Prevalence of hypertension varies from country to country, population to population and rural to urban areas. Based on this, a systematic study was established to identify the genetic basis of essential hypertension in rural population of Haryana.

3.1 Population under investigation

People of India do not constitute a single homogenous community. The country of more than one billion people is the most diverse place in the world and constitutes about 4655 identifiable communities diverse in biological traits, languages, physical appearance, dress patterns, forms of worship, occupation, food habits and kinship patterns. A high prevalence of hypertension in both rural and urban areas of India has been reported (Hazarika et al., 2004). Besides, a marked difference in prevalence has been observed among inhabitants of rural and urban area. Although a few studies are available in rural population of Haryana but these are populations, inhabiting very close to cities like Delhi or Chandigarh. The population selected in the present study resides in Mullana, a sub divisional rural area of Ambala district (Haryana), and 21 villages of this rural area (Figure 2)

![Map of interior rural region of Ambala district (Haryana)](image)

Figure 2: Map of interior rural region of Ambala district (Haryana)
3.2 Recruitment of Study Subjects

Mullana, a small sub-divisional rural area under district Ambala in the state of Haryana had been the place of survey. The place has tropical climate. The population is stable and usually consumes high fat diet, alcohol, salt and saturated fat which are a main cooking medium. The study was carried out in M. M. Institute of Medical Sciences and Research, Mullana, which is the only source of quality health care in the rural region near Ambala. The patients come for treatment from adjoining 21 villages roughly covering a radius of 30 km. According to a cross sectional survey conducted by M M Institute of Medical Science & Research, Mullana (Haryana) there are around 35,000 inhabitants residing in 21 villages of this rural area (data validated also by the latest Census report of India). The survey recorded a total of 1890 individuals afflicted with essential hypertension and these patients will be referred to as hypertensive (HT) in the text of this thesis. The patients with any form of secondary hypertension were spared. Patients who agree to participate were explained the nature and the objectives of the study, and informed consent was obtained individually. The study was approved by the ethical review committee (IEC/22) of M M University. The information about patient’s identity was not included with other data and only consulting physician had the access to this information.

3.3 Design of study

As per the DBT guidelines (http://dbtindia.nic.in/Pharmacogenomics%20-%20GUIDELINES.doc) to conduct pharmacogenomic study, the following principles were considered:

1. National relevance of study, which is dictated by:
   a) prevalence of disease in study population
   b) Drugs used for the treatment of the disease
   c) Patient’s variations in response to drugs.

A hospital (M M Institute of Medical Science & Research, Mullana, Haryana) based retrospective study was conducted during 2003-2006 to evaluate the prevalence of essential hypertension followed by a cross sectional survey in the 21 villages of this
rural area. Information regarding drug prescription was collected through prescription audit and a case-control prospective study was conducted to identify fresh hypertensive patients for the pharmacogenomic study to understand the molecular basis of the variation in response to prescribed anti-hypertensive drugs. This study was undertaken by targeting three candidate genes polymorphisms implicated in the etiology of hypertension.

1) **Hospital based Prevalence study**

a) A hospital based study was conducted retrospectively at M. M. Institute of Medical Sciences and Research, Mullana, to find out the prevalence rate of HT among other diseases during June 2003 to September 2006 period. A total of 2295 patient, (1446 males and 849 females) visited the OPD of department of Medicine. The patient population were further divided into two major sub-groups on the basis of their underlying etiology:

   i) Individuals with only essential hypertension (n =876).

   ii) Other diseases (n = 1419), individuals with other acute and chronic diseases.

b) For prospective case-control study, the demographic details with disease history of hypertensive patients were investigated, during December 2006 to June 2007, to identify various risk factors involved in it. Sample size was calculated employing an online site ([http://www.pivotalresearch.ca/resources-sample-calc.php](http://www.pivotalresearch.ca/resources-sample-calc.php)).

