MATERIALS AND METHODS

Determination of genetic damage and the polymorphic nature of CYP2D6 (∗2, ∗4, ∗10) and of GST (T1, P1, M1) genes was carried out under informed consent (Annexure I) in 200 patients diagnosed with essential hypertension and 200 normotensive healthy controls after getting approval of the present study from the Institutional Ethics Committee.

Information on demographic variables was recorded on a pre-designed questionnaire (Annexure II); physiometric and some anthropometric measurements were taken as recommended and biochemical analysis for estimation of lipid and oxidant stress levels was carried out using standard protocols. DNA damage was assessed using the alkaline single cell gel electrophoresis (SCGE) assay (Singh et al., 1994). The genetic profile of GST and CYP2D6 genes was ascertained as per methods available in literature (Girisha et al., 2004; Theophilus et al., 2006).

The details about the participants and of the methodology used to fulfill the study objectives are described in this chapter under appropriate sub-headings.

3.1 Study Design

A case-control approach was adopted for the present study because such a design allows for a direct comparison between the groups with any observed differences associated with the causes of disease under study (Checkoway et al., 2007). Following this approach, genetic damage as well as biochemical and molecular genetic investigations were made on 200 essential hypertensive cases/patients and 200 normotensive healthy controls to trace the types and levels of biomarkers associated with essential hypertension.

3.2 Study Sub-group

The patients (cases) and controls are Punjabi Jat Sikhs constituting the single largest group in the state of Punjab and are the majority (60%) among the Sikhs. Ibbetson referring to Major Todd states that Jats are from major Rajput tribes which in turn belong to the Aryan tribes though as per General Cunningham, they are immigrants
from the north-west having Scythian race as ancestors (Ibbetson, 1984). According to an ancient Sanskrit work and first Persian account “Mujmat ut-tawarikh (1026)” Jats are an ancient tribe of Sind while the Persian chronicler ‘Firishta’ has documented that Jats lived in northwest Punjab near the Koh-i-jud river with one of the Jat rulers, “Jit Salindra” first started their colonization in Punjab near Multan. But according to Fuchs (1974) Jats were considered as Central Asian nomadic group that was immigrated into Northwest India and in the 17th century they become Sikh followers (Khanna, 2003) then they were known as Jat Sikhs. Jat Sikhs also follow endogamy practices at caste level (Singh et al., 2008). Jats share some common haplotypes with other populations of Indus valley (http://www.jatland.com/home/Jat_History accessed on June 20, 2015). Some molecular genetic studies on D21S11 and D21S215 loci (Sidhu et al., 2003), 15 biparental and 17 Y–chromosome Short tandem repeats (STRs) (Giroti and Talwar, 2013) and SNP-19 of Calpain 10 (CAPN10) (Sharma et al., 2013) genes have been carried out. Limited biochemical analysis on lipid profile (Bishnoi et al., 2010; Thukral et al., 2012) and oxidative stress (Thukral et al., 2012; Kaur et al., 2014) as well as few anthropometric investigations (Singh et al., 1988; Gaur et al., 2008; Singla et al., 2011) and epidemiological studies comprise the research work on Jat Sikhs. The present study is a comprehensive study of its kind, hence purports to provide novel information on Jat Sikhs.

3.3 Sample-size Calculation

An appropriate sample size ensures the statistical validity of the results and as in the present study, the analysis of a number of biomarkers in patients for genetic damage, biochemical and molecular genetic analysis was to be performed, therefore sample size was calculated separately for each biomarker on the basis results obtained from a pilot study. As discussed in the literature, an adequate sample size was determined (Schork, 2002).

Sample size was calculated with a statistical power of 80% and alpha value of 0.05 (Annexure V). For postulated significant increase of p≤0.05 on Students’ t-test analysis (after Albertini et al., 2003), sample size calculated for genetic damage parameters was 131-175 and 110-172 was for oxidative stress biomarkers. Based upon global minor
allele frequencies of the six single nucleotide polymorphisms (SNPs) the effective
sample size ranged from 143-177 per group. Hence, considering the effective sample-
size requirements varying from 131 to 177 a 10% for contingency increase for non-
response, recording-error, illness, withdrawal from study etc. a rounded figure of 200
each, in patients and control groups was considered sufficient, comprising a total study
group of 400 individuals.

