PHARMACOGENETICS OF HYPERTENSION; A STUDY OF THE SINGLE NUCLEOTIDE POLYMORPHISMS OF THE CYP2D6 AND BETA-2 AR GENE.

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THESIS SUMMARY

Single Nucleotide Polymorphisms may influence a patient's response to an anti hypertensive medication. A polymorphism occurring in the coding region of a gene can result in a qualitative alteration in protein structure. Metoprolol, a widely used antihypertensive drug, has its metabolism mediated by the CYP2D6 enzyme and at least some of its adverse effects are related to interference with beta-2-receptor mediated functions and may in susceptible patients induce bronchospasm.

A cross sectional study was undertaken to assess whether there is an association between CYP2D6 and beta-2 AR gene polymorphisms, and the antihypertensive effect of metoprolol. The incidence of metoprolol-induced bronchospasm in this group of hypertensive patients was estimated and possible association with the genetic polymorphisms of beta-2 AR gene tested.

The incidence of bronchospasm in the local population was high (12%). The majority of patients who developed bronchospasm carried the Gly16 variant of the Beta-2 AR gene. The presence of Gly16 polymorphism markedly influenced the ability of the beta-2 receptor to desensitize, and in the absence of Arg16, the ability to prevent bronchoconstriction on exposure to Beta2 antagonists appears to be reduced. An association was also found between haplotype 1 and bronchospasm (χ^2 13.92; df = 8; p = 0.047), and between haplotype 2 and bronchospasm (χ^2 14.39; df = 8; p = 0.048).

Half of the patients who experienced bronchospasm were either heterozygous or mutant for CYP2D6 *10, which expresses an enzyme with impaired metabolizing capacity. There was no association between CYP2D6 genotype and bronchospasm, defined as a ratio of FEV1/FVC of less than 0.7, or assigned phenotype and bronchospasm (χ^2 0.17 with 1 degree of freedom; p = 0.675). There was no poor metabolizer phenotype within our study population.

A systematic review was undertaken to examine the possible association between genetic variation in the beta-2 AR gene and essential hypertension. No association was found between hypertension and codon 16 polymorphism of the beta-2 AR gene. The pooled odds ratio based on 6706 patients was 0.965 [95% CI (0.770 – 1.210)].

Keywords: Pharmacogenomics, Genetic variability, Bronchospasm, Adverse Drug Reaction, Metoprolol

TO MY WIFE LIZA, MY CHILDREN ZAHIM, ANNISSA AND ALIYA AND MY PARENTS.

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ABBREVIATIONS

AMP = Adenine Di-phosphate
Arg = Arginine
AUC = Area under the curve
Beta-1 AR = Beta-1 adrenergic receptor
Beta-2 AR = Beta-2 adrenergic receptor
cDNA = complementary DNA
COAD = Chronic Obstructive Airway Disease
CTU = Clinical Trial Unit
CYP = cytochrome P450 enzymes
DD = Detectable Difference
DME = Drug Metabolizing Enzyme
DNA = Deoxyribonucleic Acid
EDTA = Ethylene-diamine-tetra-acetic
EM = Extensive Metabolizer
FEV1 = Forced Expiratory Flow Rate in 1 second
FVC = Forced Vital Capacity
Gln = Glutamine
Glu = Glutamate
Gly = Glycine
HCL = Hydrochloric Acid
Ile = Isoleucine
IM = Intermediate Metabolizer
kb = kilo base pair
LFT = Liver Function Test
M = Molar
MR = Metabolic Ratio
mRNA = Messenger Ribonucleic Acid
NaCl = Sodium Chloride
NADPH = Nicotinamide Adenine Dinucleotide Phosphate
ORF = Open Reading Frame
PE = Physical examination
PEFR = Peak Expiratory Flow Rate
PM = Poor Metabolizer
R/N = Registration Number
RFT = Renal Function Test
RNA = Ribonucleic Acid
SNP = Single Nucleotide Polymorphism
TBE = Tris-Borate-EDTA buffer
TE = Tris-EDTA buffer
TGGE = Temperature Gradient Gel Electrophoresis
Thr = Threonine
UM = Ultra Rapid Metabolizer

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INTRODUCTION

AND

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 HYPERTENSION

Hypertension is a common disorder, afflicting millions of people worldwide. While the final phenotype of elevated blood pressure might be similar from patient to patient, underlying hereditary determinants of blood pressure elevation are polygenic and heterogeneous (Sharma, Fatibene et al. 2000).

It has now become clear that essential hypertension is not a Mendelian single-gene disorder but rather a complex trait resulting from the interaction of genes and environment. Alleles at many different loci are likely to contribute to the ultimate disease trait, and specific combinations of causative alleles vary from person to person (Gavras, Manolis et al. 1999).

Hypertension is a major public health problem because of its consequences. Its treatment is crucial and goals include decreasing morbidity and mortality by decreasing blood pressure using drugs that are effective, have good tolerance and dosing convenience, and are cost-effective. As many antihypertensives are now available, it is important to choose the most appropriate drug in terms of efficacy and with least side-effects in order to improve compliance and patient's quality of life.

Physicians tend to base their antihypertensive prescription decisions on personal experience as well as clinical guidelines such as *the Clinical Practice Guidelines on the Management of Hypertension 2002* by the Malaysian Hypertension Guidelines Working Group (Ministry of Health Malaysia, Academy of Medicine of Malaysia and *Persatuan Hypertensi Malaysia*) or the *British Hypertension Society Guidelines for Hypertension Management* (http://www.bhsoc.org/pdfs/Summary%20Guidelines%202004.pdf). These guidelines, however,

are based on hypertension management in populations of patients with unknown and likely differing heritable predispositions.

The response of any particular patient to a specific medication is not easily predictable, and causes of inter-individual variation in blood pressure responsiveness to medications are largely unknown (Chapman, Schwartz et al. 2002). As a result, traditional hypertension management has consisted of a `trial and error' approach, wherein patients are prescribed medication, reassessed, and then placed on different or additional drugs as needed. Overall adequacy of blood pressure control in clinics remains low, intolerance of medication side-effects is common, and polypharmacy is the norm (Burt, Whelton et al. 1995; Cohen 2001).

Thus, choosing the most effective blood pressure medication for an individual patient with hypertension has long been an imperfect endeavour, in which physicians use past experience and the various generalizations regarding ethnicity and age to guide therapy.

The completion of the Human Genome Project (Venter, Adams et al. 2001) has brought growing enthusiasm for applying the knowledge of genomic determinants of drug responsiveness to derive more `personalized' medication regimens, directed to the specific pathophysiology of each patient. Rather than empirically treating hypertension based on clinical experience and broad generalizations regarding such demographics as ethnicity or age (Buhler and Hulthen 1982; Cubeddu 1986; Preston, Materson et al. 1998), physicians eventually may perform genotyping of their patients, perhaps enabling identification of not only specific hereditary mechanisms of a patient's disease, but also more specific and rational therapies for that individual.

This branch of human genetics that focuses on the heritable determinants of drug responses is known as Pharmacogenomics. This emerging field of pharmacogenomics may change how this common disease is approached therapeutically. As understanding of hypertension genetics progresses, the potential to apply mechanism-based therapies that match the specific pathophysiology of each individual patient also increases.

1.2 DEFINITIONS

1.2.1 PHARMACOGENOMICS

Pharmacogenomics, can be broadly defined as the study of the impact of genetic variation on the efficacy and toxicity of drugs, or the study of how genetic makeup determines the response to a therapeutic intervention (Peet and Bey 2001). As the volume of high quality genetic and genomic information for predicting the response to drugs become available, better clinical trials and more targeted drug development may ensue.

Pharmacogenomics has the potential to revolutionize the practice of medicine by individualisation of treatment through the use of novel diagnostic tools. This new science should reduce the trial-and-error approach to the choice of treatment and thereby limit the exposure of patients to drugs that are not effective or are toxic for them.

PHARMACOGENETICS

The term pharmacogenetics is often used interchangeably with pharmacogenomics, but is used more generally to describe the study the effect of heredity on drug response (Peet and Bey 2001). Most of the variations or polymorphisms described occur in the drug metabolizing enzymes (DMEs) or cytochrome P450 enzymes. However, polymorphisms of drug transportation genes

and genes that encode protein receptors and other effectors also lead to variations in drug response.

1.2.2 PHARMACOGENOMICS VS. PHARMACOGENETICS

The many interactions involving cytochrome P450 illustrate the importance of pharmacogenomics. Likewise, we now have extensive compilations of clinically relevant polymorphisms (SNPs) that influence other drug metabolism pathways. For example, it is estimated that 2-10% of the population is homozygous for non-functional CYP2D6 mutant alleles, leading, for example, to an inability to activate some opioid analgesics. This would explain why there is great variability in pain relief experienced by patients receiving the same dose of codeine.

Pharmacogenomics alludes to the emerging possibilities of genome-wide approaches to understanding the effects of heredity on any phenotype. The power and promise of pharmacogenomics as a tool lies in its potential to predict a patient's response to available remedies prior to administration, and to a degree not previously possible.

1.3 SINGLE NUCLEOTIDE POLYMORPHISMS (SNP)

SNPs are variants in the DNA sequence, resulting from replication errors, in which one nucleotide is substituted for another e.g. purine-for-purine (**Figure 1.1)** (e.g. `A' appearing instead of `G') or pyrimidine-for-pyrimidine (C/T) transitions (Wang, Fan et al. 1998; Doris 2001).

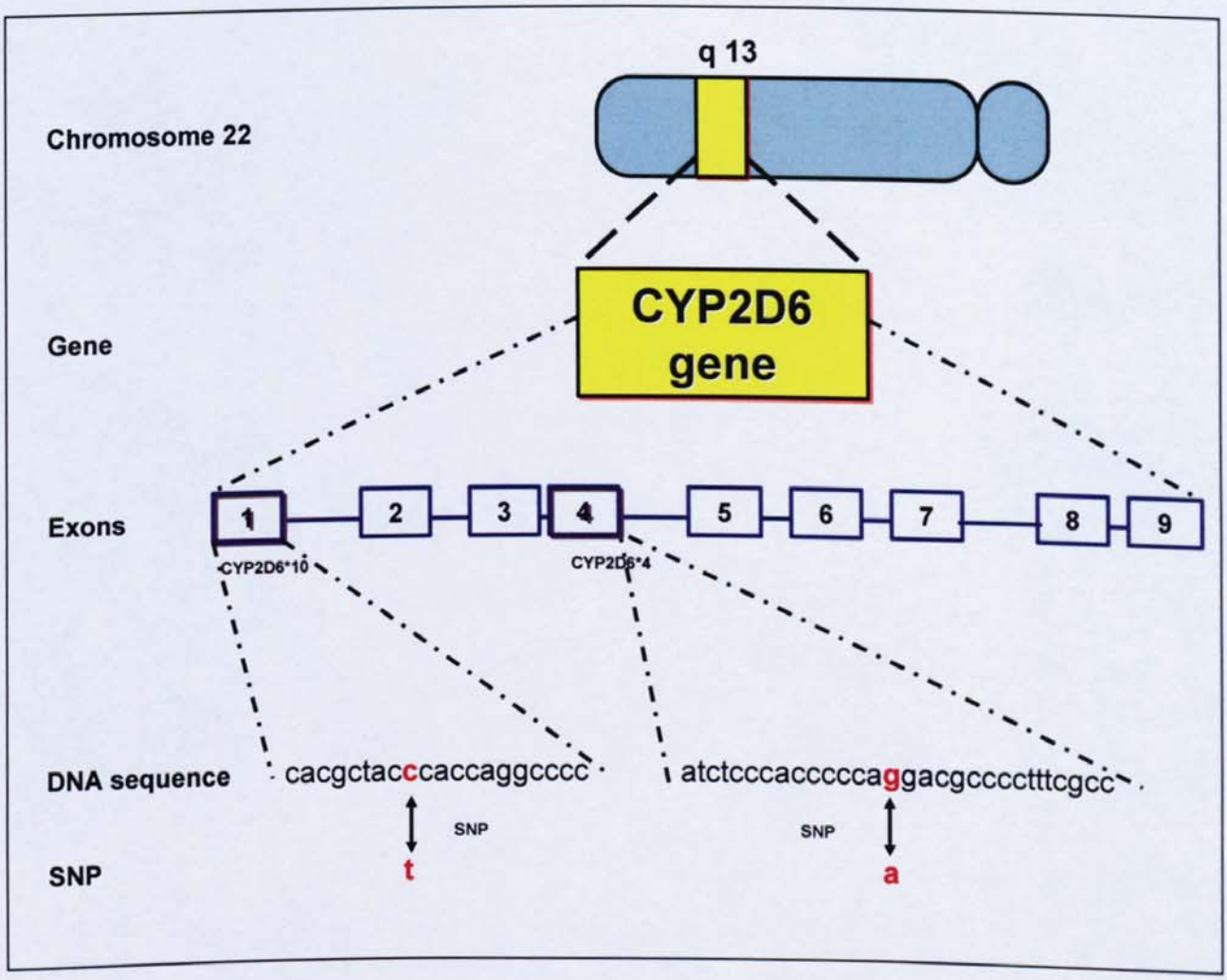

Figure1.1 CYP2D6 gene has 9 exons with more than 60 SNPs identified. Two of the SNPs are shown here (alleles CYP2D6*10 and CYP2D6*4)

Every individual carries two copies of each gene. Copies of a specific gene present within a population may not have identical nucleotide sequences. These single nucleotide changes are scattered throughout the genome of all species and forms the basis for human diversity. SNP occur in humans every 300-2000 base pairs along the genome (Lindpaintner 1999; Gray, Campbell et al. 2000). In principle, they may occur at any nucleotide, and for genetic epidemiological study, those that are relatively common will be of greatest interest.

The vast majority of SNPs are functionally silent, occurring in non-coding or non-regulatory regions of the genome. However, some of the SNPs lead to altered protein structure or expression. These biologically functional SNPs are considered the essence and substrate of human diversity in both health and disease.

Pharmacogenetics studies have until recently focused on SNPs that affected drug metabolism and demonstrated Mendelian heritability. SNPs might influence a patient's response to medication in a number of ways. A polymorphism occurring in the coding region of a gene (so-called `coding SNP') can result in a qualitative alteration in protein structure (and hence function), if the coding SNP changes the amino acid specified by a codon (so-called `nonsynonymous' or `replacement' coding SNPs). It has been estimated that as many as ~20-30% of nonsynonymous coding SNPs might result in altered protein function (Chasman and Adams 2001; Ng and Henikoff 2002). Even polymorphisms that occur in noncoding regions of DNA, however, may prove important, since such SNPs might occur in DNA motifs crucial for appropriate transcription, or correct RNA splicing, or efficient translation of messenger RNA. In addition, marker SNPs may occur in `linkage disequilibrium' with (that is, near to) other functional, as-yet-unidentified alterations in genes, thereby representing potentially useful genetic markers for disease states or drug responses.

There is an ongoing process of identifying these common, biologically relevant SNPs, in particular those that are associated with the risk of disease or response to particular drugs. Once identified and characterized, this SNP-based 'genetic profile', may be viewed as a 'fingerprint', useful in defining the risk of an individual's susceptibility to various illnesses and response to drugs.

1.4 PHARMACOGENOMICS, PHARMACOKINETICS AND PHARMACODYNAMICS

Pharmacogenomics is founded on the central principle that the patient's response to any pharmaceutical is influenced by variations in proteins encoded by the genome. The mechanisms by which these proteins determine drug effect may be divided broadly into the two categories: `pharmacokinetic' and `pharmacodynamic'.

1.4.1 Pharmacokinetics

Pharmacokinetic factors are processes that influence a drug's delivery to and arrival at its destination (i.e. its target or receptor); such factors include absorption, distribution, metabolism and excretion.

1.4.2 Pharmacodynamics

By contrast, pharmacodynamic processes determine a drug's effect at or after it reach its point of action. Hence, genetic approaches to pharmacodynamic focus on the genetic blueprints for such drug targets as enzymes or membrane receptors, as well as post-receptor signalling pathways to ultimate cellular effectors, and other intracellular mechanisms by which drugs produce changes in cell function. For example, the effects of the beta-1 -adrenergic agonist isoproterenol at the sinus node on heart rate might be influenced by qualitative or quantitative variability in any of the gene products in the drug's signalling pathway.

1.5 GENOTYPES VS. PHENOTYPES

Humans can, with some drugs, be categorized as either `extensive, slow, ultra-rapid or intermediate metabolizer' of a specific drug. Such phenotypic traits are often readily recognized during plasma or urine drug assays in study populations.

The term "poor metabolizer" can be used to describe individuals who are unable to metabolize certain drug substrates due to absence of a particular cytochrome P450 enzyme. This is due to the presence of inactivating mutations in both copies of gene. "Extensive metabolizer" is those who are able to metabolize particular drugs normally and have either one (heterozygous) or two (homozygous) normal copies of the gene. Heterozygous individuals often show slower metabolism than those with two normal copies and are sometimes referred to as "intermediate metabolizer". In some instances, at the extreme of the EM phenotype, gene amplification gives rise to the so-called 'ultra-rapid' metabolizer (UM). These individuals carry replicate functional copies of the genes. The rate at which many drugs are metabolized may vary 10-100 fold between PMs and EMs.

1.6 CYTOCHROME P450

Cytochrome P450 enzymes were first described over 40 years ago (Omura and Sato 1964). They owe their name to the spectrophotometric absorption peak they share at or near 450 nm when bound and reduced by carbon monoxide (Gonzalez 1988). These enzymes metabolize endogenous and exogenous substances in the body, providing important chemicals to sustain life as well as providing protection against unwanted chemicals. They comprise a superfamily of haemoproteins that serve as terminal oxidases of the mixed function oxidase system. Each P450 isoenzyme consists of a single protein and one haem group as the prosthetic moiety. The haem prosthetic group binds oxygen after electron transfer reactions from the reduced form of

nicotinamide adenine dinucleotide phosphate (NADPH) and this reaction incorporates one atom of molecular oxygen into the substrate (Kerremans 1996). From the initial reports, suggesting that P450 was a single enzyme, it later became apparent that this haemoprotein exists in multiple forms, the products of discrete genetic loci.

Nelson, Koymans et al. 1996 proposed a general nomenclature based on the presence of common amino acid sequence for these enzymes. This system groups the enzymes and genes into families and subfamilies with the prefix 'CYP' to designate cytochrome P450 enzymes in all species (except Drosophila and mouse gene where 'Cyp' is used). Fourteen families and 26 subfamilies exist in all mammals. Over sixty variants of the CYP2D6 gene have now been reported (Bradford, 2002 and Sistonen et al., 2007) and a regularly updated list is available (http:/www.cypalleles.ki.se/cyp2d6.htm).

Twenty of the subfamilies have been mapped to the human genome (http://www.icgeb.org/~p450srv/CEP450s.html).

Families are defined as having more than 40% homology in their amino acid sequence and are designated by an Arabic numeral, and subfamilies have more than 55% homology and are designated by a capital letter. An Arabic numeral after the letter denotes the individual enzyme and the gene associated with the enzyme is denoted in italics. For example, the CYP2 family has several subfamilies such as CYP2C, CYP2D and CYP2E (Nelson, Koymans et al. 1996). The individual enzyme is denoted by a numeral, as in CYP2D6, and the gene is denoted as *CYP2D6.*

Enzyme	Drug substrates
CYP1A2	Acetaminophen, theophylline, caffeine,' clozapine, R-warfarin, tacrine
CYP2C9	dapsone, diazepam, phenytoin, S-warfarin, tolbutamide
CYP2C19	amitriptyline, clomipramine, diazepam, imipramine, omeprazole, phenytoin, propranolol
CYP2D6	Amiodarone, beta-blockers, debrisoquine, dextromethorphan, omeprazole, tricyclic antidepressants, amitriptyline, clomipramine, codeine, desipramine, dextromethorphan, encainide/flecainide, fluvoxamine, imipramine, mexilitine, perphenazine, propafenone, thioridazine
CYP2E1	Acetaminophen, ethanol, chlorzoxazone, halothane, methoxyflurane
CYP3A4	cisapride, corticosteroids, cyclosporine, dapsone, dextromethorphan, diazepam, diltiazem, enalapril, itraconazole, ketoconazole, lovastatin, macrolides, midazolam, nifedipine, quinidine, tacrolimus (FK506), terfenadine, theophylline, triazolam, warfarin, astemizole, carbamazepine, diltiazem, erythromycin, felodipine, lidocaine, lovastatin, midazolam, nifedipine, quinidine, tacrolimus, tamoxifen, terfenadine, valproic acid, verapamil

Table 1.1 Drug Substrates for Important P450 Enzymes
In bold: the enzyme and drug substrate studied.

1.7 CYP2D6

The cytochrome P450 enzyme, CYP2D6, affects the oxidative metabolism of many clinically important medicines, including metoprolol (Mahgoub, Idle et al. 1977; Eichelbaum, Spannbrucker et al. 1979; Eichelbaum and Gross 1990).

CYP2D6 polymorphism was first reported as debrisoquine genetic polymorphism by Mahgoub et al. (1977), who showed than some subjects given the antihypertensive agent excreted comparatively very little 4-hydroxy-debrisoquine in their urine. This was subsequently shown to

be the same as the sparteine genetic polymorphism described by Eichelbaum et al., in 1979. Sequencing of the polymorphic gene was undertaken by Kimura et al., in 1989. Since then more than 60 CYP2D6 alleles have been reported (http://www.cypalleles.ki.se/cyp2d6.htm), and an internationally-accepted nomenclature for human alleles established (Daly et al., 1996).

The CYP2D6 alleles have been defined at the CYP2D locus on chromosome 22q13, of which at least 15 encode non-functional gene products are a result of aberrant splicing, gene deletion, premature translation termination, or deleterious amino acid substitutions (Daly, Brockmoller et al. 1996; Meyer and Zanger 1997).
(Human CYP Allele Nomenclature web-site: http://www.imm.ki.se/CYPalleles).

Non-functional or null alleles (0) are caused by altered splicing sites (e.g. CYP2D6*4, *11), frameshift mutations (e.g. CYP2D6*3, *6, *13, *15), deletion of the gene (5), premature stop codons (8) or missense mutations (7). Other alleles like CYP2D6*9, *10 and *41 with alterations of the amino acid sequence (substitutions e.g. CYP2D6*10, *41 or deletions e.g. CYP2D6*9) are associated with a reduction of in-vivo enzymatic activity (Sachse, Brockmoller et al. 1997; Griese, Zanger et al. 1998; Raimundo, Fischer et al. 2000).

A PM phenotype results if all inherited alleles are null alleles. Thus PM are either homozygous for a particular null allele or inherit two different null alleles (compound heterozygous). In Caucasians, CYP2D6 homozygous null genotypes are present in about 7% of the population (Sachse, Brockmoller et al. 1997; Griese, Zanger et al. 1998). The lack of metabolizing capacity impairs elimination of drugs, which are dependent on this oxidative pathway of metabolism. As a consequence, metoprolol plasma concentrations in PM were found to be elevated 3–10-fold after short-term administration (Freestone, Silas et al. 1982; Deroubaix, Lins et al. 1996).

Alleles with reduced function contribute to the occurrence of intermediate phenotypes in particular when inherited in conjunction with 0 alleles. In this setting, the effects of these alleles are most prominent, since they are not balanced by the presence of a functional allele (Sachse, Brockmoller et al. 1997; Griese, Zanger et al. 1998; Raimundo, Fischer et al. 2000). The changes of the amino acid sequence of reduced function alleles result in diminished activity of the enzyme and/or reduced expression levels of the functional holoenzyme (Zanger, Fischer et al. 2001).

Carriers of two non-functional alleles are phenotypically PM because they have a severely impaired metabolism of CYP2D6 substrates. The CYP2D6 phenotype, defined by the urinary metabolic ratio (MR) of a suitable probe drug such as sparteine, typically shows a bimodal or trimodal distribution among European populations, with the poor metabolizers (MRs > 20) representing 5-10%. The metabolic ratio distribution of EM shows extensive deviations from a normal distribution and, depending on the probe drug used, often separates into two modes comprising the major 'normal' EM mode (MRs < 1.2; 75-85%) and a subgroup of approximately 10-15% of the population with MRs between 1.2 and 20, the IMs (Bock, Schrenk et al. 1994).

Bertilsson, Meese et al. (1993) found the heterogeneity in genotypes is not confined to just the debrisoquine PM individuals. Metabolic ratio (MR) for debrisoquine varied tremendously among EMs. Another subgroup of EMs having MR < 0.1 are termed "ultra rapid metabolizer (UM)" (Bertilsson, Meese et al. 1993). The phenotypes have been linked to some recently identified mutations. They occur at different frequencies in different populations.

It was shown that the metabolic clearance of intermediate metabolizers (IM) for sparteine oxidation is five-fold lower on average compared to EMs (Griese, Zanger et al. 1998). In another

study with debrisoquine, no difference in the mean AUC (0-8 h) was found between five PMs and five heterozygous IMs (Dalen, Dahl et al. 1998). While it has possible to identify poor metabolizers with a high level of certainly by CYP2D6 genotyping, for a decade or so (Chen et al., 1996; Sache et al., 1997; Griese et al., 1998), it was only recently that intermediate metabolizers could be identified more reliably because in addition to being heterozygous with respect to a null allele, they also appeared to carry additional non-functional alleles. In particular, carriers of the *41 allele show impaired expression of CYP2D6 (Toscano et al., 2006).

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The CYP2D6-C variant (*9) has a deleted codon 281, coding for lysine (Tyndale, Aoyama et al. 1991). Although it was initially thought that the allele was associated with the PM phenotype, this allele is now linked to the IM phenotype (Broly and Meyer 1993). It occurs at an average frequency of 1% in whites (Dalen, Dahl et al. 1998) although a higher frequency was reported in Spain (Agundez, Martinez et al. 1994). Another allele, the C188T mutation, which causes a Pro to Ser amino acid change at position 34, was at first identified as one of several amino acid substitutions in the CYP2D6*4 alleles (Hanioka, Kimura et al. 1990; Kagimoto, Heim et al. 1990). The mutation decreased CYP2D6 enzyme function (Kagimoto, Heim et al. 1990) but does not cause the PM phenotype. C188T was first observed in Asian populations and were termed J (for Japanese) or Ch (for Chinese) (Yokota, Tamura et al. 1993; Johansson, Oscarson et al. 1994; Wang, Fan et al. 1998) alleles. Alleles with this mutation lack the detrimental G1934A transition found in CYP2D6*4. They also cause the IM phenotype (Yokota, Tamura et al. 1993; Armstrong, Fairbrother et al. 1994; Johansson, Oscarson et al. 1994). They are infrequent in Whites but are common in Asians.

1.7.1 CYP2D6 Gene

1.7.1.1 Mapping

By using the polyclonal antibody against rat P450dbl, a full-length human cDNA was isolated and sequence analysis revealed a distinct, novel member of the CYP2D subfamily (Gonzales, Basu et al. 1988).

Eichelbaum and colleagues (Eichelbaum, Spannbrucker et al. 1979)(Eichelbaum *et al*, 1979b) found strong linkage of polymorphic sparteine oxidation to the P I blood group, which had been mapped to the long arm of chromosome 22. Subsequently, the *CYP2D* locus (22q 13.1) was also mapped by using the *CYP2D6* cDNA (Gough, Smith et al. 1993).

In humans, the *CYP2D6* wild-type locus contains three highly homologous genes, CYP2*D8P*, *CYP2D7P*, and *CYP2D6* located in 5' to 3' orientation on a contiguous region of about 45 kb (Kimura, Umeno et al. 1989; Gaedigk, Blum et al. 1991). The *CYP2D* genes consist of nine exons and eight introns (**Figure 1.1**). *CYP2D8P* is a pseudogene, contains multiple deletions and insertions, and causes a highly disrupted open reading frame (Kimura, Umeno et al. 1989). The CYP2D7P gene resembles CY*P2D6* more than it does *CYP2D8P*. Its coding sequence indicates only a single inactivating mutation, an insertion of T_{226} in the first exon. No specific mRNA product has however been detected in the RNA from human livers suggesting that it also is a pseudogene (Kimura, Umeno et al. 1989).

1.7.1.2 Nomenclature

Based on recommendations for human genome nomenclature, Daly, Brockmoller et al. 1996 proposed that alleles at this locus be designated by *CYP2D6* followed by an asterisk and a combination of Roman letters and Arabic numerals distinct for each allele. The number will specify the key mutation and, where appropriate, a letter specifying additional mutations. Criteria for classification as a separate allele and protein nomenclature were also presented (**Table 1.2**). This nomenclature has been adopted for this thesis.

Table 1.2 Allele nomenclature for CYP2D6 proposed by Daly, Brockmoller et al. 1996. CYP2D6 alleles with corresponding single nucleotide changes and enzyme activity

1.8 BETA-2 ADRENERGIC RECEPTOR GENE

The human beta-2 adrenergic receptor (beta-2 AR) gene is situated on the long arm of chromosome 5 (5q31-33) and codes for an intronless gene product of just over 1200 base pairs (Kobilka, Frielle et al. 1987). The promoter region of the gene, which is 200-300 base pairs 5' to the translation initiation codon, has also been characterized (Emorine, Marullo et al. 1987). Receptor transcripts have a 5' leader region harbouring an open reading frame (ORF) that encodes a 19-amino acid peptide. *In vitro* study done by Parola and Kobilka 1994, showed that this peptide impedes translation of beta-2 adrenergic receptor mRNA, and thus regulates cellular expression of the receptor.

Thirteen different polymorphisms were identified by Drysdale, McGraw et al. 2000. All of these differed from the accepted wild type sequence by a single base change at different positions in the coding sequence of the gene. Due to the redundancy in the amino acid code, a number of these polymorphisms are functionally silent. Thus, although there was a base change in the coding sequence, the resultant receptor had the same amino acid sequence as the wild type form.

Reihsaus, Innis et al. 1993 screened the open-reading frame of the beta-2 AR gene for polymorphisms, using the technique of temperature gradient gel electrophoresis (TGGE). Nine polymorphisms were identified, five of which were degenerate. Four resulted in a single amino acid (AA) change in the receptor, corresponding to nucleic acid residues 46 (A-G), 79 (C-G), 100 (G-A), 491 (C-T). These result in the substitution of glycine for arginine at AA position 16 (Arg-Gly 16), glutamate for glutamine at AA position 27 (Gln-Glu 27), methionine for valine at AA position 34 (Val-Met 34), and isoleucine for threonine at AA position 164 (Thr-Ile 164) respectively (**Figure 1.2**).

Figure 1.2: Structure of the beta-2AR polymorphisms showing identified polymorphisms.

(Dewar, Wilkinson et al. 1997)

Four polymorphisms resulting from single base changes were identified which altered the amino acid sequence of the receptor protein, all resulting in amino acid substitutions. They are codon 16, 27, 34 and 164. Three of these polymorphisms (codon 16, 27 and 164) have been studied in individuals exposed to circulating catecholamines or exogenously administered beta-2 agonist such as salbutamol and appeared to alter the functional properties of the receptor (Hall 1996).

Polymorphism of the human beta-2 AR gene has been detected using the restriction enzyme Ban-1, which reveals two alleles but these were not defined (Ohe et al. 1995). Ban-1 polymorphism of the beta-2 receptor gene is unrelated to allergic disease (Dewar et al. 1998). In a group of 51 patients with asthma and 56 normal subjects, nine different point mutations in nucleic acid sequence were detected. The most frequent of which were arginine16 to glycine (Arg16->Gly) and the glutamine27 to glutamic acid (Gln27->Glu) (Reihsaus, Innis et al. 1993). The frequencies of the common genotypes of the beta-2 adrenergic receptor in 178 individuals from US (Liggett 1997) are shown in **Table 1.3**

Table 1.3: Frequencies of the common genotypes of the beta-2 adrenergic receptor in normal and asthmatic subjects

Results are from 178 individuals pooled from several studies. The Val34 → Met polymorphism is rare (< 1%) and is not included in the table **(Liggett 1997).**

The main clinical interest in beta-2 receptor polymorphisms lies in the possibility that these polymorphisms may alter the way in which the receptor down-regulates in the airways and as such may modify a patient's response to a particular drug, altering both efficacy and adverse

effects. Thus, beta-2 adrenergic receptor polymorphisms could potentially affect airway responses by altering the expression and coupling of beta-2 receptor in airway cells.