2. **Pharmacoepidemiology study**: Prescription audit was conducted during June 2005-September 2006 and January 2010 to July 2011 to assess the prescribing pattern of antihypertensive therapy and compared them with JNC-7 treatment guidelines.
3. **Pharmacogenomics study:**

a) Candidate gene polymorphisms: A case-control study was conducted on 106 patients /110 controls. The sample size calculation was based on the survey that in a population of 35,000 (inhabitants residing in 21 villages of the rural area in Mullana, Haryana) with a disease prevalence of 5.4%. A sample of 79 patients will be sufficient to represent the hypertensive population residing in the rural area under investigation.

b) Response of the patients to antihypertensive therapy: A total of 281 HT patients were randomly selected and their response to full dosages of the drugs (Ramipril 5mg/day), Hydrochlorothiazide (25 mg/day) and Metoprolol (50 mg/day) after 4 weeks of treatment was monitored. All the participants were informed about the aims and objectives of this study. Patients were sub grouped based on physician prescription:

- ACE inhibitor (Ramipril 5mg) (Monotherapy), n=106
- Diuretics (Hydrochlorothiazide 25mg) (Monotherapy), n=32
- β1 blockers (Metoprolol 50mg) (Monotherapy), n=95
- ACE inhibitor (Ramipril 2.5mg & 5mg) with Diuretics (Hydrochlorothiazide 12.5 mg) or β1 blockers (Metoprolol 25mg) (Combination therapy), n=48

a) Blood samples were collected to study the candidate gene polymorphisms *ACE I/D*, *ADD1 Gly460Trp* and *β-1 ADR Ser49Gly* polymorphisms

b) To find out correlation, if any, between the genetic polymorphisms in the candidate genes and the response of the patients to antihypertensive therapy. Additionally, comparisons were made to study variation, if any, in the distribution of various genotypes between the patients and controls.
3.4 Essential hypertension Questionnaire

A questionnaire was prepared for the sample collection which included the details of patients: Sociodemographic profile (age, sex, education, occupation, family history of essential hypertension), anthropometric variables (body weight, height, waist circumference, hip circumference, abdominal obesity, waist hip ratio and body mass index) and other information like smoking, alcohol consumption as well as details of drug treatment prescribed (name, dosage form, frequency and duration of administration) were recorded.

HT was defined as systolic blood pressure >140 mmHg and diastolic blood pressure >90 mmHg. Anyone smoking at least one cigarette per day for the minimum past six months was considered as smoker, and others were classified as non-smokers. For Alcohol intake, subjects were categorized as abstainer who never consumed alcohol, and the subject who consumed alcohol were categorized as alcoholic. All subjects who have passed 10th class were categorized as educated. Individuals performing sitting jobs in offices like clerical, peon, laboratory attendant and shopkeepers were considered sedentary.

3.5 Blood pressure measurement (Chobanian et al., 2003)

The blood pressure recordings were taken by the physicians in OPD. Patients were made to sit quietly for at least 5 minutes in a chair. Intake of caffeine, exercise, and smoking were avoided for at least 30 minutes prior to the measurement. Two readings were taken at 5 min interval, and average was recorded. If there was gross variation in them a third reading was obtained and used for the diagnosis of hypertension. Blood Pressure was measured in both arms and in the event of any difference, the higher reading was taken. An appropriately sized cuff (cuff bladder encircling at least 80 percent of the arm) was used to ensure accuracy in palpatory obliteration of radial pulse pressure was obtained before auscultating for BP measurement. By auscultatory method appearance and disappearance of Korotkoff sound were taken as indicator of systolic and diastolic BP, respectively.
3.6 Anthropometric assessment

Body weight was measured (to the nearest of 0.5kg) with the participant in standing position on weighing scale, feet about 15 cm apart and equally distributing weight on both limbs with minimum clothes and no foot wear. Height was measured (to the nearest 0.5cm) using anthropometric rod with the subject in standing position and with the head positioned so that the top of the external auditory meatus was level with the inferior margin of the bony orbit. The waist circumference (WC) was measured in a horizontal plane, midway between the inferior margin of the ribs and the superior border of the iliac crest and the suggested waist circumference cutoffs are 90 cm for men and 80 cm for women (Wildman et al., 2004). The hip circumference (HC) was measured at the fullest point around the buttocks with a metallic tape. WC (cm) was divided by HC (cm) to calculate waist to hip ratio (WHR) suggested cutoffs 0.88 for men and 0.80 for women. Body mass index (BMI) (kg/m$^2$) was calculated by dividing weight (in kilograms) by the square of height (in meters), as a measure of total adiposity. BMI values >22.9Kg/m$^2$ and > 29.9Kg/m$^2$ are traditionally classified as overweight and obese, respectively (Snehalatha et al., 2003). However, WHO has recommended lower BMI thresholds WC and WHR for Asians, listed in table 3 and table 4.