3.4 Power of the Study

Calculated retrospectively for various end-points, the power of the study is 86.53%. For
genetic damage parameters 85%, 88.75% for oxidative stress parameters and 86.25%
for molecular genetic analysis. (Annexure V)

3.5 Proforma Preparation

A detailed questionnaire after perusal of literature was prepared for recording
demographic and relevant information about hypertensive patients and normal control
individuals (Annexure II). Other pertinent details as required for biomonitoring studies
were recorded as per recommendations of Carrano and Natraj (1988).

3.6 Patient Identification

Patients confirmed as hypertensive and on a single drug (monotherapy) treatment
(Atenolol- a beta blocker) by the attending physicians at the Mata Kaulan Bandhi Chod
Charitable Hospital, Tarn Taran Road, Amritsar formed part of the study group. All
belong to rural areas (Chhaba, Chatiwind, Manawala and Basarke villages) and had
been visiting this hospital for treatment purposes. A total of 275 patients were initially
contacted. Of these, 200 patients belonging to the Jat Sikh sub-group comprised the
patient group.

3.7 Controls

An age-, sex- and area-matched group comprising 200 unrelated, healthy, normotensive
individuals belonging to the same population sub-group, who were accompanying
various patients and belonging to same villages or from the general rural population,
comprised the control group.
**Inclusion criteria**- The essential hypertensive patients belonging to *Punjabi Jat Sikh* sub-population group, above 40 years of age and on treatment with the beta-blocker Atenolol (50mg/day) formed the study group. Age-, sex- and socio-economic status-matched normotensive healthy individuals, belonging to same population sub-group, were included in the control group.

**Exclusion criteria**- The patients and normal individuals belonging to other population sub-groups or those with secondary hypertension or other cardiovascular, renal, cerebrovascular complications and those on antihypertensive treatments other than with Atenolol, were excluded from the study group.

### 3.8 Information Gathering

After explaining the nature and details of the study and after obtaining voluntary written consent (Annexure I), an interview-based method was adapted to record required information of each participant on the specially-designed questionnaire (Annexure II). Besides demographic information, physiometric and anthropometric measurements were also recorded for the patient and control groups. Specific queries on the state of hypertension, medication, genetic history and lifestyle variables were also noted. A three-generation pedigree was recorded for each participant for sub-group verification and in order to find any familial incidence/history of hypertension.

### 3.9 Demographic and General Information

Demographic details and general information related to physical activity, socioeconomic status and occupational group were also recorded for the patient and control groups. As standard of living and health status also depends upon the socioeconomic status of the individual and its community, patients and controls were classified into five main categories of socioeconomic status viz. upper class (i), upper middle class (ii), middle class (iii), lower middle class (iv), lower class (v) as per Kumar *et al.* (2012) based on calculated scores obtained from the information on educational status, type of occupation and monthly family income. The study participants self-classified themselves as those doing mild, moderate or heavy physical activity. As the patients and controls were involved in agrarian practices, and therefore had similar occupational exposures.
3.10 Blood Pressure Measurements

For taking blood pressure measurements of participants, the method recommended by Indian Hypertension Guidelines (IHG III, 2013) was followed. Each participant was seated comfortably and after a rest of 10 minutes, systolic (SBP) and diastolic (DBP) blood pressure measurements using a mercury sphygmomanometer were taken. A stethoscope was used to hear Korotkoff sound of the brachial artery. The averages of three readings were recorded as the SBP and DBP values for each participant. Categorization of the participants into different hypertension categories was then carried out (optimal, normal, high normal, hypertension stage I, II or III).

3.11 Anthropometric Measurements

Anthropometric measurements needed for assessment of the obesity status of each participant in the patient and control groups was made using standard methodology and anthropometric tools (Weiner and Lourie, 1981). General obesity was determined by body mass index (BMI) and abdominal/central obesity by waist-hip-ratio (WHR), waist-height-ratio (WHtR), waist circumference (WC) measurements. Calculations were based on measurements taken for height, weight, hip circumference (HC) and waist circumference (WC).

**Height**- Using standard method, height (cm) was measured of each participant standing barefoot and erect against a wall with feet together and the head in the ear-eye plane.

**Weight**- For taking the weight (kg), the participant stood erect on a weighing machine with minimal clothes and without shoes.