1.8.1 Allele Frequency of Beta-2 Adrenergic Receptor and ethnicity

The allelic frequency of beta-2 adrenergic receptor polymorphism is greatly influenced by ethnicity. For Gly16 and Glu27 variants, the allele frequencies among Asians are significantly lower compared to Caucasians. Generally, Ile164 is a very rare allelic variant that is seldom found in all of the populations studied. Only a few studies have been done on codon 19 and nucleotide position –20 polymorphism (**Table 1.4**).

Table 1.4: Occurrence of beta-2 AR polymorphisms among different ethnic groups

Study	Population	Allele Frequency				
		Gly16	Gln27	Thr164	-47T	20T
(Aynacioglu, Cascorbi et al. 1999)	Unrelated Turkish subjects (n = 104)	0.60	0.68	0.995	-	-
(Buscher, Eilmes et al. 2002)	Cystic fibrosis German (n = 126)	0.39	0.61	0.98	-	-
	German healthy subjects (n = 153)	0.51	0.61	0.98	-	-
(Hamdy, Hiratsuka et al. 2002)	Unrelated Egyptian subjects (n = 240)	0.43	0.76	-	-	-
	Chinese subjects with COPD (n = 65)	0.49	0.73	-	-	-
	Chinese healthy subjects (n = 41)	0.40	0.71	-	-	-
(Ho, Harn et al. 2001)	Japanese obese subjects (n = 108)	0.46	0.86	100.0	-	-
	Japanese non-obese subjects (n = 400)	0.50	0.93	100.0	-	-
(Ishiyama-Shigemoto, Yamada et al. 1999)	Hypertensive Japanese (n = 842)	0.52	0.94	-	0.91	-
	Normotensive Japanese (n = 633)	0.50	0.92	-	0.90	-
(Kato,	Unrelated Korean subjects (n = 440)	0.47	0.90	-	-	-

Sugiyama et al. 2001)	Hypertensive African Caribbeans (n = 136)	0.85	-	-	-	-
(Kim, Lee et al. 2002)	Normotensive African Caribbeans (n = 81)	0.67	-	-	-	-
(Kotanko, Binder et al. 1997)	European Caucasian asthmatics (n = 96)	0.69	0.48	-	0.60	-
	Children with 2 Caucasian parents (n = 188)	0.63	0.61	-	-	-
		0.61	0.66	-	-	-
(Lipworth, Koppelman et al. 2002)	Children with 1 Caucasian and 1 Hispanic parent (n = 40)	0.59	0.73	-	-	-
(Martinez, Graves et al. 1997)	Children with 2 Hispanic parents (n = 41)					
(Timmermann, Mo et al. 1998)	Children with hypertensive Caucasian parents (n = 18)	0.42	0.67	-	0.69	
		0.67				
	Children with normotensive Caucasian parents(n = 16)	0.72	0.56	-	0.56	
		0.56				
	Bronchohyperreactive German (n = 152)					
(Ulbrecht, Hergeth et al. 2000)	Bronchonormoreactive German (n = 295)					
		0.61	0.52	0.993	-	-
		0.65	0.57	100.0	-	-
	Chinese subjects (n = 128)					
	Chinese healthy subjects (n = 136)					
(Wang and Feng 2001)		0.38	0.92	-	-	-
	Caucasian non-asthmatics (n = 84)	0.49	0.91	-	-	-
	Caucasian asthmatics (n = 160)					
(Weir, Mallek et al. 1998)	Black non-asthmatics (n = 62)	0.61	0.60	-	-	-
	Asian asthmatics (n = 15)	0.61	0.56	-	-	-
		0.50	0.73	-	-	-
	Japanese obese subjects (n = 124)	0.40	0.80	-	-	-
	Japanese non-obese subjects (n = 450)					
(Yamada, Ishiyama-Shigemoto et al. 1999)		-	-	-	0.82	0.82
		-	-	-	0.89	0.89

Drysdale, McGraw et al. 2000 reported on 13 SNP's of the beta-2 AR gene. Polymorphisms at codon16, 27 and 164 have been reported to cause variation in the encoded amino acids (**Table 1.5**).

Allele, the two nucleotide possibilities at each SNP position;
Ca, Caucasian; A-A, African-American; As, Asian; H-L, Hispanic-Latinos; 5', 5' upstream of beta-2 adrenergic receptor gene ORF; syn, synonymous SNP.

Table 1.5: Localization of SNPs and identification of haplotypes of the beta-2 AR gene.

(Drysdale, McGraw et al. 2000)

Within the 5' leader region, two SNPs, at nucleotide position –47 and –20, were found to alter the beta-2 receptor expression.

The SNP at position –47 (T to C change), resulting in either Arg (previously denoted wild-type) or Cys at amino acid position 19, is involved in translation regulation of the beta-2 adrenergic receptor gene (McGraw, Donnelly et al. 1998).

The SNP at nucleotide position -20 (T to C change) has been studied by Timmermann, Mo et al. 1998 in Caucasians while Yamada, Ishiyama-Shigemoto et al. 1999 studied this polymorphism in Japanese population.

Other SNPs remain to be investigated for their effect on the gene function or expression.

Out of these 13 reported SNPs, we have chosen to study five. These 5 polymorphisms (codon 16, 27, 164, 19 and nucleotide position –20) have been shown to be functionally significant in influencing the beta-2 receptor expression.

1.8.2 Codon 16 polymorphism and bronchial responsiveness

In an interesting prospective study, Israel et al., 2004, tested the hypothesis that a genetic polymorphism of the beta2-adrenergic receptor that results in homozygosity for arginine rather than glycine at amino acid residue 16, influenced the response and adverse effects to the beta2 agonist albuterol in asthmatic patients. During the randomized treatment period, patients with the Gly/Gly genotype, treated with regular scheduled albuterol, showed an increase in morning peak expiratory flow rate (PEFR) relative to placebo. In contrast, patients with the Arg/Arg genotype had a decreased PEFR. The latter group of patients also showed an increase in morning symptom score relative to placebo while patients with the Gly/Gly genotype reported a decrease.

The wild type Arg16 is associated with more favourable effect on the airway responsiveness than Gly16. This genotype was found to be more likely to respond to inhaled beta-2 agonist compared to the Gly16 carriers (homozygous and heterozygous) (Martinez, Graves et al. 1997). The homozygous Arg16 has also been shown to develop less tolerance to the beta-2 agonist compared to the homozygous Gly16 (Martinez, Graves et al. 1997).

A study done by Lima, Thomason et al. 1999 on a group of 16 stable moderate asthmatics, suggested that the homozygous Arg16 responded more rapidly and to a far greater extent at equivalent plasma albuterol level compared to the Gly16 carriers (homozygous and heterozygous).

However, a larger trial by Israel, Drazen et al. 2000 found a significant reduction in pulmonary function (both morning and evening peak expiratory flow values) in patients homozygous for Arg16 when exposed to regular use of albuterol. Taylor, Drazen et al. 2000 found that a higher incidence of exacerbation of asthma in persons who were homozygous for Arg16 than in those

who were heterozygous or homozygous for Gly16 during long-term treatment with inhaled beta-2 agonist.

The presence of Gly16 allele markedly influenced the ability of beta-2 receptor to desensitize. Gly16, compared to Arg16, down regulates to a much greater degree following exposure to a beta2 agonist in both transfected cell systems and primary cultured human airway smooth muscle cells (Reihsaus, Innis et al. 1993).

Green and Liggett 1994 studied the mechanisms by which the variants of the beta-2 AR gene may alter the receptor function in vitro. Site-directed mutagenesis and recombinant combinations of homozygous forms of the beta-2-adrenergic receptor codon 16 and 27 polymorphisms were performed on Chinese hamster fibroblasts, which normally do not express any adrenergic receptors. They showed no difference in agonist binding between genotypes, but the Gly-16 variant showed markedly increased agonist-promoted down regulation of receptor expression when compared with the Arg-16 variant. These effects of Gly-16 were independent of the allele 27 variant with which the allele 16 variant co-expressed. The Arg-16/Glu-27 combinant showed complete absence of agonist-promoted receptor down regulation, whereas Gly16/Glu27 showed the same level of enhanced down regulation associated with the Gly-16 variant, which prevails over the opposite effect of Glu-27 when both variants are concomitantly expressed in homozygous form. Green, Turki et al. 1995 in another study also examined the beta-2 adrenergic receptor function in primary cultures of human airway smooth muscle. Their study confirmed previous reports of Gly-16 being associated with markedly increase agonist-driven down regulation of beta-2 adrenergic receptor. A rare haplotype (ArgGly/Glu/Glu) was studied and it was shown that homozygous for Glu-27 showed very little agonist-driven down regulation of the beta-2 adrenergic receptor.

Martinez, Graves et al. 1997 also showed that carriers of Gly-16 had decreased beta-2 adrenergic responsiveness when compared with carriers of Arg-16 allele, but codon 27 was found to be unrelated to beta-2-adrenergic responsiveness.

A study by Turki, Pak et al. 1995 found Gly 16 was associated with nocturnal asthma. Of the 23 patients with nocturnal asthma, 16 (69.6%) were homozygous Glyl6, while 8 of 22 (36.4%) of the non-nocturnal cohort were homozygous for Glyl6 ($p= 0.038$ and odds ratio of 4.0).

While Turki, Pak et al. 1995 failed to demonstrate the relationship between these polymorphisms with the severity of asthma, Reihsaus, Innis et al. 1993 in his study found a significant association between Glyl6 polymorphism and severe asthma phenotype.

Tan, Grove et al. 1997 in their study of 22 moderately severe stable asthmatic patients suggested that in the homozygous Gly16, the bronchodilator desensitization occurs only when long-acting beta-2 agonists (formoterol) are used on a regular basis.

1.8.3 Codon 27 polymorphism and bronchial responsiveness

In contrast to Glyl6, Glu 27 appears to protect against down regulation in both transfected and non-transfected cells systems (Green and Liggett 1994; Green, Turki et al. 1995). Using primary cultured human airway smooth muscle cells, they found down regulation of the receptors, as assessed by changes in receptor number, occurred at a lesser extent than the Gln 27 form following prolonged exposure to beta-2 agonists.

In addition, a similar relative resistance to down regulation was observed using beta-2 agonist mediated cyclic AMP formation as a more functionally relevant end-point for receptor coupling.

On the basis that individuals with the Glu 27 form of the beta-2 adrenergic receptor might be protected from bronchoconstrictor influences, Hall, Wheatley et al. 1995 studied airway reactivity in a group of 65 mild to moderate asthmatics and found that individuals with the Glu 27 form of the receptor were more resistant to agonist-promoted down regulation. These individuals had four times less reactive airways than those with the Gln 27 form of the receptor when assessed using metacholine challenge.

Tan, Grove et al. 1997 in their study of 22 asthmatic patients who received regular long acting beta-2 agonist found the Gln27 group of patients developed greater desensitization than the Glu27 group. This finding is in contrast from earlier reports, which found Glu27 confers greater bronchoprotective effect against agonist-induced down regulation. Since all of the 6 homozygous Glu27 cohorts were also Glyl6 homozygous, the authors suggested that due to the dominant effects of Glyl6, the bronchoprotective effect ofGlu27 were nullified.

In another study, Weir, Mallek et al. 1998 found the prevalence of the Gln27 allele, in particular the Glyl6/Gln27 variant, were significantly higher in the subgroup of non-fatal asthmatics, who had moderately severe asthma than in those who had mild asthma. The positive association between the Gln27 allele and moderate asthma support the suggestion that they are associated with enhanced bronchial hyperresponsiveness (Hall, Wheatley et al. 1995) and elevated IgE levels (Dewar, Wilkinson et al. 1997) among asthmatics.

1.8.4 Codon 164 polymorphism and bronchial responsiveness

The third polymorphism is at amino acid 164, which can either be a Threonine (Thr) or an Isoleucine (Ile). This polymorphism is rare compared to the polymorphisms at amino acid 16 or 27 with an allelic frequency of about 1% (Reihsaus, Innis et al. 1993). However, it is particularly

interesting that amino acid 164 is situated in the fourth transmembrane spanning domain of the receptor and is adjacent to a serine at position 165, which has been predicted to interact with the beta-carbon hydroxyl group of adrenergic ligands.

This polymorphism has been studied in a transfected cell system and has been shown to alter the agonist binding properties of the receptor. Cells expressing Ile 164 were found to have approximately four times less affinity for ligands containing this hydroxyl group, whereas binding of the ligands such as dobutamine which lack this hydroxyl group were unaltered (Strader, Sigal et al. 1989). This alteration in binding affinity was reflected in a reduced capacity for the receptor to activate adenylyl cyclase relative to the wild type (Thrl64) form of the receptor. The rarity of this polymorphism has precluded clinical studies from being performed to date.

1.8.5 Polymorphisms at nucleotide positions -47 and –20 and bronchial responsiveness.

In the Bergen Blood Pressure Study, Timmermann, Mo et al. 1998 studied the offspring's of hypertensive parents and compared them to the offspring's of normotensive parents. Twenty-three hypertensive and 22 normotensive families were recruited and the DNA samples from the first born of hypertensive family-history offspring and normotensive family-history offspring were analyzed specifically for the promoter and the N-terminal portion of the beta-2 AR gene. Four genetic variants, at position –47, -20, +46 and at +79 were found. Haplotypes for the four intragenic variants were constructed and significant linkage disequilibrium was found. In particular, they found the 5' leader cistron mutant with the wild type alleles at the other loci was significantly more frequent in offspring of hypertensive parents, compared to offspring of normotensive parents.

Yamada, Ishiyama-Shigemoto et al. 1999 screened the 5'-untranslated region of the beta-2 AR gene from 40 obese subjects by the PCR-direct sequencing technique and found two polymorphic sites; a T-->C substitution at -47 and a T-->C substitution at -20. They further analyzed the association of the polymorphisms with obesity in 574 subjects by PCR and restriction digestion and found the substitution at -47 was in tight linkage disequilibrium with that at -20. The polymorphisms were also in linkage disequilibrium with codon 16 and codon 27 polymorphisms. Subjects carrying the -47C/-20C allele had greater body mass index and higher serum triglyceride levels than -47T/-20T homozygotes. Thus, the exchange at -47 may alter the expression level of the beta2-adrenergic receptor gene, because the nucleotide substitution at -47 results in a Cys-->Arg exchange at the C terminal end of the leader peptide. They concluded the -47C/-20C allele might be associated with genetic predisposition to obesity and obesity-related metabolic disorders.

While the nucleotide position -47 polymorphism causes an amino acid substitution in the leader sequence, the -20 polymorphism, which is also in the part of the gene coding for the leader sequence, does not cause any amino acid substitution.

As codon 16 and 27 polymorphisms have been shown to be associated with bronchial hyper-responsiveness, we postulate that nucleotide position –47 and –20 polymorphisms could play a role in altering the bronchial response in view of its linkage disequilibrium with codon 16 and 27.

1.9 METOPROLOL

Metoprolol is a cardio-selective beta – 1 – adrenergic receptor antagonist. It acts by causing vasodilatation of the cardiac vessels and reduces the cardiac output. It is widely used world- wide as an anti-hypertensive agent. In Malaysia, it is used as the first-line treatment of hypertension in most hospitals. Its metabolism is mediated by the polymorphic CYP2D6 that exhibits large inter-ethnic differences (Kimura, Umeno et al. 1989; Meyer 1994).

As most of its adverse reactions could be due to excessive plasma concentrations, its use among the Malaysian population may therefore be associated with adverse effects due to the high frequency of the *CYP2D6*10* allele, resulting in reduced capacity of the local population to metabolize the drug (Ismail, Hussein et al. 2000; Teh, Ismail et al. 2001).

Most adverse effects of metoprolol are related to interference with beta-2-receptor mediated functions including bronchodilation and vasodilation. Metoprolol, may in susceptible patients induce bronchospasm and worsen or precipitate asthma (Katzung 2001). Severe bronchoconstriction may be induced even in 'mild' asthmatics. This is due to the incompleteness of the beta-1 cardioselectivity. The larger the dose and the higher the plasma and tissue concentrations, the greater the effect on beta 2-receptor sites (Kendall 1990).

CHAPTER

2

GENETIC POLYMORPHISMS OF CYP2D6 & BETA-2 AR GENE; A PHARMACOGENETICS AND CLINICAL STUDY OF METOPROLOL INDUCED BRONCHOSPASM

CHAPTER 2

2.1 INTRODUCTION

Metoprolol is widely used world-wide as an antihypertensive agent. It has been proven to be effective in the treatment of hypertension (Wikstrand *et al.* 1991) and chronic heart failure (MERIT-HF, 1999). In Malaysia, it is used as first-line treatment of hypertension in most hospitals. Metoprolol undergoes extensive hepatic first-pass metabolism (Borg *et al.* 1975), and CYP2D6 is the major enzyme involved (Lennard *et al.* 1982, McGourty *et al.* 1985). These polymorphic CYP2D6 enzymes exhibit large inter-ethnic differences in distribution (Meyer, 1994, Kimura *et al.* 1989). As most of its adverse effects could be due to excessive plasma concentrations, its use among the Malaysian population may therefore be associated with more frequent adverse effects due to the high frequency of the *CYP2D6*10* allele, resulting in reduced capacity of the local population to metabolize the drug (Teh *et al.* 2001, Ismail *et al.* 2000). The Malaysian population is highly heterogeneous with at least three distinct ethnic groups: Malays, Chinese and Indians. Teh et al. (2001) reported that while the CYP2D6*10 allele occurred with a frequency of 49.5%, in the Chinese population, estimates range from 64.7 to 87.8%. Therefore although the Malays share a closer common ancestry with the Chinese than the Caucasians, as a result of their relative historical geographical migration patterns, they can be genetically quite different with respect to genetic polymorphisms.

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The molecular genetics of *CYP2D6* is complex. More than 70 different alleles have been described, several of which encode non-functional gene products (null alleles, **0*) (Daly, Brockmoller et al. 1996; Sachse, Brockmoller et al. 1997; Griese, Zanger et al. 1998). A CYP2D6 poor metabolizer phenotype results when a person inherits null alleles only.

CYP2D6 poor metabolizers exhibit several-fold higher plasma concentrations of metoprolol than extensive metabolizers with normal CYP2D6 activity (Kimura, Umeno et al. 1989; Kendall,

Maxwell et al. 1991; Daly, Brockmoller et al. 1996). Poor metabolizers are therefore more susceptible to adverse effects than extensive metabolizers at standard doses of metoprolol.

As most adverse effects of metoprolol are related to interference with beta-2-receptor mediated functions including bronchodilation, a hypertensive patient when prescribed metoprolol, can develop bronchospasm and this can worsen or precipitate asthma (Katzung 2001). This is due to metoprolol's incomplete beta-1 cardioselectivity. The larger the dose and the higher the plasma and tissue concentrations, the greater the effect on beta 2-receptor sites (Kendall 1990).

The genetic polymorphisms of CYP2D6 gene have been discussed in detail in the previous chapter. In this chapter, seven polymorphisms of the CYP2D6 gene namely CYP2D6*3, CYP2D6*4, CYP2D6*6, CYP2D6*9, CYP2D6*10, CYP2D6*14 and CYP2D6*17 were studied. CYP2D6*3, CYP2D6*4 and CYP2D6*14 are associated with enzyme with poor metabolizing capacity while CYP2D6*6, CYP2D6*9, CYP2D6*10 and CYP2D6*17 are associated with enzyme of intermediate or extensive metabolizing capacity (Daly, Brockmoller et al. 1996).

The genetic polymorphisms of the beta2 adrenergic receptor gene have also been discussed in detail in the previous chapter. Thirteen different polymorphisms of the beta2 adrenergic receptor gene were identified by Drysdale, McGraw et al. (2000). Out of these, five polymorphisms were chosen for this study, namely those at codon 16, 27, 164 and at nucleotide positions -47, -20. These polymorphisms were chosen as they were found to be functionally significant (Timmermann, Mo et al. 1998; Aynacioglu, Cascorbi et al. 1999; Ishiyama-Shigemoto, Yamada et al. 1999; Yamada, Ishiyama-Shigemoto et al. 1999; Ulbrecht, Hergeth et al. 2000; Kato, Sugiyama et al. 2001; Buscher, Eilmes et al. 2002; Hamdy, Hiratsuka et al. 2002; Lipworth, Koppelman et al. 2002). One of the polymorphisms, allele -47, was also found to be in tight

linkage disequilibrium with that at -20 (Yamada, Ishiyama-Shigemoto et al. 1999). These polymorphisms were also in linkage disequilibrium with codon 16 and codon 27 polymorphisms. While codons 16 and 27 have been studied quite extensively, alleles -20 and -47 are not.

2.2 OBJECTIVES

1) To genotype for common polymorphisms in the CYP2D6 and beta2 adrenergic receptor genes in hypertensive patients.
2) To determine the association between CYP2D6 and beta2 adrenergic receptor gene polymorphisms and the response to blood pressure control with metoprolol.
3) To determine the incidence of metoprolol induced bronchospasm in this group of hypertensive patients and its association with the genetic polymorphisms of beta2 adrenergic receptor gene.

2.3 CLINICAL METHODS

This study was approved by the Research & Ethics committee, Universiti Sains Malaysia Kelantan, Malaysia.

STUDY DESIGN

2.3.1 Sample size

2.3.1.1 Sample size calculation

To calculate the required the required sample size, previous estimates of genotype frequencies were used. Where possible these were based on previous data on Malaysian populations. For the CYP2D6 study, the frequency of those homozygous for CYP2D6*10 and those heterozygous for inactive alleles was approximately 30% (Teh et al., 2000). It was predicted that 50% of patients to be studied might show poor control of hypertension. Based on these numbers, it was calculated that 126 patients would be needed to show an increased incidence of 20% in poor control of

hypertension in the 50% of patients with impaired CYP2D6 activity with 80% statistical power and alpha equal to 0.05.

For Beta-2 AR polymorphisms, local data is not available. Based on published data for other Asian populations (Drysdale et al., 2000), 20% of the population under study were expected to be homozygous for the Gly16-encoding form which has been suggested to be a risk factor for hypertension and poorer drug response. If 10% of all patients were found to show bronchospasm on treatment with metoprolol, 175 patients would be needed to show an increase incidence of 20% in bronchospasm in homozygous Gly16 patients with 80% statistical power and alpha equal to 0.05.

2.3.2 Statististical Analysis

The descriptive statistics of the samples were presented with frequency and percentage for categorical data.

Association between the genotype and phenotype, and side-effects were tested using the Chi Squared test.

All analyses were done using STATA version 7.0.

2.3.3 Patients' recruitment

2.3.3.1 Visit 1

The hypertensive patients were enrolled consecutively from the outpatients' clinic at Hospital USM (HUSM) and Physicians Clinic at Hospital Kota Bharu (HKB) during the study period.

Inclusion criteria

Male adults aged between 18 – 65 years.

Diagnosed as suffering from hypertension and received metoprolol as treatment.

Exclusion criteria

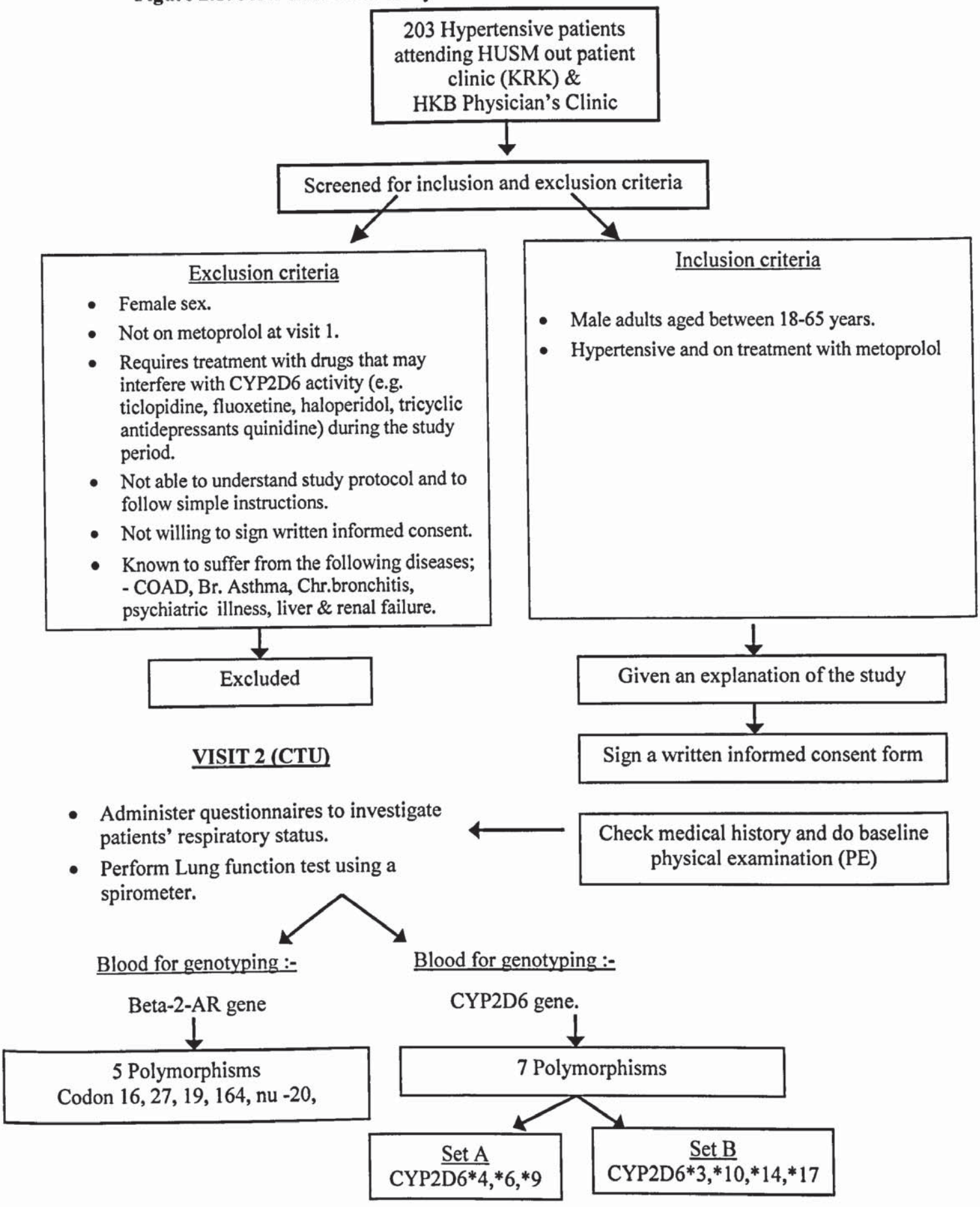
1. Female. The hormonal changes during the menstrual cycle affect the CYP2D6 enzyme activities.
2. Not on metoprolol treatment during visit 1.
3. Known to suffer from the following diseases: chronic obstructive airway disease, bronchial asthma, pulmonary disease (affecting the lung function tests), psychiatric illness (patient may not be able to follow instruction and give consent), liver and renal failure (affect metabolism and excretion of metoprolol).
4. Requires treatment with drugs that may interfere with CYP2D6 activity during the study period. (e.g. ticlopidine, fluoxetine, haloperidol, tricyclic antidepressants, quinidine)
5. Unable to understand the study protocol and unwilling to follow instructions

A baseline physical examination was done that included examination of the cardiovascular, respiratory, gastrointestinal, renal and central nervous systems.

Flow chart of the study

Figure 2.1 summarises the study.

Figure 2.1: Flow chart of the study



Blood Pressure measurement

Blood pressure was measured in both right and left upper limbs but only the highest reading was recorded. Subsequent blood pressure measurements were made on the same upper limb.

The same sphygmomanometer was used to measure the blood pressure throughout the study.

The radial pulse rate was also recorded.

Metoprolol therapy was continued and patients were reviewed 2 weeks later at the clinical trial unit (CTU) HUSM.

2.3.3.2 Visit 2

At the CTU, the patients were given an explanation of the study and asked to sign a written informed consent form.

Baseline physical examination was done including measurement of blood pressure according to the above protocol. Relevant clinical history including concurrent medical illness, past medical illness, family history of related illness were taken and recorded.

Blood was taken for the following investigations:-

(1) Renal function test (RFT)

(2) Liver function test (LFT)

(3) CYP2D6 genotyping

(4) Beta-2 AR genotyping

(5) Metoprolol drug level.

Metoprolol was then administered to these patients according to their usual morning dose.

Patients were asked questions from a standard questionnaire (Table 2.1) to determine if they had experienced difficulty in breathing and wheezing.

SOAL SELIDIK TENTANG MASALAH PERNAFASAN

3

1. Pernahkah anda mengalami sesak nafas, atau mendengar bunyi seperti siulan dari dada pada bila – bila masa yang lalu?	YA TIDAK	☐ ☐
2. Pernahkah anda mengalami sesak nafas, atau mendengar bunyi seperti siulan dari dada dalam masa 12 bulan yang lepas?	YA TIDAK	☐ ☐
3. Pernahkah anda mengalami sesak nafas, atau mendengar bunyi seperti siulan dari dada sejak anda mengambil ubat metoprolol?	YA TIDAK	☐ ☐
4. Berapa kali anda mengalami sesak nafas dalam masa 12 bulan yang lepas atau selepas mengambil ubat metoprolol?	TIADA 1 HINGGA 3 KALI 4 HINGGA 12 KALI LEBIH DARIPADA 12 KALI	☐ ☐ ☐ ☐
5. Dalam masa 12 bulan yang lepas, berapa kali, secara purata, sesak nafas mengganggu tidur anda?	TIDAK PERNAH TERJAGA KERANA SESAK NAFAS 1 HINGGA 3 MALAM SETIAP BULAN 4 ATAU LEBIH MALAM SETIAP BULAN	☐ ☐ ☐
6. Dalam masa 12 bulan yang lepas, pernahkah anda mengalami sesak nafas yang begitu teruk hingga anda hanya dapat bercakap 1 atau 2 perkataan setiap tarikan nafas?	YA TIDAK	☐ ☐
7. Pernahkah anda menghidapi penyakit asma?	YA TIDAK	☐ ☐
8. Dalam masa 12 bulan yang lepas, pernahkah dada anda berbunyi seperti siulan semasa atau selepas senaman?	YA TIDAK	☐ ☐
9. Dalam masa 12 bulan yang lepas, pernahkah anda batuk pada waktu malam, semasa anda **tidak mengalami** deman selsema atau jangkitan paru – paru?	YA TIDAK	☐ ☐

Table 2.1 Questionnaire to determine if patients had experienced difficulty in breathing (Malay version)

Questionnaire On The Respiratory Symptoms Experienced By Patients	
1. Have you ever experienced difficulty in breathing or wheezing at any time in the past?	☐ Yes ☐ No
2. Have you ever experienced difficulty in breathing or wheezing over the last 12 months?	☐ Yes ☐ No
3. Have you ever experienced difficulty in breathing or wheezing since you took metoprolol?	☐ Yes ☐ No
4. What was the frequency of shortness of breath or wheezing over the last12 months or since you took metoprolol?	☐ Never ☐ 1-3 times ☐ 4-12 times ☐ more than 12 times
5. Over the last 12 months, how often was your sleep disturbed because of the shortness of breath or wheezing?	☐ Never ☐ 1-3 times per month ☐ 4 or more per month
6. Over the last 12 months, have you ever experienced severe difficulty in breathing or wheezing resulting in ability to speak only 1 to 2 words on inspiration?	☐ Yes ☐ No
7. Have you ever suffered from Asthma?	☐ Yes ☐ No
8. Over the last 12 months, have you ever experienced wheezing during or after exercising?	☐ Yes ☐ No
9. Over the last12 months, have you ever experienced nocturnal cough even when you are not suffering from upper respiratory tract infection or pneumonia?	☐ Yes ☐ No

Table 2.2 Questionnaire to determine if patients had experienced difficulty in breathing (English version)

2.3.4 Lung Function Test

A portable Spirometer (Schiller Spirovid SP/1) was used to perform the Lung function tests. The following values were recorded: FEV1 , FVC , PEFR and FEV1/FVC

2.3.4.1 Bronchospasm

Bronchospasm was defined by a FEV1/FVC ratio of less than 0.7.

Figure 2.2 Portable Spirometer (Schiller Spirovid SP/1)

2.3.4.2 Spirometer

All patients who performed the Lung function test using a spirometer were asked to follow the following steps after a demonstration was given on how to perform the Lung function test using the Spirometer.

1) Stand erect while performing the test.

2) Loosen tight clothing or necktie.

