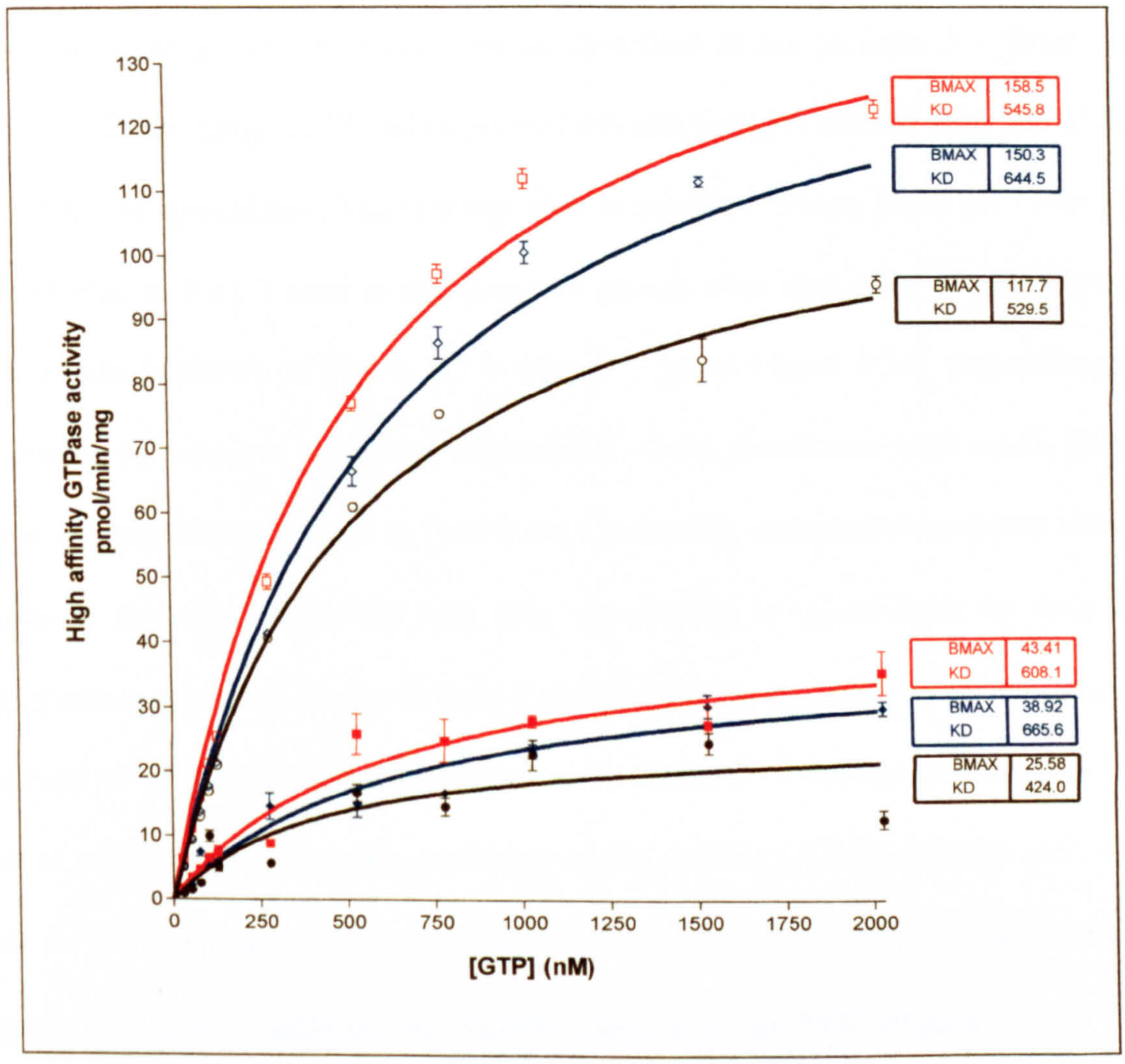


**Figure 8.3-4 GTPase assay - $\alpha_{2A}G_i$ cotransfected with $\beta_3$ or $\beta_{3-s}$ and $\gamma_5$**

Agonist stimulation of high affinity GTPase activity of $a_{2A}G_i$ fusion protein. Membrane fractions from pertussis toxin treated HEK293 cells cotransfected with $a_{2A}G_i$ fusion protein, $G\beta_3$ or $G\beta_{3-s}$ and $\gamma_5$ subunits were used to measure GTPase activity.High affinity GTPase activity measured for varying concentrations of GTP in membranes of pertussis toxin treated transfected HEK293 cells. In membranes expressing $\alpha_{2A}G_i$: ●- Basal GTPase activity, ○- GTPase activity in the presence of $1x10^{-4}$M epinephrine. In membranes expressing $\alpha_{2A}G_i$ $\beta_3\gamma_5$: ■- Basal GTPase activity, □- GTPase activity in the presence of $1x10^{-4}$M epinephrine. In membranes expressing $\alpha_{2A}G_i$ $\beta_{3-s}\gamma_5$: ◆ Basal GTPase activity, ◇- GTPase activity in the presence of $1x10^{-4}$M epinephrine.

### 8.3.3 Calcium signalling in EF88 cells cotransfected with $\alpha_{2A}G_i$, $\beta_3/\beta_{3-s}$ and $\gamma_5$

EF88 cells were transfected with $\alpha_{2A}G_i$ alone, $\alpha_{2A}G_i$ with $\beta_3\gamma_5$ and $\alpha_{2A}G_i$ with $\beta_{3-s}\gamma_5$. The cells were grown on cover slips as described above [*section 3.7 Single cell (EF88) calcium* (page 227)] and single cell calcium signals recorded after stimulating with 100 μM epinephrine. The readings were taken from at least 3 cell per cover slip. The change in Fura-2 ratio in the three cell groups over time after stimulation with epinephrine is shown in Figure 8.3-6. Figure 8.3-6 to Figure 8.3-8 summarises the results of the calcium signalling experiments. Cells transfected with $\alpha_{2A}G_i$ do not show any significant change in Fura-2 ratio indicating undetectable calcium release. However the cells transfected with $\beta_3\gamma_5$ in addition to $\alpha_{2A}G_i$ show an early and significantly increased change in Fura-2 ratio indicating activation of the downstream pathway of G protein signalling releasing Ca from the endoplasmic reticulum. The rate of calcium release in cells expressing $\alpha_{2A}G_i$ alone was 0.0010±0.0001 $sec^{-1}$ while that for cells expressing $\beta_3\gamma_5$ in addition was 0.004±0.0005 $sec^{-1}$ ($p<0.001$) and cells expressing $\beta_{3-s}\gamma_5$ in addition was 0.003±0.0002 $sec^{-1}$ ($p<0.01$) (Figure 8.3-8). Cells expressing $\alpha_{2A}G_i$ alone took 334.43 ± 19.74 seconds to reach peak calcium signal. This was significantly higher than cells expressing $\alpha_{2A}G_i$ along with $\beta_3\gamma_5$ (170.59 ± 6.82 sec) or $\beta_{3-s}\gamma_5$ (162.81 ± 10.21 sec) (Figure 8.3-5). The lag between addition of epinephrine and appearance of a calcium signal was shorter in the cells expressing $\beta_3\gamma_5$ (27.57 ± 1.51 sec) or $\beta_{3-s}\gamma_5$ (30.24 ± 1.73sec) compared to cells expressing $\alpha_{2A}G_i$ alone (40.30 ± 2.41 sec) but the differenece was only significant with the cells expressing $\beta_3\gamma_5$ (Figure 8.3-7). Though there was no difference between cells expressing β+ and $\beta_{3-s}$ in terms of onset delay of calcium