**Waist circumference**- Waist circumference in cm was recorded using a steel tape which was placed around the participant’s iliac crest and lower rib.

**Hip-circumference**- Hip circumference (cm) measurement was also carried out using a steel tape which was placed at the level of maximum protrusion of the hip area of the participants.

**Body mass index (BMI)** - As a measure of general obesity, BMI was mathematically calculated (BMI (kg/m²) = weight (kg) / height² (m²)).
**Waist-hip-ratio (WHR)** - Abdominal obesity was assessed by comparing the ratio of waist to hip circumferences with standard cut-offs (Snehalatha et al., 2003).

**Waist-to-height ratio (WHtR)** - Chobanian et al. (2013) had reported that waist-to-height ratio is a better obesity index for hypertension. Hence it was also calculated (WHtR= waist circumference (cm)/height (cm)).

### 3.12 Assessment of Obesity

The patients and controls were assessed for both, general (BMI basis) and central/abdominal obesity (WC, WHR and WHtR bases). The WHR and WC obesity cut-offs were as given by Snehalatha et al. (2003). The BMI classification was performed as per WHO (2004), and by Mishra et al. (2009). WHtR classification for assessment of abdominal obesity was made according to Hsieh and Muto, (2004).

The details of the obesity categories are:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Category</th>
<th>Range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>Below Normal</td>
<td>&lt;18</td>
<td>Misra et al. (2009), WHO (2004)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>18-22.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High Normal</td>
<td>23-24.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Obese (25)</td>
<td>≥25</td>
<td></td>
</tr>
<tr>
<td>WHR</td>
<td>Normal</td>
<td>&lt;0.88 (M), &lt;0.81 (F)</td>
<td>Snehalatha et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Obese</td>
<td>&gt;0.88 (M), &gt;0.81 (F)</td>
<td></td>
</tr>
<tr>
<td>WC(cm)</td>
<td>Normal</td>
<td>&lt;85 (M), &lt;80 (F)</td>
<td>Snehalatha et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Obese</td>
<td>&gt;85 (M), &gt;80 (F)</td>
<td></td>
</tr>
<tr>
<td>WHtR</td>
<td>Normal</td>
<td>&lt;0.5</td>
<td>Hsieh and Muto, 2004</td>
</tr>
<tr>
<td></td>
<td>Obese</td>
<td>&gt;0.5</td>
<td></td>
</tr>
</tbody>
</table>

BMI- body mass index; WHR- waist-to-hip ratio; WC- waist circumference; WHtR- waist-to-height ratio

### 3.13 Collection of Venous Blood Samples

From each participant, ~10ml intravenous blood was drawn by a certified technician; it was aliquoted into separate pre-labelled vials containing either anticoagulants EDTA or
heparin while a portion was retained without any anticoagulant. The samples were transported to the laboratory on ice. The EDTA-containing sample was stored at -20º C for later molecular genetic analysis. Samples with heparin for carrying out the single cell gel electrophoresis (SCGE) assay for genetic damage assessment, and those without anticoagulant for serum separation required for biochemical analysis, were processed within 2-3 hours of blood collection.

3.14 Preparation of Solutions

For genetic damage studies, biochemical analysis and molecular genetic work, glassware, equipment and preparation of reagents are given in annexure III The reagents prepared as per standard protocols were stored and used within recommended time interval of 1-2days for genetic damage studies, 5-7days for biochemical analysis and 10days for molecular genetic work.

3.15 Genetic Damage Studies

The alkaline single cell gel electrophoresis (SCGE/comet) assay was performed to assess for damage at DNA level in single cells (peripheral blood leukocytes). Cell-viability for each sample was checked prior to performing the SCGE assay.

3.15.1 Cell Viability

With the standard Trypan Blue Dye-Exclusion test (Doyle et al., 1995) the number of viable cells present in the samples was determined. The intact cell membranes of live cells exclude the dye whereas dead cells take-up the strain. Only samples with viable cells 80-85% were processed for SCGE assay. To perform the Trypan-Blue dye exclusion test, 100µl of Trypan-Blue dye was added to a cell suspension comprising 850µl phosphate-buffered saline (PBS) and 50µl of blood sample. The sample was thoroughly mixed and allowed to stand for 15 minutes so that dead cells could take-up the dye. The sample was then transferred on to a haemocytometer and a cover-slip was carefully placed over it. Each of the four corner squares and a center square were examined under the microscope for the presence of viable and non-viable cell counts. The percent viable cell count was then calculated (Percent cell viability= total viable cells/ total viable and non-viable cells X 100).
3.15.2 The Single Cell Gel Electrophoresis (SCGE/Comet) Assay

The assay is based upon the principle (Collins et al., 2008) that breaks in DNA relaxes the DNA super coiling and on electrophoresis, the broken ends are pulled towards the anode forming a tail (appearance of comet) while lack of free ends and the large size prevents migration of the intact DNA molecule.