3) Breathe out fully through the mouth and NOT through the nose. (Encouragement was given at all time by the investigator).

4) Rest for 2 minutes and then repeat 2 times (unless failed to perform the task). The best (defined as the highest) reading was taken.

2.3.4.3 Recording the Lung Function Tests:-

Each patient was asked to perform the test 3 times (unless unable to do so) and the readings were recorded. The best (defined as highest) reading was taken.

Attempt 1

FVC	(l/s)
FEV_1	(l/s)
PEF	(l/s)

Attempt 2

FVC	(l/s)
FEV_1	(l/s)
PEF	(l/s)

Attempt 3

FVC	(l/s)
FEV_1	(l/s)
PEF	(l/s)

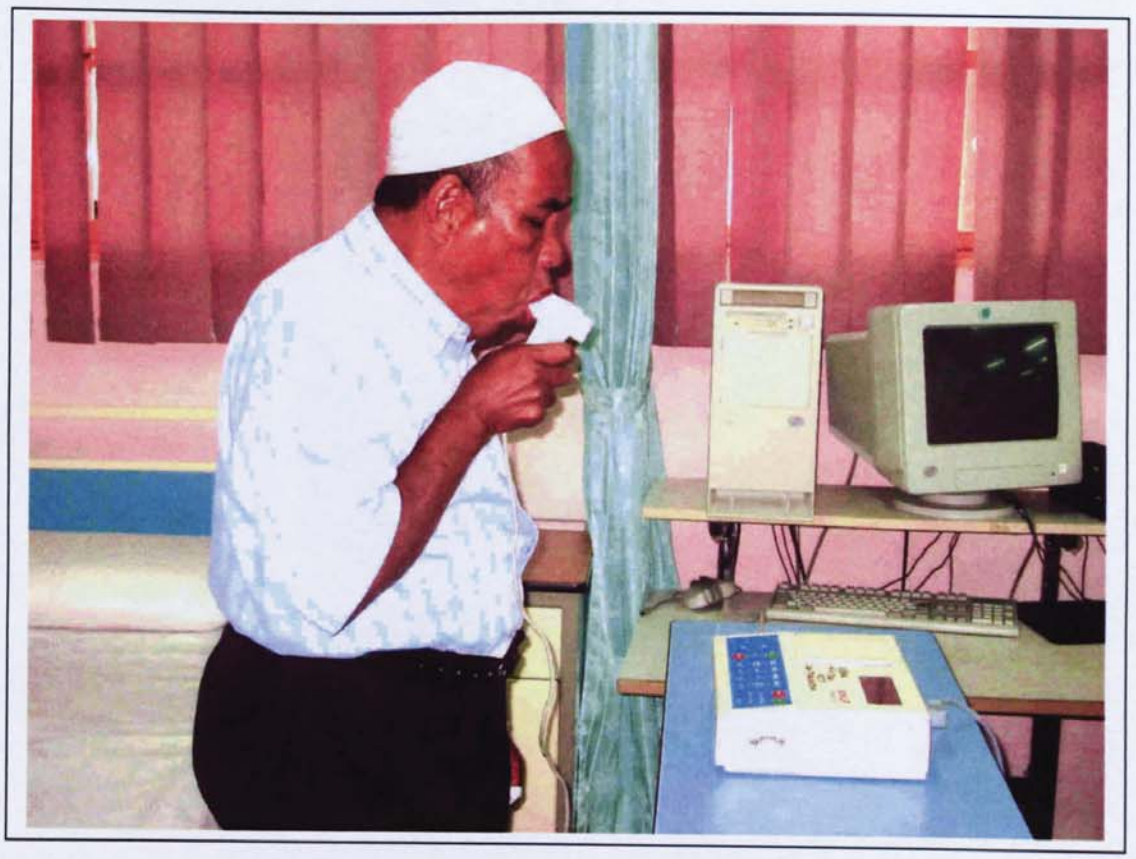


Figure2.3 Performing the lung function test using a Spirometer

2.4 GENOTYPING METHODS

2.4.1 Extraction Of DNA

Extraction of DNA was done by standard alkali lysis method (salting out technique).

(Miller et al., 1988)

2.4.1.1 Sample collection

10 ml of venous blood were drawn from the ante-cubital vein (or any other vein which is easily accessible in the upper limb), after cleaning the area with 70% alcohol. The blood was then transferred into a sodium citrate container and labelled. The following particulars of the patients were written on the label: -

- λ Name
- λ Age
- λ Patient's registration number (R/N)
- λ Date of collection

2.4.1.2 Recording of sample in laboratory

Each specimen was recorded upon arrival

The following information was entered into the record book:

- λ Date received
- λ Name
- λ Age
- λ Diagnosis
- λ R/N
- λ Hospital / clinic
- λ Test
- λ Note / remark
- λ Laboratory number

2.4.1.3 Reagents used for DNA extraction: -

1) Red Cell Lysis Buffer

100 ml of 1x Lysis buffer containing:
0.64 M Sucrose
0.02 M Tris-HCL
2% Triton X-100

For 0.5x Lysis buffer, the 1x lysis buffer was diluted fresh 1: 1 with autoclaved Milli-Q water.

Preparation of the lysis buffer: -

- 21.9 g sucrose was dissolved in 75 ml of autoclaved Milli-Q water (by gentle heating of the solution)
- 1 ml of 2 M Tris (pH 7.8) and 2 ml of Triton X-100 was added.
- 24 ml autoclaved Milli-Q water was added to a total of 100 ml.
- Stored at 4°C until used

2) 20% Sodium Dodecyl Sulphate (SDS)

Preparation: -

- 20 g of SDS was dissolved in autoclaved Milli-Q water, warming it on a hot plate
- The solution was made up to 100 ml
- Stored at room temperature.

3) Proteinase-K (20 mg/ml)

Preparation: -

100 mg proteinase K
+ 125 µl of 2M Tris-HCL (pH 8.0)
+ 437 µl of autoclaved Milli-Q water

- Aliquoted into 450 ul (into 10 Eppendorf tubes)
- Stored at -20°C
- 50 ml of CaCl2 stock solution was added just before use
  (to give a working concentration of 20 mg/ml proteinase-K in 50mM of Tris-HCL pH 8.0 and 10 mM CaCl2)

4) Saline EDTA (pH 8.0)

Preparation: -

0.44 g NaCl
0.89 g EDTA in 75 ml of Milli-Q water

- pH was adjusted to 8.0 by adding HCL and Sodium Hydroxide
- Volume made up to 100 ml
- Autoclaved (121°C for 20 minutes).
- Keep at room temperature until use

5) 5x Tris-EDTA Buffer (TE pH 8.0)

Preparation of 1 litre of 5x TE buffer: -

6.06 g Tris and 1.86 g EDTA were dissolved in 900 ml of Milli-Q water.
The pH of the solution was adjusted to 8.0 using hydrochloric acid and sodium hydroxide
The volume was made up to 1000 ml.
The stock solution was diluted to 1x before use by autoclaved Milli-Q water.
The solution was kept at room temperature until use.

6) 2 M Tris-HCl pH 7.8

Tris-HCl is required for the preparation of the lysis buffer.

24.23 g of Tris was dissolved in approximately 75 ml of Milli-Q water.
The pH of the solution was adjusted to 7.8 with hydrochloric acid and sodium hydroxide.
The solution was made up to the total volume of 100 ml for a 2 M stock solution, it was autoclaved and then stored at room temperature.

7) 2 M Potassium Chloride

One hundred millilitres of 2 M potassium chloride were prepared as follows: -

- 15.08 g of KCl dissolved in Milli-Q water and then autoclaved and kept at room temperature until use.

8) 0.1 M Calcium Chloride

Stock solution of 0.1 M calcium chloride dehydrate (CaCl2. 2H2O) was prepared by dissolving 0.15 g of CaCl2 2H2O in 10 ml of autoclaved Milli-Q water.

9) RNAase A 10 mg/ml

The lyophilized pancreatic RNAase A was reconstituted by adding 0.9 volume of 0.01 M sodium acetate (pH 5.2) to the stock

Then boiled at 100 °C for 15 minutes to prevent precipitation of RNAase A.

Then gradually cooled to room temperature.

pH was adjusted to 7.4 with 0.1 volume of 1 M Tris-HCl (pH 7.4).

This produced a stock with a concentration of 10 mg/ml.

Aliquots of the solution were store at -20°C to avoid freeze and thaw cycles, which would denature the enzyme.

10) Preparation of Stock Solution for DNA Extraction

Stock solution were prepared fresh and stored at 4 °C when not used. Working solutions, which were aliquots of the stock solutions, were used to prevent contamination.

2.4.1.4 Steps of DNA Extraction (Salting out technique)

DNA was extracted using the salting out technique over 2 days.

Day 1

1. Blood was thawed by leaving on ice for about 10-15 minutes.
2. 5ml of 1x Red Cell Lysis buffer was diluted 1:1 using Milli-Q water to make a freshly diluted 0.5x Lysis buffer.
3. 5ml of thawed blood was diluted with 0.5X Red cell Lysis buffer (to a total volume of 15 ml).
4. After gentle mixing, the sample was incubated on ice for 10 minutes.
5. The sample was centrifuged at 3500 rpm for 15 minutes.
6. The pellet obtained was rinsed with Tris-EDTA (pH 8.0) and centrifuged again at 3500 rpm for 15 minutes, repeating this process for three times.
7. The pellet was resuspended in 2 ml of saline EDTA (pH 8.0) to ensure good dispersion using wide bore pipette tips to break up the precipitate.
8. 100 microlitres of 20% SDS was then added.
9. RNAase A was added to a final concentration of 50 µg/ml and incubated at 37°C for 1 hour.
10. Proteinase-K was added to a final concentration of 100 µg/ml and the sample was kept at 37°C overnight (the tube shaken 2 or 3 times during incubation.)

Day 2

1. After the overnight incubation, DNA was precipitated by adding 100ml of 2 M KCl to the sample.
2. The sample was mixed in the tube gently and slowly for complete dissolution of KC1.
3. 4ml of cold 100% Ethanol was added to precipitate the DNA.
4. The DNA was removed with a plastic spatula or pipette tip and transferred to an Eppendorf tube containing 70% Ethanol.
5. Wash the DNA with Ethanol by inverting the Eppendorf tube.

   This step was repeated 3 times and left to air dry for 5-10 minutes.
6. This DNA was then reconstituted with 100-200µl TE buffer (pH 8.0) and left to dissolve in the cold room overnight.

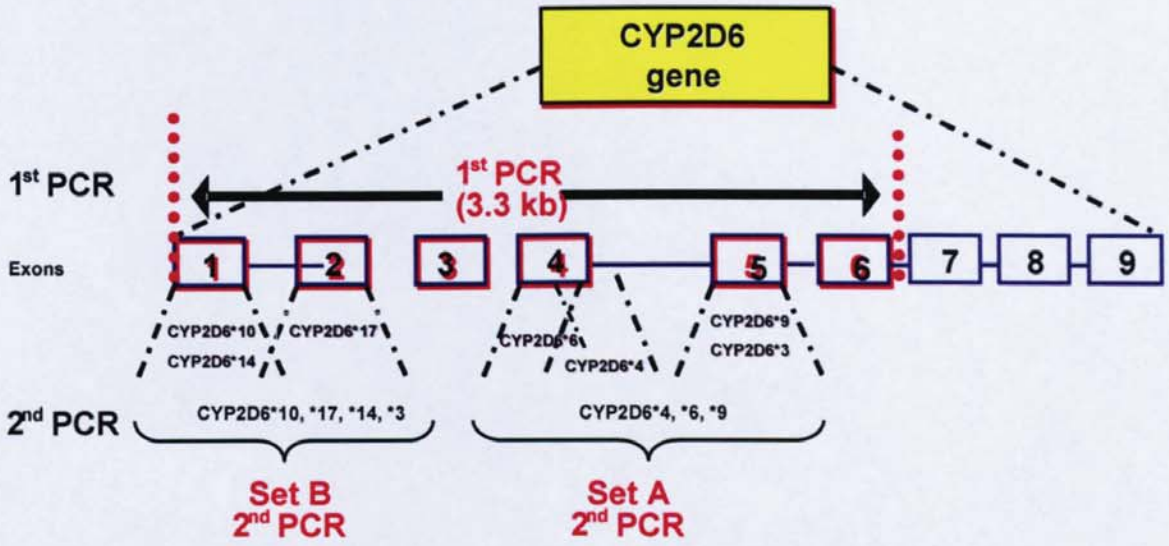
The final DNA concentration was measured by measuring the 260 nm absorbance of the DNA (value of OD = 1 (A_{260} measurement) contains ~50ug of DNA/ml)

Purity of the DNA was measured by taking the ratio of OD_{260} : OD_{280} (260:280)

2.4.2 CYP2D6

A two-step PCR was designed for studying the polymorphism of the CYP2D6 gene.

Figure 2.4 Two-step multiplex PCR for the molecular analysis of the CYP2D6 gene. The nine exons of the CYP2D6 gene and the location of the 1st and 2nd PCR Primers.



Set A:

*4
*6
*9

Set B:

*3
*10
*14
*17

For the set A alleles, a common forward primer was used (CYP2D6 P1 Fw), paired with the 3 individual reverse primers (**Table 2.3**).

For the set B alleles, a common forward primer was used (JFw), paired with the 4 individual reverse primers (**Table 2.4**).

2.4.2.1 PRIMERS FOR 1st PCR FOR CYP2D6

Allele	Primer Sequence	14 Location (bp)	Tm (°C)
Cyp2D6 Fw	5' CCA GAA GGC TTT GCA GGC TTC A 3'	1279-1300	68
Cyp 2D6 Rv	5' ACT GAG CCC TGG GAG GTA GGT A 3'	6371-6350	70

Table 2.3 Primers for 1st.PCR for CYP2D6

2.4.2.2 PRIMERS FOR 2nd PCR FOR CYP2D6 SET A

Simultaneous detection of alleles *CYP2D6 *4, *6, *9*

Allele	Primer Sequence	15 Location (bp)	Tm (°C)
Forward primer:		**16**	**17**
P1 (Fwd)	5' ATT TCC CAG CTG GAA TCC 3'	2916-2933	54
Reverse primers:			
CYP2D6*4WT	5' GGG CGA AAG GGG CGT CC 3'	3481-3465	60
CYP2D6*4MUT	5' GGG CGA AAG GGG CGT CT 3'	3481-3465	58
CYP2D6*6WT	5' GCC TCC TCG GTC ACC CA 3'	3342-3327	60
CYP2D6*6MUT	5' GCC TCC TCG GTC ACC CC 3'	3342-3327	60
CYP2D6*9WT	5' GGC AGC CAC TCT CAC CTT 3'	4251-4234	58
CYP2D6*9MUT	5' GGC AGC CAC TCT CAC CC 3'	4251-4236	58

Table 2.4 Primers for 2nd multiplex PCR for CYP2D6 set A alleles.

2.4.2.3 PRIMERS FOR 2nd PCR FOR CYP2D6 SET B

Table 2.5 shows the primer sequence for 2nd PCR of set B.

Allele	Primer Sequence	18 Location (bp)	19 Tm (°C)
Forward primer:		**20**	**21**
JFw	5' ACC AGG CCC CTC CAC CGG 3'	1336-1353	60
Reverse primers:			
CYP2D6*3WT	5' TGG GTC CCA GGT CAT CCT 3'	4185-4168	58
CYP2D6*3MUT	5' TGG GTC CCA GGT CAT CCG 3'	4185-4169	60
CYP2D6*10MUT	5' GGC AGG GGG CCT GGT GA 3'	1735-1720	60
CYP2D6*11WT	5' CGA AGC GGC GCC GCA AC 3'	2518-2502	60
CYP2D6*11MUT	5' CGA AGC GGC GCC GCA AG 3'	2518-2502	60
CYP2D6*14WT	5' CTT CTG CCC ATC ACC CAC C 3'	3395-3377	62
CYP2D6*14MUT	5' CTT CTG CCC ATC ACC CAC T 3'	3395-3377	60
CYP2D6*17WT	5' CCG AAA CCC AGG ATC TGG G 3'	2660-2642	62
CYP2D6*17MUT	5' CCG AAA CCC AGG ATC TGG A 3'	2640-2642	60

Table 2.5 Primers for 2nd. multiplex PCR for CYP2D6 set B alleles.
Simultaneous detection of alleles *CYP2D6 *3, *10, *14 & *17*

The list of references for the primers is given in Table 2.6:-

Primer	References
P1	Heim & Meyer, 1990
3WT	Heim & Meyer, 1990
3MUT	Heim & Meyer, 1990
4WT	Heim & Meyer, 1990
4MUT	Heim & Meyer, 1990
JFW	McLellan *et al.*, 1997
10WT	Johansson *et al.*, 1994
10MUT	Johansson *et al.*, 1994
17WT	Masimirembwa *et al.*, 1996
17MUT	Masimirembwa *et al.*, 1996
9WT	Tyndale *et al.*, 1991
9MUT	Tyndale *et al.*, 1991
CYP2D6-F	Lundqvist *et al.*, 1999

Table 2.6:- References for the CYP2D6 gene primers.

2.4.2.4 Procedures

2.4.2.4.1 First PCR

The first PCR involves the following steps:

Preparation of Master Mix (M1stPCR)

Master Mix (label as "M1stPCR")	1X (ul)	34X (ul)
Water for PCR	9.5	323.0
Reaction buffer (10X)	2.5	85.0
$MgCl_2$ (50mM)	1.0	34.0
dNTPs (10mM each)	0.5	17.0
Total	13.5	459.0

Preparation of Primers Mix (P1stPCR)

Primers Mix (label as "P1stPCR")	1X (ul)	34X (ul)
Water for PCR	5.5	187.0
2D6FW (5pM)	1.25	42.5
Ex6Rv (5pM)	1.25	42.5
Total	8.0	272.0

Preparation of *Taq* polymerase

Biotools *Taq* polymerase (1U/ul) (label as "Taq 1stPCR")	1X (ul)	34X (ul)
	1.5	51.0

q

Preparation of genomic DNA (DNA concentration = 100 micrograms per ml)

Genomic DNA (32 different sample)	2.0ul each

Total volume PCR = 25 µl

1) The master mix (M1stPCR), primers mix (P1stPCR) and the genomic DNA samples (healthy volunteer), were left to thaw on ice.
2) The amount of master mix (~0.5ml) and primers mix (~0.3ml) were checked to ensure there are enough for the PCR preparation procedures.
3) The samples and reagents were kept on ice once they have been thawed to prevent degradation, which may affect the PCR reaction.

4) The master mix (M1stPCR) and primers mix (P1stPCR) were mixed by gently tapping 5-10 times and centrifuged.
5) 8ul of primers mix (P1stPCR) were added into each labelled PCR tube.
6) The genomic DNA was added into the respective PCR tube. The PCR tube was gently tapped (5-10 times) and centrifuged.
7) Start the PCR machine and subject the PCR tube heat at 80°C for 5 minutes.
8) Add 51 ul of Biotools Taq into the master mix (M1stPCR), mixed by gently tapping 10-15times and spin the master mix for not more than 5 seconds.
9) After the hot start, take out all the PCR tubes and put it on ice.
10) The master mix (M1stPCR) was mixed by pipetting in and out, then 15.0ul was aliquoted into each PCR tube.
11) Mixing was done by gently tapping 5-10 times followed by a quick spin.
12) The PCR tubes were then placed in the PCR machine. The PCR conditions were programmed as below and the machine started:

Pre-denaturation	94°C, 90s	
Denaturation	94°C, 45s	35 cycles
Annealing	68°C, 90s	
Extension	72°C, 240s	
Final extension	72°C, 480s	
Hold	20°C, 350s	

2.4.2.4.2 Second PCR

2.4.2.4.2.1 Preparation of the primer mix:

The primer mix was in 2 sets; set A and set B.

Primer Mix for set A (each PCR tube)

Component Set A	Concentration (from stock)	Volume	Check (WT)	Check (MT)
H2O		7.50ul		

P1 FW	5pM	2.50ul		
*9 RV	2pM	1.25ul		
*6 RV	5pM	1.25ul		
*4 RV	5pM	1.50ul		
Total volume		15.0ul		

Different amount of primers were used after optimization in order to gain the specificity of the expected band.

Primer Mix for set B (each PCR tube)

Component Set B	Concentration (from stock)	Volume	Check (WT)	Check (MT)
H2O		5.50ul		
J FW	5pM	2.50ul		
*3 RV	5pM	2.50ul		
*17RV	5pM	1.25ul		
*14RV	2pM	1.25ul		
*10RV	5pM	1.00ul		
Total volume		15.0ul		

1) All the reagents and the first PCR product were left to thaw on ice.
2) All the reagents and primers were double-checked to ensure the amount of reagents were enough for the PCR preparation procedures. Each tube was for single use only (for 48 reactions; in 96 wells Thermal Cycler).
3) The check list (PCR2-CYP2D6 check list) was marked before the reagents or primers were loaded into the PCR tube and the volume double-checked before being loaded.
4) All the PCR tubes were labelled as below:

Label on the PCR tube	Description
1A	Sample 1, Set A, wild type tube
1B	Sample 1, Set A, mutant type tube
1X	Sample 1, Set B, wild type tube
1Y	Sample 1, Set B, mutant type tube

5) All the primers and reagents were mixed by gently tapping 5-10 times and centrifuged. The primers and reagents were arranged in a consecutive sequence according to the sequence of the reagents to be loaded and were put aside after using it.

6) Set A and set B were prepared for both wild type and mutant tubes (each sample will be used in 4 tubes) as below:

Set A	Wt (ul)	Mut(ul)	60X (ul)
Water for PCR	6.5	6.5	390
P1 (common primer) (5pM)	2.5	2.5	150
*9 (2pM)	1.25	1.25	75
*6 (5pM)	1.25	1.25	75
*4 (5pM)	1.5	1.5	90
1st PCR product	1.0	1.0	-

Set B	Wt (ul)	Mut (ul)	60X (ul)
Water for PCR	5.5	5.5	330
JFW (common primer) (5pM)	2.5	2.5	150
*3 (5pM)	2.5	2.5	150

*17 (5pM)	1.25	1.25	75
*14 (2pM)	1.25	1.25	75
*10 (5pM)	1.0	1.0	60
1st PCR product	1.0	1.0	-

7) 8.5ul of master mix were loaded into each tube after the preparation of the primers mix. The master mix (M2ndPCR) contained:

Master Mix (label as "M2ndPCR")	1X (ul)	52X (ul)
Water for PCR	4.5	234
Reaction buffer (10X)	2.5	130
$MgCl_2$ (50mM)	1.0	52
dNTPs (10mM each)	0.5	26
Total	8.5	442

8) 1.5ul (1unit/ul) of Biotools Taq (Taq 2ndPCR) were added into each PCR tube.

9) The total volume for each PCR tube was 25 µl

10) The mixture was mixed by gently tapping 5-10 times and centrifuged for a few seconds (not more than 5 seconds).

11) All the PCR tubes were put in the PCR machine.

The PCR condition was programmed as below and the machine started:

	Denaturation	94°C, 90s	
	Annealing	66°C, 90s	} 15 cycles
	Extension	72°C, 140s	
Hold		20°C, 350s	

2.4.3 Beta-2 AR Gene

A two-step PCR was designed to study the polymorphisms of the Beta-2 AR gene.

2.4.3.1 Nomenclature of Beta-2 AR gene

The reference sequence used for Beta-2 AR gene was retrieved from the Gen Bank (accession no. M15169). [http://www.ncbi.nih.gov/genebank] The first nucleotide of the open reading frame (ORF) was referred to as nucleotide 1, which corresponded to nucleotide 1, 488 of M15169; while the 5` UTR began at -1 and proceeded in the negative direction (upstream from nucleotide 1).

2.4.3.2 Beta-2 AR Gene Sequence and the 5 polymorphisms

```
BASE COUNT      790 a    873 c    895 g    893 t
ORIGIN
        1 cccgggttca agagattctc ctgtctcagc ctcccgagta gctgggacta caggtacgtg
       61 ccaccacacc tggctaattt ttgtattttt agtagagaca agagttacac catattggcc
      121 aggatctttt gctttctata gcttcaaaat gttcttaatg ttaagacatt cttaatactc
      181 tgaaccatat gaatttgcca ttttggtaag tcacagacgc cagatggtgg caatttcaca
        //
     1321 ggcgagggca gttcccctaa agtcctgtgc acataacggg cagaacgcac tgcgaagcgg
     1381 cttcttcaga gcacgggctg gaactggcag gcaccgcgag cccctagcac ccgacaagct
     1441 gagtgtgcag gacgagtccc caccacaccc acaccacagc cgctgaatga ggcttccagg
     1501 cgtccgctcg cggcccgcag agccccgccg tgggtccgcc Cgctgaggcg cccccagcca
     1561 gtgcgctTac ctgccagact gcgcgccAtg gggcaacccg ggaacggcag cgccttcttg
     1621 ctggcaccca atAgaagcca tgcgccggac cacgacgtca cgcagCaaag ggacgaggtg
     1681 tgggtggtgg gcatgggcat cgtcatgtct ctcatcgtcc tggccatcgt gtttggcaat
     1741 gtgctggtca tcacagccat tgccaagttc gagcgtctgc agacggtcac caactacttc
     1801 atcacttcac tggcctgtgc tgatctggtc atgggcctgg cagtggtgcc ctttggggcc
     1861 gcccatattc ttatgaaaat gtggactttt ggcaacttct ggtgcgagtt ttggacttcc
     1921 attgatgtgc tgtgcgtcac ggccagcatt gagaccctgt gcgtgatcgc agtggatcgc
     1981 tactttgcca ttacttcacc tttcaagtac cagagcctgc tgaccaagaa taaggcccgg
     2041 gtgatcattc tgatggtgtg gattgtgtca ggccttaCct ccttcttgcc cattcagatg
     2101 cactggtacc gggccaccca ccaggaagcc atcaactgct atgccaatga gacctgctgt
       //
     3301 ttaggcaggg atttgaggag cagcttcagt tgttttcccg agcaaaggtc taaagtttac
     3361 agtaaataaa atgtttgacc atgccttcat tgcacctgtt tgtccaaaac cccttgactg
     3421 gagtgctgtt gcctccccca ctggaaaccg c
```

Figure 2.5 Beta-2 AR gene sequence and the 5 polymorphisms.

Bold, italic & underlined = the ORF.
Red = polymorphism (SNP)
Capital A = nucleotide position 1

2.4.3.3 Design of Allele Specific Multiplex-PCR primers

Primers specific for a 2-step nested PCR reaction were designed to detect allelic variants for amino acid changes in ORF at positions 16, 27, 164 and at 5'UTR region for nucleotides at position -20 and –47.

The primers for the first PCR (Beta2-Fw and Beta2-Rv) span the region of intronless gene from 5' UTR to ORF to produce fragment A of size 710 bps (**Figure2.4**).

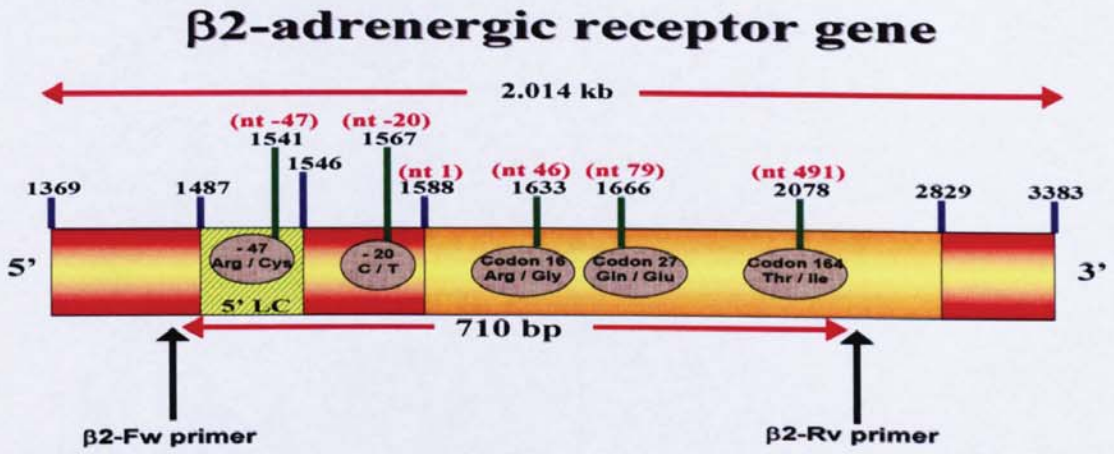


Figure 2.6 The primers for the first PCR (Beta2-Fw and Beta2-Rv) and the amplified region of the Beta-2 AR gene.

To improve assay specificity, primers for the second PCR were designed to have specific 3' ends that were manipulated to differentiate single nucleotide changes at the specific locus during PCR amplification (**Tables 2.6, 2.7, 2.8 & 2.9**).

The 'BLAST" program at http://www.ncbi.nlm.nih.gov/blast was used to confirm the specificity of the primers. These primers were also designed to have similar annealing temperatures and appropriate length as well as GC contents for multiplexing reactions.

Tables 2.5 to 2.9 lists the sequences of the primers used for both the first and the second PCR reactions.

Sequence of the primers used in Beta-2 AR for both the first and the second PCR reactions

Primer	Sequence	Tm (°C)	Size (bp)	Primer concentrations (μM)
Beta2-Fw	5` CAC CAC AGC CGC TGA ATG AGG 3	68	710	1.25
Beta2-Rv	5` GGC TTG GTT CGT GAA GAA GTC 3	64	710	1.25

Table 2.7 Primers used in Beta-2 AR for both the first PCR reactions.

Primer	Sequence	Tm (°C)	Size (bp)	Primer concentrations (μM)
Forward primer:	**SET A**			
Beta2-Fw	5` CAC CAC AGC CGC TGA ATG AGG 3`	68		0.5
Reverse primers:				
Beta 16°	5` GTC CGG CGC ATG GCT TCT 3`	60	179	0.5
Beta utr 20C	5` CGC GCA GTC TGG CAG GTG 3`	62	114	0.5

Table 2.8 Primers used in Beta-2 AR for both the second PCR reactions Set A.

Primer	Sequence	Tm (°C)	Size (bp)	Primer concentrations (μM)
Forward primer:	**SET B**			
Beta2-Fw	5` CAC CAC AGC CGC TGA ATG AGG 3`	68		0.5
Reverse primers:				
Beta 16G	5` GTC CGG CGC ATG GCT TCC 3`	62	179	0.5
Beta utr 20T	5` CGC GCA GTC TGG CAG GTA 3`	60	114	0.5

Table 2.9 Primers used in Beta-2 AR for both the second PCR reactions Set B.

Primer	Sequence	Tm (°C)	Size (bp)	Primer concentrations (μM)
Forward primer:	**SET C**			
Beta2-Fw	5` CAC CAC AGC CGC TGA ATG AGG 3`	68		0.5
Reverse primers:				
Beta 27C	5` CCA CAC CTC GTC CCT TTG 3`	58	212	0.3
Beta2utr 47C	5` CTG GGG GCG CCT CAG CG 3`	62	86	0.7
Beta 164C	5` CTG AAT GGG CAA GAA GGA GG 3`	62	624	0.15

Table 2.10 Primers used in Beta-2 AR for both the second PCR reactions Set C.

Primer	Sequence	Tm (°C)	Size (bp)	Primer concentrations (μM)
Forward primer:	**SET D**			
Beta2-Fw	5` CAC CAC AGC CGC TGA ATG AGG 3`	68		0.5
Reverse primers:				
Beta 27G	5` CCA CAC CTC GTC CCT TTC 3`	58	212	0.3
Beta2utr 47T	5` CTG GGG GCG CCT CAG CA 3`	60	86	0.7
Beta 164T	5` CTG AAT GGG CAA GAA GGA GA 3`	60	624	0.15

Table 2.11 Primers used in Beta-2 AR for both the second PCR reactions Set D.

2.4.3.4 Method Optimization of the Allele Specific Nested Multiplex PCR

Fragment A was first amplified using PCR conditions and compositions determined empirically. The PCR protocols were then further optimized for the PCR compositions and reaction conditions to produce specific bands. The final optimized protocol included a reaction mixture comprising 1X Biotools® PCR Buffer (B & M Labs, SA), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs (Promega, Madison), 1.25 µM of primers (Invitrogen The Netherlands), 1.5 U Biotools® *Taq* and 200 ng of DNA in a total volume of 25 µl.