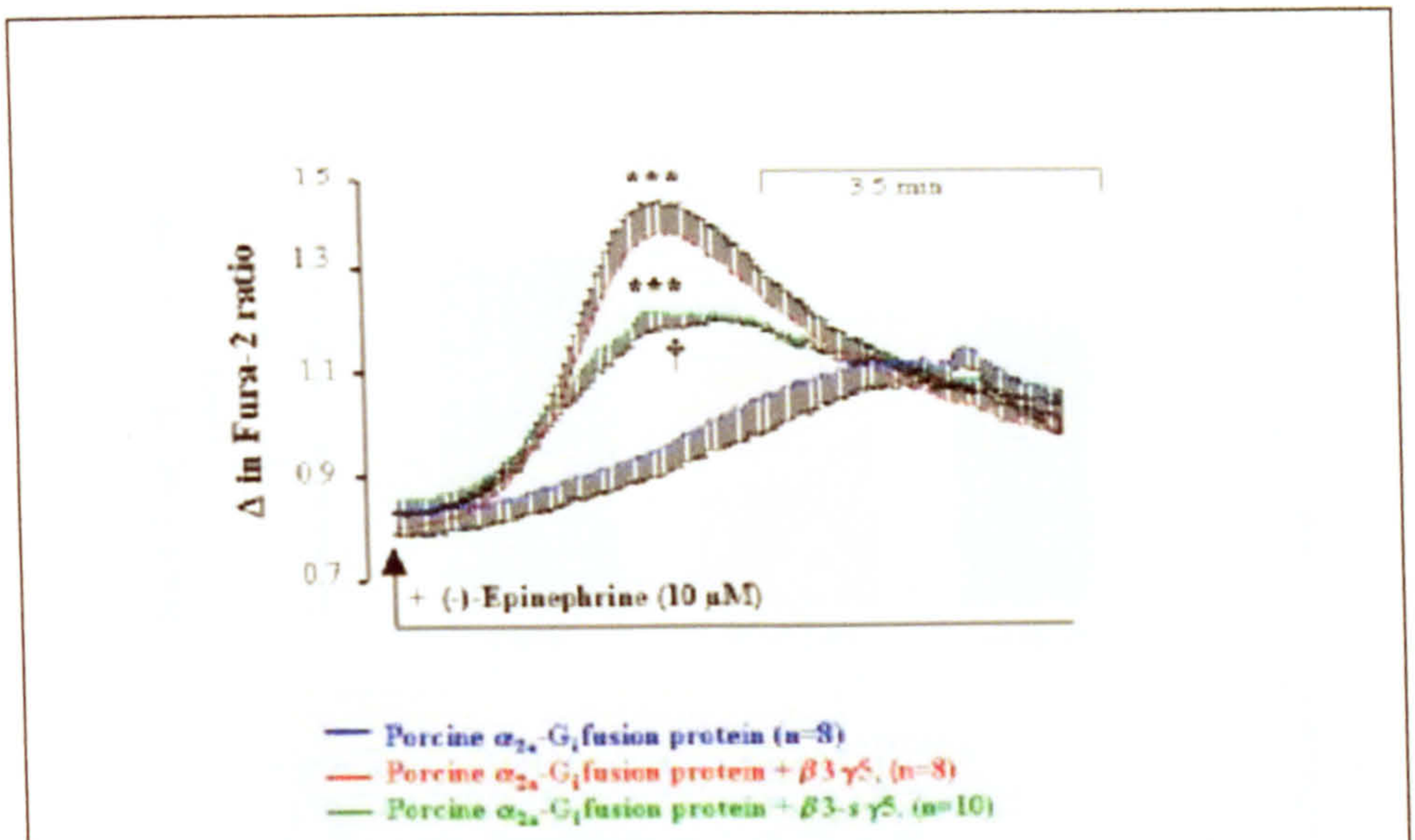


**Figure 8.3-6 Graph showing the change in intracellular Fura-2 ratio after addition of Epinephrine to EF88 cells transfected with $\alpha_{2A}G_i$ with or without $\beta_3/\beta_{3\text{-}s}$ and $\gamma_5$ subunits.**

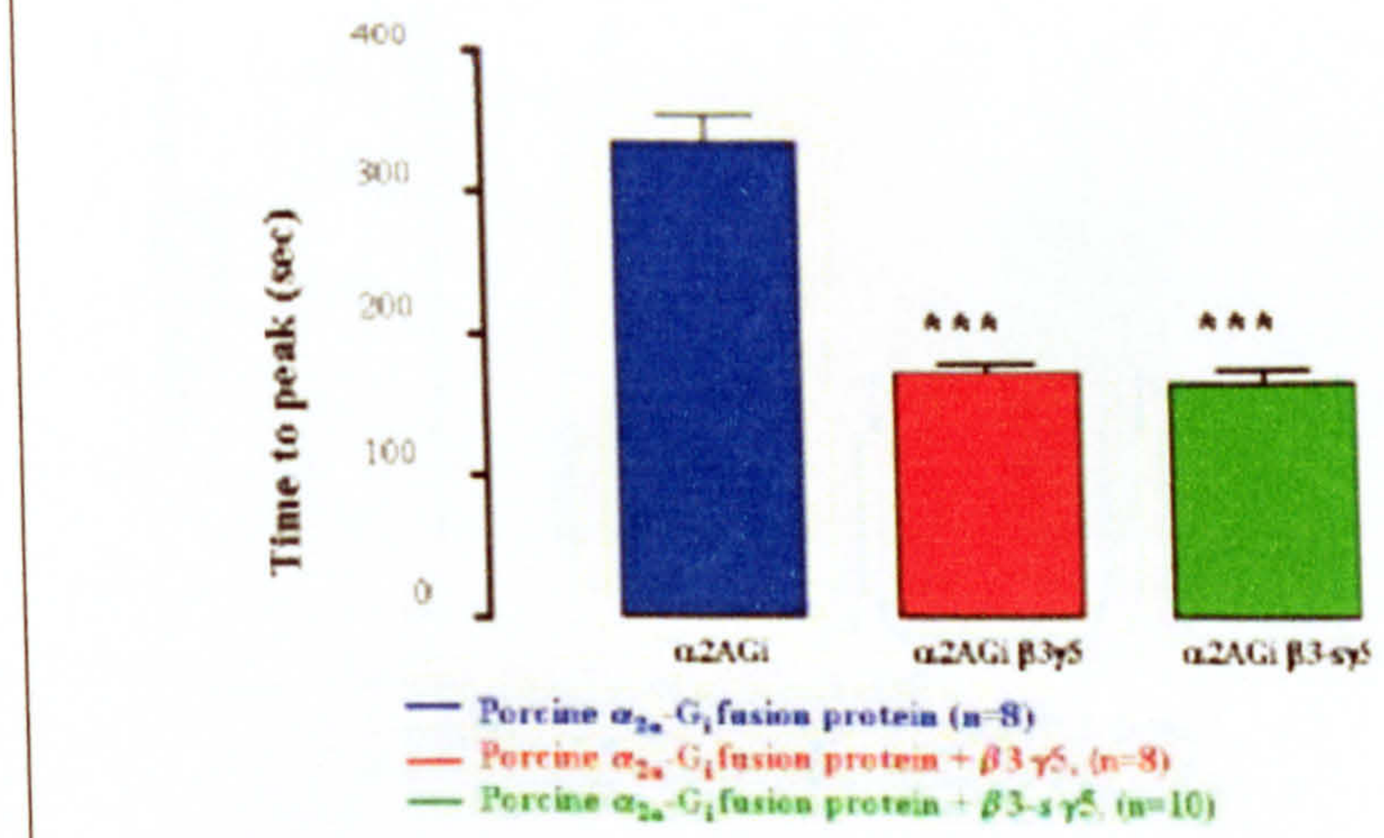


**Figure 8.3-5 Graph showing the time to attain peak calcium signal after addition of Epinephrine to EF88 cells transfected with $\alpha_{2A}G_i$ with or without β3/β3-s and γ5 subunits.**