Table 3: Cutoffs of anthropometric variables in Asian population (WHO, 2004)

<table>
<thead>
<tr>
<th>Waist Circumference (WC)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt; 90 cm</td>
<td>&lt; 80 cm</td>
</tr>
<tr>
<td>Obese</td>
<td>&gt; 90 cm</td>
<td>&gt; 80 cm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Waist Hip Ratio (WHR)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt; 0.9</td>
<td>&lt; 0.8</td>
</tr>
<tr>
<td>Obese</td>
<td>&gt; 0.9</td>
<td>&gt; 0.8</td>
</tr>
</tbody>
</table>
Table 4: Classification of BMI (Kg/m$^2$) in Asian population (WHO, 2004)

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI (Kg/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>15.0 to 18.4</td>
</tr>
<tr>
<td>Normal</td>
<td>18.5 to 22.9</td>
</tr>
<tr>
<td>Overweight</td>
<td>23.0 to 27.5</td>
</tr>
<tr>
<td>Obese</td>
<td>27.6 to 40.0</td>
</tr>
<tr>
<td>Morbidly Obese</td>
<td>&gt; 40.0</td>
</tr>
</tbody>
</table>

3.7 Inclusion criteria

**For Patients:**

- Physicians diagnosed patients with HT (>140mmHg (SBP) and > 90mmHg (DBP)) who were untreated with antihypertensive therapy.
- The HT patients who were between 18 and 80 years of age.
- Patients residing in rural area for more than two generations but occasionally commuting to city for employment related purposes for less than a year.
- HT patients who were treated with diuretics, β$_1$-blockers and ACE inhibitors alone or combination with ACE inhibitor.
- Patients belonging to only Haryanvi Jats ethnicity.

**For Control:**

- Individuals who were having blood pressure < 120 mmHg (SBP) and < 89mmHg (DBP) were considered as non hypertensive or normotensive.
- Individuals between the age of 18 and 80 years.
Individuals who were not receiving any antihypertensive therapy.

Individuals belonging to only Haryanvi Jats ethnicity.

3.8 Exclusion criteria (Patients and Controls):

- All individuals with the age below 18 years or those with age above 80 years.
- HT patients who were taking drugs other than diuretics, β-blockers and ACE inhibitors alone or in combinations (ACE inhibitor with diuretics or β-blockers).
- Patients with hypertension due to diabetes mellitus, metabolic syndrome, renal failure or other cardiovascular diseases.
- Individuals residing in the rural area but commuting to city on daily basis in connection with their earnings for more than a year.
- Individuals with ethnicity other than Haryanvi Jats.

3.9 Criterion for starting treatment phase

The choice of antihypertensive therapy was based on physician’s clinical acumen and past experience of 20 years in the field. The study was conducted cross-sectionally and recruitment of the patients was randomly selected and treated with low dose to high dose of antihypertensive therapy. As per our protocol, the blood pressure of patients treated with the final doses of 5mg of Ramipril, 25mg of Hydrochlorothiazide and 50 mg of Metoprolol were monitored after 4 weeks to assess the response of the therapy. If the patients showed significant decrease in blood pressure then the same therapy was continued otherwise additional drug was added.

3.10 Criterion for identifying responders and non responders

Patients were classified as responders to antihypertensive treatment if they had a fall in mean arterial blood pressure of 10% or greater in their both Systolic blood pressure (SBP) and Diastolic blood pressure (DBP) after 4 weeks of anti-
hypertensive therapy (Johnson et al., 2003). If the patients failed to respond to either of the mentioned drugs at the listed doses, it was recorded as a non-responder and a second drug was added to the treatment profile of the patient.