PCR amplification was performed using Gene Amp PCR System 2400® Perkin Elmer (Applied Biosystems, Foster City) with pre denaturation (hot start) for 1 minute 30 seconds, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 68°C for 1 minute 30 seconds, and extension at 72°C for 4 minutes. A final extension at 72°C for 8 minutes was included.

Ten µl of the first PCR products were analyzed on 1% agarose gel (staining with Ethidium bromide) in 1X TBE after electrophoreses with 80 volt for 1 hour.

Subsequent to a successful first PCR, 2 parallel allele-specific reactions, one with a wild-type specific primer and the other with a mutation-specific primer, were carried out in separate single PCR reactions for each of the polymorphic loci.

The empirical compositions first attempted comprised 1.5 mM $MgCl_2$, 0.5 U *Taq* Polymerase, 0.2 mM dNTPs, 2.0 µl (1:50) template and 0.3 µM of each primer.

Fifteen cycles of 2 minutes at 94°C, 30 seconds at 69°C and 30 seconds at 72°C were performed.

The expected fragment sizes for each allele are listed in Tables 3.6 to 3.9.

The 5 primer sets were then combined into one single allele specific multiplex PCR.

The following parameters were examined to optimized multiplex PCR assay for the alleles: annealing temperature, magnesium concentrations and *Taq* Polymerase concentrations. The amount of $MgCl_2$, *Taq* and primers were varied stepwise until specific bands were seen.

The cycling conditions were optimized to obtain the highest yield and specificity for the five different alleles studied. The reaction conditions were chosen to allow amplification when there was a perfect match between the primer and the template DNA.

The final conditions and parameters that gave desired results comprised a duplex (set A/B: Arg16/Gly16 and -20T/C) and triplet (set C/D: Gln27/Glu27, -47T/C and Thr164/Ile164) multiplex reactions.

The PCR compositions were the same for both and comprised 1X Biotools® PCR Buffer, 2.0 U of Biotools *Taq*, 1.5 mM $MgCl_2$ and 0.2 mM of each dNTPs and specific primers (concentration listed in Tables 3.6 to 3.9) in a total volume of 25 µl. Twenty cycles of PCR was performed at 94°C for 90 seconds, 62.5°C for 35 seconds and 72°C for 35 seconds. Second PCR products were separated using 3% agarose gels electrophoresis for 1 hour and 20 minutes at 90 volt to get the best separation.

The results obtained from multiplex PCR were checked against the results produced by individual single PCR reaction approach to avoid false positive or false negative findings. Reactions were repeated if the bands were inconclusive.

This method was used to screen DNA samples from patients. Some samples were selected and sent for sequencing to confirm the genotypes.

2.4.4 Gel Electrophoresis

2.4.4.1 Preparation and casting of Gel

For the 1st PCR gel electrophoresis, 1% agarose gel was used while for the 2nd PCR gel electrophoresis, 3% agarose gel was used.

1. The correct amount of agarose powder required to prepare the appropriate gel was calculated depending on the volume of gel tank and the resolution required.
2. The correct amount of agarose powder was weighed and transferred into a clean, autoclaved beaker.
3. 5X TBE stock was diluted to 1X TBE using milliQ water.
4. An appropriate amount of 1X TBE buffer was added to a beaker containing the correct amount of weighted agarose. The amount of TBE buffer depends on the size of gel needed (50 ml for mini gel and 400 ml for maxi gel).
5. The beaker was swirled slowly to mix the agarose.
6. The mouth of the beaker was covered with a plastic wrapper and heated in a microwave oven for 2 min until the agarose was dissolved.
7. The beaker was left to cool under room temperature to 60°-70°
8. 2µl of (10 mg/ml) ethidium bromide solution was added and mixed thoroughly but gently to avoid bubbles.
9. If bubbles were present, the agarose solution was heated in the microwave oven for 1 min to remove them.
10. The gel tray was set and the rubber band was inserted on both ends of the tray. The comb was adjusted so that the distance between the end of comb did not touch the surface of the gel tray by using the tray ruler
11. The agarose solution was poured into the tray and the casting comb placed into their slots.
12. The gel was covered with a piece of paper to protect it from light.

13. The gel was left for approximately 30 to 45 min to allow it to set.
14. The casting comb was carefully removed to ensure the wells were not damaged.
15. The rubber was removed from the ends of the gel tray and the gel tray was placed in a horizontal electrophoresis apparatus.
16. 1x TBE buffer was added to the reservoir until the buffer just covers the agarose gel (about 1mm depth from gel surface).
17. The gel was placed in the tank such that the end for sample loading was on the negative (black) side of the tank.

2.4.4.2 Loading of Gel.

1. The chemicals required were left to thaw.
2. 6x loading dye were added to DNA sample on a small piece of parafilm tape. The amount of loading dye used depended on the volume of sample (2 microlitres plus 10 microlitres of sample or 4 microlitres plus 20 microlitres of the sample). Mixing was done by pipetting the solutions in and out.
3. All the solutions were sucked up using a pipette and loaded carefully into the wells.
4. DNA ladder (consisting of DNA fragments of 100 base pairs) was then loaded.
5. The electrophoresis apparatus was closed after ensuring that the electrodes are located correctly.
6. The leads were then connected to the power pack.

2.4.4.3 Electrophoresis

1. The power pack was turned on and the voltage set at 1-5 v/cm (the distance measured between the positive and negative electrodes) volts for 45 to 60 min or until the dye is 1 cm from the bottom end of the gel
    - Minigel tank: 80 -100 V (tank length = 20 cm)
    - Maxigel tank: 150 to 175 V (tank length = 35 cm)
2. Check to ensure that the electrophoresis is proceeding by observing bubbles being generated at the electrodes.
3. Once the samples had run sufficiently, the power pack was turned off and the lid removed from the tank.
4. The gel was removed from the electrophoresis apparatus and placed onto a UV light box.
5. The UV light box was switched on after ensuring complete darkness in the room.
6. The camera was switched on; the exposures adjusted, focused and zoom to obtain a clear image of the correct size and brightness.
7. The image was then captured.

Photographs of the gel electrophoresis result are shown in figures 2.12, 2.13, 2.14 & 2.15

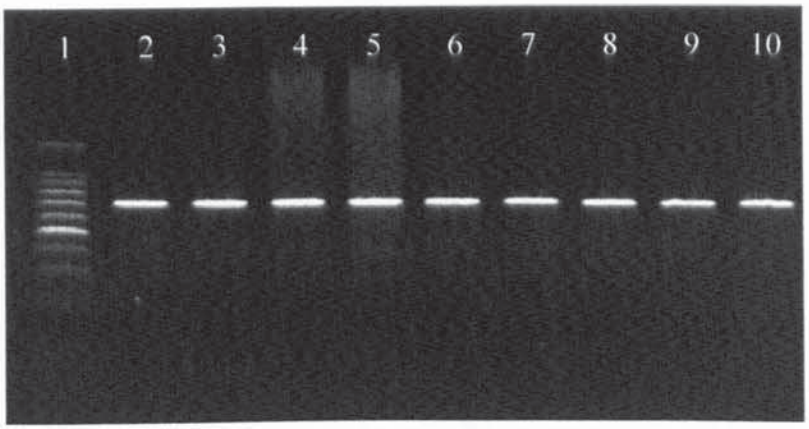


Figure 2.7: 1st Beta-2 AR PCR products of 710 bp fragments.

Lane 1: DNA marker (100 bp)

Lane 2-10: 1st PCR products (710 bp) from different DNA samples. All the 9 samples were successfully amplified.

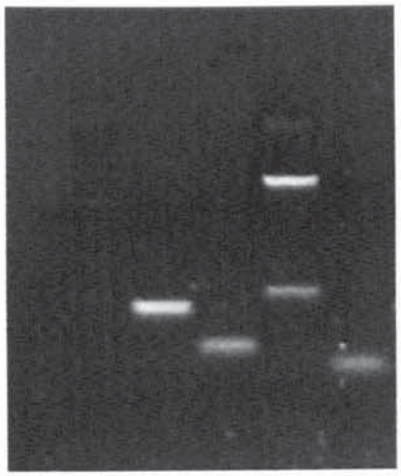


Figure 2.8:- Amplification of Beta-2 AR SNP in a single reaction tube

The PCR products in various lanes are as follows.

Lane 1 = DNA marker (100 bp).

Lane 2 = alleles 16A (179 bp).

Lane 3 = 20T (114 bp).

Lane 4 = -27C (212 bp), 164C (624 bp).

Lane 5 = -47T (86 bp).

This gel electrophoresis picture showed = Homozygous 16A, -20T, 27C, -47T and 164C

Amplification of the CYP2D6 gene

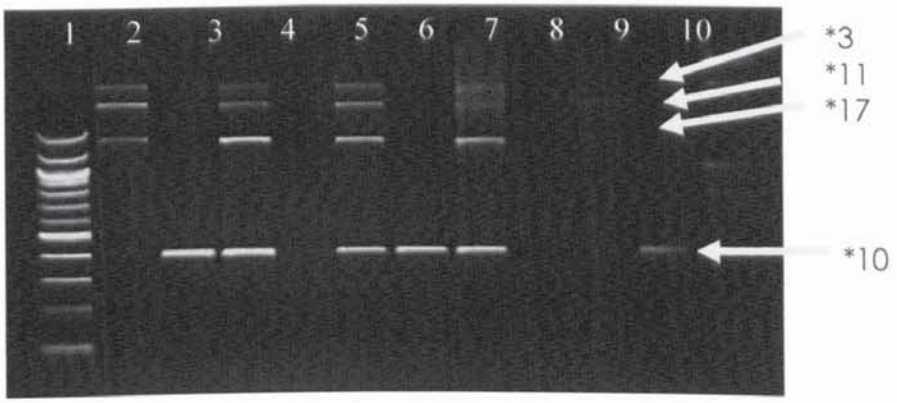


Figure 2.9:- Amplification of the CYP2D6 gene; second PCR set A.

Lane 2, 4, 6, 7, 10: Wild Type sample 1

Lane3, 5, 7, 9, 11: Mutant samples

Lane 1 : 100bp Ladder

Lane 12 : 1kb Ladder

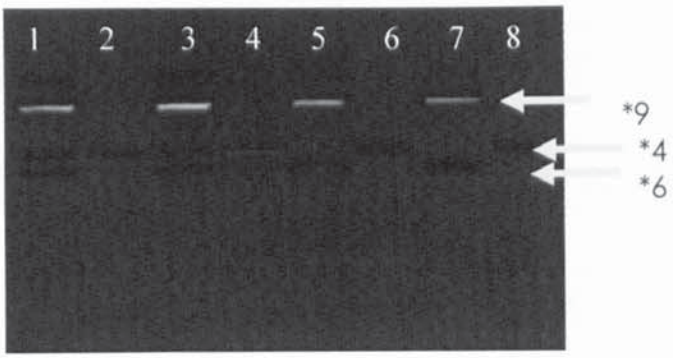


Figure 2.10:- Amplification of the CYP2D6 gene; second PCR set B.

Lane 1, 3, 5, 7: Wild Type sample

Lane 2, 4, 6, 8: Mutant sample

2.4.3.5 DNA sequencing

DNA sequencing was performed to further confirm the PCR results.

First PCR products from subjects genotyped by the described method as homozygote for wild-type, as well as heterozygote and homozygotes for the investigated mutations were chosen at random and purified by QIAGEN QIA quick PCR Purification Kit® (Qiagen, Hilden, Germany). The purified product was processed for DNA sequencing using standard Kit of ABI PRISM Big Dye Terminator. DNA sequences were analyzed on ABI PRISM 3100 DNA Analyzer v3.0 (Applied Biosystem, USA).

Some examples of the DNA sequencing result are shown in figures 2.11- 2.15

Figure 2.11: DNA sequencing trace of a patient sample with heterozygous Arg16/Gly16

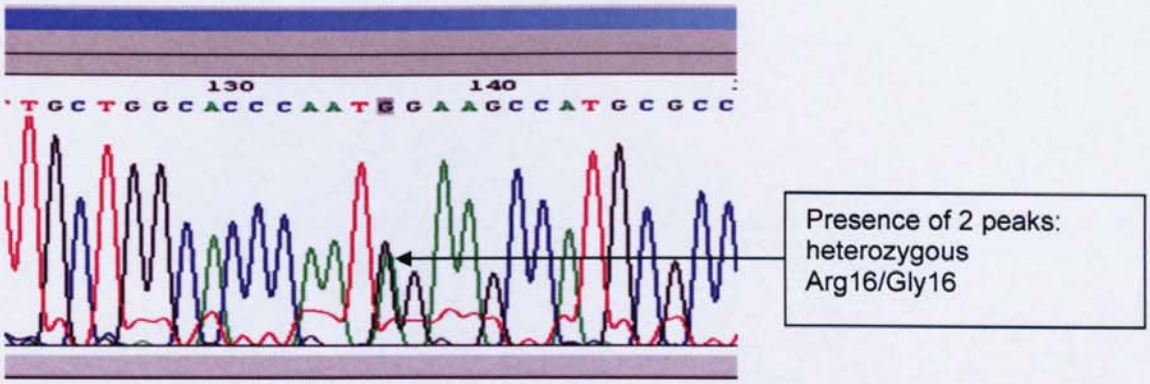


Figure 2.12: DNA sequencing trace of a patient sample with heterozygous C-20/T-20

sequencing results that identified heterozygous Arg/Gly at codon

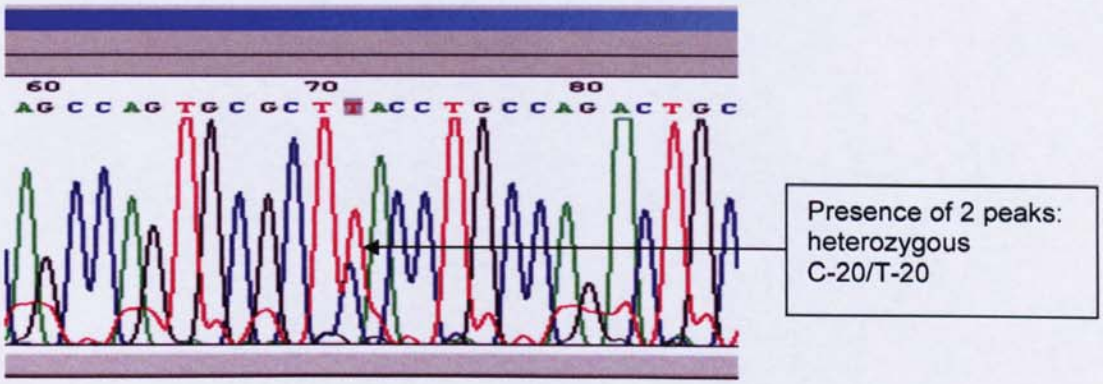
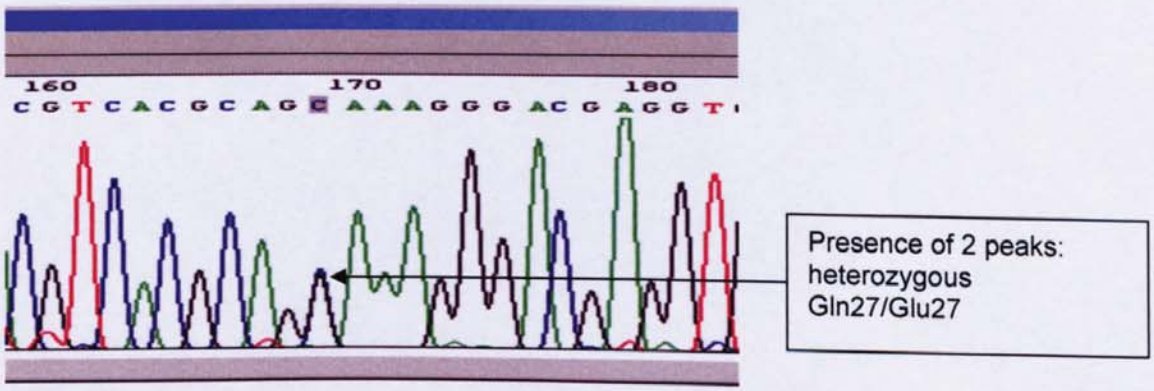


Figure 2.13: DNA sequencing trace of a patient sample with heterozygous Gln27/Glu27



C/T at codon -20

Figure 2.14: DNA sequencing trace of a patient sample with homozygous Thr164/Thr164

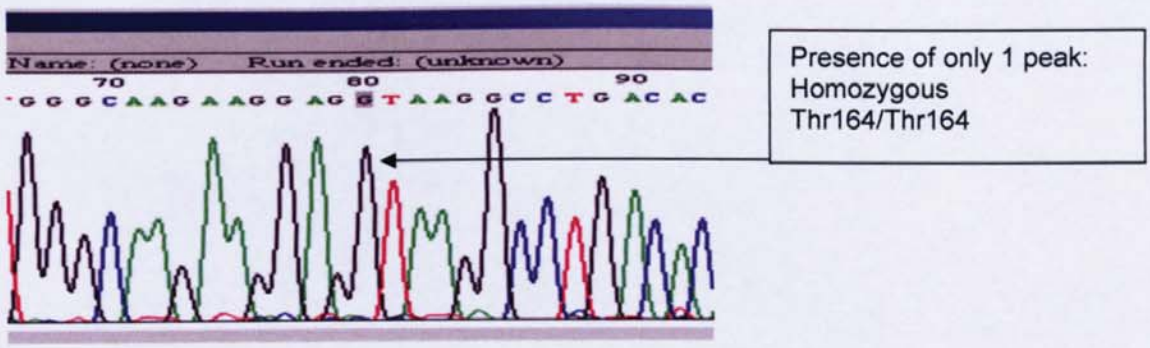
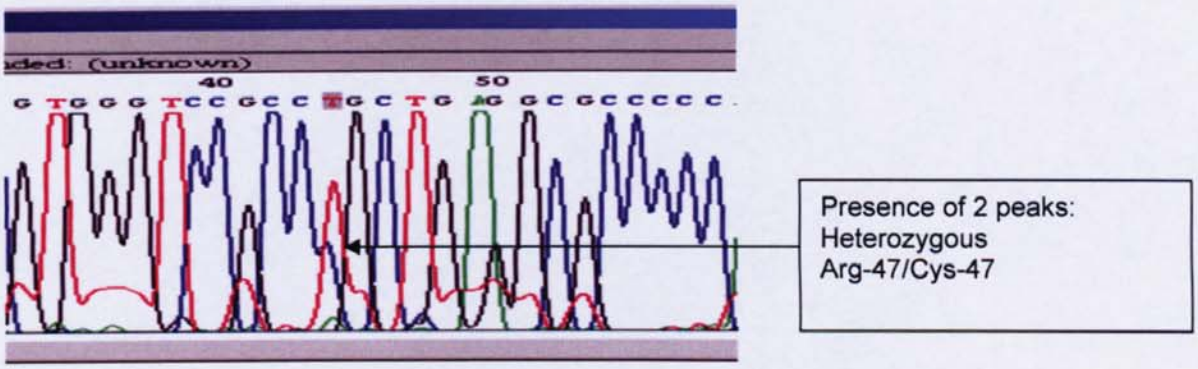


Figure 2.15: DNA sequencing trace of a patient sample with heterozygous Arg-47/Cys-47



2.5 RESULTS

2.5.1 Clinical Results

Two hundred and three (203) patients were enrolled during the study period. Out of these, 9 patients were excluded from the study during the second visit, as they were no longer taking metoprolol. Therefore the final number of patients recruited was 194. This was in excess of the estimates calculated in the sample size calculation (section 2.3.1.1). The larger number of patients meant the power of the study to detect an association when there is one between high blood pressure or bronchospasm and genotype was better than planned.

2.5.1.1 Demographic Data Of The Patients

The majority was more than 55 years of age. The youngest patient was 37 years old. The mean age was 57 (SD=6.804). The patients' age distribution is shown in Figure 2.16.

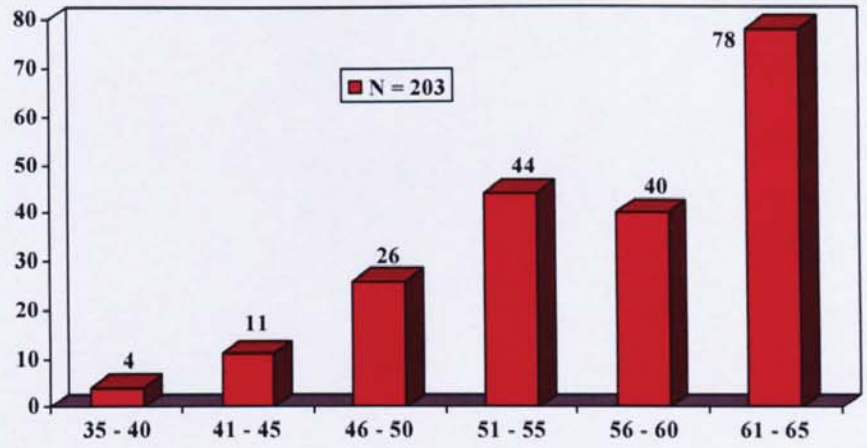


Figure 2.16: The age distribution of all the patients

Most of the patients were either overweight or obese with an average body mass index (BMI) of 25.88 (SD= 3.68). The majority of the patients (59%) weighed between 61 to 75 kg. Figure 2.17 shows the weight distribution of the patients and Figure 2.18 the BMI distribution.

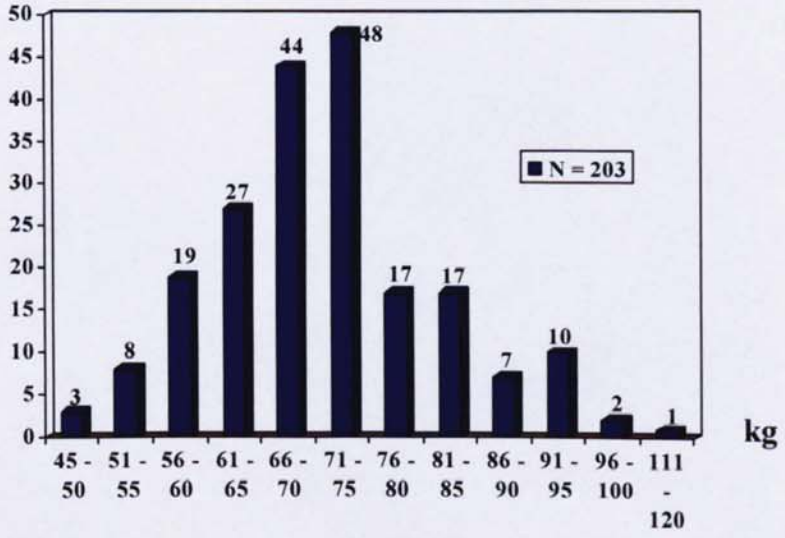


Figure 2.17: The weight distribution of the patients

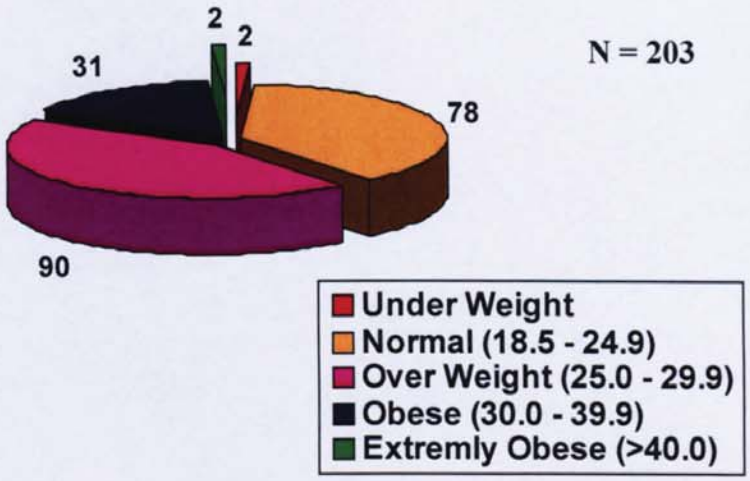


Figure 2.18: The Body Mass Index (BMI) distribution of the patients

2.5.1.2 The Ethnic Groups of the patients

Most of the patients were Malays (76.3%). A Malay patient is defined as one who is a Muslim, speaks the Malay language as his or her first language and whose both parents and grandparents are Malays.

A Chinese or Indian patient is identified by their name and race as they appeared on their identity cards and with both parents and grandparents being of the same ethnic group.

A patient, who had parents or grandparents not of the same ethnic group, was grouped under mixed ethnic origin. Similarly, a Chinese or Indian patient whose either parent or grandparent is from a different ethnic origin, were also grouped under mixed ethnic origin (Others-Chinese).

Twenty-five (27) patients or 13.3%, were grouped under mixed ethnic origin. The rest were Chinese (8.4%), Indians (1.5%) and Thai (0.5%) **(Table 2.12).**

Ethnic Group	No.	(%)
Malay	155	(76.4)
Chinese	17	(8.4)
Indian	3	(1.5)
Thai	1	(0.50
Chinese – Malay	1	(0.5)
Others (mixed) – Thai	13	(6.4)
Others (mixed) – Bangladesh	1	(0.5)
Others (mixed) – Arab	1	(0.5)
Others (mixed) – Cambodia	1	(0.5)
Others (mixed) – Chinese	1	(0.5)
Others (mixed) -Dutch – Thai	1	(0.5)
Others (mixed) – Pakistan	2	(1.0)
Indian – Indonesian	1	(0.5)
Indian – Cambodia	2	(1.0)
Others (mixed) – Indonesian	1	(0.5)
Others (mixed) – Cambodia	1	(0.5)
Others (mixed) – Malay – Sumatra	2	(1.0)
Total	203	

Table 2.12:- Breakdown of the patients into different ethnic groups. (N = 203)
* Others = Malays whose origin are mixed.

2.5.1.3 Metoprolol dosage

The average metoprolol dose given was 135 mg per day and most of the patients were on low doses (100mg per day and 200mg per day). Only one patient received 400mg per day and 5 received 300mg per day.

Nine patients discontinued metoprolol therapy at the second visit. These patients developed adverse drug reactions (headache, impotence and insomnia) after taking metoprolol and requested that their medication be changed.

2.5.1.4 Concurrent illness of the patients

The majority of the patients (64%) had concurrent Ischemic Heart Disease (IHD).

Twenty-five percent also had Diabetes Mellitus (DM), 9% also had Cerebral-Vascular Accident (CVA), 3.5% also had Transient Ischemic Attacks (TIA) and 1.5% also had Cardiac Arrhythmias. Only 13% of the patients had no concurrent illnesses.

2.5.1.5 Bronchospasm

After performing the Lung Function Tests, 25 patients (12%) showed FEV1/FVC of less than 0.7, the criterion used for objectively validated presence of bronchospasm.

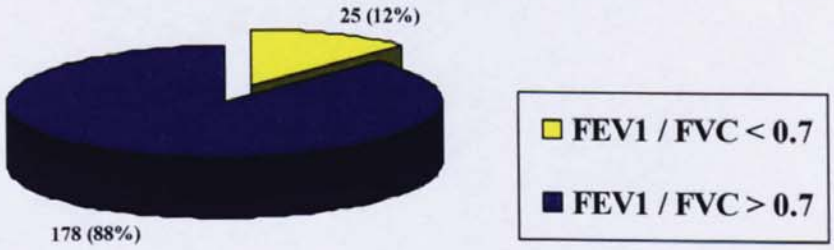


Figure 2.19 Objective Bronchospasm (FEV1/FVC < 0.7)

2.5.1.5.1 Objective Bronchospasm

Most of the patients (88%) had objective mild bronchospasm with FEV1/FVC ratio between 0.6 to 0.7. Only 2 patients (8%) had severe bronchospasm (FEV1/FVC ratio less than 0.5). Figure 2.19 shows the distribution of FEV1/FVC ratio.

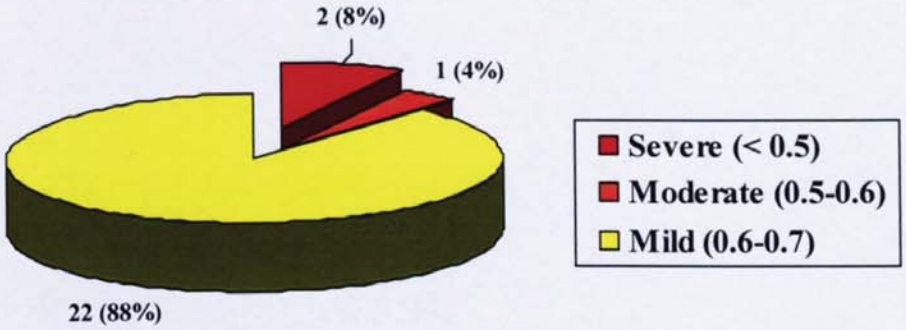


Figure 2.20 Objective Bronchospasm; severity

2.5.1.5.2 Clinical Bronchospasm

From the Questionnaire, patients were asked if they had experienced any difficulty in breathing after taking metoprolol. Thirty-one (15%) had and out of these, only 6 (19%) had FEV1/FVC ratio of less than 0.7 .Out of the 172 patients who did not experience difficulty in breathing after taking metoprolol, 19 (11%) showed FEV1/FVC ratio of less than 0.7.

2.5.1.5.3 Objective Bronchospasm in relation to Metoprolol dosage.

None of the patients who showed objective bronchospasm were on dosage of more than 200mg. daily. Two were on a dose of 50mg.daily, 14 were on 100mg.daily and 9 patients were on 200mg.daily.

2.5.1.5.4 Bronchospasm in relation to hypertension control

Out of 194 patients, 105 (54.1%) had uncontrolled systolic hypertension (> 140 mmHg) and 86 (44.3%) had uncontrolled diastolic hypertension (> 85 mmHg). Sixty eight (35.1%) had both

systolic and diastolic uncontrolled hypertension. Figure 2.21 shows the distribution of systolic and diastolic blood pressure in the patient group.

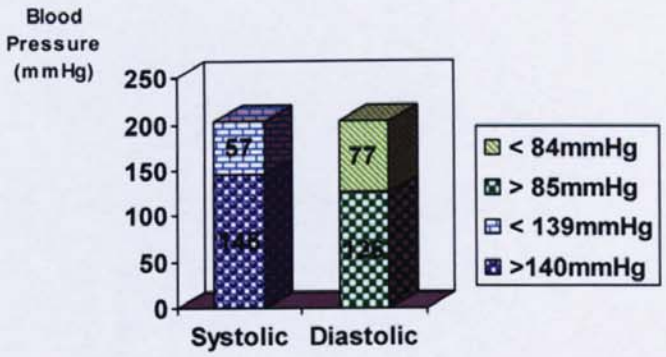


Figure 2.21: Uncontrolled Hypertension

The intervals between the start of metoprolol and measurement of BP values ranged from six weeks to a few years. Unfortunately, because of the observational nature of the study, this could not be controlled by us during the investigation.

Out of the 25 patients who had bronchospasm, 22 (88%) had uncontrolled systolic hypertension and 16 (64%) diastolic hypertension. Out of 25 patients who experienced bronchospasm, 16 (64%) had both systolic as well as diastolic uncontrolled hypertension (**Figure 2.22**).

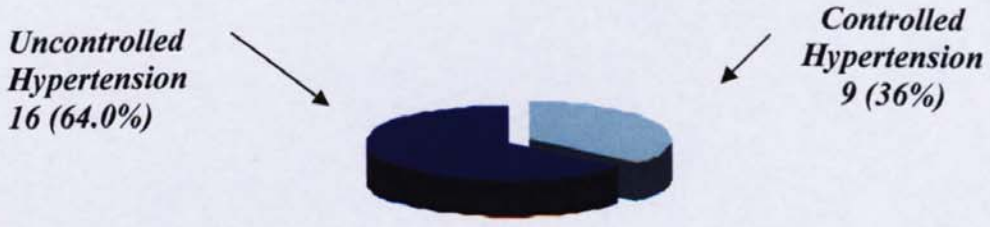


Figure 2.22: Bronchospasm and control of hypertension. Out of 25 patients who experienced bronchospasm, 16 had both systolic as well as diastolic uncontrolled hypertension.

2.5.2 Genotyping Results

2.5.2.1 CYP2D6 Genotyping

Seven alleles of the CYP2D6 gene were studied. These are CYP2D6*3, *4, *6, *9, *10, *14 and *17. All the patients were genotyped for these 7 alleles and their genotype frequencies were calculated and grouped into heterozygous, homozygous wild type and homozygous mutant.

