MATERIALS AND METHODS

The present case-control study comprised a total of 400 subjects of which 200 were coronary artery disease (CAD) patients and 200 age- and sex-matched healthy controls belonging to the Ramgarhia Sikh population sub-group of the Amritsar city and of adjoining villages. Among the patients were 48.50% (n=97) males and 51.50% (n=103) females compared to 49.00% (n=98) males and 51.00% (n=102) females in the control group. For each of the study participants, general information, demographic details and disease-specific information along with the exposure, medical and family histories were recorded on a specially designed questionnaire and as per the recommended monitoring criteria for genetic damage assessment by an interview-cum-questionnaire based method. A three-generational pedigree was drawn and only those participants with no inter-caste marriages formed the study group.

3.1. Study Group

The Ramgarhia Sikh population sub-group traces its ethnic origin from Indo-Aryan ethnic tribe of the Punjab region in South Asia and is named after Jassa Singh Ramgarhia (the leader and founder of the Ramgarhia Misl) (www.ramgarhialko.org accessed on April 18, 2013) Ramgarhia Sikh were originally engaged in carpentry and wood work, blacksmithmanship and masoning (www.indianetzone.com accessed on April 18, 2013). The group practices endogamy (www.sikhiwiki.org accessed on April 18, 2013) and congregated collectively as Ramgarias generally practicing Sikhism (www.sikhnet.com accessed on April 18, 2013). Genetic studies on this group have included, investigations of ABO, Rh, Haptaglobin and transferrin types in Ramgarhias and Ramdasias of Punjab (Sehajpal et al., 1981), HLA class I antigen distribution in Sikh population included Ramgarhia Sikhs from Punjab (Babaita and Usha, 2004); hemoglobin variants have also been studied in North Indian populations inclusive of Ramgarhia Sikhs (Chahal and Bansal, 2005). Genotyping of glyoxalase I (GLO) polymorphism in North-west India was also carried out in the Ramgarhia Sikhs (Chahal et al., 1986) and SNPs analysis of HLA-C and CCHCR1 genes in Psoriasis patients from four different ethnic groups have included the Ramgarhia Sikhs of Punjab (Gandhi et al., 2011).
Patients- The patients were contacted from local hospitals (Sri Guru Ram Das Medical College and Hospital, Randhawa Hospital and Fortis (Escort) Hospital) in Amritsar. The attending physicians diagnosed the patients as having CAD on the basis of either/and electrocardiogram findings, echocardiography, computed tomography scan, stress-testing and angiography.

Controls- Healthy individuals from the general population belonging to the same population sub-group with neither past/present exposure history nor any recent or past illness were matched for sex, age and socioeconomic status with the patient group.

3.2. Design of the Study

A case-control study design was adopted after ethical clearance from the Institutional Ethics Committee. All participants provided details about their demographic information, family history and disease-related information on a questionnaire by the interview method. Anthropometric measurements were taken to assess general and central obesity and blood pressure measurements to provide information about presence/absence of hypertension. Venous blood samples (aliquoted with and without anti-coagulant) were processed in the laboratory for genetic damage assessment, lipid profiling, oxidative stress estimation and molecular genetic analysis using standard methodologies.

Observations were documented and results were analyzed using appropriate statistical tests. List of equipment/glassware, list of chemicals used and preparation of glassware/reagents are given in appendices III-V. The details of the methodology are explained under various sub-headings.

3.3. Sample Size Calculation and Power of the Study

The sample size for the genetic damage (per cent DNA in tail) and oxidative stress (TOS) was calculated using the following formula (Albertini et al., 2000) of \( N = 2 \times \sigma^2 (Z_{\alpha} + Z_{1-\beta})^2 / D^2 \) where \( Z_{\alpha}, Z_{1-\beta}, \sigma \), an standard deviation (estimated), and D is the difference in means of per cent DNA in tail and oxidative stress. Taking \( p < 0.05 \) as acceptable error and 80% power and using 1.96 for \( Z_{\alpha} \) and 0.8416 for \( Z_{1-\beta} \). The standard deviation (based on the data from a pilot study on Ramgarhia Sikhs. For difference in means, an increase
from the mean (pilot study) significant at 0.05 was calculated was considered. The effective sample size therefore on basis of per cent DNA in tail and TOS was calculated as 38 and 23 respectively. However 200 samples each of patients and controls were investigated and the power of the study was 84% at 5% level of significance. The 3-5% prevalence of CAD in Punjab (Gupta et al., 2008) requires 175 individuals and based on allele frequencies of the seven gene variants obtained from SNPedia and literature, the calculated effective size was from 93-172. Hence a size of 175 participants each was considered optimal for cases and controls of the present study. An additional 10% contingent on account of drop-outs was added and rounded-off to 200 cases and 200 controls for the present study.