The standard alkaline SCGE protocol of Singh et al. (1998) with slight modifications under prevalent laboratory conditions was generally adhered to. Indian analytical chemicals, agarose-coated slides (Ahuja and Saran, 2001) and silver nitrate staining were other differences from the standard protocol.

Pre-cleaned slides coated with normal melting point agarose (1% NMPA) were used; a second layer with blood sample mixed in low melting point agarose (0.75% LMPA) was poured and the gel was allowed to set; this was followed by pouring a third layer of (0.05%) LMPA. The sandwiching of the blood sample permits embedding of the cells in the agarose layer. The slides were then treated with lysing solution (2.5 M NaCl, 100 mM Na₂-EDTA; 10 mM Tris–HCl, pH 10–10.5; 1% Triton X-100 and 10% DMSO) for 1-2 hours for the removal of red blood cells (RBCs), outer walls and nucleated proteins under light-protected condition. Alkaline treatment (pH ≥ 13.0) was given in the electrophoresis buffer (0.3mol⁻¹ NaOH, 1mmol⁻¹ Na₂-ETDA) for DNA unwinding and then the slide preparations were subjected to electrophoresis (25V, 300mA for 25 minutes; 1V/cm). After electrophoresis, slides were treated with neutralization buffer (0.4 M Tris, pH 7.5), fixed in fixing solution (15% trichloroacetic acid, 5% zinc sulfate and 5% glycerol) followed by staining with silver nitrate (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilisic acid, 0.15% v/v formaldehyde, 5% w/v sodium carbonate). The slides were coded and allowed to air-dry. Coded slide preparations were subsequently observed for DNA damage under a binocular transmission light microscope.

3.15.3 Scoring for DNA Damage

For each sample, two slides were prepared and 50 cells/slide were captured using a E420 Olympus camera for scoring DNA damage (Plate I). The Comet Assay Software Program-CASP (http://http://www. casplab.com/download) which is a freely-available
image analysis programme, was used for scoring DNA damage. The various genetic damage indices recorded were per cent tail DNA, Tail Moment (TM), Olive Tail Moment (OTM). On the basis of per cent tail DNA, damage index (DI) and damage frequency (DF) were calculated (Garcia et al., 2007). The percent tail DNA is considered the best parameter for genetic damage as it is linearly related to break frequency; this is followed by tail moment which expresses both, tail length and tail intensity (Collins et al., 2008). Olive tail moment is the product of DNA length and fraction of DNA in tail (Cotelle and Ferrard, 1999). The frequency distribution of cells with tail is known as damage frequency (Franke et al., 2005). Comets were classified into four categories representing different tail intensities and summing the scores from 100 comets gave overall damage index (Collins et al., 2008).

3.16 Biochemical Analysis

Biochemical analysis was performed for lipid profile assessment and oxidative stress measurements on blood sera samples of study participants. Serum samples were prepared by allowing the blood (~3ml) to clot for half-an-hour followed by centrifuging at 5000g for 10 min. The supernatant (comprising the serum) was transferred to a fresh tube and stored at -20º C for biochemical analysis to be carried out within two months (Matthan et al., 2010).

3.16.1 Lipid Profile

As the atenolol-prescription required its intake early morning after breakfast, and since all patients belonged to rural areas and travelled for treatment to the local hospital (Mata Kaulan ji Charitable Hospital, Amritsar), only non-fasting sampling was feasible. Lipid profile was hence carried out on non-fasting samples. Such a sampling is effective for lipid profiling and has been supported in literature. A cross-sectional study by Sidhu and Naugler (2012) has documented that fasting samples for lipid analysis are not necessary as most people consume much less fat in ordinary diet in comparison to that in the fat-tolerance test. Some studies have shown that triglycerides showed a strong predictive relationship with cardiovascular risk when measured after 4-hours of food intake (Kannel and Vasan, 2009). Langsted et al. (2008) had also observed that the change in lipid profile is minimal after normal diet-intake. The levels of total
cholesterol (TC), high density lipoproteins (HDL-C) and triglycerides (TG) were ascertained on a semi-automated analyzer (Clinical chemistry analyzer, Erba, CHEM-7, India) using standard kits (Angstrom, Vadodra, India) and following the manufacturer’s instructions.