2.5.2.2 Genotype Frequency of CYP2D6 Alleles

Two hundred patients (98.5%) were homozygous for wild type *3. None was heterozygous or homozygous mutant. Genotyping for 3 patients (1.5%) failed. One hundred and ninety eight patients (97.5%) were homozygous wild type *4. One patient (0.5%) was homozygous mutant and another patient (0.5%) heterozygous. Genotyping for 3 patients (1.5%) failed. **(Table 2.13)**

	CYP2D6 Genotype Frequency		
CYP2D6	Homo-wild type	Hetero	Homo – Mutant
*3	200 (100%)	-	-
*4	194 (97.0%)	6 (3.0%)	
*6	192 (96.0%)	8 (4.0%)	-
*9	200 (100%)	8 (4.0%)	-
*10	83 (41.5%)	76 (38.0%)	41 (20.5%)
*14	198 (99.0%)	1 (0.5%)	1 (0.5%)
*17	199 (99.5%)	1 (0.5%)	-

Table 2.13: Genotype frequency of the 7 SNP of the CYP2D6 gene that were studied.

One hundred and ninety two patients (94.6%) were homozygous wild type *6.

Eight patients (3.9%) were heterozygous while none were homozygous mutant. Genotyping for 3 patients (1.5%) failed. **(Table 2.13)**

Two hundred patients (98.5%) were homozygous wild type CYP2D6*9.

None were heterozygous or homozygous mutant. Genotyping for 3 patients (1.5%) failed. **(Table 2.13)**

Eighty-three patients (40.9%) were homozygous wild type *10.

Seventy-six (37.4%) were heterozygous and 41 (20.2%) were homozygous mutant. Genotyping for 3 patients (1.5%) failed. **(Table 2.13)**

One hundred and ninety eight patients (97.5%) were homozygous wild type *14. One patient (0.5%) was homozygous mutant and another patient (0.5%) was heterozygous. Genotyping for 3 patients (1.5%) failed. **(Table 2.13)**

One hundred and ninety nine patients (98%) were homozygous wild type *17.

One patient (0.5%) was heterozygous while none were homozygous mutant. Genotyping for 3 patients (1.5%) failed. **(Table 2.13)**

2.5.2.3 Genotype and Phenotype Frequency

The frequency distribution of the various genotypes, and assigned extent of metabolism phenotypes are shown in Table 2.14. There was no poor metabolizer phenotype within our study population.

	Genotype and Frequency						
Genotype	*1/*1	*1/*3	*1/*6	*1/*10	*4/*10	*6/*10	*10/*10
Frequency	78(39%)	1 (0.5%)	2 (1%)	68(34%)	8 (4%)	7 (3.5%)	36(18%)
Phenotype	EM	IM	IM	EM	IM	IM	IM

Table 2.14:- Frequency distributions of the various CYP2D6 genotypes and phenotypes. (N = 200; genotyping failed for 3 patients)

2.5.2.4 Association Between Genotype Frequency And Bronchospasm

There was no association between CYP2D6 genotype and bronchospasm, defined as a ratio of FEV1/FVC of less than 0.7 or assigned phenotype and bronchospasm ($\chi^2 = 0.17$ with 1 degree of freedom; $p = 0.675$).

2.5.2.5 Association Between Genotype Frequency And Hypertension Control

We compared the 7 CYP2D6 SNP with the control of hypertension in our patients. The control of hypertension was grouped as:

i) Systolic & diastolic control (hypertension control)
ii) Systolic BP control
iii) Diastolic control

Good control of hypertension is defined as:

Systolic BP < 140

Diastolic BP < 85

We then compared the genotypes of each of the SNP (wild type vs. mutant vs. heterozygous) against these 3 groups.

Genotypes and the overall blood pressure control

Phenotype	Good	Poor
EM	**58**	**87**
IM	**21**	**35**

Chi Square 0.1058; df = 1, *p-value* = 0.745

Table 2.15: Distribution of the various genotypes and phenotypes (EM & IM) and the control of overall BP.

Genotypes and the systolic blood pressure control

Phenotype	Good BP control	Poor BP control
EM	68	78
IM	27	29

Chi Square 0.0436; df = 1, *p-value* = 0.835

Table 2.16: Distribution of the various genotypes and phenotypes (EM & IM) and the control of systolic BP.

Genotypes and the diastolic blood pressure control

Phenotype	Good	Poor
EM	85	62
IM	30	25

Chi Square 0.1754; df = 1, *p-value* = 0.675

Table 2.17: Distribution of the various genotypes and phenotypes (EM & IM) and the control of diastolic BP.

2.5.2.6 Beta-2 AR Genotyping Results

2.5.2.6.1 Beta-2 AR Genotyping

Five alleles of the Beta-2 AR gene were studied. These are codon 16, 27, 164 and nucleotide positions -47 and -20. All the patients were genotyped for these 5 alleles and their genotype frequencies were calculated and grouped into heterozygous, homozygous wild type and homozygous mutant.

2.5.2.6.2 Genotype frequency of Beta-2 AR alleles.

The genotype frequency of each of the alleles is given below:

2.5.2.6.2.1 Genotype frequency for codon 16 of the Beta-2 AR gene.

One hundred and thirty four (66.7%) patients carried the heterozygous allele 16.

Forty-two (20.9%) carried the homozygous wild type allele and 25 (12.4%) carried the homozygous mutant allele. Genotyping for 2 patients failed **(Table 2.18).**

2.5.2.6.2.2 Genotype frequency for nucleotide position -20 SNP of the Beta-2 AR gene.

Twenty-seven (13.4%) patients carried the heterozygous allele 20.

Thirty-seven (18.4%) carried the homozygous **c** allele and 137 (68.2%) carried the homozygous **t** allele. Genotyping for 2 patients failed **(Table 2.18).**

2.5.2.6.2.3 Genotype frequency for codon 27 of the Beta-2 AR gene.

Twenty-nine (14.4%) patients carried the heterozygous allele 27.

One hundred and sixty nine (84%) carried the homozygous wild type allele and 3 (1.5%) carried the homozygous mutant allele. Genotyping for 2 patients failed **(Table 2.18).**

2.5.2.6.2.4 Genotype frequency for codon 164 of the Beta-2 AR gene.

Two hundred and one (100%) patients carried the homozygous wild type allele. Genotyping for 2 patients failed **(Table 2.18).**

2.5.2.6.2.5 Genotype frequency for nucleotide position -47 SNP of the Beta-2 AR gene.

Seventeen (8.5%) patients carried the heterozygous allele 47. Three (1.5%) carried the homozygous wild type allele and 181 (90%) carried the homozygous mutant allele. Genotyping for 2 patients failed **(Table 2.18).**

	Beta2 Genotype Frequency		
Codon & Nucleotide position	Homo-wild type	Hetero	Homo – Mutant
16	42 (20.9%)	134 (66.7%)	25 (12.4%)
27	169 (84%)	29 (14.4%)	3 (1.5%)
164	201 (100%)	-	-
-47	3 (1.5%)	17 (8.5%)	181 (90.0%)
-20	37 (18.4%)	27 (13.4%)	137 (68.2%)

Table 2.18: Genotype frequency of the 5 SNPs of the Beta2 AR gene.

The genotypes were in Hardy-Weinberg equilibrium (Chi Squares value = 5.61, 10 df and p = 0.85).

2.5.2.6.2.6 Genotypes of the patients who experienced Bronchospasm

Sixteen patients experienced bronchospasm after taking metoprolol. The CYP2D6 and Beta2 adrenergic receptor genotypes and haplotypes of these patients are shown in **Table 2.19.**

	CYP2D6				Beta -2		
Patient	Genotype	Phenotype	BP control	FEV1/FVC	Codon 16 Genotype	Haplotype 1	Haplotype 2
1	*1/*1	EM	poor	.69	Hetero	T-T-A-C	T-T-G-C
2	*1/*6	IM	poor	.61	Hetero	T-T-A-C	T-T-G-C
3	*10/*10	IM	poor	.52	Mutant	T-C-A-C	T-C-A-C
4	*1/*10	EM	poor	.65	Hetero	C-C-G-C	T-T-A-C
5	*1/*10	EM	poor	.44	Hetero	T-T-A-C	T-T-G-C
6	*4/*10	IM	poor	.68	Mutant	T-T-A-C	T-T-A-C
7	*1/*1	EM	poor	.69	Mutant	T-T-A-C	T-T-A-C
8	*1/*10	EM	poor	.69	Hetero	T-T-A-C	T-T-G-C
9	*1/*10	EM	poor	.69	Wild Type	T-C-G-C	T-C-G-C
10	*10/*10	IM	poor	.66	Hetero	T-C-A-C	T-C-A-G
11	*1/*1	EM	poor	.60	Hetero	T-T-A-C	T-T-G-C
12	*1/*10	EM	poor	.68	Hetero	T-C-A-G	T-C-G-C
13	*1*1	EM	poor	.68	Hetero	T-T-A-C	T-T-G-C
14	*1/*10	EM	poor	.42	Hetero	T-C-A-C	T-C-G-G
15	*1/*10	EM	poor	.69	Wild Type	T-T-G-C	T-T-G-C
16	*1/*1	EM	poor	.69	Hetero	T-T-A-C	T-T-G-C

Table 2.19: Genotype, phenotype and haplotype of the 16 patients who experienced bronchospasm.

Alleles 3, 4, 6 and 14 are associated with poor metabolizing capacity of the enzyme while alleles 9, 10 and 17 are associated with intermediate or extensive metabolizing capacity of the enzyme.

The haplotype frequencies by ethnic group are given in table 2.20.

					Total	Chinese	Malay	Others
Nucleotide	**-47**	**-20**	**16**	**27**				
Allele	**T/C**	**T/C**	**G/A**	**C/G**				
Position	**AA19 BUP**	**-**	**AA16**	**AA27**				
	C	C	A	C	0	0	0	0
	C	C	G	C	0.00255	0	0.00336	0
	C	C	G	G	0.05205	0.08824	0.04879	0.03571
	C	T	G	G	0.00261	0	0.00341	0
	T	C	A	C	0.05197	0.03606	0.05253	0.08241
	T	C	A	G	0.01107	0.0565	0.00362	0.01786
	T	C	G	C	0.12252	0.11331	0.11758	0.13188
	T	C	G	G	0.01108	0.00001	0.01596	0
	T	T	A	C	0.48423	0.43686	0.49941	0.41759
	T	T	G	C	0.25167	0.2373	0.24869	0.2967
	T	T	G	G	0.01026	0.03172	0.00666	0.01786

Table 2.20: Haplotype frequencies by ethnic group
N = 200; Malay 153, Chinese 17, Others (mixed ethnicity) 30

The haplotypes frequencies of those who experienced bronchospasm and those who did not, are given in table 2.21 and 2.22. An association was found between haplotype 1 and bronchospasm (χ^2 13.92; df = 8; p = 0.047), and between haplotype 2 and bronchospasm (χ^2 14.39; df = 8; p = 0.048).

Haplotype 1	Bronchospasm	No Bronchospasm
C-C-G-C	2	2
C-T-G-G	1	2
T-C-A-C	5	24
T-C-A-G	1	2
T-C-G-C	1	13
T-T-A-C	13	117
T-T-G-C	2	4
C-C-G-G	0	13
C-T-A-G	0	1
Total	**25**	**178**

Chi Square 13.92; df = 8, *p-value* = 0.047
Table 2.21 Haplotype 1 frequencies of those who experienced bronchospasm and those who did not.

Haplotype 2	Bronchospasm	No Bronchospasm
C-C-G-C	0	2
T-C-G-G	0	5
T-C-A-C	2	2
T-C-A-G	1	0
T-C-G-C	3	24
T-T-A-C	5	49
T-T-G-C	12	90
T-T-G-G	1	6
C-C-G-G	1	0
Total	**25**	**178**

Chi Square 14.39; df = 8, *p-value* = 0.048

Table 2.21 Haplotype 2 frequencies of those who experienced bronchospasm and those who did not.

2.6 DISCUSSION

All the patients were recruited from the outpatients' clinic and physicians' clinic of Hospital USM and Hospital Kota Bharu. These two hospitals are the referral hospitals (tertiary centre) for the whole of Kelantan state. Kelantan is a state in the north-eastern part of Malaysia (figure2.15), neighbouring Thailand. It has a population of 947,375 with the majority of its population being Malays (94.55%). Ethnic Chinese make up 4.4% of the population while ethnic Indians and 'Others' (mainly ethnic Thais and those of mixed origin) make up 0.25% and 0.8% respectively.

Figure 2.23 Map of peninsula Malaysia. Kelantan is a state at the north-eastern part of Malaysia bordering Thailand.

In our sample population, the ethnic compositions of our patients were mainly Malays (76.3%), with Chinese and Indians forming 8.4% and 1.5% of the samples respectively. There was one Malaysian of Thai origin (0.5%). About thirteen percent (13.3%) of the patients were of mixed ethnic origin, which is much higher than that of the local population. This higher percentage of mixed-ethnicity is due to the strict criteria that we adhere to when we defined Malays. A Malay is defined by the country's constitution as one who is a Muslim, speaks the Malay language as his or her first language at home and practices the Malay culture and customs. However, our definition of Malay involves confirmation of the pedigree by three generations. Thus, a Malay by our country's constitution may not be a three-generation Malay. Therefore, when we traced the origin of our Malay patients for 3 generations, we found that some of them had mixed parentage (e.g. Malay-Chinese, Malay-Arab) thus making them not a true Malay by our definition.

Almost half of the patients with mixed parentage were of mixed Malay-Thai origin, a reflection of the close geographical location of the state with Thailand. The other patients with mixed ethnicity have a background of a mixture of the various ethnic groups (e.g. Malay-Chinese, Chinese-Indian) and they have been grouped under 'Others', reflecting the heterogeneity of the Malaysian population.

Chinese and Indians are less heterogeneous compared to Malays. A Chinese or Indian patient is identified by their name and race as it appeared in their identity card and with both parents and grandparents also of the same ethnic group. Unlike the Malays, we did not face any problem tracing these two races over 3 generations.

The heterogeneous local population makes studying their genetic polymorphisms a challenging task. The various SNPs of the CYP2D6 and Beta2 adrenergic receptor gene are known to vary according to ethnicity. As the local population was very heterogeneous and our samples were limited, we could not correlate their genotype with their ethnicity.

Out of the 203 patients recruited, 3 samples could not be genotyped (failed genotyping).

Most of the patients were more than 55 years of age, with a mean age of 57 (SD=6.804). As hypertension is an age-related disease, this result was not unexpected. Most of the patients were above middle age. The youngest patient was 37 years old.

We limited our study to adults for the relative ease of obtaining consent compared to children. We defined adults as those above 18 years but had a cut-off age of 65 years for inclusion.

Most of the patients were either overweight or obese with an average body mass index (BMI) of 25.88 (SD= 3.68). Hypertension is also known to be obese related disease and therefore this result is not unexpected.

The majority of the patients (64%) had concurrent Ischemic Heart Disease (IHD). Twenty-five percent also had Diabetes Mellitus (DM), 9% also had a Cerebral-Vascular Accident (CVA), 3.5% also had Transient Ischemic Attacks (TIA) and 1.5% also had Cardiac Arrhythmias. Only 13% of the patients had no concurrent illnesses. Concurrent illnesses in these patients were expected as the majority was in an age group where age-related diseases are common. However, we excluded those who have concurrent Congestive Heart Failure as this would be a confounding factor for the assessment of bronchospasm.

We defined good control of hypertension based on *the Clinical Practice Guidelines on the Management of Hypertension 2002* by the Malaysian Hypertension Guidelines Working Group (Ministry of Health Malaysia and Malaysia. 2002).

Good control of hypertension is defined as:
Systolic BP < 140
Diastolic BP < 85

For discussion of the control of hypertension, we grouped these patients as those with good:
i) Systolic & diastolic control (hypertension control)
ii) Systolic BP control
iii) Diastolic control

Out of 194 patients, 105 (54.1%) had uncontrolled systolic hypertension (> 140 mmHg) and 86 (44.3%) had uncontrolled diastolic hypertension (> 85 mmHg). Seventy (36.1%) had both systolic and diastolic uncontrolled hypertension.

Good BP control was not achieved for the majority of these patients despite being on an anti hypertensive drug (metoprolol). This could be due to several reasons. First, this could be due to the patients' poor compliance. While only 9 out of the 203 patients recruited (at the first visit) requested their managing physician to change their drug after developing adverse drug reactions (severe headache, insomnia and impotency), other patients might not have revealed these problems to their physician because the reactions were mild and they did not want to changed to another drug again. Measuring metoprolol drug level (pharmacokinetics) could have helped us to confirm compliance but unfortunately we did not do so due to limitation of resources.

Failure to optimize the dose of prescribed metoprolol could be another explanation for the poor BP control in these patients. The duration that these patients were on prescribed metoprolol varied. Some had had the drug for more than a year while most had it for only a few weeks.

The CYP2D6*14 is associated with poor metabolizer. However, the majority of the patients (99%) were homozygous wild type. Only 0.5% was homozygous mutant and 0.5% heterozygous.

For allele 3 and 4 (the other 2 alleles associated with poor metabolizers), all the patients were wild-type for these two alleles. For allele 6, the majority (96%) of the patients were also wild-type for this allele.

Kirchheiner et al., 2004, investigated the influence of CYP2D6 genotypes on the pharmacokinetic and pharmacodynamics of a single 100mg dose of metoprolol. Although the median clearances of the drug in ultra rapid and poor metabolizers differed by more than10-fold, the pharmacodynamic differences were less marked. The corresponding reduction in heart rates were 18 and 32 beats

per min. Blood pressure showed no difference between genotypes (Kirchheiner, Heesch et al. 2004).

Nozawa et al., 2005, also observed similarly modest pharmacodynamic effects of genotype on isoproterenol-induced increase in heart rate in patients receiving metoprolol on a routine bases. Patients who were homozygous for CYP2D6*10 showed both higher trough and peak blood levels of metoprolol. This translated into a lower relative increase in the isoproterenol effect on heart rate at the trough level but not at the peak levels.

Overall therefore, the evidence suggests that while genotype may induce marked effects on the pharmacokinetics of metoprolol, the effects on the extent of adrenergic inhibition are more modest.

We divided bronchospasm into objective bronchospasm (as measured by FEV1/FVC) and clinical bronchospasm (as assessed from their answers to the questionnaire).

25 patients (12%) experienced objective bronchospasm. This figure is much higher than that quoted in the product literature (<1%) and from a study conducted by (Marina 2001) where she found the incidence of metoprolol-induced bronchospasm in Kelantan population to be at 7%. However, in her study, bronchospasm was assessed using a questionnaire (clinical bronchospasm) which might not have been accurate. Our assessment of bronchospasm was done by measuring the FEV1/FVC ratio (which we called objective bronchospasm) as well as by a set of questionnaires (clinical bronchospasm). The use of lung function test (FEV1/FVC ratio) has allowed us to pick up more patients with bronchospasm compared to the use of only questionnaires.

With the Questionnaire, patients were asked if they had experienced any difficulty in breathing after taking metoprolol. Thirty-one (15%) answered 'Yes". Out of these, only 6 (19%) had FEV1/FVC ratio of less than 0.7. In other words, assessment of bronchospasm is more effective using the spirometer than using a questionnaire and this could be the reason why we identified more patients with bronchospasm.

When compared to the previous study by (Marina 2001) who conducted her study in the same local population, clinical bronchospasm in our study was found to be twice higher. This could be due to our more detailed questionnaire which allowed better assessment of the symptoms of airway obstruction. A factor, which could have contributed to the high incidence of bronchospasm in our patients, is age as most were more than 55 years old. Higher age was found to be related to reduced lung function (Ismail, 1992).

Eight out of the 16 patients (50%) who developed bronchospasm were either heterozygous or mutant for CYP2D6*10. Fourteen of these patients (88%) carried the Gly16 variant of the Beta2 AR gene. The presence of Gly16 allele markedly influenced the ability of beta-2 receptor to desensitize. Gly16, compared to Arg16, down regulates to a much greater degree following exposure to a beta2 agonist in both transfected cell systems and primary cultured human airway smooth muscle cells (Reihsaus, Innis et al. 1993).

(Martinez, Graves et al. 1997), also found this variant associated with a less favourable effect on airway responsiveness as compared to the Agr16 variant. This genotype is less likely to respond to inhaled beta-2 agonist compared to Argl6 carriers. Their findings are supported by (Lima, Thomason et al. 1999) who studied a group of 16 stable moderate asthmatics, and suggested that

the homozygous Argl6 patients responded more rapidly and to a far greater extent at equivalent plasma albuterol level compared to Glyl6 carriers (homozygous and heterozygous). While this study was conducted on asthmatics treated with beta2 agonists, our study was done on hypertensives on beta2 antagonists. Our results suggest that the presence of Arg16 variant is associated with reduced sensitivity to bronchoconstriction while Gly16 tends to increase bronchoconstriction. In other words, in the absence of Arg16, the ability to prevent bronchoconstriction on exposure to beta2 antagonists appears to be reduced.

Haplotyping was done for all our samples. However, our haplotyping takes account of only a subset of SNPs considered by Drysdale et al. and therefore strict comparison of the two data sets is not possible.

Eight out of the 16 patients (50%) who experienced bronchospasm were either heterozygous or homozygous mutant for CYP2D6*10, which is predicted to result in impaired metabolism of metoprolol. This rate, when compared with other populations, is high and could be the other reason for the high incidence of bronchospasm in the local population. However, there was no significant difference in the frequency of CYP2D6*10 carriage between those with bronchospasm and other patients in this population. Also, we could not find any association between the other CYP2D6 genotypes and bronchospasm (χ^2 0.17 with 1 degree of freedom; p = 0.675).

Out of the 25 patients who had bronchospasm, a majority (64%) had both systolic as well as diastolic uncontrolled hypertension (**Figure 2.20**). All our patients were either extensive metabolizers or intermediate metabolizers. There was no poor metabolizer. Theoretically, a poor metabolizer would have resulted in accumulation of the drug in their blood,, poor control of their

hypertension and development of bronchospasm. However, there were 28% patients with impaired metabolizing capacity (IM) and these patients were the possible cause why there was high incidence of bronchospasm in our patients.

The 2 patients (patient 14 and patient 16), who experienced bronchospasm and had the homozygous wild-type beta2 codon 16 (Arg16), were also wild-type for CYP2D6 *10. However, these 2 patients carried the mutant Beta2 allele 20. As codons 16 and 27 have been shown to be associated with bronchial hyper-responsiveness, we postulated that allele –20 (as well as allele -47), could play a role in bronchial response in view of its linkage disequilibrium with codon 16 and 27.

The average metoprolol dose given was 135 mg per day and most of the patients were on low doses (100mg per day and 200mg per day). Only one patient received 400mg per day and 5 received 300mg per day.

All the 203 patients recruited were prescribed metoprolol during their first visit. However, 9 patients discontinued metoprolol at their second visit as they developed adverse reactions (headache, impotence and insomnia) and requested that their medication be changed. These 9 patients became ineligible for the subsequent analyses.

All the patients who experienced bronchospasm were on low dosage of the drug. This contradicts the conventional teaching of dose-related development of iatrogenic bronchospasm (Kendall 1990). An explanation for this high incidence of bronchospasm could be the high prevalence of CYPD2D6*10 mutant allele in our patients (**Table 2.13**). Our results supports the findings by Teh, Ismail et al. (2001) and Ismail, Hussein et al. (2000) who also found a high prevalence of

CYP2D6*10 allele in the Malaysian population. The presence of this allele results in an individual having a reduced capacity to metabolize the drug, and hence accumulation of the drug in their blood.

As this is a cross-sectional study we did not have the baseline BP values before the patients started on metoprolol. The cut –off point for the control of BP was taken as a single value (140mm.Hg for systolic and 90mm.Hg for diastolic) and these patients were broadly grouped into controlled and uncontrolled BP. Some of these patients might have had a baseline BP higher than these cut-off values, thus wrongly grouping them into the uncontrolled group. However, a mean BP was taken from all these patients to minimize classification errors arising from one-off measurement.

We also did not have any baseline pre-metoprolol values for the lung function tests, especially the FEV1/FVC ratio. Definition of bronchospasm was based on a single value (above 80 was taken as no bronchospasm) and patients were grouped into those had bronchospasm and those who did not. The lung function tests were done on these patients after they were started on metoprolol. Some of the patients might have had a baseline FEV1/FVC ratio higher than the cut-off value leading to misclassification.

We were unable to study the pharmacokinetics of metoprolol in the individual subjects due to the limitation of resources. However, this may not be necessary as metabolic profile can be predicted from the genotype.

CHAPTER

3

SYSTEMATIC REVIEW OF GENETIC POLYMORPHISMS OF BETA-2 AR GENE AND HYPERTENSION

CHAPTER 3
Systematic Review & Meta-Analysis

3.0 INTRODUCTION

Hypertension is a complex trait to which genetic and environmental factors contribute interactively in causing the disease. Investigations of the genetic basis of hypertension have drawn considerable interest and several candidate genes have been identified that associate with the disease.

Alpha-adducin (ADD1) gene, the beta(1)-adrenergic receptor (Beta-1 AR) and beta(2)-adrenergic receptor (Beta-2 AR), endothelial nitric oxide synthase (NOS3), and components of the renin-angiotensin-aldosterone system (angiotensinogen [AGT], angiotensin converting enzyme [ACE], the angiotensin type I receptor [AGTR1], and aldosterone synthase [CYP11B2]) are among the candidate genes identified to date.

	Candidate Genes	**Abbreviations**
1	Alpha-adducin	ADD1
2	Beta-1-adrenergic	Beta-1 AR
3	Beta-2 adrenergic	Beta-2 AR
4	Endothelial nitric oxide synthase	NOS3
5	Renin-angiotensin-aldosterone (angiotensinogen)	AGT
6	Angiotensin converting enzyme	ACE
7	Angiotensin type 1 receptor	AGTR1
8	Aldosterone synthase	CYP11B2

Table 3.1: The candidate genes associated with hypertension.

These genes contribute to the risk of hypertension and screening for these genes may enable early identification of individuals at high risk of developing the disease and hence institute prophylactic measures. Identification of those genes may also lead to the development of more efficacious treatments.

Among the candidate genes, the genes from the renin-angiotensin-aldosterone system have been the most widely studied. The renin-angiotensin-aldosterone system plays a central role in maintenance of vascular tone and in salt and water homeostasis. Experimental evidence demonstrated that stimulation of the renin-angiotensin-aldosterone system raises blood pressure (BP) and that inhibition has the opposite effect. The substrate for this system is angiotensinogen, which is secreted by the liver and the level of which is influenced by estrogens, glucocorticoids, and thyroid hormones (Clauser, Gaillard et al. 1989). Renin cleaves angiotensinogen to produce angiotensin I, which is converted by angiotensin-converting enzyme (ACE) to angiotensin II (Sealy 1990) an active peptide that causes vasoconstriction (Lifton 1996).

The gene that encodes angiotensinogen is found on chromosome 1q42 to 43 (Gaillard, Clauser et al. 1989; Fukamizu, Takahashi et al. 1990) where a tyrosine for cytosine substitution in the second exon results in the substitution of the threonine for methionine at amino acid position 235 (Met235Thr) in the translated protein. In humans, the Thr235 variant has been associated with both hypertension and elevated plasma angiotensinogen levels (Jeunemaitre, Soubrier et al. 1992; Caulfield, Lavender et al. 1994). In a meta-analysis, the ThrThr genotype was associated with an approximately 32% increased risk of hypertension in White but not in non-White subjects (Staessen, Kuznetsova et al. 1999).

The sympathetic nervous system represents an important regulator of blood pressure through alterations in vascular responsiveness, renin release, sodium handling and cardiac output. Beside the genes from the renin-angiotensin-aldosterone system, genetic variation in either the alpha-adrenergic receptor, leading to enhanced vasoconstriction, or beta2-adrenergic receptor, leading to attenuated vasodilatation, might be important for increasing total peripheral resistance and hence blood pressure.

Apart from the genes from the renin-angiotensin-aldosterone system, the beta-2 AR gene has also drawn substantial interest. Because the beta-2 adrenergic receptor is involved in diverse physiologic pathways that are relevant to the control of BP (Modan, Halkin et al. 1985; Haffner, Mitchell et al. 1992; Insel 1996; Chruscinski, Rohrer et al. 1999), it is an attractive candidate gene for susceptibility to hypertension. Beta-2 adrenergic receptor belongs to a family of receptors that mediate their action by stimulating guanine nucleotide binding regulatory proteins. Beta-2 adrenergic receptor agonists promote a rise in intracellular cAMP concentration, which, through smooth cell relaxation, leads to marked vasodilatation. Other potential blood pressure regulating effects of beta-2 adrenergic receptor include their action on renal sodium excretion and renin release.

The advances of the Human Genome Project have led to an increased appreciation that variations in genetic background may underlie a substantial portion of the clinical heterogeneity apparent in cardiovascular disease, including hypertension. Any "functional" molecular variations of the beta-2 adrenergic receptor gene may cause attenuated vasodilatation, leading to increased total peripheral resistance and hence ultimately hypertension.

17 SNPs in the promoter and coding regions of beta-2 adrenergic receptor gene have been reported (Halushka, Fan et al. 1999). Within the beta-2 adrenergic receptor gene open reading frame, four amino acid polymorphisms have been reported all due to single base substitutions, although only two of these polymorphisms, the substitutions at codon 16 and 27, are, both common in the general population and functionally important. Indeed, several studies implicated the polymorphisms at these two codons in the susceptibility to hypertension (Kotanko, Binder et al. 1997; Timmermann, Mo et al. 1998; Gratze, Fortin et al. 1999; Bray and Boerwinkle 2000; Busjahn, Li et al. 2000). Other studies, however, failed to find an association between these single nucleotide polymorphisms (SNP) and hypertension (Candy, Samani et al. 2000; Herrmann, Buscher et al. 2000; Jia, Sharma et al. 2000; Xie, Stein et al. 2000). A variant in the promoter region (T-47C) was shown by an in vitro study to regulate receptor expression at the translational level (McGraw, Donnelly et al. 1998) while the Ile(164) variant demonstrates decreased responsiveness to agonist activity both in vitro and in animal models. In studies of patients with congestive heart failure, this variant has been associated with poor functional capacity and decreased survival.

Among the SNPs of the beta-2 AR gene, the variant at codon 16 is the most widely studied. It has been reported that the Glyl6 variant is associated with the presence of hypertension in African Caribbeans (Kotanko, Binder et al. 1997) whereas the alternate allele, Argl6, is associated with increased BP values in the Bergen Blood Pressure Study (Timmermann, Mo et al. 1998) and the German twin study (Busjahn, Li et al. 2000). Also, a few studies have provided evidence suggestive of linkage and association at (or near) the beta-2 AR gene locus in Black (Svetkey, Timmons et al. 1996) and White subjects (Svetkey *et al*, 1997) whereas a lack of association has been recently reported in a black African population (Candy, Samani et al. 2000).

Since genotype-phenotype relationships may be complex and weak, association studies of large sample sizes are needed to detect an association between genetic variation in the beta-2 AR gene and essential hypertension. Most studies had small sample sizes with not enough power to confirm an association. Pooling of data through systematic reviews and meta-analysis would be helpful to find out if indeed there is an association between genetic variation and essential hypertension.

3.1 OBJECTIVE

To examine systematically the possible association between genetic variation in the beta-2 AR gene and essential hypertension.

3.2 METHODS

3.2.1 Search strategy for the identification of studies

A search strategy was designed to identify studies that have been conducted on possible association between hypertension and beta-2 AR gene polymorphism.

3.2.2 Data Sources and study selection

Electronic literature search of MEDLINE, and the Cochrane Library database plus manual reference checks of all articles on beta-2 AR gene AND hypertension published in English language up to July 2004.

A two-level screen strategy was adopted.

3.2.3 Level 1 screening

The search term used for the computerized search was:

hypertension AND beta-2 adrenergic receptor gene

The other terms used for the computerized search are listed in **Table 3.2**

	Search Terms (MEDLINE)
Hypertension:	Hypertension
Beta-2 Adrenergic Receptor gene:	beta-2 AR gene
Publication types:	Not specified
Publication dates:	10 years (1995 to 2005)
Languages:	English only
Age group:	All
Gender:	both male and female

Table 3.2 Terms used for computerized search (MEDLINE)

Exclusion criteria

The following studies were excluded:

1) Publications which were

   (i) Abstracts only
   (ii) Case reports
   (iii) Letters
   (iv) Narrative reviews

2) Included fewer than 50 subjects

3) Non human (animal) studies

4) Studies that did not involve genotyping of the Beta 2 AR gene allele(s).