3.4. Ethical Clearance and Informed Consent

The study was approved by the Institutional Ethics Committee (IEC) of Guru Nanak Dev University. All the participants were explained the nature of the research problem and those who voluntarily gave written informed consent (Appendix I) formed the study group.

3.5. Designing of Questionnaire and Collection of Information

A detailed questionnaire was designed (Appendix II) after studying the relevant literature thoroughly. Details of demography, lifestyle and disease-relevant information were documented therein and other records were also maintained as per the recommendation of Carrano and Natarajan (1988) using a face-to-face interview method singly with the study participants.

3.6. Inclusion and Exclusion Criteria

3.6.1. Inclusion Criteria

Patients above 35 years of age and with electrocardiographic (ECG) changes and with echocardiographic evidence of myocardial infarction (MI) and positive treadmill test as diagnosed by any of the attending physicians were included in the study. Healthy
persons from the general population with no present or past history of CAD or any other chronic disease or exposures were identified as the control group. All participants were unrelated *Ramgarhia Sikhs* with no inter-caste marriages up to three generations.

### 3.6.2. Exclusion Criteria

Patients with negative treadmill test and with renal, liver, lung and thyroid or any other disease and those receiving lipid-lowering drugs were excluded from the study. Relatives of patients and controls were not included among the study participants.

### 3.7. Physical Activity

Patients and controls were categorized for physical activity (self reported) into four categories viz; sedentary, light, moderate and vigorous.

### 3.8. Socioeconomic Status

The socioeconomic status of the study participants was calculated on the basis of education, occupation and monthly income (Kumar, 2012). The scores for education (7 for professional or honours, 6 for graduate or post graduate, 5 for intermediate or post-high school diploma, 4 for high school, 3 for middle school certificate, 2 for primary school and 1 for illiterate), occupation (10 for professional, 6 for semi-professional, 5 each for clerical job, shop owner and farmer, 4 for skilled worker, 3 for semi-skilled worker, 2 for unskilled worker and 1 for unemployed) and monthly family income in rupees (12 for \( \geq 32050 \) Rs, 10 for \( 16020 – 32049 \) Rs, 6 for \( 12020 – 16019 \) Rs, 4 for \( 8010 – 12019 \) Rs, 3 for \( 4810 – 8009 \) Rs, 2 for \( 1601 – 4809 \) Rs and 1 for \( \leq 1600 \) Rs) were used to calculate a total score which was then used for categorization as belonging to upper-I (26-29), upper-middle-II (16-25), middle/lower-middle-III (11-15), lower/upper-lower-IV (5-10) and Lower (V) (5) socioeconomic status.

### 3.9. Anthropometric Measurements for Obesity and Nutritional Assessment

Height, weight, waist circumference and hip circumference measurements were taken according to standard methodology, using an anthropometric rod, weighing scales and
steel tape, respectively (Weiner and Lourie, 1981). General and central obesity was then assessed.

*Height*- Participants stood upright without shoes with their heels joint, buttock and upper back in contact with the wall and head in Frankfurt Horizontal (F-H) plane. The anthropometric rod was positioned in mid-sagittal plane of the subject and the horizontal movable arm of the anthropometric rod was brought to touch the vertex after the subject had stretched up to his/her full height and taken a deep breath. Height was recorded to the nearest 0.1 cm.

*Weight*- The participants stood in the centre of the weighing machine without shoes and the weight was noted to the nearest 0.5 kg.

*Waist Circumference*- Waist circumference (WC) was measured using a non-stretchable measuring steel tape. The participant was asked to stand erect in stress-free position, arms at the side and both feet together. Waist circumference was measured as a smallest horizontal circumference between the costal limits and the iliac crest at the end of normal expiration.