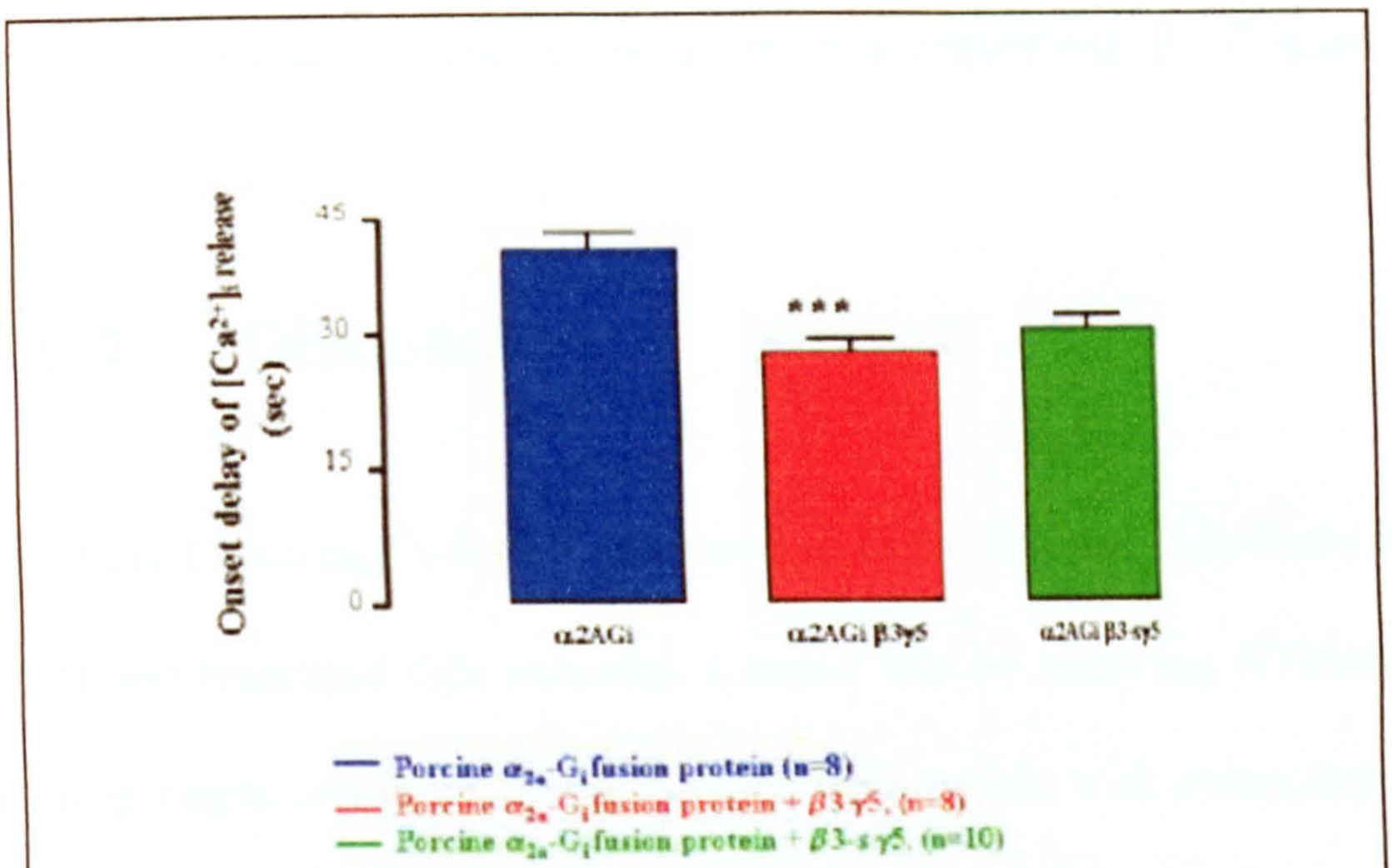


**Figure 8.3-7 Graph showing the delay in first detecting a calcium signal after addition of Epinephrine to EF88 cells transfected with $\alpha_{2A}G_i$ with or without $\beta_3/\beta_{3-s}$ and $\gamma_5$ subunits.**

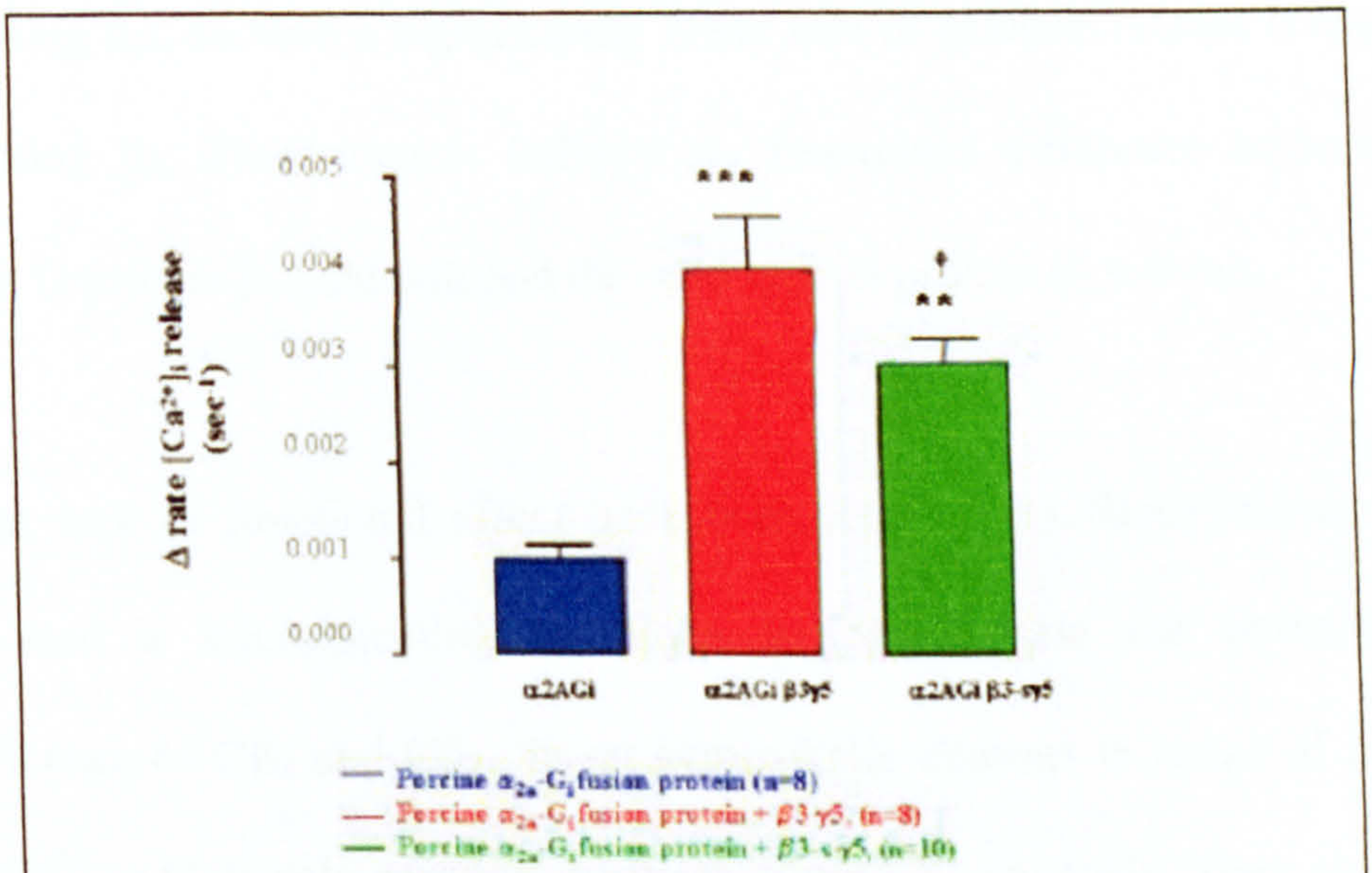


**Figure 8.3-8 Graph showing the rate of calcium release after addition of Epinephrine to EF88 cells transfected with $\alpha_{2A}G_i$ with or without $\beta_3/\beta_{3-s}$ and $\gamma_5$ subunits.**