3.11 Collection of blood sample

The blood samples of 106 HT patients and 110 random samples of age and sex matched normal, healthy individuals, inhabiting the same rural area as control group were collected randomly. The patients and their controls belonged to Haryanvi Jat ethnicity. It is prudent to mention that patients come regularly for their treatment and the follow up is up to date. The compliance was good because free bus service was available to fetch and leave patients to their respective villages. The blood samples were collected in vacuum container tubes containing EDTA as an anticoagulant. The samples were transported in ice to the laboratory and were processed on the same day for further analysis.

3.12 Blood Sample Collection and Storage

Some basic considerations were taken care of during the study and were as follows:

- Blood samples were collected in sterile vials, pre-coated with ethylene diamine tetra acetate (EDTA) (2.5mM), as an anti-coagulant.
- Fresh, sterile syringe with wide bore needle was used for each participant, during collection of the blood samples.
- The samples were transported from site of collection to the work bench on ice or cool bags.
- Care was taken to perform all steps for sample processing at low room temperatures, while all centrifugation segments were executed at 4°C.
- Sample storage was performed, after quick freezing in liquid nitrogen, at -20°C.
• Glassware used, during sample processing, was thoroughly washed and double autoclaved to ensure neat and sterile environment.

• Fresh sterile plastic-ware was used during sample processing and any kind of reuse of plastic-ware was strictly avoided.

• All reagents were prepared in Milli-Q (MQ) water.

• All disposable material, including plastic was autoclaved prior to discarding them.

3.13 Reagents Required

• **Tris (hydroxymethyl) aminomethane-chloride (Tris-Cl)** (1 M; pH 8.0): 75 ml of sterile MQ water was used to dissolve 12.11 gm of Tris base. pH was set to 8.0 with 1N HCl and the final volume was made to 100 ml with MQ water. The solution was filtered through Whatmann filter paper and stored in a sterile tight screw capped reagent bottle.

• **Tris-Cl** (1 M; pH 7.3): As prepared above, 12.11 gm of Tris base was dissolved in 75 ml of sterile MQ water and the pH was set to 7.3 with 1N HCl. The final volume of the solution was made to 100 ml with MQ water. The solution was filtered and stored in a sterile tight screw capped reagent bottle.

• **Ammonium chloride (NH₄Cl)** (1M): Ammonium chloride (5.35 gm) was dissolved in 80 ml of MQ water and final volume of the solution was made 100 ml.

• **Di-sodium ethylene diamine tetra acetate (Na₂EDTA)** (0.5 M; pH 8.0): Added 18.61 gm of EDTA salt to 50 ml of MQ water in a 250 ml flask and placed it on a magnetic stirrer. Simultaneously, supplemented the solution with 10 M NaOH drop wise, until the pH reached at 8.0. Allowed the salt to dissolve and then made the final volume of the solution to 100 ml with MQ water.

• **Red Blood Cell (RBC) Lysis buffer** (10 mM Tris, pH 8.0; EDTA, 1mM; NH₄Cl, 125mM; pH 8.0): 10 ml Tris (1 M; pH 8.0), 2 ml of EDTA (0.5 M) and
125 ml of NH₄Cl (1 M) were mixed in MQ water to obtain a final volume of 1000 ml.

- **Tris-EDTA (TE) buffer** (Tris, 10 mM; EDTA, 1mM; pH 8.0): TE buffer was prepared by mixing 10 ml Tris-Cl (1 M; pH 8.0) and 2 ml of EDTA (0.5 M) in 700 ml MQ water and the final volume was made to 1000 ml.

- **Tris-EDTA (TE) buffer** (Tris, 10 mM; EDTA, 1mM; pH 7.3): As above, 10 ml Tris-Cl (1 M; pH 7.3) and 2 ml of EDTA (0.5 M) were mixed in MQ water to obtain a final volume of 1000 ml.

- **Sodium dodecyl Sulphate (SDS) 10%**: Dissolved 10 gm of SDS salt in 70 ml of warm MQ water and the final volume was made to 100 ml.