**Total cholesterol (TC)**- The serum sample (25µl) was mixed with the reagent (enzyme chromogen including cholesterol esterase, cholesterol oxidase and peroxidase) and double-distilled water (1.5ml of each) and was incubated for 15 minutes at 37ºC while the blank lacked the serum sample. Absorbance of the mixture was recorded at 505nm. Level of TC in sample was determined by comparing absorbance of sample with that of standard (Total cholesterol (mg/dl) = absorbance of sample/absorbance of standard X 200).

**High density lipoprotein cholesterol (HDL-C)**- The absorbance of the centrifuged supernatant (5000g for 15 minutes), which had been obtained from mixing 200µl each of serum sample and precipitating reagent (phosphotungstic acid and magnesium chloride), and of the blank was measured at 505nm. The concentration of HDL cholesterol (mg/dl) was estimated as absorption of the sample/absorption of standard X concentration of standard X 2 (Lopez Virella *et al.*, 1977).

**Triglycerides (TG)**- Triglycerides were measured from the estimations of coloured complexes (formed by H₂O₂ which is a by-product of glycerol oxidation). The intensity of coloured complexes directly depends on the intensity of triglycerides. The concentration of the triglycerides was estimated by comparing its absorbance with that of a standard.

**Very Low density lipoprotein cholesterol (VLDL) and low density lipoprotein cholesterol (LDL-C)**- Levels of low density lipoprotein cholesterol (VLDL-C= TGX1/5) and LDL-C (LDL-C=TC-HDL-VLDL) were calculated as per the equations given by Friedwald *et al.* (1972).

**Cardiac risk indices**- From the values obtained for TC, HDL-C, LDL-C and TG, the coronary risk (TC/HDL) and artherogenic risk (LDL/HDL) and TG/HDL indices were calculated (Ugwuja *et al.*, 2013) as these have greater predictive values for vascular risk compared to isolated parameters (Millan *et al.*, 2009). Changes in the TC/HDL and
LDL/HDL ratios are also better predictors for coronary heart disease as compared to the isolated lipid or lipoprotein levels (Pereira, 2012).

### 3.16.2 Oxidative Stress Measurements

Oxidative stress biomarkers included total antioxidant capacity (TAC), total oxidant status (TOS), and of malondialdehyde (MDA) level as a function of lipid peroxidation. These were assessed in blood serum samples by standard methodology of Erel (2004, 2005) for estimation of total antioxidant capacity and of Beuge and Aust (1978) for MDA. The oxidative stress index (OSI) was obtained as a ratio of TOS and TAC (Demirbag et al., 2007).

**Total Antioxidant Capacity and Total Oxidant Status** - Spectrometric analysis of total antioxidant capacity and total oxidant status in blood serum samples was carried out as per Gupta et al. (2009). Analysis of TAC was made at the wavelength of 444nm taking Trolox as the standard (10mM, pH 7.4) solution (Erel, 2004) while TOS was analyzed at a wavelength of 560nm using H$_2$O$_2$ as standard. Total antioxidant capacity and total oxidant status was calculated as Factor*(ΔA blank - ΔA sample), where Factor is concentration of the standard/(ΔA blank-ΔA standard). Total antioxidant capacity was measured as mmol Trolox Equivalent/l and total oxidant status was measured as μmol H$_2$O$_2$ Equivalent/L.

**Oxidative stress Index** - The oxidative stress index (OSI) in arbitrary units (AU) was obtained as per Demirbag et al. (2007) by taking the ratio of TOS to TAC. Increase in OSI indicates increase in total oxidant status and decrease in antioxidant capacity (Bolukbas et al., 2005).

**Lipid peroxidation** - Malondialdehyde is a by-product of oxidative degradation of lipids (lipid peroxidation). On reacting with thiobarbituric acid, it forms a pink-coloured complex that absorbs light at 532nm; the color-intensity indicates its concentration.