release and time to peak of calcium signal, cells expressing $\beta_{3\text{-}s}$ showed a significantly lower rate of calcium release compared to cells expressing $\beta_3$. (Figure 3.7-1,Figure 8.3-8).

## 8.4 Discussion

In this study I used $\alpha_{2A}G_i$ fusion constructs to determine any functional differences in the wild and truncated $G\beta_3$ subunits. I tested this by assaying GTPase activity and measuring single cell calcium signals after stimulation with epinephrine. There was no significant difference between $\beta_3$ or $\beta_{3\text{-}s}$ transfected cells in terms of GTPase activity after stimulation with epinephrine. In terms of calcium signalling experiments, though there was no difference between cells expressing $\beta_3$ and $\beta_{3\text{-}s}$ in terms of onset delay of calcium release and time to peak of calcium signal, cells expressing $\beta_{3\text{-}s}$ showed a significantly lower rate of calcium release compared to cells expressing $\beta_3$. These results indicate no functional difference between the splice variant G protein $\beta_{3\text{-}s}$ subunit and the wild type G protein $\beta_3$ subunit.

Similar lack of functional effect have been reported by Ruiz-Velasco and Ikeda[767] who used a microinjection technique to demonstrate the potential functional significance of $G\beta_3$ and $G\beta_{3\text{-}s}$ in rat sympathetic neurons in terms of modulating N-type $Ca^{2+}$ or G-protein-gated inwardly rectifying $K^+$ channels. They also investigated whether $G\beta_{3\text{-}s}$ can couple to a pertussis toxin-insensitive $G_{i2}$ mutant or to $\alpha_2$-adrenoceptors and whether $G\beta_{3\text{-}s}$ can form a heterodimer with $G\gamma_2$. Dimerisation of G protein β-subunits with Gγ is required for interaction with all known effectors, as the β-subunits form a functional monomer.[811] Their findings suggest that $G\beta_{3\text{-}s}$ is

functionally inactive. First, in contrast to $G\beta_3/G\gamma_5$ and $G\beta_3/G\gamma_2$, neither $G\beta_{3\text{-}s}/G\gamma_5$ nor $G\beta_{3\text{-}s}/G\gamma_2$ evoked any basal facilitation of $Ca^{2+}$ currents or enhancement of $Ca^{2+}$ current. Second, no functional effect of $G\beta_{3\text{-}s}$ on $K^+$ channels could be found.

There are conflicting views regarding the association of the C825T allele and vascular disorders. Various studies, which have shown an association between C825T and disease states, have regarded $G\beta_{3\text{-}s}$ as a functional protein with an enhanced activity.[675;723;812] Nonetheless, the actual effect $G\beta_{3\text{-}s}$ exerts on G protein signalling mechanisms remains unresolved and little direct evidence exists that demonstrates $G\beta_{3\text{-}s}$ possesses modulatory activity.

Evidence available in the literature, which suggests $G\beta_{3\text{-}s}$ is a functional and overactive G protein subunit, is rather indirect. Overall, there are three main observations that have led investigators to this conclusion. First, G protein signalling pathways in neutrophils and platelets were found to be enhanced in individuals carrying the T allele compared with noncarriers. That is, neutrophils of C825T allele carriers had a significantly greater N-formyl-methionyl-leucyl-phenylalanine (fMLP)- and interleukin-8-induced chemotaxis.[675;812] Chemotaxis in all three genotypes was PTX sensitive, suggesting coupling through either $G\alpha_i$ or $G\alpha_o$. Moreover, a significantly greater epinephrine-induced platelet aggregation in those persons carrying the C825T allele was also reported.[723] Unfortunately, no direct evidence was provided to show $G\beta_{3\text{-}s}$ caused both the enhanced chemotaxis and platelet aggregation. These findings are in contrast to those of others that showed a significant effect on various cell functions and signal transduction components due to overexpressing $G\beta_{3\text{-}s}$. Overexpression of $G\beta_{3\text{-}s}$ in COS-7 (African green monkey

kidney) cells stimulated with lysophosphatidic acid was associated with a significantly increased chemotactic migration index (compared with the expression of $G\beta_3$).[675] Functional expression of $G\beta_{3\text{-}s}$ in COS-7 cells (confirmed by Western blot analysis) evoked a significantly enhanced activation of G proteins as monitored by GTPS binding over that observed in mock- or $G\beta_3$ -transfected cells. When Sf9 insect cells were infected with $G\beta_3$ and $G\beta_{3\text{-}s}$ along with $G_{i2}$, $G\gamma_5$, and the $m_2$-muscarinergic receptors, carbachol-stimulated binding of GTPS was enhanced 10-fold in those cells expressing $G\beta_{3\text{-}s}$ compared with cells expressing $G\beta_3$. These differences were not explained by large differences in the expression levels of $G\beta_3$ or $G\beta_{3\text{-}s}$.[813] These findings would suggest that $G\beta_{3\text{-}s}$ is capable of forming a functional heterodimer with $G\gamma_5$. This is in contrast to the findings of Rosskopf et al[681] who showed when $G\beta_{3\text{-}s}$ was overexpressed in HEK-TS cells, there was a spontaneous activation of the mitogen-activated protein kinase (MAP kinase) pathway, which was three times more pronounced than that following overexpression of $G\beta_1$, $G\beta_3$, or $G\beta_4$. A straightforward interpretation of these results suggests that the truncated $G\beta_{3\text{-}s}$ not only interacts with G subunits but is also capable of activating intracellular effector pathways with a greater efficacy than wild-type $G\beta_3$.[767] However, two recent studies have reported that in the same expression system (i.e., Sf9 cells), the expressed $G\beta_{3\text{-}s}$ could not be purified and thus not reconstituted with several $\gamma$-subunits.[814;815] These differences may be a result of different assay conditions employed. In this context, the experiments carried out in this study suggest no difference between the splice variant and the wild type $\beta_3$ subunit, while Ruiz-Velasco and Ikeda[767] report lack of function of the $G\beta_{3\text{-}s}$ subunit.