- **Ammonium acetate** (7.5 M): Dissolved 28.9 gm of ammonium acetate salt in 20 ml of MQ water and the final volume was adjusted to 50 ml.

- **Chilled dehydrated ethyl alcohol**: Undiluted dehydrated ethyl alcohol stored in -20°C deep freezer.

- **Ethanol (70%)**: 70 ml of dehydrated ethanol was added to 30 ml of sterile MQ water to obtain a final volume of 100 ml.

- **Proteinase K solution** Lyophilized powder was dissolved at a concentration of 20mg/ml in sterile 50mM Tris (pH 8.0) containing 1.5 mM calcium acetate. The stock solution was divided into small aliquots and stored at -20°C. Aliquots were thawed and used for assay.

### 3.14 Protocol for isolation of Genomic DNA

Genomic DNA was extracted from the blood samples (Buffy coat) using modified salting out procedure as described by Miller *et al.*, (1988).

- 2 ml of blood sample were collected from each participant in sterile vials, pre-coated with ethylene diamine tetra acetate (EDTA) (2.5mM), as an anticoagulant.
To 300 µl of blood sample, added RBC lysis buffer (three times the volume of blood sample taken) and kept for incubation on a rocker, to permit perpetual shaking at room temperature (RT) until the RBCs completely lysed.

Centrifuged the solution at 13,000 rpm for 1 minute to obtain a creamish white blood cells (WBC) pellet.

The supernatant was discarded and the WBC pellet was thoroughly suspended in 300 µl TE buffer (pH 8.0), using a vortexing machine. Thereafter, 20 µl of 10% SDS solution (final concentration (Fc) = 0.62%) was added to the above solution and the mixture was incubated at 56ºC for 30 minutes on a dry-bath.

Subsequently, added proteinase K solution and incubated the mixture at 56ºC for another 2 hours.

Added 150 µl of 7.5M ammonium acetate (Fc = 2.4 M) and mixed vigorously for about 1 minute per sample, on a vortexer. Centrifuged the mixture at 13,000 rpm, at RT, for 15 minutes, thereby resulting in separation of the precipitated proteins as a pellet.

The clear supernatant was transferred to a fresh sterile microcentrifuge tube. To this added chilled absolute ethyl alcohol (twice the volume of clear supernatant). The tube was gently rocked a couple of times to allow precipitation of genomic DNA.

The genomic DNA precipitates were centrifuged at 13,000 rpm for 10 minutes to pellet at the bottom of the tube. The latter were subsequently washed in 150 µl of 70% ethanol and air dried at room temperature.

100 µl of TE buffer (pH 7.3) was used to dissolve the dried DNA pellet by incubating at 65ºC for 10 minutes. The dissolved DNA was finally stored at -20ºC till further use.
3.15 Agarose Gel Electrophoresis (Sambrook and Russell, 2001)

The isolated genomic DNA was subjected to qualitative analysis by standard agarose gel electrophoresis.

3.16 Reagents Required

- **Tris-Acetic acid-EDTA (TAE) buffer (50x)**: 242 gm of Tris base was dissolved in 500 ml of MQ water. This solution was then supplemented with 57.1 ml of glacial acetic acid followed by 100 ml of 0.5 M EDTA (pH 8.0). Final volume of the solution was made to 1000 ml with MQ water and the same was filtered and diluted to a working concentration of 1x before use.

- **Agarose**: 0.8% agarose gel was prepared in 1xTAE buffer for qualitative analysis of genomic DNA samples.

- **Ethidium bromide (10 mg/ml)**: 10 mg of ethidium bromide was added to 1 ml of MQ water and dissolved by gentle mixing before storing in an amber bottle at room temperature.

- **Gel loading Dye (6x)**: 0.025 % bromophenol blue (BPB) with 40% sucrose in water was used for analysis of genomic DNA.

- **Gel loading dye (10x)**: 0.5% (w/v) xylene cyanol, dissolved in distilled water, was mixed with equal volume of glycerol. The same was used for PCR product analysis.