Malondialdehyde (MDA) levels were determined as per Devasagayam et al. (2004). For determination of MDA levels, 100μl of serum sample was mixed with 100μl of each Tris-HCl (150mM, pH7.1), ascorbic acid (1.5mM) and ferrous ammonium sulphate (1.0mM). The final volume of the mixture was made to 1ml by double-distilled water.
The mixture was incubated at 37°C for 15 minutes. To the solution, 1ml of trichloroacetic acid (10%) and 2ml of thiobarbituric acid (0.375%) were added. After mixing, the solution was kept in a boiling water bath for 15 minutes and centrifuged at 10000rpm for 15min. The absorbance of the supernatant solution was then measured at 532nm against a sample blank (saline solution instead of serum). MDA levels in µmol/L were then calculated as per Jyothi et al. (2008) by the equation E=kCL and C=E/K×L (µmol/L), where K is the molar the extinction coefficient, E is the extinction/absorbance, C = Concentration in moles/litre and L= Length of the cuvette used (1 cm).

3.17 Molecular Genetic Investigations

Molecular genetic investigations were made to study the genotypic status of GST (T1, M1 and P1 alleles) and CYP2D6 (*2, *4 and *10 alleles) and to identify whether these polymorphisms have an association with the state of hypertension and with genetic damage. For this part of the study, the isolation and quantification of genomic DNA from intravenous blood samples was carried out followed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis.

3.17.1 DNA Isolation and Purification

Genomic DNA was isolated from white blood cells using the organic method (Gill et al., 1984). Cells were lysed using a detergent and the lysed cells were mixed with phenol, chloroform and isoamyl alcohol to extract the contaminants (proteins, organic and lipid wastes) into the organic phase. High molecular weight naked DNA remained in aqueous phase, which was then recovered by alcohol precipitation.

For DNA isolation, 1ml of whole blood was suspended in 5ml lysis buffer-1 (155mM ammonium chloride, 10mM potassium bicarbonate and 0.1mM EDTA) to lyse red blood cells (RBCs) at 4ºC (in the refrigerator) for 12-15 minutes. The samples were centrifuged at 4000rpm for 15 minutes, during which the pellet of WBCs (white blood cells) was formed. After discarding the supernatant, 5ml of lysis buffer was added to the pallet and the contents were incubated overnight at 37ºC. Following centrifugation at the WBC pellet was resuspended in 1ml of lysis buffer-II (WBC lysis buffer: 400mM
sodium chloride, 10mM Tris and 2mM EDTA) to which 65µl of detergent (10% Sodium Dodecyl Sulphate) had been added and the suspension was incubated for 3h in a water bath set at 57ºC. To this, 1ml of the organic solvent mixture (25 phenol: 24chloroform:1isoamyl alcohol) was added and the contents were mixed gently and a milky emulsion was formed after centrifugation at 4000 rpm for 15 minutes. To the aqueous phase containing DNA, 1ml of 24chloroform:1isoamyl alcohol mixture was added, and again centrifugation was carried out at 4000 rpm for 15 minutes. Chilled ethanol (3ml) was added to the supernatant and the tube containing the supernatant was gently inverted several times until the DNA precipitated. The DNA was washed with 70% ethanol, dried at room temperature and dissolved in 50µl of TE (10mMTris, 1mM EDTA) buffer.

3.17.2 Quantification of Isolated DNA by Agarose Gel Electrophoresis

Quantity of the isolated DNA (2µl) was checked on 0.8% agarose gel. The gel was prepared in TBE (Tris, Boric acid, EDTA) by heating it in a microwave oven. On cooling, the gel was poured in a casting tray in which a comb had been placed so that wells formed on the solidification of the gel and after removal of the combs. The gel was then placed in an electrophoresis chamber filled with buffer (TBE) and DNA samples (Dye=3µl and DNA=2 µl) were loaded in the wells. Following electrophoresis at 75V for 30 minutes, the gel was visualized under UV transilluminator and bands obtained were compared to that of Lambda DNA (250 ng/µl) which had been loaded in the first well as the standard marker (Plate III). Only on obtaining high molecular weight genomic DNA samples (~200ng/µl), were the samples used for amplification.