While only speculation can explain such divergent results, it must be emphasized that the original findings from Siffert et al.[579] may be regarded as artificial as they were all obtained in systems in which $G\beta_{3\text{-}s}$ was strongly overexpressed, e.g., in Sf9 insect cells or COS-7 and HEK TS cells, respectively. It appears reasonable to assume that such high expression levels greatly exceed those occurring under physiological conditions. An alternative explanation for the results of Ruiz-Velasco and Ikeda[767] would be that $G\beta_{3\text{-}s}$, due to its structural deletion, has lost its ability to interact with $Ca^{2+}$ and $K^+$ channels but retains its ability to stimulate the MAP kinase pathway. This latter hypothesis can seemingly be ruled out, as Ruiz-Velasco and Ikeda[767] also reported lack of dimerisation of labelled $G\beta_{3\text{-}s}$ with $G\gamma$ subunits and lack of formation of a G protein heterotrimer using fluorescence resonance energy transfer (FRET). Coprecipitation experiments of in vitro translated $G\beta$ subunits and epitope-tagged $G\gamma$ subunits suggested dimerisation of $G\beta_{3\text{-}s}$ with $G\gamma_{12}$ and $G\gamma_5$.[813] However, the amounts of these latter products were significantly reduced compared with those obtained after in vitro translation of wild-type $G\beta_3$ together with these tagged G subunits. It can, therefore, not be ruled out that $G\beta_{3\text{-}s}$ /$G\gamma$ dimers form exclusively in artificial systems in which all components are strongly overexpressed. A definitive proof for the dimerisation and functional activity of $G\beta_{3\text{-}s}$ with G subunits would require the purification and functional reconstitution of G$\beta$3-s.[767] While the lack of dimerisation could explain the non-functionality of the $G\beta_{3\text{-}s}$ shown by Ruiz-Velasco and Ikeda,[767] in my experiments there is definite evidence of functional activity suggesting that functional G protein heterotrimers were formed in the cell systems studied and resulted in nearly similar functional activity. Though I could not assay the expression level of the transfected G protein subunits, the use of pertussis toxin resistant $\alpha_{2A}G_i$ fusion constructs and measurements performed after treatment with pertussis toxin

would indicate that the signalling pathway studied is predominantly mediated through the transfected subunits. Even if the results are due to overexpression, they are different from the original findings from Siffert et al,[579] who also used similar similar systems with overexpression of the $G\beta_3$ subunits.

One hypothesis to explain the lack of function associated with the $G\beta_{3\text{-}s}$, and its variable association with clinical phenotypes is the inability of the $G\beta_{3\text{-}s}$ mRNA to form a functional protein in vivo and the resultant degradation of the $G\beta_{3\text{-}s}$ mRNA. Thus, the 825T allele carriers would lack the functional wild-type $G\beta_3$, and the effect should be stronger in homozygous compared with heterozygous 825T allele carriers. Unfortunately, $G\beta_3$ tissue distribution has rarely been studied on the protein level and available antibodies lack sensitivity and specificity. However, the difficulties of demonstrating $G\beta_{3\text{-}s}$ expression by means of Western blot analysis compared with the ease by which $G\beta_{3\text{-}s}$ mRNA is detected by means of RT-PCR adds supports to this hypothesis.[767] Aso in support of this hypothesis is the finding of significant expression of $G\beta_3$ but a lack of $G\beta_{3\text{-}s}$ in fat cells regardless of GNB3 genotype by Western Blot, resulting in individuals with the TT genotype with the lowest and individuals with the CC genotype with the highest amount of wild-type $G\beta_3$ in the fat cells and cells from CT individuals displaying intermediate levels.[816] This resulted in a blunted response toward the lipolytic effects of $\beta_1$- or $\beta_2$-adrenoceptor agonists in fat cells from 825T allele carriers despite unchanged levels of adrenoceptors and $G_i/G_s$ proteins.

A consistent interpretation would strongly imply that the expression level of wild-type $G\beta_3$ correlates relatively strictly with, or may even determine the efficacy of, signal transduction via a variety of G protein-coupled receptors. Nevertheless, the amount of

expressed $G\beta_3$ wild-type protein may have an impact on the delicate balance between the different G protein β- and -γ subunits and their specific combination with heptahelical receptors. One way to assess the hypotheses mentioned above would be to express increasing amounts of $G\beta_3$ in cells and to measure the effect of this manipulation on specific cell responses or signal transduction events. It would not be too surprising if alternative splicing generating "mRNA junk" would result in a reduced amount of functional wild-type protein. Another question that need to be answered is whether $G\beta_{3\text{-}s}$ subunits are stable and whether they can dimerise with G subunits and form coordinated Gβ complexes capable of combining with heptahelical receptors. Finally in addition, if $G\beta_{3\text{-}s}$ can be detected as protein and their tissue distribution determined, it would shed light on the mechanisms and potentially explain the contrasting phenotypes associated with the C- and T-alleles.

# Chapter 9 - CONCLUSIONS

G protein activation is the key event in intracellular signal transduction, so it is logical to assume that the C825T polymorphism may have an impact on a variety of disease processes, signal transduction in human cells and tissues, as well as responses to common drugs. Previous studies have shown the 825T allele in the white human population of European descent to variably increase the risk for obesity,[706;708;710] hypertension,[689;690] coronary heart disease,[718;719] stroke,[713;735] and depression[736;737]. In contrast, the association of the 825T allele with these disorders in non-Caucasian ethnicities remains controversial.[695;697-699;738] Furthermore, other reports have suggested that the 825T allele may serve as a pharmacogenetic marker for response to cardiovascular agents such as diuretics,[704] clonidine,[739] angiotensin II,[732] and endothelin-1.[732] However these have not been replicated. It is not clear if this is a causal association, as functional effect of the polymorphism has not been proven unequivocally. Despite a large number of studies after the initial publication suggesting its association with hypertension, the molecular and biochemical mechanisms underlying these associations remained obscure.

In this thesis,I have addressed the important question of whether the GNB3 C825T polymorphism is a candidate gene for hypertension and LV hypertrophy by studying association of blood pressure and LV mass phenotypes using both a family-based and population-based approach in independent groups. In addition I have studied the functional aspects of this polymorphism specifically looking for evidence supporting its pathogenic role in a physiologically meaningful way.

The case-control study design is a highly efficient method for detecting association and the family based approach is less prone to errors stemming from admixture, given its use of unaffected family members as internal controls. The population I studied were not

ascertained for hypertension but were drawn from the west of Scotland region with high cardiovascular risk. The twin study confirms the genetic determination of blood pressure and LV mass in an independent population. For effects conferred by a single gene variant to be evident, they must either be very strong or found at a very high frequency to be statistically detectable. These analytic strategies have consistently and categorically demonstrated that the GNB3 gene does not play a significant independent role in hypertension and LV mass phenotypes.

Further studies looking at epistatic interactions with this polymorphism would facilitate the genetic dissection of these phenotypes. In support of this I have demonstrated a significant gene-interaction interaction between GNB3, ACE and aldosterone synthase polymorphisms determination of ECG LV mass. This interaction is biologically plausible as G proteins are signal transduction molecules through which the actions of the renin-angiotensin-aldosterone system and the adrenergic system are mediated. Thus GNB3 may exert a major effect only in association with variation in these genes. However these findings need to be further tested in large association studies, especially with ascertainment for high blood pressure which can identify individuals in the extremes of LV mass distribution, as this can give power at the stage of defining genotype–phenotype association.[169] The significant heritability of ECG indices of LV mass that I have shown would recommend the use of electrocardiographic phenotypes rather than echocardiographic left ventricular mass, as it is inexpensive and practical in many thousands of individuals needed in large population genetic studies.

I have shown that 825T allele has no significant functional effect on Gi mediated signalling as measured by platelet aggregation. Epinephrine-induced platelet aggregation

is considered to be exclusively mediated by Gi2 after activation of the α2A adrenoceptor,[797] ADP mediates platelet activation through two G-protein-coupled receptor subtypes Gq and Gi,[798] and PAF stimulation activates Gq coupled PLC and mobilisation of intracellular calcium. Given the activation of PTX-sensitive G proteins by epinephrine together with the observation that the α2A adrenoceptor activates G protein heterotrimers containing Gβ3,[800] epinephrine-mediated platelet aggregation should have bee enhanced in GNB3 825T allele carriers, in contrast to platelet activation evoked by ADP. The absence of this relationship excludes any major functional effect of the GNB3 polymorphism in biological systems.