3.17 Procedure

a. For a 30 ml gel, 0.24 gm of agarose was weighed and added to 30 ml TAE buffer (1x). Agarose was allowed to melt by gently heating in a microwave oven. Ethidium bromide solution (10 mg/ml) was added to the agarose solution (Fc = 0.5 μg/ml) when the same had cooled to about 45-55°C. Thereafter, the agarose solution was poured into a previously set electrophoresis tray and allowed to solidify at room temperature.
b. 8-10 µl of genomic DNA sample and 1-2 µl of gel loading dye were mixed before loading into the agarose gel and the mixture was electrophoresis in 1xTAE buffer (pH 8.0) at 100 volts.

c. Xylene cyanol front was monitored and the run was terminated as the dye front moved approximately 8.0 cm from the loading point.

d. The amplified products were visualized on a UV-transilluminator after the gel was stained with ethidium bromide solution.

3.18 Photo Documentation

a. When the agarose gel was visualized on a UV-trans-illuminator, double-stranded DNA was visible as fluorescent bands, as a result of intercalation of ethidium bromide into it.

b. The agarose gel was documented as a photograph (.jpg format) with the help of Digital Imaging System (Ultra lum, Inc., USA) and Photoshop software (Adobe Photoshop 7.0).

3.19 Spectrophotometric Analysis of genomic DNA (Sambrook and Russell, 2001)

The isolated genomic DNA was checked for purity and quantified by determining its absorbance at 260 nm and 280 nm, as follows.

3.19.1 Reagents/Material Required

- MQ water
- TE buffer (pH 7.3)
- Quartz cuvettes
- Micropipettes

3.19.2 Procedure

a. Optical density of the diluted DNA solution was determined, concurrently, at 260 nm and 280 nm, using TE buffer as diluent, as well as blank.

b. DNA concentration in the analyzed sample was calculated using the formula for double stranded DNA.
Concentration of DNA (µg/ml) = \(A_{260\text{nm}} \times \text{constant} \times \text{dilution factor}\)

Here,

- \(A_{260\text{nm}}\) represents the absorbance value of the solution, under analysis, when observed in UV light at 260 nm wavelength;
- Constant is represented by the value 50, as an absorbance of 1 at 260 nm is represented by 50 µg/ml of DNA; and
- Dilution factor represents the times for which the solution has been diluted before observing its absorbance value.

The values thus obtained represented the concentration of DNA in the analyzed samples.

c. The isolated DNA was checked for purity from the ratio of absorbance values at 260 nm and 280 nm, respectively.

d. DNA was isolated afresh for samples revealing a ratio <1.60 or >1.90.

e. Quantified DNA samples were then diluted to a working concentration of 40 or 20 ng/µl in TE buffer (pH 7.3) and stored at -20°C, until analyzed for genetic polymorphisms in \(ACE, ADD1, \beta-1 ADR\) gene by polymerase chain reaction (PCR).

### 3.20 Genotyping \(ACE I/D\) Polymorphism (Agarwal et al., 2004):

The human \(ACE\) gene is located on chromosome 17q23 and includes 26 exons. The insertion/deletion polymorphism is located intron 16 and consist of a 287 base pair sequence which is either present (insertion) or absent (deletion). The \(ACE I/D\) polymorphism (rs 1799752) were detected by the polymerase chain reaction using the primers flanking a 287 bp insertion sequence (Figure 3).
Material and Methods

Master mix for every PCR analysis was prepared fresh and various reaction components for a single reaction, of volume 25µl, are given below table 5. The various PCR cycling parameters were optimized to a total of 35 cycles (Table 6).

Table 5: Concentration of reaction components used for amplification

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Volume (in µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq DNA polymerase (3U/µl)</td>
<td>0.1</td>
</tr>
<tr>
<td>10X Buffer B</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>1.5</td>
</tr>
<tr>
<td>FP and RP (10µM)</td>
<td>1.0</td>
</tr>
<tr>
<td>dNTPs (10µM)</td>
<td>0.4</td>
</tr>
<tr>
<td>DNA (20ng/µl)</td>
<td>2</td>
</tr>
<tr>
<td>Sterile water</td>
<td>17.5</td>
</tr>
<tr>
<td>Total volume</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 6: PCR Protocol for *ACE* genotype