3.2.4 Level 2 screening

The studies that passed Level 1 screening must furthermore meet the following inclusion criteria.

Inclusion criteria

1) Full text articles
2) Association studies of Beta 2 gene polymorphism(s) with Hypertension. The information on the results of the allele frequency must be given in the article.
3) Case-control studies
4) Samples size > 50
5) Human studies.

Except for criteria no.3, meeting the exclusion criteria has automatically led to meeting all these inclusion criteria.

3.2.5 Cross validation

After extraction of the following data, cross-validation was done independently by my supervisor. Any discrepancies were resolved by discussion.

a) Study identification

b) The design of the study (case-control studies)

c) The participants (hypertensive with or without treatment), number of patients involved in the studies.

d) The controls (population-based, hospital-based, matched / unmatched).

e) The population / ethnic group that were studied.

f) The number of beta-2 AR gene alleles studied.

g) The genotyping method used by the researchers.

h) The statistics used in each of the studies. If Odds Ratio (OR) was calculated and given in the studies.

i) Outcome measures used and main result obtained

Data was extracted systematically for the patient and control characteristics, the alleles studied and results. The screening of studies, selection and data extraction were performed independently by my supervisor and me. Disagreements in the evaluation of individual's trials due to extraction errors were resolved through discussion.

3.2.6 Odds ratio of frequency of different alleles

The Odds Ratio (OR) from each of the studies was determined and the results were pooled together to obtain a combined OR for the 8 studies.

3.2.7 Calculation of Odds ratio

The OR was calculated as follows:

Table 3.3 Calculation of Odds Ratio

	Hypertensive	Normotensive
Wild Type	A	b
Mutant	C	d

$$OR = \frac{ad}{bc}$$

3.3 RESULTS

38 studies examining the association of beta-2 AR gene with hypertension were identified (**Table 3.4**).

Table 3.4 List of articles obtained after electronic literature search of MEDLINE, and the Cochrane Library database. **In bold** = studies that passed Level 1 screening.

	Name	Title	Journal
1.	**(Candy, Samani et al. 2000)**	Association analysis of beta2 adrenoceptor polymorphisms with hypertension in a Black African population.	J Hypertens. 2000 Feb; 18(2):167-72.
2.	(Busjahn, Li et al. 2000)	Beta-2 adrenergic receptor gene variations, blood pressure, and heart size in normal twins.	Hypertension. 2000 Feb; 35(2):555-60.
3.	(Zhang, Bui et al. 2000)	Antisense inhibition of beta (1)-adrenergic receptor mRNA in a single dose produces a profound and prolonged reduction in high blood pressure in spontaneously hypertensive rats.	Circulation. 2000 Feb 15; 101(6):682-8.
4.	(Clare Zhang, Kimura et al. 2000)	New beta-blocker: prolonged reduction in high blood pressure with beta (1) antisense oligodeoxynucleotides.	Hypertension. 2000 Jan; 35(1 Pt 2):219-24.
5.	(Xu, Huang et al. 2000)	Beta2-adrenergic receptor gene polymorphisms in myasthenia gravis (MG).	Clin Exp Immunol. 2000 Jan; 119(1):156-60.
6.	**(Gratze, Fortin et al. 1999)**	Beta-2 Adrenergic receptor variants affect resting blood pressure and agonist-induced vasodilation in young adult Caucasians.	Hypertension. 1999 Jun; 33(6):1425-30.
7.	(Chruscinski, Rohrer et al. 1999)	Targeted disruption of the beta2 adrenergic receptor gene.	J Biol Chem. 1999 Jun 11; 274(24):16694-700.
8.	(Luft 1998)	Molecular genetics of human hypertension.	J Hypertens. 1998 Dec; 16(12 Pt 2):1871-8. Review.
9.	(Kawahira, Sawa et al. 1998)	In vivo transfer of a beta 2-adrenergic receptor gene into the pressure-overloaded rat heart enhances cardiac response to beta-adrenergic agonist.	Circulation. 1998 Nov 10; 98(19 Suppl):II262-7; discussion II267-8.
10.	(O'Byrne and Caulfield 1998)	Genetics of hypertension. Therapeutic implications.	Drugs. 1998 Aug; 56(2):203-14. Review.
11.	(McIlveen, White et al. 1997)	Autonomic control of bronchial circulation in awake sheep during rest and behaviour.	Clin Exp Pharmacol Physiol. 1997 Dec; 24(12):940-7.
12.	(Svetkey, Chen et al. 1997)	Preliminary evidence of linkage of salt sensitivity in black Americans at the beta 2-adrenergic receptor locus.	Hypertension. 1997 Apr; 29(4):918-22.
13.	(Freedman, Yu et al. 1997)	Genetic linkage analysis of growth factor loci and end-stage renal disease in African Americans.	Kidney Int. 1997 Mar; 51(3):819-25.
14.	**(Svetkey, Timmons et al. 1996)**	Association of hypertension with beta2- and alpha2c10-adrenergic receptor genotype.	Hypertension. 1996 Jun; 27(6):1210-5.
15.	(Bohm, Flesch et al. 1996)	Role of G-proteins in altered beta-adrenergic responsiveness in the failing and hypertrophied myocardium.	Basic Res Cardiol. 1996; 91 Suppl 2:47-51. Review.
16.	(Castellano, Beschi et al. 1993)Castellano M *et al*	Gene expression of cardiac beta 1-adrenergic receptors during the development of hypertension in spontaneously hypertensive rats.	J Hypertens. 1993 Aug; 11(8):787-91.

17.	(Umemura, Hirawa et al. 1993)	Molecular biology of alpha-adrenergic receptor and essential hypertension.	Nippon Rinsho. 1993 Jun; 51(6):1591-601. Review. Japanese.
18.	(Malczewska-Malec, Wybranska et al. 2004)	Analysis of candidate genes in Polish families with obesity.	Clin Chem Lab Med. 2004 May;42(5):487-93.
19.	(Romaino, Teh et al. 2004)	A simple and rapid genotyping method for beta-2 receptor (beta2 AR) gene using allele specific multiplex PCR.	J Clin Pharm Ther. 2004 Feb;29(1):47-52.
20.	(Pereira, Floriano et al. 2003)	Beta2 adrenoceptor functional gene variants, obesity, and blood pressure level interactions in the general population.	Hypertension. 2003 Oct;42(4):685-92. Epub 2003 Aug 4.
21.	(Heckbert, Hindorff et al. 2003)	Beta2-adrenergic receptor polymorphisms and risk of incident cardiovascular events in the elderly.	Circulation. 2003 Apr 22;107(15):2021-4. Epub 2003 Apr 7.
22.	(Snieder, Dong et al. 2002)	Beta2-adrenergic receptor gene and resting hemodynamics in European and African American youth.	Am J Hypertens. 2002 Nov;15(11).973-9.
23.	(Kim, Lee et al. 2002)	The effects of beta2 adrenoceptor gene polymorphisms on pressor response during laryngoscopy and tracheal intubation.	Anaesthesia. 2002 Mar;57(3):227-32.
24.	**(Herrmann, Nicaud et al. 2002)**	Polymorphisms of the beta2 -adrenoceptor (ADRB2) gene and essential hypertension: the ECTIM and PEGASE studies.	J Hypertens. 2002 Feb;20(2):229-35.
25.	(Lin, Ericsson et al. 2001)	Association of beta2-adrenoceptor Gln27Glu variant with body weight but not hypertension.	Am J Hypertens. 2001 Dec;14(12):1201-4.
26.	**(Ranade, Shue et al. 2001)**	The glycine allele of a glycine/arginine polymorphism in the beta2-adrenergic receptor gene is associated with essential hypertension in a population of Chinese origin.	Am J Hypertens. 2001 Dec;14(12):1196-200.
27.	**(Herrmann, Buscher et al. 2000)**	Beta2-adrenergic receptor polymorphisms at codon 16, cardiovascular phenotypes and essential hypertension in whites and African Americans.	Am J Hypertens. 2000 Sep;13(9):1021-6.
28.	**(Xie, Stein et al. 2000)**	Human beta2-adrenergic receptor polymorphisms: no association with essential hypertension in black or white Americans.	Clin Pharmacol Ther. 2000 Jun;67(6):670-5.
29.	**(Jia, Sharma et al. 2000)**	beta2-adrenoceptor gene polymorphisms and blood pressure variations in East Anglian Caucasians.	J Hypertens. 2000 Jun;18(6).687-93.
30.	(Meirhaeghe, Helbecque et al. 2000)	Impact of polymorphisms of the human beta2-adrenoceptor gene on obesity in a French population.	Int J Obes Relat Metab Disord. 2000 Mar;24(3):382-7.
31.	**(Kato, Sugiyama et al. 2001)**	Association analysis of beta2 adrenoceptor polymorphisms with hypertension in a Black African population.	Hypertension ; 2001; 37 (2): 286-92
32.	(Xu, Huang et al. 2000)	beta2-adrenergic receptor gene polymorphisms in myasthenia gravis (MG).	Clin Exp Immunol. 2000 Jan;119(1):156-60.
33.	(Chruscinski, Rohrer et al. 1999)	Targeted disruption of the beta2 adrenergic receptor gene.	J Biol Chem. 1999 Jun 11;274(24):16694-700.
34.	**(Kato, Hyne et al. 1999)**	Complete genome searches for quantitative trait loci controlling blood pressure and related traits in four segregating populations derived from Dahl hypertensive rats.	Mamm Genome. 1999 Mar;10(3):259-65.
35.	(Bohm, Flesch et al. 1997)	Beta-adrenergic signal transduction in the failing and hypertrophied myocardium.	J Mol Med. 1997 Nov-Dec;75(11-12):842-8. Review.

36.	(Lowes, Minobe et al. 1997)	Changes in gene expression in the intact human heart. Down regulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium.	J Clin Invest. 1997 Nov 1;100(9):2315-24.
37.	**(Kotanko, Binder et al. 1997)**	Essential hypertension in African Caribbeans associates with a variant of the beta2-adrenoceptor.	Hypertension. 1997 Oct;30(4):773-6.

Out of these 38 studies, 28 were excluded based on the exclusion criteria.

The list of the remaining 10 clinical studies are given in **Table 3.5**

	Articles
1	**(Svetkey, Timmons et al. 1996); Hypertension. 1996 Jun;27(6):1210-5**
2	**(Kotanko, Binder et al. 1997); Hypertension. 1997 Oct;30(4):773-6.**
3	**(Gratze, Fortin et al. 1999); Hypertension. 1999 Jun;33(6):1425-30.**
4	**(Candy, Samani et al. 2000); J Hypertens. 2000 Feb;18(2):167-72.**
5	**(Herrmann, Buscher et al. 2000); Am J Hypertens. 2000 Sep;13(9):1021-6.**
6	**(Jia, Sharma et al. 2000); J. Hypertens. 2000 Jun;18(6):687-93.**
7	**(Xie, Stein et al. 2000); Clin Pharmacol Ther. 2000 Jun;67(6):670-5.**
8	**(Kato, Sugiyama et al. 2001); Hypertension. 2001 Feb;37(2):286-92.**
9	**(Ranade, Shue et al. 2001); Am J Hypertens. 2001 Dec;14(12):1196-200.**
10	**(Herrmann, Nicaud et al. 2002); J Hypertens. 2002 Feb;20(2):229-35.**

Table 3.5: Studies that passed Level 1 screening.

After Level 2 screening, 2 studies were excluded (Svetkey, Timmons et al. 1996; Gratze, Fortin et al. 1999) as they did not meet the inclusion criteria (**Table 3.6**).

No clear information was given by (Svetkey, Timmons et al. 1996) regarding which alleles that they studied, while the study by (Gratze, Fortin et al. 1999) was not a case-control study.

	(Svetkey, Timmons et al. 1996)Svetkey P Hypertension 1996; 27 (6):1210-5	(Gratze, Fortin et al. 1999)Gratze G Hypertension 1999; 33 (6):1425-30
Polymorphism studied	Information not available	16
Population Studied	Americans Black + White	Caucasians
Sample size;	175 [96 black] [79 White]	57 [G16/G16= 15] [A16/A16= 12] [A16/G16= 27]
1) Cases	86 HT	57 (20-30 yrs.old)
2) Control	86 NT	
Genotyping Methods (methods)	RFLP	PCR (Allele-sp)
Results	Information on the genotype frequency not available	Information on the genotype frequency not available
Reason for rejection	**No information in the text about which allele was studied**	**Not a case-control study**

Table 3.6: Studies that were excluded at Level 2 screening.

Studies that passed Level 2 screening.

Eight studies passed level 2 screening (table 3.7 & 3.8).

	Kotanko Hypertension 1997; 30 (4): 773-6	Candy J. Hypertension 2000; 18 (2): 167-72	Herrmann AM. J. Hypertension 2000; 13 (9): 1021-6	Jia J. Hyperten 2000; 18 (6): 687-93	Xie Clin Pharmaco Ther 2000; 67 (6): 670-5	Kato Hypertension 2001; 37 (2): 286-92	Renade AM. J. Hypertension 2001; 14 (12): 1196-200	Herrmann J. Hypertension 2002; 20 (2): 229-35
Polymorphism studied	16	16, 27	16	16, 27	16, 27	16, 27, -47	16, 27, -47	16, 27, -47
Population Studied	Africans Caribbeans West indies	Black Africans	(1) Africans Americans (2) White (Norwegian)	East. Anglia Caucasians	Americans Black & White	Japanese	Chinese	French
Sample size;	217	315	243	596	663	1457	859	2356
1) Cases	136	192	76 (HT.) male & female	298	356 (155 Black) + (201 White)	842	595	1178
2) Control	81	123	167 (NT) male and female	298 (age matched)	307 (128 Black + 179 White)	633	264	1178
Remarks	Recruitment from primary care. +ve family hx of HT ± on drugs. BP > 95 mmHg			144 of the patient randomly assigned to beta-blockers Recruitment from local hospital		BMI vs. 525 DM		PEGASE study:- 707 cases
Genotyping Methods (methods)	PCR – (Allele-sp)	PCR (Allele-sp)	PCR – RFLP	PCR- RFLP	SSCP with seq.	PCR (Allele-sp)	PCR (Allele-sp)	PCR (Allele-sp)

Table 3.7 Studies that passed Level 2 screening; demographic data

	Kotanko Hypertension 1997; 30 (4): 773-6	Candy J. Hypertension 2000; 18 (2): 167-72	Herrmann AM. J. Hypertension 2000; 13 (9): 1021-6	JiaH J. Hyperten 2000; 18 (6): 687-93	Xie Clin Pharmaco Ther 2000; 67 (6): 670-5	Kato Hypertension 2001; 37 (2): 286-92	Renade AM. J. Hypertension 2001; 14 (12): 1196-200	Herrmann J. Hypertension 2002; 20 (2): 229-35
Result	16 WT Heter MU HT 7 28 101 NT 15 24 42	16 WT Heter MU HT 50 94 48 NT 26 67 30	**Black** 16 WT Heter MU HT 12 17 11 NT 12 42 12 **White** 16 WT Heter MU NT 26 42 33	16 WT Heter MU HT 39 152 107 NT 36 158 104	**Black** 16 WT Heter MU HT 48 72 35 NT 31 57 40 **White** 16 WT Het MU NT 35 81 61	16 WT Heter MU HT 204 403 232 NT 162 305 164	16 WT Heter MU HT 169 312 114 NT 99 110 55	16 WT Heter MU HT 153 180 63 NT 108 135 34
OR & CI (Het. Vs. WT)	2.500 (0.875 – 7.143)	0.730 (0.413 – 1.288)	0.405 (0.152 – 1.077) 1.006 (0.367 – 2.755)	0.888 (0.536 – 1.471)	0.816 (0.461 – 1.442) b 0.826 (0.483 – 1.411) w	1.049 (0.814 – 1.353)	1.662 (1.195 – 2.311)	0.941 (0.675 – 1.312)
OR & CI (Mu. Vs. WT)	5.153 (1.960 – 13.548)	0.832 (0.431 – 1.606)	0.917 (0.292 – 2.879) 1.477 (0.543 – 4.017)	0.950 (0.561 – 1.609)	0.565 (0.298 – 1.072) b 0.893 (0.510 – 1.562) w	1.123 (0.834 – 1.497)	1.214 (0.809 – 1.823)	1.308 (0.806 – 2.123)
OR & CI (nWt Vs.WT)	0.239 (0.095 – 0.6)	1.314 (0.768 – 2.245)	1.929 (0.779 – 4.778) b 0.824 (0.341 – 2.004) w	1.096 (0.677 – 1.773)	1.404 (0.829 – 2.375) b 1.17 (0.714 – 1.918) w	0.93 (0.733 – 1.18)	0.661 (0.487 – 0.898)	0.985 (0.719 – 1.349)
P value	0.0002	0.6	0.104 (B) 0.624 (W)	> 0.05	> 0.05	> 0.05	0.01	>0.05
Conclusion	Strong association	No association	No association	No association	No association	No association	Association (for Gly16 allele)	No association

Table 3.8: Studies that passed Level 2 screening; results and OR

3.3.1 Outcome Measure

Except for 2 studies (Kotanko, Binder et al. 1997; Herrmann, Buscher et al. 2000) which considered the polymorphism at codon 16 only, the other studies dealt with more than one polymorphism. **(Table 3.6)**.

We pooled all the 8 studies that finally passed Level 2 screening but decided to analyze the results of the polymorphism at codon 16 as this is the only allele which is common for all the 8 studies.

For each study, the subjects were divided into:

1) Hypertensives
2) Non Hypertensives (controls)

Genotyping results for the polymorphism at codon 16 were grouped into:

1) Homozygous Wild Type (Homo WT)
2) Homozygous Mutant (Homo Mu)
3) Heterozygous (Hetero)

3.3.2 META ANALYSIS

3.3.2.1 Meta-Analysis of Odds Ratio

Meta analysis of the OR was done for the following data using a fixed or random – effects model as appropriate heterozygous allele frequency vs. WT allele frequency

1) Mutant allele frequency vs. WT allele frequency
2) Non-WT allele frequency vs. WT allele frequency

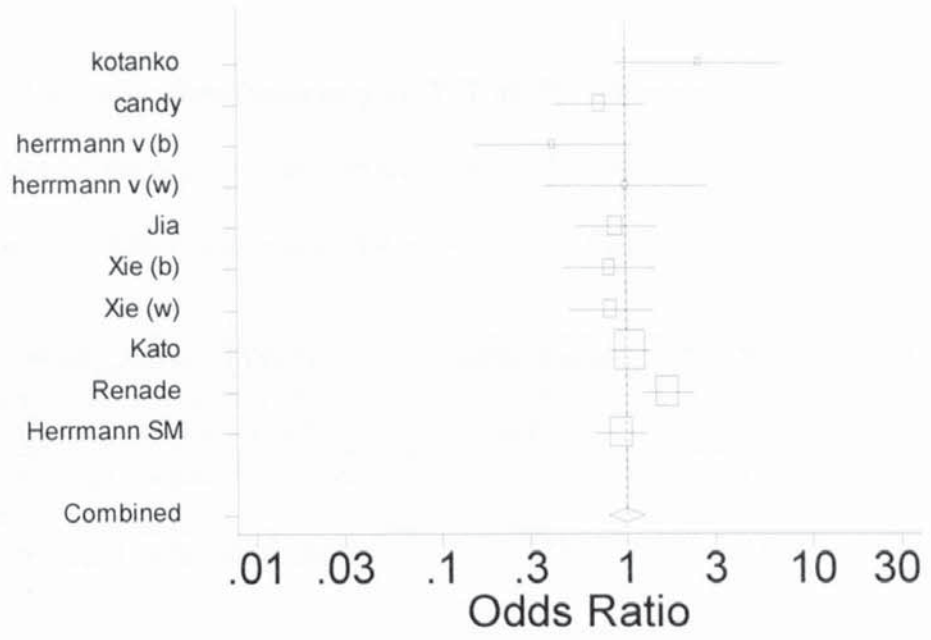
3.3.2.2 Heterozygous allele frequency vs. WT allele frequency

The OR for the occurrence of the heterozygous allele versus the WT allele in the hypertensive and control subjects from the 8 studies is shown in **Table 3.9** and **Figure 3.1** along with the pooled estimate.

Study Author (Year)	Odds Ratio	L95% Limit	U95% Limit
(Kotanko, Binder et al. 1997)	2.500	0.875	7.143
(Candy, Samani et al. 2000)	0.730	0.413	1.288
(Herrmann, Buscher et al. 2000) black	0.405	0.152	1.077
(Herrmann, Buscher et al. 2000) white	1.006	0.367	2.755
(Herrmann, Nicaud et al. 2002)	0.941	0.675	1.312
(Jia, Sharma et al. 2000)	0.888	0.536	1.471
(Xie, Stein et al. 2000) black	0.816	0.461	1.442
(Xie, Stein et al. 2000) white	0.826	0.483	1.411
(Kato, Sugiyama et al. 2001)	1.049	0.814	1.353
(Ranade, Shue et al. 2001)	1.662	1.195	2.311
Pooled Estimate	0.995	0.797	1.243

Table 3.9: Odds ratio for heterozygous and wild-type alleles among hypertensive and control subjects

Figure 3.1: Odds ratio for occurrence of heterozygous versus wild type alleles among hypertensive and control subjects.




```
. meta exp_theta ll ul, ci gr(r) eform xlab(.01 .03 .1 .3 1 3 10 30)
cline id(study) xline(1)

Meta-analysis (exponential form)

       |  Pooled      95% CI          Asymptotic      No. of
Method |     Est   Lower   Upper  z_value  p_value   studies
-------+-----------------------------------------------------
Fixed  |   1.041   0.904   1.199    0.560    0.575      10
Random |   0.995   0.797   1.243   -0.044    0.965

Test for heterogeneity: Q= 17.635 on 9 degrees of freedom (p= 0.040)
Moment-based estimate of between studies variance =  0.055
```

The test for heterogeneity showed a ***p*** value of 0.040, which means that the data show significant heterogeneity. Therefore the random effects model is more appropriate giving a corresponding pooled OR of 0.995 [95% CI 0.797 – 1.243]. This was not significantly different to 1 under the null hypothesis. Therefore, the data do not provide any support

for a difference in distribution of two allele variants between the hypertensive and control patients.

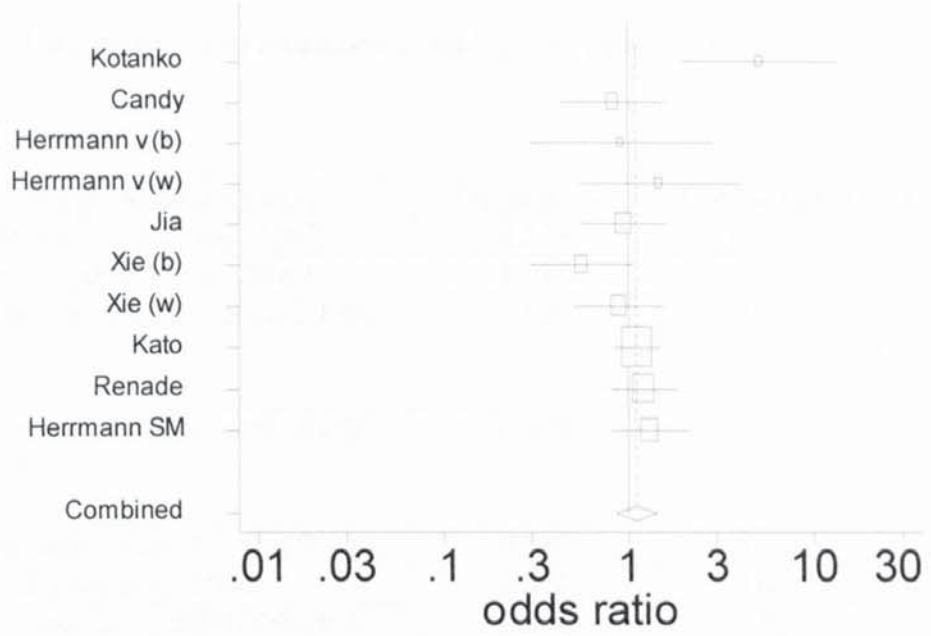
3.3.2.3 Mutant allele frequency vs. WT allele frequency

The OR for the mutant allele frequency vs. WT allele frequency of the 8 studies were pooled and analyzed to obtained a combine OR (figure 3.2 and table 3.10).

Study Author (Year)	Odds Ratio	L95% Limit	U95% Limit
(Kotanko, Binder et al. 1997)	5.153	1.960	13.548
(Candy, Samani et al. 2000)	0.832	0.431	1.606
(Herrmann, Buscher et al. 2000) (black)	0.917	0.292	2.879
(Herrmann, Buscher et al. 2000) (white)	1.477	0.543	4.017
(Herrmann, Nicaud et al. 2002)	1.308	0.806	2.123
(Jia, Sharma et al. 2000)	0.950	0.561	1.609
(Xie, Stein et al. 2000) (blacks)	0.565	0.298	1.072
(Xie, Stein et al. 2000) (white)	0.893	0.510	1.562
(Kato, Sugiyama et al. 2001)	1.123	0.834	1.497
(Ranade, Shue et al. 2001)	1.214	0.809	1.823
Pooled Estimate	1.098	0.930	1.295

Table 3.10: Odds ratio for mutant and wild-type alleles among hypertensive and control subjects

Figure 3.2: Combined OR; Mutant vs. Wild Type




```
. meta oddsr llim ulim if code==2, ci gr(r) eform xlab(.01 .03 .1 .3 1
3 10 30) cline id(study) xline(1)

Meta-analysis (exponential form)

       |  Pooled      95% CI          Asymptotic      No. of
Method |     Est   Lower   Upper  z_value  p_value   studies
-------+-----------------------------------------------------
Fixed  |   1.098   0.930   1.295    1.101    0.271        10
Random |   1.101   0.859   1.411    0.760    0.447

Test for heterogeneity: Q= 16.655 on 9 degrees of freedom (p= 0.054)
Moment-based estimate of between studies variance =  0.066
```

.

The test for heterogeneity showed a ***p*** value of 0.054, which means that the data is not significantly dispersed. Thus, when pooled together the data for allele frequency of allele 16 mutant and wild type, the **combined OR** was 1.098 (fixed pooled estimate) [95% CI (0.930 – 1.295)].

The **combined *p*** value is = 0.271

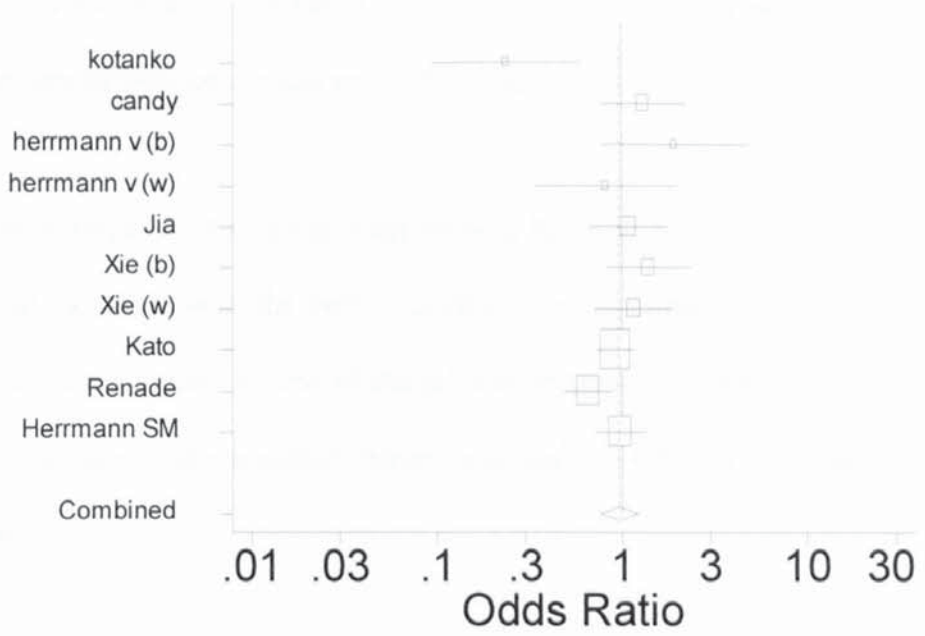
3.3.2.4 Non-WT allele frequency vs. WT allele frequency

The OR for the non-WT allele frequency vs. WT allele frequency of the 8 studies were pooled and analyzed to obtained a combine OR (**figure 3.3 & table 3.11**).

Study Author (Year)	Odds Ratio	L95% Limit	U95% Limit
(Kotanko, Binder et al. 1997)	0.239	0.095	0.096
(Candy, Samani et al. 2000)	1.314	0.768	2.245
(Herrmann, Buscher et al. 2000) (blacks)	1.929	0.779	4.778
(Herrmann, Buscher et al. 2000) (white)	0.824	0.341	2.004
(Herrmann, Nicaud et al. 2002)	0.985	0.719	1.349
(Jia, Sharma et al. 2000)	1.096	0.677	1.773
(Xie, Stein et al. 2000) (blacks)	1.404	0.829	2.375
(Xie, Stein et al. 2000) (white)	1.17	0.714	1.918
(Kato, Sugiyama et al. 2001)	0.93	0.733	1.18
(Ranade, Shue et al. 2001)	0.661	0.487	0.898
Pooled Estimate	0.965	0.770	1.210

Table 3.11: Odds ratio for non wild type and wild-type alleles among hypertensive and control subjects

Figure 3.3: Combined OR; Non-Wild vs. Wild Type




```
. meta oddsr llim ulim, ci gr(r) eform xlab(.01 .03 .1 .3 1 3 10 30)
cline id(study) xline(1)

Meta-analysis (exponential form)

       |  Pooled      95% CI          Asymptotic      No. of
Method |     Est   Lower   Upper  z_value  p_value   studies
-------+-------------------------------------------------------
Fixed  |   0.937   0.821   1.068   -0.975    0.329      10
Random |   0.965   0.770   1.210   -0.308    0.758

Test for heterogeneity: Q= 21.035 on 9 degrees of freedom (p= 0.012)
Moment-based estimate of between studies variance =  0.066
```

The test for heterogeneity showed a ***p*** value of 0.012, which means that the data is significantly dispersed. Thus, when pooled together the data for allele frequency of allele 16 non-wild type and wild type, the **combined OR** was 0.965 (random pooled estimate) [95% CI (0.770 – 1.210)].

The **combined *p*** value = 0.758

3.4 DISCUSSION

The practice of evidence-based medicine requires reliable summaries of the vast and expanding volume of clinical research available.

Evidence-based medicine has been defined by (Sackett 1997) as 'integrating individual clinical expertise with the best available external clinical evidence from a systematic search.' A systematic review of the relevant external evidence provides a framework for the integration of the research, while meta-analysis offers a quantitative summary of the results.

In many cases a systematic review includes a meta-analysis, although there are some situations when this is impossible due to lack of data or inconsistencies between studies. In this systematic review, meta-analysis was possible due to the availability of a common outcome measure among the studies.

The Cochrane Collaboration, launched in 1993, has been influential in the promotion of evidence-based medicine. This is an international network of individuals who are committed to preparing, maintaining and disseminating systematic reviews of research on the effects of health care. Their reviews are made available electronically in the Cochrane Database of Systematic Reviews, part of the Cochrane Library (http://www.update-software.com/cochrane).

Prior to the start of our systematic review, we searched the Cochrane Database of Systematic Reviews for any review of studies on possible associations between hypertension and the beta-2 adrenergic receptor gene. We found none. The most closely related reviews were 3 systematic reviews on hypertension and anti-hypertensive beta-blockers, but none of these reviews focused on the genetic aspect of the disease.

Despite its functional importance in the pathophysiology of hypertension, there have surprisingly been relatively a very limited number of studies done on the beta2 adrenergic receptor gene.

Only MEDLINE and the Cochrane library were searched because other electronic databases were unavailable. From our MEDLINE search we found thirty-nine studies on the association of beta2 adrenergic receptor gene with hypertension.

Using just one electronic database, our search was limited in its scope and breadth of potential trials. (Nieminen and Isohanni 1999) reported an under representation of European journals on MEDLINE. In addition, only studies published in English were included and a number of potential trials published in other languages, may also have been missed.