These findings are supported by molecular studies where I used pertussis toxin resistant α2AGi fusion constructs which provides a robust measure of G protein activity as I performed measurements after treatment with pertussis toxin restricting the signalling pathway studied to that predominantly mediated through the transfected subunits. I found no significant difference between β3 or β3-s transfected cells in terms of GPTase activity after stimulation with epinephrine. But the β3 or β3-s transfected cells showed a two-fold higher GTPase activity compared to cells transfected with only the α2A-Gi fusion construct. This suggests formation of the functional heterotrimer with the transfected β3/β3-s and γ5 subunits, and pre-treatment with pertussis toxin would have neutralised any endogenous G protein activity. I used the single cell calcium signalling experiment to study the downstream effect of different $\beta_3$ subunits. Cells expressing either β3 or β3-s subunit showed significant differences in the measures of calcium signalling compared to cells not expressing them. Though there was no difference between cells expressing β3 and β3-s in terms of onset delay of calcium release and time to peak of calcium signal, cells expressing β3-s showed a significantly lower rate of calcium release compared to

cells expressing β3. These results indicate that the β3-s subunit is functional and that its functional effect is only slightly decreased compared to the wild type G protein β3 subunit.

These findings are different from that reported by Ruiz-Velasco and Ikeda,[767] who studied calcium signalling in terms of modulating N-type $Ca^{2+}$ and G-protein-gated inwardly rectifying $K^{+}$ channels, while my experiments assessed the activation of IP3 calcium channels in the sarcoplasmic reticulum in response to epinephrine which are activated by PLC-β through both Gα and Gβ3γ5 subunits independently. Previous functional studies have shown three times more pronounced spontaneous activation of MAPK pathway in HEK-TS cells expressing Gβ3-s compared to Gβ3 and a10-fold increase in carbachol-stimulated binding of GTPS Sf9 insect cells expressing Gβ3-s compared with cells expressing Gβ3.[681;813] These results along with our findings would suggest that the truncated Gβ3-s not only interacts with G subunits but is also capable of activating intracellular effector pathways like the wild-type Gβ3.[767] However this study does not support an enhanced activity of the Gβ3-s, and in contrast to experiments which have shown an enhanced activity, I have controlled for the upstream factors in the signalling cascade by using the fusion construct and thus measuring functional activity specifically through the transfected subunits and thus these results are probably more accurate.

The problems with genetic association studies need to be addressed if meaningful results are to be obtained. Critics have conceded that findings from many genetic association studies are inconsistent and cannot be replicated, and that these studies should be restricted to the study of polymorphisms that have been shown to have a direct effect on gene function.[817] In fact, in a recent review of genetic association studies, Hirschhorn et al[186]

found that over 600 positive associations between common gene variants and disease have been reported. Of the putative associations which have been studied three or more times, only 3.6% have been consistently replicated. Recently, more stringent criteria for the evaluation of these studies have been proposed.[722] The guidelines say that association studies should have "large sample sizes, small p values, report associations that make biological sense and alleles that affect the gene product in a physiologically meaningful way. In addition, they should contain an initial study as well as an independent replication, the association should be observed both in family-based and population-based studies, and the odds ratio and/or the attributable risk should be high.[722] The analytic strategies undertaken in this study reflects the recommendations of these criteria and demonstrate the breadth of the analyses and the clear conclusions reached about the candidature of GNB3 as a putative gene for hypertension. Ultimately, studies that meet these criteria will help to dissect clinically relevant genetic variants from single nucleotide polymorphisms without biological function in the system under investigation.

Despite the perception that gene interactions are important, it is increasingly clear that existing strategies are inadequate to deduce the type of interactions. Most analyses of risk factors focus on identifying agents that have a consistent effect regardless of an individual's context, but context dependent genetic effects which may involve gene-gene interactions (epistasis) as well as gene environment interactions are important.[818] Increasing evidence indicates that the genes contributing to the risk of hypertension do not have the same effects across time, in different environments, or in different genetic backgrounds. Current efforts in humans and other species to generate dense genetic maps using many polymorphic markers, such as SNPs, can be used to reconstruct common genomewide haplotypes. In future, it should then, at least theoretically, be possible to

obtain sufficient population sizes by sampling individuals from the general population and using highdensity SNP maps to reconstruct haplotypic relationships. It should be possible to perform genome scans for direct associations between haplotypes at one location or combinations of haplotypes at two locations with trait variation. The haplotypes could be used to reconstruct the genetic relationship between individuals, both at individual loci and for combinations of loci. QTL effects can then be predicted by using the variance-component approach to estimate the proportion of the genetic variance that results from the effects of individual loci and from the interactions between them.[818] The designs might therefore not be cost-effective for detecting novel epistatic patterns until the large-scale collection of haplotype data becomes feasible. An integration of biological knowledge with genetic associations is essential in interpreting as well as testing hypotheses about causal pathways, rather than just modelling the joint effects of genotype and environment.

# Chapter 10 - Appendix - Quantitative Genetics

## 10.1 Phenotypic variation

For quantitative traits, the phenotype of a particular individual is called its phenotypic value. Two parameters are of interest for any quantitative trait, namely the mean and the variance. The mean ($\mu$) is defined as the expected phenotypic value across all individuals in the population. The variance ($\sigma^2$) is defined as the expected value of the squared deviation of each phenotypic value from the population mean. The standard deviation equals the square root of the variance.

Thus, for continuously measurable traits, it is simplest to characterize gene effects in the context of the gaussian distribution. If we consider a single locus *L* with two variant alleles *A* and *a* with population frequencies *p* and *q* = 1 - *p* respectively, there are two important measures we can define – displacement and genetic variance.[819]

### *10.1.1 Displacement*

Displacement (*t*), is the number of standard deviations difference between the mean values of the two homozygotes *AA* and *aa* (assuming the variance within genotype is the same for each genotype). There is an additional parameter, *d*, representing the mean value of heterozygotes *Aa* relative to the two homozygotes. Thus, a value of *d* = 1 corresponds to equal means for genotypes *AA* and *Aa* (that is, *A* is dominant), whereas *d* = 0 corresponds to equal means for genotypes *Aa* and *aa* (that is, *A* is recessive). A value of *d* = 0.5 corresponds to the heterozygotes being exactly intermediate between the two homozygotes, a situation often described as additive.

### *10.1.2 Genetic Variance*

The second measure of gene effect is the population variance attributable to segregation of the gene. A major assumption of quantitative genetics is that genetic and environmental effects on complex traits are additive. This assumption cannot be verified explicitly because the individual factors are not directly observable. But it allows the phenotypic value of any individual to be written as the sum of three terms:

- The mean $\mu$ of the entire population
- A deviation from the population mean due to the genotype of the individual
- A deviation from the population mean due to the environment of the individual

The additivity assumption is written in symbolic form as

$$P_i = \mu + G_i + E_i$$

Where $P_i$ is the phenotypic value of the *i*th individual of the population, $\mu$ is the mean of the population, $G_i$ is the deviation of the *i*th individual from the population mean due to genetic factors, and $E_i$ is the deviation of the *i*th individual from the population mean due to environmental factors.