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total number of cycles: 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial Denaturation</td>
<td>94°C for 3 minutes</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>94°C for 30 seconds</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>58°C for 20 seconds</td>
</tr>
<tr>
<td>4. Extension</td>
<td>72°C for 20 seconds</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C for 3 minutes</td>
</tr>
<tr>
<td>End</td>
<td>4°C</td>
</tr>
</tbody>
</table>

The PCR products were resolved in 2% agarose gel and visualized following ethidium bromide staining. All samples, identified as DD after initial amplification, were reconfirmed with an insertion-specific primer pair (Gen bank accession number GQ449380, GQ449383):

Forward primer: 5′GCCACTACGCCGGCTAAT-3′
Reverse primer: 5′GATGTGGCCATCACATTCGTCAGAT-3′

Each set of amplification contained known positive and negative controls. Amplified product appears at 490bp for II genotype, 190 for DD genotype and both 490 and 190bp PCR products for ID genotype.

**Photo documentation**

The PCR products were visualized by placing the gel on an UV-transilluminator and photographed using digital imaging system.
3.21 Genotyping α-adducin (ADD1) Polymorphism (Shin et al., 2004)

A G-to-T substitution polymorphism located at nucleotide position 614 of exon 10 of the ADD1 gene (Gen bank accession number L29294) resulted in the genetic variant of amino acid residue 460. The Gly460-Trp polymorphism of ADD1 (rs 4961) was detected using an Amplification Refractory Mutation System polymerase chain reaction (PCR) as described in figure 4. Briefly, two allele-specific primers and their constant complementary strand primer were mixed and used for the PCR amplification in a single reaction. Deliberate differences were introduced into the allele-specific primers in addition to the base substitution, and they were able to drastically reduce cross-reactions between two allelic PCRs in a mixed reaction. The following primer sets were used:

F614G, 5′GGGGCGACGAAGCTCCGAGGTAG-3′

F614T 5′GCTGAACTCTGGCCAGCGACGAAGCTTCCGAGGATT-3′

R614 5′CCTCCGAAGCCCGACCTACCCA-3′

PCR was performed in thermal cycler (Bio Rad, Japan) in a 25 µl reaction mixture in 0.2 ml thin-wall PCR tubes (Axygen Scientific, Inc., CA) containing 2 µl genomic DNA solution (40ng/µl), 10x buffer B 2.5 µl, 1 µl (25mM MgCl2), 0.5 µl (10 mM) each of the dGTP, dATP, dTTP and dCTP (Promega, Madison, WI), 1 µl (F614G primer, F614T primer and common primer (R614)), 0.12 µl AmpliTaq Gold polymerase and 17.88 µl sterile water. The cycling conditions comprised a hot start at 94°C for 10 minutes, followed by 35 amplification cycles at 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by final extension step at 72°C for 3 minutes. The size of PCR products were 220 bp and 234 bp for the 460Gly and 460Trp alleles, respectively, and were clearly resolved on 4% agarose gel (NuSieve, 3:1 agarose, FMC Bioproducts).
Material and Methods

Figure 4: Presentation of ARMS PCR method for genotyping of ADD1 polymorphism

3.22 Genotyping of β-1 ADR Polymorphism (Fragoso et al., 2005)

The β-1 ADR polymorphisms fragments containing sequences were amplified by polymerase chain reaction (PCR) as shown in figure 5. The sense and antisense primers were for the β-1 ADR gene (Gen bank accession number C_8898494_10 for Ser49Gly) codon 49 (nucleotide145):

5’CCGGGCTTCTGGGGTGTTCC3’ and
5’GGCGAGGTGATGGCGAGGTAGC3’

The PCR conditions were initial denaturation step at 94°C for 5 min, followed by 30 cycles (denaturation at 94°C for 30 seconds, annealing 61°C extension at 72°C for 30 seconds) a final extension step at 72°C for 10 minutes. The β-1 ADR gene codon polymorphism (rs1801252) was detected using restriction fragment length polymorphism (RFLP).
Figure 5: Presentation of RFLP method for genotyping of (Ser49Gly) \(\beta-1\) ADR polymorphism

Briefly the 564 bp fragment obtained by PCR containing the \(\beta-1\) ADR codon 49 polymorphism was digested by \(Eco\ 0109I\), giving fragments of 345 and 219 bp for Gly49.