*Hip Circumference*- Hip circumference (HC) was taken as the greatest circumference at the level of greater trochanter on both sides. The measurement was made to the nearest 0.1 cm.

*General Obesity and Nutritional Status*- Body Mass Index (BMI) was used to assess general obesity and nutritional status using the criteria of WHO (2004) for Asian Indians and also as given by Misra *et al.* (2009). BMI was calculated as weight in kilograms divided by height in meters squared (m\(^2\)). BMI of <18.5 kg/m\(^2\) implies underweight, BMI between 18.5-22.9 kg/m\(^2\) is normal, BMI between 23.00-24.90 kg/m\(^2\) is considered as overweight and ≥ 25 kg/m\(^2\), obese.

*Abdominal Obesity*- Waist circumference (WC), Waist-hip-ratio (WHR) and waist-to-height-ratio (WHtR) were used for assessing abdominal/central obesity.

WC - Using the gender specific WC cut-offs (Snehalatha *et al.*, 2003), male participants were categorized obese if WC ≥ 80 cm and females with WC ≥ 85 cm.
Waist-Hip-Ratio- Waist-hip-ratio (WHR) is an indicator of central obesity and is calculated as waist circumference (cm) divided by hip circumference (cm). According to Snehalatha et al. (2003), WHR cut-off values for females and males are ≥0.81 cm and ≥0.85 cm, respectively.

Waist-to-Height Ratio- Waist-to-height ratio (WHtR) is also an indicator of abdominal obesity and was calculated as waist circumference (cm) divided by height (cm). Individuals with WHtR ≥0.5 are considered as obese (Hsieh and Muto, 2005).

3.10. Physiometric Measurements for Hypertension Status

Blood pressure measurements were taken (after the participants had relaxed for about ten minutes) from the left arm with the help of a mercury sphygmomanometer and a stethoscope as per the recommendations of I.G.H-III (2013). Three concurrent readings were taken at 5-10 minutes interval and their averages were considered as systolic and diastolic blood pressure (SBP, DBP) values. On the basis of blood pressure readings, participants were classified as normotensive or hypertensive as per Indian Hypertensive guidelines III (I.G.H III, 2013) and the JNC 7 guidelines (Chobanian et al., 2003). The subjects with SBP <120 mmHg and DBP <80 mmHg were considered as normotensive; those with SBP 120-139 mmHg and DBP 80-89 mmHg were considered as pre-hypertensive, SBP of 140-159 mmHg and DBP 90-99 mmHg includes the hypertensive stage I and those with SBP ≥ 160 mmHg and DBP ≥ 100 mmHg are in the hypertensive stage II category.

Derived Blood Pressure Parameters- Pulse pressure (PP) and mean arterial pressure (MAP) were also determined. PP was obtained by subtracting DBP from SBP while MAP was calculated by using a formula (MAP = DBP + (SBP-DBP)/3) as given by Pérusse et al. (1989).

3.11. Metabolic Syndrome

Occurrence of metabolic syndrome in the study group was assessed on the basis of the following criteria:
1. The NCEP–ATP-III guidelines (2001) - Existence of any three of the following traits in the same individual would meet the criteria for metabolic syndrome (MS) with slight modification for the obesity parameter using WC cut-off for Asian Indians.
   
a. Abdominal obesity as defined by WC ≥80 cm in men and ≥85cm in women (as per Snehalatha et al. (2003) for Asian Indians).
   
b. Serum triglycerides ≥160 mg/dl
   
c. High density lipoprotein cholesterol <30 mg/dl in males and <35 mg/dl in females
   
d. Blood pressure levels of ≥130/85 mmHg

2. International Diabetes Federation (IDF, 2005) - Abdominal obesity as defined by WC should be considered compulsory attribute in addition to any of the other two features in ATP-III guidelines for the MS criteria.

3.12. Blood Sample Collection

Venous blood sample (~5ml/individual) was obtained by a certified technician; it was aliquoted and transferred to the laboratory for analysis. About 3ml of blood sample was transferred to a vial containing 30µl of 0.5M EDTA for molecular genetic analysis, 0.5µl was transferred into a heparinized vial containing sodium heparin for genetic damage assessment. About 2ml of blood was transferred to a tube without any anticoagulant to separate the serum for use in biochemical and oxidative stress analysis. The samples were transported to the laboratory in an ice-box. The single cell gel electrophoresis (SCGE/comet) assay was performed within 3-4 hours of sample collection while the residual blood and blood sera samples (after separation) were stored at -20°C and -80°C for molecular and biochemical analyses, respectively. The blood samples from both patients and controls were withdrawn and processed on the same day.