3.17.3 DNA Amplification by Polymerase Chain Reaction

Polymerase chain reaction (PCR) enables selective amplification of specific DNA sequences using forward and reverse oligonucleotide primer sequences which can be obtained from documentations in literature and in silico (www.genome.ucsc.edu/cgi-bin/hgPcr).

Variants of GST (T1, M1 and P1) (Girisha et al., 2004) and of CYP2D6 (*2, *4 and *10) (Theophilus et al., 2006) were amplified by PCR. The conditions and
requirements for PCR were obtained from the literature and are presented in table. The standard PCR mixture comprised 50 ng/µl of isolated and quantified DNA, 2.5µl 10X reaction buffer (500M KCl, 100mM Tris-HCl pH8.3, 15mM MgCl2), 1µl of 2.5mM dNTP’s, 20pmol each of forward and reverse primers and 2U of Taq DNA. The Thermal cycler used was MastercylerR Gradient (Eppendorf, Germany). On completion of the cycles for the reaction, the products were checked on agarose gel electrophoresis and gel documentations were made.

3.17.4 Multiplex PCR

Multiplex PCR is a widespread method to detect multiple deletions and duplications using many primer combinations (Markoulatos et al., 2002). For the GSTT1 and GSTM1 genes, multiplex PCR using an internal control (HLADRB1) was performed as per the method of Girisha et al. (2004). The GSTT1 amplified products of 459 bp implied GSTT1 present genotype. In the case of GSTM1 amplification, the present allele was visualized as a 206bp fragment while internal control showed as a 796bp fragment (Plate VII).

3.17.5 PCR-RFLP

For GST P1 (rs1695) and CYP2D6 (*2, *4 and 10*), the PCR-RFLP analysis was carried out. The details of the PCR mix, amplification conditions, restriction digestion and probable genotyping results are presented below:

3.17.6 Restriction Digestion

The PCR products of the GST (P1) and CYP2D6 (*2, *4 and *10) genes were subjected to restriction digestion. Each reaction mixture contained the master mix buffer, 10X BSA with one of the relevant restriction enzymes (BSmA1 for GST P1 gene, and HhaI for CYP2D6*2, BstNI for CYP2D6*4 and HphI for *10 alleles. On incubation for 16 hours in a water bath (65ºC) to complete the restriction digestion, the GST P1 products were checked on 2.00% and CYP2D6 alleles on 1.50% agarose gel. Genotyping was based on observing the restriction pattern of the amplified products.
<table>
<thead>
<tr>
<th>Gene variant</th>
<th>Table.</th>
<th>PCR-RFLP details of CYP2D6 (*2, *4 and *10) and (GST T1, M1 and P1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST T1M1</td>
<td>For GST T1</td>
<td>F 5'TTCCTTACTGTCTACATCTC 3' R 5'TCACGGGATCTAGGCCA3'</td>
</tr>
<tr>
<td>For GST M1</td>
<td>F 5'GAA CTC CTT GAA AAG CTAAG C3' R5' GTT GGG CTC AAA TAT ACG GTG G-3'</td>
<td>Restriction Details</td>
</tr>
<tr>
<td>GST P1</td>
<td>F 5‘ACCCCAAGGGCTCTATGGA3’ R5’TGAGGGCACAAGAAGCCC3’</td>
<td>Multiplex PCR</td>
</tr>
<tr>
<td>CYP2D6*2</td>
<td>F5’-GCTGCGG66GCTGAGA6CT3’ R5’-GGCTATCTACCCAGGTCGTGCT3’</td>
<td>PCR-RFLP</td>
</tr>
<tr>
<td>CYP2D6*4</td>
<td>F5’-TCGCCGCTTTCGCAACCACCT3’ R5’-TGCCCTGTCAGACTCTTCTC3’</td>
<td>PCR-RFLP</td>
</tr>
<tr>
<td>CYP2D6*10</td>
<td>F 5‘GTGCGCTGAGAAGTGCTGTCG3’ R 5’ CACCCACCACATCCATGTTTGC3’</td>
<td>PCR-RFLP</td>
</tr>
</tbody>
</table>

**Method of detection**: PCR

**Composition of reaction mixture**: Taq polymerase (10X), dNTPs (10mM), forward primer (1X), reverse primer (1X), Taq polymerase (3U/µl), DMSO (10%), Genomic DNA (50ng/µl), PCR water

**Thermal cycler conditions**: 95°C/5min, 95°C/45s, 61°C/45s, 72°C/45s, 72°C/10min, 22°C/hold

**PCR Product**: Genomic DNA (50ng/µl)
3.18 Statistical Analysis

All the statistical analysis was performed using SPSS software version 16.00 (SPSS Inc., Chicago, Illinois, USA). The data on continuous variables are presented as mean±standard error of mean (S.E.M.) and of categorical variables as numbers and percentages. The statistical significance was set at $p \leq 0.05$.