This implies that the variance in phenotypic value (the phenotypic variance, $\sigma_p^2$) can be partitioned into one component due to variation among genetic factors, which is called the genotypic variance, $\sigma_g^2$, and another component due to variation among environmental factors, which is called the environmental variance, $\sigma_e^2$. The basis of this partitioning is the definition of the variance in terms of the expected value of the squared deviations:

$$\sigma_p^2 = E(P_i - \mu) = E(G_i^2) + E(E_i^2) = \sigma_g^2 + \sigma_e^2$$

In a genetically uniform population, $\sigma_g^2$ must equal zero, and the observed variance in that population provides an estimate of $\sigma_e^2$. Whereas the observed variance of a randomly bred population provides an estimate of $\sigma_g^2 + \sigma_e^2$. An estimate of $\sigma_g^2$ can be obtained by subtraction, since $\sigma_g^2 = (\sigma_g^2 + \sigma_e^2) - \sigma_e^2$. This method of separating the genotypic and environmental variance has also been used in the studies of monozygotic twins in human populations, as they have shared identical genotypes but different environmental experiences.

## 10.2 Average effect

For a quantitative trait, the mean phenotypes of *AA*, *Aa*, *aa* are denoted *a*, *d* and *–a*, which are measured as deviation from the average of the homozygous genotypes. The symbols *a* and *d* represent the effect of the alleles. The quantity *2a* measures the difference between the means of the homozygous genotypes, because *a-(-a)=2a*, and *d/a* serves as a measure of dominance

- *d=a* means that *A* is dominant to *a*
- *d*=0 implies additivity (*Aa* exactly intermediate between *AA* and *aa*)
- *d=-a* means that *a* is dominant to *A*

If *A* and *a* have allele frequencies *p* and *q*, then in a random mating population the overall mean phenotypic value is given by

$$\mu = p^2a + 2pqd + q^2(-a) = (p-q)a + 2pqd$$

To obtain $G_i$ values, the mean phenotype of each genotype needs to be expressed as a deviation from the overall population mean.

$$G_1 = a-\mu = 2q[a+(q-p)d]-2q^2d = 2q\alpha - 2q^2d$$
$$G_2 = d-\mu = (q-p)[a+(q-p)d]+2pqd = (q-p)\alpha + 2pqd$$
$$G_3 = -a-\mu = -2p[a+(q-p)d]-2p^2d = -2p\alpha - 2p^2d$$

Each deviation can be expressed in terms of the quantity $a+(q-p)d$, which appears so often in the equations of quantitative genetics that it is assigned a special symbol $\alpha$ and given the special name average effect.

$$\alpha = a+(q-p)d$$

The average effect can also be written as $q[d-(-a)]+p(a-d)$, which more easily shows its biological meaning. It is the average change in mean phenotype that would result from choosing an allele at random (in whatever genotype it happens to be) and changing it to an *A* allele.

The gaussian model described above has a direct extension to discrete outcomes (for example, affected/unaffected). In this case, the quantitative genetic variable is latent (unobserved) and often described as the genetic 'liability'. Superimposed on the genetic-liability distribution is a risk function, so that risk of disease also increases continuously with liability. If one assumes a gaussian form for this risk function, the model can be characterized as a 'threshold' model, wherein total liability is defined as the sum of genetic and non-genetic liabilities, and disease occurs when an individual's total liability exceeds a threshold *T*. As in the case of continuous outcomes, a single locus can influence the distribution of liability, where individual genotypes have different mean liabilities. The same measures used for quantitative outcomes can also be used here — namely displacement and proportion of variance explained, measured on the scale of liability. Alternatively, one can conceptualize measures of gene effect on the scale of risk rather than liability. For example, classical measures from epidemiology, such as the relative risk, can be used to quantify the risk of disease for one genotype (say *AA*) compared to another (say *aa*), a concept termed the genotype relative risk or genotypic risk ratio (*GRR*).[820] The *GRR* is analogous to displacement, in that it measures the effect of a particular allele or genotype, independent of its frequency. Another useful, more complex measure is the sibling relative risk ($\lambda_s$) attributable to locus *L* (ratio of risk to sibs of an affected case to the population prevalence).[821] If the *GRR* for genotype *AA* is $g_2$ and that for genotype *Aa* is $g_1$, and the frequency of allele *A* is *p* and $q = 1 - p$, $\lambda_s$ can be calculated as $1 + (1/2V_A + 1/4\ V_D)/K^2$, where $K = p^2g_2 + 2pqg_1 + q^2$, $V_A = 2pq(p(g_2 - g_1 + q(g_1 - g_0))^2$ and $V_D = p^2q^2(g_2 + g_0 - 2g_1)^2$. Note that these formulas are analogous to the continuous case except that the displacement *t* is now replaced by the *GRR* $g_2$.

## 10.3 Components of genotypic variation

The genotypic variance can be expressed as $V_G(L) = V_A(L) + V_D(L)$, where $V_A(L)$ is the 'additive' genetic variance and $V_D(L)$ is the 'dominance' genetic variance. Thus

$$\sigma_g^2 = p^2[2q\alpha - 2q^2d]^2 + 2pq[(q-p)\alpha + 2pqd]^2 + q^2[-2p\alpha - 2p^2d]^2 = 2pq\alpha^2 + (2pqd)^2$$

The first term in the equation is termed the additive genetic variance given by

$$\sigma_a^2 = 2pq\alpha^2$$

The second term in the equation dominance variance given by

$$\sigma_d^2 = (2pqd)^2$$

Whereas the additive variance depends both on *a* (twice the difference between homozygous genotypes) and *d* (the dominance effect), the dominance variance depends only on *d*. Both variance components also depend on allele frequencies.

When multiple loci are considered, $2pq\alpha^2$ can be replaced by the sum of such terms, one for each locus, and likewise $(2pqd)^2$ can be replaced with a sum of similar terms. Each locus may have different values of $p,q,a,d$. There is also a term corresponding to nonadditive interactions between genotypes at different loci, which is called the interaction variance, symbolised $\sigma_i^2$. And in still more general models in which assortative mating is allowed, there is a term due to assortative mating symbolised by $\sigma_{am}^2$. The term assortative mating means that there is a positive correlation between the phenotypes of mating pairs, as occurs in human populations for height. With all these taken into account, the genotypic variance can be written as

$$\sigma_g^2 = \sigma_a^2 + \sigma_d^2 + \sigma_i^2 + \sigma_{am}^2$$

In principle one could also partition the environmental variance similarly.

The proportion of total variance attributable to a locus ($h^2_L$), is then given by $V_G(L)/(1 + V_G(L))$, assuming the variance within genotype to be 1.0. It is important to note that $h^2_L$ is a function of both displacement $t$ and the allele frequency $p$. Thus, a rare gene with large displacement (that is, mendelian) may contribute the same proportion to variance as a common gene with modest displacement (that is, non-mendelian). In addition, because $h^2_L$ is a function of $p$, its value can vary from one population to another when $p$ varies, even when the displacement $t$ is the same.

## 10.4 Broad sense heritability

For a complex trait, one measure of the aggregate effect of all genetic factors combined is the broad sense heritability ($H^2$), defined as the ratio of the genotypic variance to the phenotypic variance:

$$H^2 = \frac{\sigma_g^2}{\sigma_p^2} = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$$

Hence if $H^2$=0, all of the phenotypic variance is attributable to differences in environment, and if $H^2$=1, all of the phenotypic variance is attributable to differences in genotype. The variance components have a very limited utility and apply only to differences in phenotype within populations and not between populations.