Manual search was done on the references quoted in each of the studies. We checked the references written at the end of each of the studies but we could not find any new studies related to hypertension and beta2 adrenergic receptor gene.

Another limitation of our systematic review was our inability to make contact with the researchers during the study period to enquire about missing data or further clarification regarding missing data, in particular, the 2 studies that did not pass Level 2 screening (Svetkey, Timmons et al. 1996; Gratze, Fortin et al. 1999).

Unpublished studies were not included in our systematic review and this could have resulted in a number of unpublished trials being missed.

Results which were not positive for an association between hypertension and beta2 adrenergic receptor gene may not have been published. The small sample size in each of the study could have individually resulted in the lack of power to detect an association, but pooling of these data would have added more power for an association.

Out of the 10 studies which passed Level 1 screening, we found only 3 studies (30%) which showed an association but this figure could have been higher had we contacted the experts in the field of hypertension for the unpublished trials.

Search terms "hypertension AND beta2 adrenergic receptor gene" were used for the MEDLINE search which resulted in thirty eight studies being retrieved. These studies were conducted from 1994 till July 2004.

The broad search term retrieved many unrelated studies. Among these are studies on beta2 adrenergic receptor gene and myasthenia gravis, obesity, linkage analysis of the

beta2 adrenergic receptor gene loci, the beta2 gene expression, signal conduction and targeted disruption. There was also a study of the effect of beta2 adrenergic receptor polymorphisms on pressor response during tracheal intubation, animal studies, twin studies as well as review articles on the beta2 adrenergic receptor gene. Studies on other genes related to hypertension, such as the alpha adrenergic receptor gene and beta1 adrenergic receptor gene, were also retrieved.

A broad search term was specifically conducted to include as many related studies as possible. The two level screening was then used to find only the studies that were related to our objective.

We limited our search to English publications only. While this strategy has limited our search for a higher number of studies, it consequently resulted in reduced retrieval of unrelated studies.

Only 8 studies finally passed the Level 2 screening. The inclusion and exclusion criteria enabled us to obtain only the studies that met our objective.

The 2 studies that passed Level 1 but not the Level 2 screening, (Gratze, Fortin et al. 1999) and (Svetkey, Timmons et al. 1996), were excluded.

(Gratze, Fortin et al. 1999) investigated the beta2 agonist mediated in vivo vasodilatation in normotensive Austrian Caucasians and analysed the results with respect to the codon 16 polymorphisms. Fifty-seven normotensive men aged between 20 to 32 years, were genotyped for the beta2 adrenergic receptor codon 16 and these subjects were then given

a graded infusion of the selective beta2 adrenergic receptor salbutamol while the stroke volume and blood pressure were determined continuously. The last 4 minutes of each infusion were evaluated statistically. While their results provided additional evidence that the beta2 adrenergic receptor allele 16 is intimately related to blood pressure regulation, it did not meet the objective of our study, as it was not a case-control study, and therefore had to be excluded from this study.

(Svetkey, Timmons et al. 1996) measured untreated blood pressure in unrelated individuals and characterized each subject as hypertensive (cases) and normotensive (controls). These subjects were then genotyped for variants at the beta2 adrenergic receptor locus. However, the variants were referred to as 3.7kb genotype and 3.4kb genotype, departing from the usual classification of beta2 adrenergic receptor alleles (e.g. alleles 16, 27, 164, -47 etc). We excluded this study from our systematic review as the authors defined the beta2 adrenergic receptor genotypes by means of RFLP and no information concerning the site of the RFLP was revealed. A direct comparison with the results obtained in other studies was therefore not possible.

Cross validation of the extracted data was done independently by my supervisor. Discrepancies were minimal and were resolved through discussions.

Out of the 17 SNPs (alleles) of the beta2 adrenergic receptor gene that have been reported to date, only 4 (codons 16, 27, 164 and nucleotide position -47) have been widely

studied. However, only allele 16 is commonly studied in all the 8 trials that finally passed Level 2 screening and we have taken this allele as our outcome measure.

We were able to perform a meta-analysis for the data that we have extracted from our systematic review, taking the results of allele 16 as our outcome measure.

Out of the 8 studies that finally passed Level 2 screening, only one study gave the Odds Ratio of their results. We used *STATA* software (*STATA Statistical Software:* Release 7.0, Stata Corporation 2001, Texas) to calculate the individual and the combined Odds Ratio.

While each study gave the individual *p* value, the combined *p* value had to be calculated using this software.

The **combined *p*** value for allele frequency of allele 16 **heterozygous and wild type** was 0.965, which was not statistically significant.

The **combined *p*** value for allele frequency of allele 16 **mutant and wild type** was 0.271, which was also not statistically significant.

The **combined *p*** value for allele frequency of allele 16 **non-wild type and wild type** was 0.758, which was also not statistically significant.

Taken together, the overall results showed that there was no association between hypertension and the beta2 adrenergic receptor allele 16.

However, the following confounding factors have to be taken into consideration: the small number of studies that were obtained, the limitation of our search strategy, the publication bias and the polygenic inheritance of the disease, where the many different

candidate genes interact with environmental factors to determine an individual's susceptibility to developing hypertension. Studying the effect of only one candidate gene may therefore not yield any association between the gene and the disease.

Various studies have indicated that variation in blood pressure is genetically determined but only to some extent. It seems likely that variation in the many different candidate genes, each making a small contribution, determine an individual's susceptibility to developing hypertension. The relatively small effect of an individual gene, coupled with the complexity of the phenotype make the search for genes that affect BP variation a challenging task. Studying the effect of only one candidate gene may therefore not yield the expected association, if any, between the gene and the disease.

Studies of molecular genetics of hypertension have shown that SNPs modify the relevant gene function to some extent by disrupting the function of single genes, thereby leading to distinct phenotypes. However, most of the association studies that used a candidate-gene design used a small number of SNPs (typically one). As some of the SNPs are in linkage disequilibrium with each other, studying more SNPs may yield more favourable results. (Yamada, Ishiyama-Shigemoto et al. 1999) found the substitution at nucleotide position -47 was in tight linkage disequilibrium with that at nucleotide position -20. The polymorphisms were also in linkage disequilibrium with codon 16 and codon 27 polymorphisms. Only 3 studies studied more than 2 SNPs, while most studied only one or two SNPs. Studying more SNPs may allow better understanding of the role of this gene in causing the disease.

One possible explanation why consistent results have rarely been found when a particular candidate gene is studied is because individual genes play a modest role in the pathogenesis of hypertension, and the confounding influences of nongenetic factors may decrease or increase the chance of identifying a causative relation between the genes and hypertension. It also depends on the populations studied. Considerable care should be exercised when evaluating in one population the relevance of certain gene polymorphisms to hypertension that has been implied in another population.

The impact of hypertension varies among ethnic groups (Kaplan 1994). It is more prevalent and is associated with more severe complications in populations of West African ancestry (Anderson, Myers et al. 1989) than in any other ethnic group. This has prompted speculation that there may be ethnic differences in the genetic basis of hypertension. This hypothesis is supported by observations in people of African origin that the blood pressure response to sodium loading or mental and physical stress is enhanced.

In most surveys, black children have higher pressures than white children, partly because they mature earlier and have a larger body size. In the UK, blacks of West Africa or Caribbean descent living in London have higher BP than Europeans. These higher pressures probably reflect lower socioeconomic status and a lower standard of health care. The findings were further supported by (Cruickshank, Jackson et al. 1985) who conducted a study among factory workers of equal social class in Birmingham UK, found

blood pressures in blacks were similar to those in whites and Asians. The importance of environmental forces is further illustrated by the frequent rise in blood pressure seen when rural blacks migrate to urban areas where they adopt a westernized diet and experience additional cultural stresses (Poulter, Khaw et al. 1990).

Families share both genes and environment and similarity may result from either. Heritability is the proportion of the total variance of a phenotype that can be explained by genes. It is inferred from the comparison of blood pressure correlations between relatives; the closer the relative, the closer the blood pressure and the higher the heritability estimate. However, calculations of heritability are relevant only to the specific population and environment from which the information was gathered. Heritability may vary between populations if they differ in the prevalence or the nature of genes affecting blood pressure. Even in the absence of genetic differences, important environmental differences can alter estimates of heritability as a result of gene-environment interaction. Extrapolation from one population to another can therefore be misleading. Thus, when conducting a study on the genetics of hypertension, one should therefore attempt to reduce genetic heterogeneity by focusing on a relatively homogenous population.

Unfortunately, the sample populations for our systematic review were quite heterogeneous. Out of the 8 studies that we included in our systematic review, 2 studied on black populations, 2 on Caucasians, 1 Japanese, 1 Chinese and there were 2 studies that compared white and black subjects (Herrmann, Buscher et al. 2000; Xie, Stein et al. 2000). In view of the heterogeneity of these sample populations, one could argue that the

results from these studies should not have been pooled together and our results would have been different had we focused on one population.

3.5 CONCLUSION

Hypertension is a polygenic trait. It is likely that several candidate genes, either by acting together or alone, and contributions of a number of alleles within each of these genes, contribute to the disease. Interaction of these genes with the environment in producing the disease has further complicated our understanding of this disease.

In this systematic review, we could not find any association between hypertension and codon 16 of the beta2 adrenergic receptor gene. Taking into account the limitations of our study and the fact that we studied only one of the many candidate genes, this result probably did not reflect the overall picture of the disease association.

Certainly, when conducting a study on such a disease, one has to consider the contribution of various genetic and non-genetic factors that interact to produce the disease.

CHAPTER

4

GENERAL DISCUSSION

&

CONCLUSION

Hypertension is a worldwide problem and drug therapy is a major approach to reducing its impact. At present, hypertension is pharmacologically managed with an empirical trial-and-error or 'one-size-fits-all' approach. However, this approach fails to adequately address the therapeutic needs of many patients, and pharmacogenetics has been offered as a tool for enhancing patient-specific drug therapy.

The goal of pharmacogenetics in hypertension is to apply knowledge of genetic predictors of treatment response to improve efficacy and reduce toxicity before initiation of therapy.

Pharmacogenetics study of hypertension is still in its infancy. The main challenge is to develop the knowledge base of genes and genetic polymorphisms affecting blood pressure control and translating this knowledge into effective and specific antihypertensive therapies. . More data is necessary before routine clinically-informative pre-prescription genotyping becomes a reality. Beside genetics, other factors such as age, gender, and diet may also play a significant role in determining antihypertensive drug response. Therefore, more studies examining how hypertension gene polymorphisms interact with environmental determinants to affect antihypertensive drug-response, will be needed for meaningful translation of pharmacogenomics hypotheses into useful clinical interventions.

Another challenge is to determine the applicability of pharmacogenetics findings across various population groups. A large number of publications report differences in prevalence and severity of hypertension in people of different racial categories.

Pharmacogenetics studies on one population may not be applicable to other population groups. This may further contribute to the inconsistencies noted across studies. More studies will be required to better define the frequency of genetic polymorphisms and their pharmacogenetics effects in multiple populations.

Another challenge is the development of pharmacogenetics screening tests and its availability to general medical practitioners. Recently, there have been tremendous advances in high-throughput genotyping and these have resulted in reduction in the cost of genotyping. However, there are no universal laboratory standards for genetic tests compared to other commonly used laboratory measurements. Medical practitioners need to be trained and be knowledgeable about the general principles of genetics in order to understand the performance of new pharmacogenetics screening tests so that they may make appropriate choices.

The combined use of DNA microarray technology and automated DNA sequencing has permitted the detection of specific SNPs in several genes involved in drug response. Groups of SNPs or haplotypes inherited together in a stretch of DNA make correlation of genotypes with specific disease or drug response phenotypes more easily attainable. These advances are likely to improve the clinical diagnosis and drug selection for patients suffering from apparently the same disease.

These high throughput technologies for discovering DNA polymorphism and SNP genotyping, coupled with broad academic and commercial initiatives to characterize

genetic variation, genome-wide, are resulting in catalogs of variants that can be used in large-scale clinical studies. It is now possible to simultaneously genotype up to hundreds of thousands of SNPs in samples from a large number of individuals. Certainly, only a few years ago, this would not have been possible. 'Haplotype blocks', or correlated patterns of SNPs that can be adequately represented by fewer SNPs, have the promise of reducing the amount of genotyping required for genome-wide searches.

While the pharmacogenetics of hypertension is still in its infancy, the concept of personalized medicine has shown more rapid progress in areas such as infectious diseases and oncology.

Mallal *et al.*(2002), conducted a study on the pharmacogenetics of abacavir, an HIV-1 nucleoside reverse transcriptase inhibitor. They implicated MHC alleles in the hypersensitivity shown by about 5% of the HIV cases receiving the drug. Their findings suggest that screening patients for the presence of the predisposing MHC haplotype could reduce the incidence of hypersensitivity to abacavir from 9% to 2.5%. While this study is small, it adds to the literature on several other variants, including those in MDR1, the multidrug transporter P-glycoprotein and CYP2D6 that correlate with response to, or pharmacokinetics of, protease inhibitors and non-nucleoside reverse transcriptase inhibitors (Fellay J *et al*, 2002).

Genetic polymorphisms in chemokines and chemokine receptors, including RANTES, MIP-1α and CCR5, have also been found to correlate with both susceptibility to HIV-1

infection and progression of the disease (Ioannidis JP et al, 2001). Taken together, these findings may lead to the development of a panel of polymorphisms that could help personalize HIV therapy, by determining when to initiate therapy and which compounds to choose to maximize efficacy and minimize adverse effects.

Pharmacogenetics studies have also successfully characterized polymorphisms that correlate with response to anti-asthma drugs. For example, Drazen *et al.* (1999), showed that a promoter polymorphism in 5-lipoxygenase, which alters transcription levels of the gene, also correlates with response to a derivative of the drug Zileuton, a 5-lipoxygenase inhibitor. Of the individuals who did not respond to Zileuton, 20% carried rare variant alleles at this locus. By contrast, all of the responders had wild-type alleles.

Similarly in a study of genetic polymorphisms of the Beta2-adrenergic receptor, Drysdale *et al.* (2000) demonstrated that a haplotype correlated strongly with asthma patients' response to Beta2-agonists. These two examples again demonstrate the possibility of using an individual's genotype to suggest a therapeutic strategy that is more likely to be efficacious. Certainly, before such tests are incorporated into clinical practice, additional genetic markers would have to be coupled with the existing polymorphisms to make the resulting tests more highly sensitive and specific.

In the field of oncology, in which the stakes are high, as failure to cure cancer usually leads to death, drugs commonly have a narrow therapeutic index and toxicities can be severe. In one of the first examples of applications of pharmacogenetics in oncology, Weinshilboum and Sladek identified polymorphic responses to the key antileukemic

drug, 6-mercaptopurine (6-MP) in 1980. Polymorphism of the gene thiopourine S-methyl transferase (TPMT) remains one of the best understood examples of pharmacogenetics variation (Weinshilboum RM, 1980).

TPMT is a cytosolic drug-metabolizing enzyme that catalyzes the S-methylation of 6-MP and azathioprine. In their original seminal study Weinshilboum and Sladek demonstrated a very clear tri-modal frequency of TPMT activity in red blood cells from 298 unrelated control adults (Weinshilboum RM, 1980). One in 300 subjects lacked TPMT activity, and 11% had intermediate levels. Family studies showed that the frequency distribution was due to inheritance. While phenotypic studies have shown a clear tri-modal distribution, the genetic basis of phenotypic variation has proved more complex. Seventeen variant TPMT alleles have been identified to date, although 3 variant alleles account for the majority (>95%) of persons with intermediate (1 variant allele) or low (2 variant alleles) TPMT activity (Krynetski EY *et al*, 1995) (Yates CR *et al*, 1997).

Subsequent clinical studies have demonstrated very clearly that TPMT polymorphism can predict toxicity of 6-MP and consequences of therapy. Children with acute lymphocytic leukaemia (ALL) with intermediate or absent TPMT activity are at higher risk of myelosuppression when prescribed standard doses of 6-MP (Relling MV et al, 1999a). In addition, patients with low TPMT activity are at increased risk of secondary cancers (Relling MV et al, 1998) (Relling MV et al, 1999b).

As the therapy of cancer is usually complex, involving many drugs, wide variability in outcome is commonly observed. There are many factors contributing to this, including the characteristics of the malignant cell, renal and hepatic function, diet, compliance with therapy and the therapeutic protocol employed.

As pharmacogenetics moves forward with ever more powerful genome-wide technologies, the field will benefit from a cautious approach in predicting future applications; "personalized medicine for all" is not on the immediate horizon, more a distant goal. A systematic approach to the dissection of important metabolic pathways using a combination of strategies and determining the applicability and clinical importance of the knowledge gained will move us toward this goal. Use of surrogate endpoints such as minimal residual disease or pharmacodynamic endpoints may provide earlier data than use of harder outcomes although subsequent validation would still be necessary.

ETHICAL, LEGAL AND SOCIETAL CONSIDERATIONS

There is the fear by the general public that genetic data, including pharmacogenetics data, will be misused by insurance companies and employers to discriminate against individuals 'branded' as being at greater risk of disease or treatment failure. This represents a major stumbling block for both genetic research and the use of genetic data in the clinical setting. Requirements for confidentiality and limitation of access to relevant data, even in the research setting, place major constraints on the speed of progress.

At present, insurance companies ask applicants about their family history, and therefore indirectly have access to an integrated predictor of heritable risk of disease. This practice is generally well accepted. Clearly there is a need for education, information and teaching about genetics, to put the risks in perspective and to better highlight the potential benefits of the increasing knowledge about pharmacogenetics. Rather than treat genetic information as a special type of medical information, it should be controlled by the same standards of confidentiality applicable to all types of medical data, updated and redefined if necessary. Ultimately, society will benefit most from pharmacogenetics by simultaneously protecting the privacy of medical information, and perhaps more importantly, the way it is used.

This may be best accomplished by enacting appropriate laws to regulate the use of genetics. International consensus when drafting those activities by lawmakers and ethicists would be of great benefit to all.

Since the introduction of 'evidence-based medicine', which is founded on observation, validated by experiments, the personal aspects of treatment started to become endangered. The concept of randomization and the double-blind procedure introduced into clinical trials in the 20th century may not be fully consistent with the individual aspects of treatment. The physician has to take into account the personal needs and values of the patient in order to win the patient's faith.

For the scientific approach it is pivotal to level-out all the unknown, non-accountable variations of individual responses to treatment. Evidence-based medicine is asking for a standardized application of therapy that has little room for idiosyncrasy. A patient would be interested to know whether he belongs to the group of patients that are expected to respond to a particular therapy or to the other group of unfortunates that do not respond or, even worse, whether he belongs to the group that will experience unfavourable side-effects. The challenge therefore is to provide individual care on a scientific basis.

While personalized medicine is on the agenda of many research groups and pharmaceutical companies, until the challenges are overcome, it will remain an elusive dream. Laboratory tests have to be designed for biomarkers that identify subgroups of patients with a particular disease that can be targeted by specific drugs. Drugs have to be specifically designed for such subgroups. Newly developed drugs have to be tested on various subgroups of patients. For rare variants of individual biomarker profiles it might be difficult to find enough patients for a trial, or it might be too expensive to design and evaluate a new drug for such subgroups. Groups of patients characterized by less-profitable genotypes are at risk of becoming therapeutic 'orphans'. Ethical problems concerning minorities that carry certain biomarkers or have a particular ethnic background may emerge and regulations may be necessary to maintain equity and protection of privacy.

In summary, while there remains optimism that pharmacogenomics will someday play a useful role in the optimization of treatment of hypertension, the evidence to date suggests

that the path from gene–drug outcome associations to knowledge applicable to everyday clinical practice will be neither short nor direct.

CONCLUSION

Drug response is complex, resulting not only from underlying genotypic variation at many loci, but also from variation at the level of gene expression, and post-translational modification of proteins. Non-genetic factors including, drug dose, drug interactions and diet are important too.

Hypertension is only partially genetically determined. It seems likely that variation in many different candidate genes, each making a small contribution, determine an individual's susceptibility to hypertension. The relatively small effect of an individual gene, coupled with the complexity of the phenotype make the search for genes that affect inter-individual BP variation a challenging task.

Despite its functional importance in the pathophysiology of hypertension, there have surprisingly been relatively a very limited number of studies on the beta2 adrenergic receptor gene. The Cochrane Database of Systematic Reviews was searched for any review of studies on possible associations between hypertension and the beta-2 adrenergic receptor gene and none was found. Against this background, this systematic review was undertaken to study the role of the beta2 adrenergic receptor and hypertension. The results showed that there was no association between hypertension and the beta2 adrenergic receptor allele 16.

A well-conducted clinical trial of appropriate design and size is generally regarded as the gold standard for testing a drug's efficacy. The current study is cross-sectional. Despite a number of limitations, notable findings are the high incidence of metoprolol-induced bronchospasm among our hypertensive patients and the genotyping which suggested that the presence of beta2 adrenergic receptor gene Arg16 variant was associated with reduced sensitivity to bronchoconstriction, while Gly16 increased it. In other words, in the absence of Arg16, the ability to prevent beta2 antagonist-induced bronchoconstriction appears to be reduced.

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APPENDIX

CASE RECORD FORM

(CRF)

GENERAL INSTRUCTIONS

- Please write legibly. Block printing is preferred.
- All entries must be made in black ink with a ballpoint pen.
- For missing information, please enter:

N/A	if data is not applicable.
UNK	if information is unknown or not available.
ND	if procedure was not done.

- Header Information : must be completed on each CRF form

Center no	This is a 1-digit number given to the site. This number should be the same on all CRF pages. (USM Kelantan 1; USM Penang 2; UMMC 3; HKL 4, Hospital Pulau Pinang 5, IMU 6, UIA 7)	
Project No	This is a 2-digit number given to the project:	
	Project heading	Project No
	Development of Rapid Assays for Genotyping	12
	A Study of Structure-function Relationship of Cytochrome P450	13
	Genetic Epidemiology of Drug Metabolising enzymes (DME)	14
	Value Added Therapeutic Drug Monitoring (TDM)	15
	Genetic polymorphism of CYP2D6 and beta-2 receptor	16
	Understanding Gene Defects in Drug Dependence	17
	Metoprolol-Ticlopidine Interaction	18
	Human acetyltransferase polymorphisms	19
Patient no	This is a unique 3-digit number given to every patient during visit 1.	
Patient's ID	This is a subject specific code that identifies the centre at which the subject is studied, the project under which he is studied and his personal subject number. Thus the first subject at HKL enrolled for "Value Added TDM" will be identified as 4-15-001 and the 25th subject enrolled at UMMC for "Genetic Epidemiology of DME" will be identified as 3-14-025.	

Patient initials	Enter first, middle, then last initial. If patient has no middle name, enter the first initial followed by a dash and then the last initial. The last initial is the family name. e.g. Annie Lim should be initialed as A-L Yin-Lin Seow should be initialed as YLS Ahmad Bin Ali should be initialed as ABA.
	Please remember to record the same patient initials in the CRF all pages.
Dates	All dates must be entered in the format DD/MM/YYYY e.g. 25/Sep/2002; If month is unknown, record as 'UNK'; If day is unknown, record as 01. Thus if day and month is unknown, record as 01/UNK/2002.
If you make an error	Draw a single line through the entry, reenter the data correctly, and initial and date the correction. Never use whiteout or obliterate information.
Confidentiality	All references to patients must be made by initials or Subject ID number and **NOT BY NAME.** If reports or hospital records are attached to the CRF, the patient name must be blocked over completely with a black marker and the patient number and initials entered.
Signatures / Initials on CRFs	All CRF pages must be reviewed and approved by the principal Investigator or sub-investigator.

VISIT 1 (KRK)

200 Hypertensive patients attending HUSM out patient clinic (KRK)

Screened for inclusion and exclusion

Exclusion criteria

2) Female sex.
3) Does not have a diagnosis that requires chronic treatment with metoprolol.
4) Is not on metoprolol at visit 1.
5) Requires treatment with drugs that may interfere with CYP2D6 activity (e.g. ticlopidine, fluoxetine, haloperidol, tricyclic antidepressants quinidine) during the study period.
6) Not able to understand study protocol and to follow simple instructions.
7) Not willing to sign written informed consent.
8) Know to suffer from the following diseases: - COAD, Br. Asthma, Chr.bronchitis

Excluded

Inclusion criteria

1) Male adults aged between 18-65 years.
2) Hypertensive.
3) On treatment with metoprolol for at least 3 months prior to enrollment.
4) Does not require treatment with drugs that may interfere with CXP2D6 (e.g. ticlopidine, fluoxetine, haloperidol, tricyclic antidepressants quinidine) during the study period.
5) Able to understand study protocol and to follow simple instructions.
6) Willing to sign written informed consent.
7) Not know to suffer from the following diseases; - COAD, Br. Asthma, Chr.Bronchitis.

Given an explanation of the study

Sign a written informed consent form

Check medical history and do baseline PE

VISIT 2 (CTU)

2) Administer questionnaires to investigate patients' respiratory status.
3) Perform Lung function test using a spirometer.

Blood for genotyping :-

Beta-2-receptor gene &

Blood for pharmacokinetic studies :-

½ hour pre-dose
½ hour post-dose
1 2 4 6 8 hours post-dose

HPLC for metoprolol and metabolites

DETAILED INSTRUCTIONS

VISIT 1

Patients attending out patient clinic (KRK).

Step 1 : Obtain Informed Consent
Before conduction any trial related procedures, please ensure proper completion of Patient's Informed Consent.

Step 2 : Check Inclusion Criteria
Tick the corresponding "YES" or "NO" box next to each inclusion criteria 1- 8.

Note : If answers to **any** of the **inclusion criteria** question are ticked **"NO"** the **patient should not be enrolled** into the trial.

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ ☐☐☐☐ Center No. Subject No. **Subject's Initials** ☐☐☐ **Date of Exam** ☐☐☐☐☐☐☐☐	**Visit 1**

INCLUSION CRITERIA	**YES / NO**
1) Male adults aged between 18-65 years.	☐ ☐
2) Hypertensive.	☐ ☐
3) On treatment with metoprolol for at least 3 months prior to enrolment.	☐ ☐
4) Does not require treatment with drugs that may interfere with CXP2D6 (e.g. ticlopidine, fluoxetine, haloperidol, tricyclic antidepressants quinidine) during the study period.	☐ ☐
5) Able to understand study protocol and to follow simple instructions.	☐ ☐
6) Willing to sign written informed consent.	☐ ☐
7) Not know to suffer from the following diseases; - 8) COAD. Br. Asthma. Chr.Bronchitis.	☐ ☐

Note: ***All inclusion criteria should be marked "YES" for eligible patients. If one or more inclusion criteria are marked "NO", the patient will be classified as a screen failure & will not be eligible for the trial.***

Investigator's Signature: _____ **Date :** ☐☐ ☐☐☐ ☐☐☐☐
day months year

VISIT 1

Step 3: Check Exclusion Criteria

Tick the corresponding "YES" or "NO" box next to each exclusion criteria 1 – 7. Please sign & date the page to verity the authenticity of the data.
(See opposite page)

Note: If answers to **any** of the **inclusion criteria** question are ticked **"NO"** the **patient should not be enrolled** into the trial.

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ ☐☐☐☐ (Center No. / Subject No.) **Subject's Initials** ☐☐☐ **Date of Exam** ☐☐☐☐☐☐☐☐	**Visit 1**

EXCLUSION CRITERIA	**YES / NO**
1) Female sex.	☐ ☐
2) Does not have a diagnosis that requires chronic treatment with metoprolol.	☐ ☐
3) Is not on metoprolol at visit 1.	☐ ☐
4) Requires treatment with drugs that may interfere with CYP2D6 activity (e.g. ticlopidine, fluoxetine, haloperidol, tricyclic antidepressants quinidine) during the study period.	☐ ☐
5) Not able to understand study protocol and to follow simple instructions.	☐ ☐
6) Not willing to sign written informed consent.	☐ ☐
7) Know to suffer from the following diseases ;- COAD, Br. Asthma, Chr. bronchitis	☐ ☐

Note : All inclusion criteria should be marked "YES" for eligible patients. If one or more inclusion criteria are marked "NO", the patient will be classified as a screen failure & will not be eligible for the trial.

Investigator's Signature: _____ **Date :** ☐☐ ☐☐☐ ☐☐☐☐
day months year

VISIT 1

Step 4: Check Demographic Data

- **Date of Birth:** Record the patient's date of birth. *(All dates must be entered in the format DD / MMM / YYYY e.g. 25 / Sep / 2002. If part of the date is unknown, please record as 01 / UNK / 2001).*

- **Sex:** Tick the box corresponding to the patient's sex. Tick only one box.

- **Ethnic Origin:** Tick the box corresponding to the patient's ethnic origin. (Chinese, Malay, Thai or Indian). Tick "other" & specify if patient belongs to other ethnic origin.

- **Weight:** Enter the weight of the patient. (one decimal point – e.g. 056.5kg, 100.5kg)

- **Height:** Enter the height to the nearest centimetres (no decimal – e.g. : 150cm).

Step 5: Check Vital Signs

- Please conduct vital signs examination after 5 minutes sitting & record the following details.

  - Systolic Blood Pressure (in mmHg) to the nearest 2mmHg
  - Diastolic Blood Pressure (in mmHg) to the nearest 2mmHg
  - Pulse (in Beats-Per-Minute)

Blood pressure must be measured in **both right and left upper limb** and **record only the higher reading.**
Repeat twice the measurement in the arm with the higher reading (allowing the patient to rest for 5 minutes in between the measurements) and **record the average of the 3 measurements.**

For vital signs, an observation is abnormal
if systolic BP $\geq$ 190 mmHg
and/or diastolic BP $\geq$ 105 mmHg

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ ☐☐☐☐ Center No. Subject No. **Subject's Initials** ☐☐☐ **Date of Exam** ☐☐☐☐☐☐☐☐☐	**Visit 1**

Demography

Date of Birth :

Day month year

Sex : ☐ Male

☐ Female

Ethnic Origin : ☐ Chinese ☐ Thai

☐ Malay ☐ Indian

☐ Other (Please Specify) _____

Parents :	**Father**		**Mother**	
Ethnic Origin	☐ Chinese	☐ Thai	☐ Chinese	☐ Thai
	☐ Malay	☐ Indian	☐ Malay	☐ Indian
	Other (Please Specify) _____		Other (Please Specify)	
Father-Grandparents	: ☐ Chinese	☐ Thai	☐ Chinese	☐ Thai
Ethnic Origin	☐ Malay	☐ Indian	☐ Malay	☐ Indian
	Other (Please Specify) _____		Other (Please Specify) _____	
Mother- Grandparents	: ☐ Chinese	☐ Thai	☐ Chinese	☐ Thai

Ethnic Origin ☐ Malay ☐ Indian ☐ Malay ☐ Indian

Other (Please Specify)

Vital Signs*

Systolic Blood Pressure : mmHg

Diastolic Blood Pressure : mmHg

Pulse : bpm

*Sitting : After 5 mins rest

Abnormal vital signs :

- if systolic BP > 190 mmHg
- and/or diastolic Bp > 105 mmHg

VISIT 1

Step 6: Review Relevant Medical History / Current Medical Conditions

- Please list all relevant medical history and current medical conditions.

- Indicate the date of onset & state whether it is still an active problem at start of study drug.

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ Center No. ☐☐☐☐ Subject No. **Subject's Initials** ☐☐☐	**Visit 1**

Relevant Medical History / Current Medical Conditions

Clinically significant finding must be recorded below.
Is patient currently suffering from or has he/she ever suffered in the past from any significant medical or surgical conditions?

☐ No ☐ Yes → If 'Yes', please list below one diagnosis per line.
Please give diagnosis rather than the symptoms.

	History / Diagnosis *(use precise medical terminology)*	**Date of onset**	**Is it still an active problem now?**	
			Yes	**No**
1.	_____	☐☐ dav ☐☐☐ month ☐☐☐☐ year	☐	☐
2.	_____	☐☐ dav ☐☐☐ month ☐☐☐☐ year	☐	☐
3.	_____	☐☐ dav ☐☐☐ month ☐☐☐☐ year	☐	☐
4.	_____	☐☐ dav ☐☐☐ month ☐☐☐☐ year	☐	☐
5.	_____	☐☐ dav ☐☐☐ month ☐☐☐☐ year	☐	☐
6.	_____	☐☐ dav ☐☐☐ month ☐☐☐☐ year	☐	☐
7.	_____	☐☐ dav ☐☐☐ month ☐☐☐☐ year	☐	☐
8.	_____	☐☐ dav ☐☐☐ month ☐☐☐☐ year	☐	☐

Note : If concomitant medications taken, please complete the Concomitants page.