To evaluate for Gaussian/normal distribution of the data, the Kolmogrov-Smirnov test was performed (Annexure IV). Though the data did not show Gaussian distribution, but as the sample size was $\geq 30$, parametric tests (which are more robust) were performed (Garth, 2008; Fagerland, 2012).

The overall differences between patient and control groups (and the gender differences) for demographic, anthropometric, physiometric and clinical characteristics were evaluated by the Students’ t-test. The differences for biomarkers of genetic damage, oxidative stress and lipid profile were also evaluated by the Students’ t-test.

Genetic damage, oxidative stress and lipid profile parameters were also stratified according to demographic, anthropometric and disease-related variables as well as for $CYP2D6$ and $GST$ genotypes. There were also compared using the Students’ t-test.

Principal Component Factor analysis (PCA) was performed to reduce the data into principal variables that explain the maximum variability for the disease-status. The factor loading of $\geq 0.4$ and Eigen values $\geq 1$ criteria were adapted for final factor evaluation. From the various components, first the principal component combines those individual variables which in all probable linear combinations explain the maximum variance and the rotated factor matrix ($V_{\text{max}}$) explains the correlation of various risk factors for the disease under study. The Eigen values of $\geq 0.4$ are similar to 0.05 cut-off for defining significance (Sterne and Davey, 2001).

PCA was performed to evaluate the risk factors for disease-status in patients (with and without including the genetic damage parameters), for genetic damage and molecular genetic markers in patients and controls. PCA analysis was also performed on patient and control data to evaluate the most predictive gene variants for the disease-status.
The multifactor dimensionality reduction (MDR) analysis was performed to find whether any gene-gene and gene-environment combinations predict the disease status and genetic damage. The combinations with higher testing accuracy and cross validation (CV) consistency were considered as best predictor combinations.

The Pearson’s correlation, univariate followed by multivariate linear regression, and the combined multiple regression analyses were performed to find which confounding variables (singly or in combination) were significantly associated with or are the predictors of genetic damage and oxidative stress. Multiple analysis of variance (ANOVA) was also performed for understanding association of confounding variables with genetic damage end-points assessed. The association of various gene variants and genetic damage and oxidative stress parameters was also evaluated. To evaluate the odds ratio and 95% confidence interval for CYP2D6 (*2, *4 and *10) and GST (T1, M1 and P1) genotypes for disease status, logistic regression after adjustment of variables was performed.

The allelic and genotypic frequencies of CYP2D6 (*2, *4 and *10) and GST (T1, M1 and P1) were manually calculated by gene counting method and were compared by Chi-squared test between patients and controls. The genotypic frequencies were analyzed for Hardy-Weinberg Equilibrium with p>0.05 representing Hardy-Weinberg equilibrium. Odds ratio analysis was performed to find out the relative risk of genotypes for disease.

The Haploview software (http://haploview.software.informer.com/4.2/) was used to evaluate for linkage disequilibrium and haplotypic analysis. Different models of inheritance were also investigated for each gene by Web-Assotest (http://www.ekstroem.com/assotest/assotest.html).

Metaanalysis was performed to compare the genetic damage in hypertensive patients in present study with that in other studies. Also the allele frequencies of CYP2D6 (*2, *4 and *10) and GST (T1, M1 and P1) genotypes were compared with those in other populations of India by Chi-squared analysis.

### 3.19 Limitations and Shortcomings

As any hospital-based study, the present study also has certain limitations. The study group is not truly representative of the Jat Sikh population sub-group, as the target study
group belonged to the rural area. Also with a larger sample size, the power of the study could have been further increased, yet a larger sample size could not be studied because of the time-limit of the study programme. A shortcoming is that as the prevalent disease-risk factors have been identified retrospectively, causal relationships between the factors cannot be exactly explained.