## 10.5 Artificial selection and narrow-sense heritability

Genetic changes can sometimes occur slowly in traits affected by many genes in populations that are large enough, because, then selection can act on the genetic variation contributed by new mutations. Although genetic variation is essential for progress under selection, it is not sufficient. The reason is quite subtle. Only the additive genetic variance $\sigma_a^2$ contributes to the response to selection. The genotypic variance $\sigma_g^2$ is not the key quantity. In plant breeding, in most genetically heterogeneous populations, artificial selection can change phenotype well beyond the range of variation found in the original population. A total selection response of 3-5 times the original phenotypic standard deviation is not unusual, and for selection to change a population of effective size $N_e$ halfway to its selection limit typically requires about $0.5N_e$ generations.

The difference in mean phenotype between the selected parents ($\mu_s$) and the entire parental population ($\mu$) is called the selection differential and designated $S$:

$$S = \mu_s - \mu$$

The difference in mean phenotype between the progeny generation ($\mu'$) and the previous generation ($\mu$) is called the response to selection and designated $R$:

$$R = \mu_s - \mu$$

Any equation that defines the relationship between the selection differential $S$ and the response to selection $R$ is known as the prediction equation. For individual truncation selection, the prediction equation is

$$R = h^2 S$$

Where $h^2$ is called the narrow-sense heritability of the trait. As with broad sense heritability, the narrow-sense heritability is a ratio of variances, but not the ratio of genotypic variance to phenotypic variance, but the ratio between the additive genetic variance to phenotypic variance.

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2}$$

The usual way to estimate narrow-sense heritability is from correlation between relatives.

## 10.6 Correlation between relatives

Estimation of the additive genetic variance might at first seem to be very difficult, but in fact it is quite straightforward. The reason is that the theoretical covariance between certain types of relatives is a simple multiple of $\sigma_a^2$. Suppose $x$ and $y$ represent the phenotypic values of a trait between any pair of relatives , then the covariance of $x$ and $y$ is defined as

$$Cov(x,y) = E[x-E(x)][y-E(y)]$$

Just as variance is the expected squared deviation from the mean, the covariance is the product of deviations from the mean. If $x$ and $y$ are independent, then $Cov(x,y)$=0. Based on a random sample of $n$ pairs of values, the covariance is estimated as

$$Cov(x,y) = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{n-1}$$

The covariance is used in calculating the correlation coefficient $r$ in phenotypic value between $x$ and $y$:

$$r = \frac{Cov(x,y)}{\sigma_x \sigma_y}$$

where $\sigma_x$ and $\sigma_y$ are the phenotypic standard deviations of $x$ and $y$. The correlation coefficient is sometimes preferred over the covariance as a measure of relationship between random variables, because $r$ must always lie between –1 (perfect negative correlation, $x$=-$y$) and +1 (perfect positive correlation, $x$=$y$). Independence between $x$ and $y$ implies that $r$=0.

### 10.6.1 Parent-Offspring correlation

The parent-offspring correlation is often used to estimate the narrow-sense heritability because the covariance equals the additive genetic variance. The covariance can be calculated either from a single parent (usually chosen to be the father to avoid possible non-genetic maternal effects on the offspring) or from the average of the parents (called the midparent). The result is the same. The covariance is calculated as the product of the parent-offspring deviations, each weighted by the frequency of the parent-offspring pair.
Letting *Cov(PO)* be the covariance between a single parent and offspring,

$$Cov(PO) = pq\alpha^2 = \frac{\sigma_a^2}{2}$$

Alternatively, we can write the correlation coefficient between a single parent and offspring $r_{PO}$ as

$$r_{PO} = \frac{Cov(PO)}{\sigma_p^2} = \frac{\frac{1}{2}\sigma_a^2}{\sigma_p^2} = \frac{h^2}{2}$$

where $h^2$ is the narrow-sense heritability.

## 10.6.2 Heritability estimates from resemblance between relatives

Theoretical covariances for common relationships are shown below - variance terms due to interaction between loci (epistasis) are ignored.

| Degree of relationship | Covariance |
|---|---|
| Offspring and one parent | $\sigma_a^2/2$ |
| Offspring and midparent | $\sigma_a^2/2$ |
| Half siblings | $\sigma_a^2/4$ |
| Full siblings | $(\sigma_a^2/2)+(\sigma_d^2/4)$ |
| Monozygotic twins | $\sigma_a^2+\sigma_d^2$ |
| Nephew and uncle | $\sigma_a^2/4$ |
| First cousins | $\sigma_a^2/8$ |
| Double first cousins | $(\sigma_a^2/4)+(\sigma_d^2/16)$ |

*First cousins are the offspring of matings between siblings and unrelated individuals; double first cousins are the offspring of matings between siblings from two different families.

The additive genetic variance can be estimated directly from covariance between parent and offspring, midparent (the average of parents) and offspring, half-siblings, uncle-nephew (or aunt-niece), or first cousins. What these degrees of relationships have in common is that the relatives can share at most one allele at any locus. The covariances between the other degrees of relationship in the above table include a contribution due to dominance because the relatives can share two alleles at any locus. The theoretical covariance between monozygotic twins is equal to the covariance of an individual with itself, or $\sigma_g^2$.

## 10.6.3 Offspring-on-parent regression

A regression line can be drawn on a plot of parental phenotype on the x-axis and offspring phenotype along the y-axis. Parent-offspring regression is a convenient method for estimating the narrow-sense heritability because the slope of the line $b_{OP}$, can be shown to satisfy

$$b_{OP} = \frac{Cov(PO)}{\sigma_p^2} = \frac{\frac{1}{2}\sigma_a^2}{\sigma_p^2} = \frac{h^2}{2}$$

Hence $h^2$ can be estimated as $2b_{OP}$. (the regression coefficient of midparent on offspring, $b_{OM}$, equals $h^2$). It makes no difference whether the offspring and parent are considered individually or are pooled and their mean used instead.

## 10.6.4 Genotype-Environment interaction

The measures described above for effects of single loci need to be considered in the context of their genetic and/or environmental background. The gaussian model provides the simplest context whereby all other genetic factors are assumed to have small and additive effects, and the environment is also assumed to be gaussian and additive. In this case, in addition to the components of genetic variance $V_A(L)$ and $V_D(L)$ defined for locus $L$, we have the components of residual genetic variance $V_A(R)$ and $V_D(R)$, which are the additive and dominance variance summed across all other loci, with $V_G(R) = V_A(R) = V_D(R)$ . The non-genetic component is assumed to have variance $V_E$.

From the perspective of biometrical genetics, epistasis refers to non-additive interactions between gene effects (much as dominance refers to non-additive effects between alleles at a single locus). Thus, the genetic variance underlying a trait can include sources of variance involving interactive effects among any

number of loci, and these are termed epistatic variance components. Often, these are segregated into terms based on the number of loci involved in the interaction (for example, two, three or four loci).[819]

Genotype by environment interaction occurs when the genotypic and environmental effects are not additive, but differ according to which genotype is in which specific environment. A curve showing the phenotype of a genotype across the range of environments is called the norm of reaction for the genotype. Gene-environment interaction is indicated when the norms of reaction cross. The important implication of gene-environment interaction is that the deviations due to genotype are not independent of the deviations due to environment.

### 10.6.5 Genotype-Environment association

This is another cause of nonindependence of genotypic and environmental deviations. It occurs when the genotypes in a population are not distributed randomly in all the possible environments. With gene-environment association, it is difficult, if not impossible to separate the genetic and environmental causes of variation, because there is a systematic association of certain genotypes with certain environments.

### 10.6.6 Genotype-by-sex interaction

This occurs when the magnitude of a genotypic deviation depends on the sex of the individual. It is a potential problem in genetic analysis because the expression of many complex traits depends in part on developmental, hormonal, or other factors associated with sex.

# Chapter 11 - REFERENCES

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