VISIT 1

Step 7: Perform Physical Examination

► Please perform a physical examination on the following:

- General Appearance
- Skin
- Eyes
- Lung
- Heart
- Abdomen
- Extremities

Please comment on any abnormal physical examination findings.

Note:

- Information about the physical examination must be present in the source documentation at the study site.

Beta-2-Receptor and CYP2D6 Metoprolol study	ID ☐☐☐ Center No. ☐☐☐☐ Subject No. Subject's Initials ☐☐☐	Visit 1

Physical Examination

► Please perform a physical examination on the following:

Examination	Normal	Comments
General Appearance	☐ Yes ☐ No	
Skin	☐ Yes ☐ No	
Eyes	☐ Yes ☐ No	
Lungs	☐ Yes ☐ No	
Heart	☐ Yes ☐ No	
Abdomen	☐ Yes ☐ No	
Extremities	☐ Yes ☐ No	

Note: please comment on any abnormal physical examination findings.

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ Center No. ☐☐☐☐ Subject No. **Subject's Initials** ☐☐☐	**Visit 1**

Checklist Eligibility

Has the patient fulfilled the following criteria:

Informed Consent signed	☐ Yes	☐ No
Inclusion / Exclusion Criteria	☐ Yes	☐ No
Vital Signs	☐ Yes	☐ No
Physical Examination	☐ Yes	☐ No
	☐	☐

END OF VISIT 1

I have reviewed & found all data pertaining to Visit 1 to be complete & accurate:

Investigator's Signature: _____ **Date:** ☐☐ ☐☐☐ ☐☐☐☐

Day month year

VISIT 2

The patient will arrive at the Clinical Trial Unit (CTU) in the morning

Step 1: Check for Adverse Events / Concomitant Medications

► Please document all adverse events & concomitant medication information on the Adverse Event & Concomitant Medication page.

Step 2 : Perform Physical Examination & Vital Signs

► Please perform a physical examination on the following:
- General Appearance, Skin, Eyes, Lungs, Heart, Abdomen, Extremities

► Please conduct vital signs examination after **5 minutes sitting** & record the following details.
- Systolic Blood Pressure (in mmHg)
- Diastolic Blood Pressure (in mmHg)
- Pulse (in the Beats-Per-Minute)

Measure Blood pressure of **both upper limb** but **record only the higher reading. Repeat twice** the measurement in the arm with the higher reading (allowing the patient to rest for 5 minutes in between the measurements) and **record the average of the 3 measurements.**

For vital signs, an observation is abnormal
if systolic BP $\geq$ 190 mmHg
and/or diastolic BP $\geq$ 105 mmHg
and/or if there is irregular pulse rate, tachycardia (Rapid beating of heart. $\geq$ 130 Beats-Per-Minute) or bradycardia. ($\leq$ 40 Beats-Per-Minute)

Step 3: Re-check Inclusion / Exclusion criteria

► Please re-confirm patient's eligibility for study. Verify all Inclusion / Exclusion criteria during this visit.

► Patients who DO NOT meet the Inclusion / Exclusion criteria are NOT ELIGIBLE to be enrolled into the study.

Step 4: administer questionnaire

► Conduct the interview in a quiet room with only the investigator and the patient present.

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ Center No. ☐☐☐☐ Subject No. **Subject's Initials** ☐☐☐	**Visit 2**

SOAL SELIDIK TENTANG MASALAH PERNAFASAN

1. Pernahkah anda mengalami sesak nafas, atau mendengar bunyi seperti siulan dari dada pada bila – bila masa yang lalu?
   - YA ☐
   - TIDAK ☐

2. Pernahkah anda mengalami sesak nafas, atau mendengar bunyi seperti siulan dari dada dalam masa 12 bulan yang lepas?
   - YA ☐
   - TIDAK ☐

3. Pernahkah anda mengalami sesak nafas, atau mendengar bunyi seperti siulan dari dada sejak anda mengambil ubat metoprolol?
   - YA ☐
   - TIDAK ☐

4. Berapa kali anda mengalami sesak nafas dalam masa 12 bulan yang lepas atau selepas mengambil ubat metoprolol?
   - TIADA ☐
   - 1 HINGGA 3 KALI ☐
   - 4 HINGGA 12 KALI ☐
   - LEBIH DARIPADA 12 KALI ☐

5. Dalam masa 12 bulan yang lepas, berapa kali, secara purata, sesak nafas mengganggu tidur anda?
   - TIDAK PERNAH TERJAGA KERANA SESAK NAFAS ☐
   - 1 HINGGA 3 MALAM SETIAP BULAN ☐
   - 4 ATAU LEBIH MALAM SETIAP BULAN ☐

6. Dalam masa 12 bulan yang lepas, pernahkah anda mengalami sesak nafas yang begitu teruk hingga anda hanya dapat bercakap 1 atau 2 perkataan setiap tarikan nafas?
   - YA ☐
   - TIDAK ☐

7. Pernahkah anda menghidapi penyakit asma?
   - YA ☐
   - TIDAK ☐

8. Dalam masa 12 bulan yang lepas, pernahkah dada anda berbunyi seperti siulan semasa atau selepas senaman?
   - YA ☐
   - TIDAK

9. Dalam masa 12 bulan yang lepas, pernahkah anda batuk pada waktu malam, semasa anda **tidak mengalami** deman selsema atau jangkitan paru – paru?
   - YA ☐
   - TIDAK ☐

Step 5: perform lung function tests using Spirometer

- Demonstrate to the patient on how to perform the Lung function test using the Spirometer before starting.
- Ask patient to stand erect while performing the test.
- No tight clothing should be worn around the neck
- Use a nose-clip to ensure full expiration is done through the mouth and NOT through the nose
- Ensure expiration is done fully and forcefully without any leaks and with investigator giving encouragement at all times.
- Allow 2 minutes rest in between the 3 attempts.

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ Center No. ☐☐☐☐ Subject No. ☐☐☐ Subject Initials ☐☐☐☐ Randomisation Number Date of Exam : ☐☐ ☐☐☐ ☐☐☐☐	**Visit 2**

Lung Function Tests :-

Attempt 1

FVC	(l/s)
FEV_1	(l/s)
PEF	(l/s)

Attempt 2

FVC	(l/s)
FEV_1	(l/s)
PEF	(l/s)

Attempt 3

FVC	(l/s)
FEV_1	(l/s)
PEF	(l/s)

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ Center No. ☐☐☐☐ Subject No. ☐☐☐ Subject Initials ☐☐☐☐ Randomisation Number Date of Exam: ☐☐ ☐☐☐ ☐☐☐☐	**Visit 2**

Physical Examination

Please perform a physical examination. Clinically significant finding made which meet the definition of an adverse event must be recorded on the Adverse Event Section of the case Report Form

Is physical examination completed? ☐ Yes ☐ No

Vital Signs*

Weight : ☐☐☐ . ☐ kilograms

Systolic Blood Pressure : ☐☐☐ mmHg

Diastolic Blood Pressure : ☐☐☐ mmHg

Pulse : ☐☐☐ bpm

*Sitting : After 5 mins rest

Abnormal vital signs :
- if systolic BP > 190 mmHg
- and/or diastolic Bp > 105 mmHg
- and/or if there is irregular pulse rate, tachy- or bradycardia

Inclusion / Exclusion Criteria

Dose the patients meet all Inclusion / Exclusion criteria? ☐ Yes ☐ No

** Note : patient's who DO NOT meet Inclusion / Exclusion criteria are NOT ELIGIBLE for the study.*

Was **questionnaire** administered to the patient Yes ☐ No ☐

Was **Lung Function Tests** using Spirometer performed on the patient?
Yes ☐ No ☐

Adverse Events / Concomitant Medications

Did an Adverse Event occur? Were Concomitant Medications taken? ☐ Yes ☐ No

If "Yes", please document Adverse Events & Concomitant Medication Page

VISIT 2

End of Visit Checklist

- ► Please complete the checklist to indicate the completion of Visit 2.
- ► Please sign & date to verify the data for Visit 2

Beta-2-Receptor and CYP2D6 Metoprolol study	**ID** ☐☐☐ Center No. ☐☐☐☐ Subject No. ☐☐☐ Subject Initials ☐☐☐☐ Randomisation Number	**Visit 2**

End of Visit Checklist

Has the patient fulfilled the following criteria:

Inclusion / Exclusion Criteria	☐Yes	☐No
Vital Signs	☐Yes	☐No
Physical Examination	☐Yes	☐No
	☐	☐

Were the following procedures conducted:

Was questionnaire administered to the patient	☐Yes	☐No
Was lung function tests performed on the patient?	☐Yes	☐No
Was blood for DNA analysis taken from the patient?	☐Yes	☐No
Was blood for pharmacokinetic studies taken from the patient?	☐Yes	☐No
Was blood for baseline investigations taken from the patient?	☐Yes	☐No

I have reviewed & found all data pertaining to visit 2 to be complete & accurate:

Investigator's Signature : _____ Date : ☐☐ dav ☐☐☐ mont ☐☐☐☐ vear

END OF VISIT 2.

Beta-2-Receptor and CYP2D6 Metoprolol study	ID ☐☐☐ Center No. ☐☐☐☐ Subject No. Subject's Initials ☐☐☐	Lab. Result (Baseline-investigations)

Laboratory Evaluation

Name of local laboratory: _____

Date samples taken: ☐☐ ☐☐☐ ☐☐☐☐ (day month year) Time sample taken: ☐☐ ☐☐ Hrs

	Value	Unit	Comment if clinically notable
Haematology			
RBC Total			
Haemoglobin			
Haematocrit			
WBC Total			
Platelets			
Biochemistry			
Albumin			
Creatinine			
Sodium			
Potassium			
Total Protein			
Alkaline Phosphatase			
SGPT (ALT)			
SGOT (AST)			
Lactate Dehydrogenase			

Beta-2-Receptor and CYP2D6 Metoprolol study	ID ☐☐☐ Center No. ☐☐☐ Subject No. Subject's Initials ☐☐☐	Lab Result (Pharmacokinetics)

Pre / Post Dose (*hours*)	Pre / Post Dose (*time*)	Actual timing (*am/pm*)	A / RATIO	Blood Pressure (*mmHg*)	Comments
0					
0.5					
1					
2					
4					
6					
8					

Beta-2-Receptor and CYP2D6 Metoprolol study	ID ☐☐☐ Center No. ☐☐☐ Subject No. Subject's Initials ☐☐☐	Lab Result (genotyping)

* Please **tick** (✓) in the appropriate column.

Gene / Alleles	Mutant	Wild-type	Genotype
Beta-2-receptor gene			
Allele 16			
Allele 20			
Allele 27			
Allele 47			
Allele 164			
CYP2D6 gene			
* 3			
* 4			
* 6			
* 9			
* 10			
* 14			
* 17			

APPENDIX 2

CYP2D6 GENOTYPING RESULTS OF ALL THE PATIENTS AND THEIR PHENOTYPE

R/N	AGE	ETHNIC	GENDER	GENOTYPE	ALLELES		PHENOTYPE
M001	54	Malay	male	*1/*10	1	1	EM
M002	61	Malay	male	*1/10	1	1	EM
M003	64	Chinese	male	*1/10	1	1	EM
M004	62	Malay	male	*1/1	1	1	EM
M005	47	Malay	male	*10/10	10	10	IM
M006	65	Chinese	male	*1/3	1	3	IM
M007	50	Malay	male	*1/*1	10	10	IM
M008	64	Others	male	*1/*1	1	1	EM
M009	54	Chinese	male	*1/10	1	10	EM
M010	43	Others	male	*10/*10	10	10	IM
M011	55	Malay	male	*1/*10	1	10	EM
M012	60	Malay	male	*1/1	1	1	EM
M013	65	Malay	male	*1/10	1	10	EM
M014	65	Malay	male	*10/10	10	10	IM
M015	38	Indian	male	*1/*1	1	1	
M016	55	Malay	male	*1/10	1	10	EM
M017	65	Malay	male	*1/*1	1	1	EM
M018	43	Others	male	*1/*1	1	1	EM
M019	55	Chinese	male	*1/10	1	10	EM
M020	50	Others	male	*1/1	1	1	EM
M021	64	Malay	male	*1/1	1	1	EM
M022	49	Malay	male	*1/*10	1	10	EM
M023	65	Malay	male	*1/*1	1	1	EM
M024	56	Malay	male	*6/10	6	10	IM
M025	63	Chinese	male	*1/10	1	10	EM
M026	63	Others	male	*1/1	1	1	EM
M027	51	Malay	male	*10/10	10	10	IM
M028	58	Others	male	*1/*1	1	1	EM
M029	45	Malay	male	*1/*1	1	1	EM
M030	65	Malay	male	*1/6	1	6	IM
M031	65	Malay	male	*1/*1	1	1	EM
M032	64	Malay	male	*1/6	1	6	IM
M033	59	Malay	male	*1/*10	1	10	EM
M034	55	Chinese	male	*10/10	10	10	IM
M035	63	Malay	male	*1/10	1	10	EM
M036	64	Malay	male	*4/*10	4	10	IM
M037	59	Malay	male	*10/10	10	10	IM
M038	60	Malay	male	*1/*10	1	10	EM
M039	65	Malay	male	*1/10	1	10	EM
M040	47	Malay	male	*1/*10	1	10	EM
M041	58	Malay	male	*10/*10	10	10	IM
M042	61	Others	male	*10/*10	10	10	IM
M043	48	Malay	male	*1/*10	1	10	EM
M044	49	Others	male	*6/*10	6	10	IM

R/N	AGE	ETHNIC	GENDER	GENOTYPE	ALLELES		PHENOTYPE
M045	61	Malay	male	*10/*10	10	10	IM
M046	57	Malay	male	*1/*1	1	1	EM
M047	55	Malay	male	*1/10	1	10	EM
M048	61	Chinese	male	*10/*10	10	10	IM
M049	56	Malay	male	*1/*1	1	1	EM
M050	51	Malay	male	*1/1	1	1	EM
M051	54	Malay	male	*1/*1	1	1	EM
M052	54	Malay	male	*1/10	1	10	EM
M053	55	Malay	male	*4/10	4	10	IM
M054	55	Malay	male	*1/10	1	10	EM
M055	53	Malay	male	*1/*10	1	10	EM
M056	65	Chinese	male	*10/*10	10	10	IM
M057	59	Malay	male	*1/1	1	1	EM
M058	48	Malay	male	*1/1	1	1	EM
M059	51	Others	male	*1/1	1	1	EM
M060	69	Malay	male	*1/*1	1	1	EM
M061	59	Chinese	male	*10/10	10	10	IM
M062	65	Malay	male	*1/10	1	10	EM
M063	56	Chinese	male	*1/*10	1	10	EM
M064	57	Malay	male	*1/*10	1	10	EM
M065	51	Malay	male	*1/*10	1	10	EM
M066	65	Malay	male	*10/*10	10	10	IM
M067	65	Malay	male	*1/10	1	10	EM
M068	65	Malay	male	*1/10	1	10	EM
M069	52	Malay	male	*1/1	1	1	EM
M070	65	Malay	male	*4/10	4	10	IM
M071	42	Malay	male	*10/10	10	10	IM
M072	49	Others	male	*1/10	1	10	EM
M073	62	Malay	male	*1/10	1	10	EM
M074	55	Malay	male	*6/10	6	10	IM
M075	47	Malay	male	*1/10	1	10	EM
M076	62	Malay	male	*1/10	1	10	EM
M077	65	Others	male	*1/1	1	1	EM
M078	45	Malay	male	*1/*10	1	10	EM
M079	42	Malay	male	*1/*1	1	1	EM
M080	60	Malay	male	*4/10	4	10	IM
M081	49	Others	male	*1/10	1	10	EM
M082	55	Malay	male	*1/*10	1	10	EM
M083	57	Malay	male	*1/*1	1	1	EM
M084	47	Malay	male	*10/10	10	10	IM
M085	61	Malay	male	*10/*10	10	10	IM
M086	44	Others	male	*1/10	1	10	EM
M087	62	Malay	male	*1/*1	1	1	EM
M088	65	Malay	male	*1/*1	1	1	EM

R/N	AGE	ETHNIC	GENDER	GENOTYPE	ALLELES		PHENOTYPE
M089	49	Malay	male	*1/1	1	1	EM
M090	65	Malay	male	*1/10	1	10	EM
M091	65	Malay	male	*6/*10	6	10	IM
M092	52	Malay	male	*1/1	1	1	EM
M093	55	Others	male	*1/1	1	1	EM
M094	53	Malay	male	*1/1	1	1	EM
M095	53	Malay	male	*10/10	10	10	IM
M096	63	Malay	male	*10/10	10	10	IM
M097	65	Others	male	*1/10	1	10	EM
M098	55	Malay	male	*1/1	1	1	EM
M099	57	Malay	male	*10/10	10	10	IM
M100	65	Malay	male	*1/10	1	10	EM
M101	61	Others	male	*4/10	4	10	IM
M102	38	Malay	male	*1/1	1	1	EM
M103	65	Malay	male	*1/1	1	1	EM
M104	59	Malay	male	*4/10	4	10	IM
M105	46	Malay	male	*1/1	1	1	EM
M106	60	Malay	male	*1/*1	1	1	EM
M107	59	Malay	male	*6/10	6	10	IM
M108	49	Malay	male	*1/*10	1	10	EM
M109	52	Malay	male	*1/10	1	10	EM
M110	64	Malay	male	*1/10	1	10	EM
M111	61	Malay	male	*1/1	1	1	EM
M112	53	Malay	male	*10/10	10	10	IM
M113	56	Others	male	*1/*1	1	1	EM
M114	60	Malay	male	*1/1	1	1	EM
M115	51	Malay	male	*1/1	1	1	EM
M116	65	Others	male	*1/10	1	10	EM
M117	62	Malay	male	*10/10	10	10	IM
M118	59	Malay	male	*1/1	1	1	EM
M119	49	Malay	male	*4/10	4	10	IM
M120	65	Malay	male	*1/1	1	1	EM
M121	64	Malay	male	*1/1	1	1	EM
M122	62	Malay	male	*1/*1	1	1	EM
M123	64	Others	male	*1/*10	1	10	EM
M124	58	Malay	male	*10/10	10	10	IM
M125	65	Malay	male	*1/10	1	10	EM
M126	46	Malay	male	*10/10	10	10	IM
M127	48	Malay	male	*6/*10	6	10	IM
M128	49	Malay	male	*1/1	1	1	EM
M129	65	Malay	male	*1/10	1	10	EM

M130	51	Malay	male	*10/10	10	10	IM
M131	65	Malay	male	*10/10	10	10	IM
M132	59	Malay	male	*10/10	10	10	IM

R/N	AGE	ETHNIC	GENDER	GENOTYPE	ALLELES		PHENOTYPE
M133	49	Malay	male	*6/10	6	10	IM
M134	63	Malay	male	*1/1	1	1	EM
M135	57	Malay	male	*1/1	1	1	EM
M136	51	Malay	male	*1/1	1	1	EM
M137	65	Malay	male	*10/10	10	10	IM
M138	59	Malay	male	*1/1	1	1	EM
M139	49	Malay	male	*1/10	1	10	EM
M140	54	Others	male	*1/10	1	10	EM
M141	55	Malay	male	*1/10	1	10	EM
M142	57	Malay	male	*1/1	1	1	EM
M143	65	Malay	male	*1/10	1	10	EM
M144	65	Chinese	male	*1/1	1	1	EM
M145	55	Chinese	male	*1/1	1	1	EM
M146	58	Chinese	male	*1/*1	1	1	EM
M147	52	Others	male	*1/*1	1	1	EM
M148	65	Malay	male	*4/10	4	10	IM
M149	65	Malay	male	*1/1	1	1	EM
M150	62	Malay	male	*1/*1	1	1	EM
M151	54	Malay	male	*1/*1	1	1	EM
M152	60	Malay	male	*1/*10	1	10	EM
M153	58	Malay	male	*1/*10	1	10	EM
M154	65	Malay	male	*10/10	10	10	IM
M155	54	Malay	male	*1/1	1	1	EM
M156	64	Malay	male	*10/10	10	10	IM
M157	57	Malay	male	*1/10	1	10	EM
M158	64	Malay	male	*1/1	1	1	EM
M159	65	Others	male	*10/*10	10	10	IM
M160	51	Malay	male	*10/*10	10	10	IM
M161	47	Others	male	*1/*10	1	10	EM
M162	38	Malay	male	*1/*10	1	10	EM
M163	54	Malay	male	*1/*1	1	1	EM
M164	44	Malay	male	*1/*10	1	10	EM
M165	50	Malay	male	*1/*1	1	1	EM
M166	38	Malay	male	*1/*10	1	10	EM
M167	59	Malay	male	*10/*10	10	10	IM

M168	63	Malay	male	*10/*10	10	10	IM
M169	61	Malay	male	*1/*1	1	1	EM
M170	65	Malay	male	NA	NA	NA	NA
M171	50	Malay	male	*1/*1	1	1	EM
M172	54	Malay	male	*1/*1	1	1	EM
M173	61	Malay	male	*1/*1	1	1	EM
M174	55	Malay	male	*1/*10	1	10	EM
M175	65	Chinese	male	*1/*10	1	10	EM
M176	46	Others	male	*1/*1	1	1	EM

R/N	AGE	ETHNIC	GENDER	GENOTYPE	ALLELES		PHENOTYPE
M177	42	Malay	male	*1/*1	1	1	EM
M178	57	Malay	male	*1/*1	1	1	EM
M179	43	Malay	male	*1/*10	1	10	EM
M180	65	Malay	male	*1*1	1	1	EM
M181	65	Malay	male	*1/*110	1	10	EM
M182	60	Malay	male	*1/*10	1	10	EM
M183	58	Malay	male	*1/10	1	10	EM
M184	54	Malay	male	*1/10	1	10	EM
M185	54	Malay	male	NA	NA	NA	NA
M186	55	Indian	male	*1/*1	1	1	EM
M187	64	Malay	male	*1/*1	1	1	EM
M188	65	Malay	male	*1/10	1	10	EM
M189	65	Chinese	male	*1/*10	1	10	EM
M190	65	Malay	male	*1/10	1	10	EM
M191	54	Indian	male	*10/*10	10	10	IM
M192	55	Malay	male	*1/*1	1	1	EM
M193	61	Malay	male	*10/10	10	10	IM
M194	49	Chinese	male	*1/1	1	1	EM
M195	57	Malay	male	*1/*10	1	10	EM
M196	50	Malay	male	*1/*1	1	1	EM
M197	65	Malay	male	*1/*10	1	10	EM
M198	64	Others	male	*1/*10	1	10	EM
M199	60	Malay	male	NA	NA	NA	NA
M200	57	Malay	male	*1/*1	1	1	EM
M201	49	Malay	male	*1/*10	1	10	EM
M202	56	Chinese	male	*1/*1	1	1	EM
M203	65	Malay	male	*1/*1	1	1	EM

EM = Extensive Metabolizer
IM = Intermediate Metabolizer
NA = Not available (genotyping not done)

Appendix 3

HAPLOTYPE ANALYSIS OF THE BETA 2 AR GENE

ethnic	_ID_	Allele 47_1	Allele 47_2	Allele 20_1	Allele 20_2	Allele 16_1	Allele 16_2	Allele 27_1	Allele 27_2	HAPLOTYPE1	HAPLOTYPE2	PROB
Ch	1	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Ch	2	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Ch	3	T	T	C	C	G	G	C	C	T-C-G-C	T-C-G-C	1
Ch	4	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Ch	5	T	T	T	T	A	G	C	G	T-T-A-C	T-T-G-G	0.998701
Ch	5	T	T	T	T	A	G	C	G	T-T-A-G	T-T-G-C	0.001299
Ch	6	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	1
Ch	7	C	T	C	C	G	G	C	G	C-C-G-G	T-C-G-C	1
Ch	8	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Ch	9	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Ch	10	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Ch	11	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.14668
Ch	11	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.85332
Ch	12	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Ch	13	T	T	C	T	A	G	C	G	T-C-A-C	T-T-G-G	0.077759
Ch	13	T	T	C	T	A	G	C	G	T-C-A-G	T-T-G-C	0.903249
Ch	13	T	T	C	T	A	G	C	G	T-C-G-C	T-T-A-G	0.000588
Ch	13	T	T	C	T	A	G	C	G	T-C-G-G	T-T-A-C	0.018404
Ch	14	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	1
Ch	15	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Ch	16	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Ch	17	T	T	C	C	A	A	C	G	T-C-A-C	T-C-A-G	1
Mal	18	T	T	C	C	A	A	C	C	T-C-A-C	T-C-A-C	1
Mal	19	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	20	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	20	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	21	T	T	C	T	A	G	C	G	T-C-A-C	T-T-G-G	0.037993
Mal	21	T	T	C	T	A	G	C	G	T-C-A-G	T-T-G-C	0.102402
Mal	21	T	T	C	T	A	G	C	G	T-C-G-C	T-T-A-G	1.63E-05

Mal	21	T	T	C	T	A	G	C	G	T-C-G-G	T-T-A-C	0.859589
Mal	22	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	23	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	24	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	25	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	26	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	27	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	28	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	29	T	T	C	C	G	G	C	G	T-C-G-C	T-C-G-G	1
Mal	30	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	31	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	32	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	32	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	33	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	34	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	35	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	35	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	36	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	37	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	38	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	38	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	39	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	40	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	41	T	T	C	C	A	G	C	G	T-C-A-C	T-C-G-G	0.650169
Mal	41	T	T	C	C	A	G	C	G	T-C-A-G	T-C-G-C	0.349831
Mal	42	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	43	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	44	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	45	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	46	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	47	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	48	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	0.992719
Mal	48	C	T	C	T	A	G	C	G	C-T-G-G	T-C-A-C	0.007272

Mal	49	T	T	C	C	G	G	C	C	T-C-G-C	T-C-G-C	1
Mal	50	T	T	C	C	G	G	C	C	T-C-G-C	T-C-G-C	1
Mal	51	C	T	T	T	A	G	C	G	C-T-A-G	T-T-G-C	0.000258
Mal	51	C	T	T	T	A	G	C	G	C-T-G-G	T-T-A-C	0.999742
Mal	52	T	T	T	T	G	G	C	C	T-T-G-C	T-T-G-C	1
Mal	53	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	54	C	T	C	T	A	G	C	C	C-C-A-C	T-T-G-C	1E-05
Mal	54	C	T	C	T	A	G	C	C	C-C-G-C	T-T-A-C	0.99999
Mal	55	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	56	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	57	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	58	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	59	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	60	T	T	T	T	G	G	C	C	T-T-G-C	T-T-G-C	1
Mal	61	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	62	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	63	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	64	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	65	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	66	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	67	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	68	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	69	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	70	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	71	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	72	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	73	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	74	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	75	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	76	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	77	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	77	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	78	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127

Mal	78	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	79	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	80	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	80	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	81	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	82	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	83	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	84	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	85	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	86	T	T	C	T	G	G	C	C	T-C-G-C	T-T-G-C	1
Mal	87	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	88	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	89	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	90	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	91	T	T	C	C	G	G	C	C	T-C-G-C	T-C-G-C	1
Mal	92	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	93	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	94	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	95	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	96	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	97	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	98	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	99	T	T	T	T	G	G	C	C	T-T-G-C	T-T-G-C	1
Mal	100	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	101	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	102	T	T	T	T	A	G	C	G	T-T-A-C	T-T-G-G	0.999905
Mal	102	T	T	T	T	A	G	C	G	T-T-A-G	T-T-G-C	9.48E-05
Mal	103	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	104	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	105	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	106	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	107	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	108	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1

Mal	109	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	110	T	T	C	C	A	G	C	G	T-C-A-C	T-C-G-G	0.650169
Mal	110	T	T	C	C	A	G	C	G	T-C-A-G	T-C-G-C	0.349831
Mal	111	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	112	C	C	C	C	G	G	G	G	C-C-G-G	C-C-G-G	1
Mal	113	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	114	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	0.992719
Mal	114	C	T	C	T	A	G	C	G	C-T-G-G	T-C-A-C	0.007272
Mal	115	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	116	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	117	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	117	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	118	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	0.992719
Mal	118	C	T	C	T	A	G	C	G	C-T-G-G	T-C-A-C	0.007272
Mal	119	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	120	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	121	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	0.992719
Mal	121	C	T	C	T	A	G	C	G	C-T-G-G	T-C-A-C	0.007272
Mal	122	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	123	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	123	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	124	T	T	C	C	G	G	C	C	T-C-G-C	T-C-G-C	1
Mal	125	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	125	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	126	T	T	C	C	G	G	C	G	T-C-G-C	T-C-G-G	1
Mal	127	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	128	T	T	T	T	G	G	C	C	T-T-G-C	T-T-G-C	1
Mal	129	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	130	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	131	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.18127
Mal	131	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.81873
Mal	132	C	T	C	C	G	G	C	G	C-C-G-C	T-C-G-G	0.009146
Mal	132	C	T	C	C	G	G	C	G	C-C-G-G	T-C-G-C	0.990854

Mal	133	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	134	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	135	T	T	C	C	A	G	C	G	T-C-A-C	T-C-G-G	0.650169
Mal	135	T	T	C	C	A	G	C	G	T-C-A-G	T-C-G-C	0.349831
Mal	136	C	T	C	C	G	G	C	G	C-C-G-C	T-C-G-G	0.009146
Mal	136	C	T	C	C	G	G	C	G	C-C-G-G	T-C-G-C	0.990854
Mal	137	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	138	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	139	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	140	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	141	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	142	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	143	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	144	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	145	T	T	C	C	G	G	C	C	T-C-G-C	T-C-G-C	1
Mal	146	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	147	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	0.992719
Mal	147	C	T	C	T	A	G	C	G	C-T-G-G	T-C-A-C	0.007272
Mal	148	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	149	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	150	C	T	C	C	G	G	C	G	C-C-G-C	T-C-G-G	0.009146
Mal	150	C	T	C	C	G	G	C	G	C-C-G-G	T-C-G-C	0.990854
Mal	151	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	152	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	153	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	154	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	155	C	C	C	C	G	G	G	G	C-C-G-G	C-C-G-G	1
Mal	156	C	C	C	C	G	G	G	G	C-C-G-G	C-C-G-G	1
Mal	157	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	158	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	159	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	160	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	161	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1

Mal	162	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	163	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	164	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	165	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Mal	166	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Mal	167	T	T	T	T	A	G	C	G	T-T-A-C	T-T-G-G	0.999905
Mal	167	T	T	T	T	A	G	C	G	T-T-A-G	T-T-G-C	9.48E-05
Mal	168	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Mal	169	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	0.992719
Mal	169	C	T	C	T	A	G	C	G	C-T-G-G	T-C-A-C	0.007272
Mal	170	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Other	171	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Other	172	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	173	C	T	C	C	G	G	C	G	C-C-G-G	T-C-G-C	1
Other	174	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	175	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	176	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Other	177	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	178	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Other	179	C	T	C	C	G	G	C	G	C-C-G-G	T-C-G-C	1
Other	180	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	181	C	T	C	T	A	G	C	G	C-C-G-G	T-T-A-C	1
Other	182	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Other	183	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	184	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	185	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	186	T	T	C	C	A	G	C	G	T-C-A-C	T-C-G-G	0.000145
Other	186	T	T	C	C	A	G	C	G	T-C-A-G	T-C-G-C	0.999855
Other	187	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	188	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	189	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Other	190	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	191	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1

Other	192	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Other	193	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	194	T	T	T	T	G	G	C	G	T-T-G-C	T-T-G-G	1
Other	195	T	T	T	T	A	G	C	C	T-T-A-C	T-T-G-C	1
Other	196	T	T	T	T	A	A	C	C	T-T-A-C	T-T-A-C	1
Other	197	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.260887
Other	197	T	T	C	T	A	G	C	C	T-C-G-C	T-T-A-C	0.739113
Other	198	T	T	T	T	G	G	C	C	T-T-G-C	T-T-G-C	1
Other	199	T	T	C	C	A	G	C	C	T-C-A-C	T-C-G-C	1
Other	200	T	T	C	T	A	G	C	C	T-C-A-C	T-T-G-C	0.260887