3.23 Statistical analysis

Data analysis was performed using the Statistically Package for Social Sciences (SPSS 16.0) for window version 7.5. Main analysis included statistics; however relationships were explored using cross-tabulations, correlation and logistic regression.

3.23.1 Chi square test: (Sundar Rao and Richard, 2004)

Allelic frequencies between hypertensive patients and normotensives groups were compared using Chi square test. The deviation of the observed number from those specified by the hypothesis formed the basis of Chi square test. Each deviation was squared, each square divided by the expected number (E) and results were added, the Chi square was given by formula:

\[
\chi^2 = \xi \frac{(O - E)^2}{E}
\]

\(\xi\) denotes summation
Given a fixed total, the number of independent cells or cells that can be varied freely was the degree of freedom (df).

\[ df = (r-1)(c-1) \]

\( r = \) numbers of rows
\( c = \) numbers of columns

3.23.2. Hardy Weinberg equilibrium: (Guo and Thompson, 1992)

The studied population groups were tested for their Hardy Weinberg equilibrium on a contingency table of observed versus predicted genotype frequencies.

3.23.3 Unpaired t test: (Altman, 1991; Armitage and Berry, 1994)

The mean values obtained for BMI, WC, WHR, and Systolic blood pressure and Diastolic blood pressure and for hypertensive patients and normotensives population were compared by using unpaired t test.

3.23.4 Odd ratio: (Verma et al, 1993)

Odd ratio was calculated to check the probability of association of a given allele with susceptibility to hypertension.

The odd ratio is the ratio of the odds of an event occurring in one group to the odds of it occurring in another group. The term is also used to refer to sample-based estimates of this ratio. If the probabilities of the event in each of the groups are \( p_1 \) (first group) and \( p_2 \) (second group), then the odds ratio is:

\[
\frac{p_1/(1-p_1)}{p_2/(1-p_2)} = \frac{p_1 q_2}{p_2 q_1} = \frac{p_1}{p_2} \frac{q_2}{q_1}
\]

Where \( q_x = 1 - p_x \). An odds ratio of 1 indicates that the condition or event under study is equally likely to occur in both groups. An odd ratio greater than 1 indicates that the condition or event is more likely to occur in the first group. And an odd ratio less than 1 indicates that the condition or event is less likely to occur in the first group. The odds ratio must be greater than or equal to zero if it is defined. It is undefined if \( p_2 q_1 \) equals zero.
3.23.5 Relative risk (Spitalnic, 2006)

Relative risk reflects the magnitude of the difference in the frequency of an outcome between groups. For a given exposure (exposure could mean exposure to a risk factor or exposure to a treatment). The relative risk tells that proportional difference in risk between groups.

The relative risk of an outcome is calculated as:

\[
\text{Relative risk} = \frac{\text{Risk of the outcome with exposure}}{\text{Risk of the outcome without exposure}}
\]

3.23.6 Multivariate regression (Green, 1991)

Multiple regression is a statistical technique that allows us to predict someone’s scores on one variable on the basis of their scores on several other variables. Multi-regression is flexible method of data analysis that may be appropriate whenever a quantitative variable (the dependent or criterion variable) is to be examined in relationship to any other factors (independent or predictors variables).

3.23.7 ANOVA (Larget, 2003)

Analysis of Variance (ANOVA) for balanced data from a wide variety of experimental designs. In analysis of variance, a continuous response variable, known as dependent variable, is measured under experimental conditions identified by classification variables, known as independent variables. The variations in the response are assumed to be due to effects in the classification, with random error accounting for the remaining variation. Analysis of variance is used to cast inference on group means. The null and alternative hypotheses are:

\[
H_0: \mu_1 = \mu_2 = \cdots = \mu_k
\]

\[
H_1: H_0 \text{ is false}
\]

Where \( \mu_i \) represents the population mean of group \( i \)