3.13. Genetic Damage Assessment

Genetic damage was assessed using the alkaline single cell gel electrophoresis (SCGE/Comet) assay as per the protocol of Singh et al. (1988) and as modified by Ahuja and Saran (1999) on the samples which had been checked for cell viability.
Cell Viability Test - The cell viability of each blood sample was assessed with the Trypan-Blue Dye exclusion test (Coligan et al., 1997). It is based on the principle that viable cells possess intact cell membranes which exclude the dye and so remain unstained whereas non-viable cells do not possess an intact cell membrane and so get stained. For this, a mixture of Trypan-Blue (100µl) and blood (50µl) was poured into a tube containing 850µl of phosphate buffer saline (PBS), mixed thoroughly, and kept in the refrigerator (4°C) for 10 minutes. Then ~30µl of the cell mixture was placed over a haemo-cytometer and the number of stained and unstained cells were separately counted. Cell viability was calculated by dividing the number of stained cells with the total number of stained and unstained cells. All the samples had ≥90% cell viability and so were processed for the SCGE assay (Kleeberger et al., 1999).


The SCGE assay is a simple technique for assessing DNA damage in peripheral blood samples. The principle of the assay is that cells embedded in agarose gel, get lysed by detergents under high salt concentration and form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. On electrophoresis (pH≥13), loosening of supercoiling occurs and the strands become free and extend towards the anode, resembling comets (Tice et al., 2000; Collins et al., 2008).

DNA damage in peripheral blood leukocytes (PBL) of patients with coronary artery disease (CAD) and healthy controls was evaluated both, by the alkaline single cell gel electrophoresis (SCGE) assay (Singh et al., 1988) and the enzymatically-modified SCGE (Collins, 2004), the latter is more specific and sensitive and uses lesion-specific enzymes to check for apurinic/apyrimidinonic sites. Oxidized pyrimidines are assessed by Endonuclease III (Endo III) and oxidized purines with formamidopyrimidine DNA glycosylase (Fpg) as per the standard procedure (Collins et al., 1993; Dusinska and Collins, 2008).

The methodology for both, standard DNA damage and oxidized DNA damage assessment was same except for inclusion of enzymatic treatment in the latter. For each sample, two slides for the standard assay and six slides for the enzymatic assay (8 slides/sample) were prepared. Agarose coated slides were used instead of frosted slides.
(Kalantari et al., 2013) and silver staining of nucleoids instead of staining with ethidium bromide (Nadin et al., 2001; Gonzalez et al. 2012) was carried out. The steps of the SCGE assay include:

**Preparation of First Layer** - Washed and cleaned glass slides were dipped (two third) in 1% molten normal melting point agarose (NMPA). Their undersurface was cleaned with tissue paper in order to remove the agarose. The slides were kept at 37°C to allow the gel to set and then stored in a dust-proof box till needed.

**Preparation of Second Layer** - For this 110µl of 0.5% of low melting point agarose (LMPA) was mixed with 20µl of whole blood and then the mixture was poured on the pre-coated slides. Coverslips were then placed and the slide preparations were kept at 4°C for 15-20 minutes to enhance gelling of agarose.

**Preparation of Third layer** - For the third layer, the coverslips were removed gently and a third layer of agarose (120µl 1% LMPA) was poured and then coverslips were placed again over it. The slides were kept for 15-20 minutes at 4°C for allowing the gel to set.

**Lysis Treatment** - The coverslips were removed gently and slide preparations were placed for two hours at 4°C in chilled lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris buffer, 1% Triton X-100, 10% DMSO, NaOH pellets 12gm; pH 10) containing high salts and detergents. This was followed by 2-3 washings with deionized water. For the enzymatic treatment, after the lysis-treatment, and three washing (5 min each) with enzymatic buffer, the slides were incubated with Endo III and FPG for oxidative damage assessment.

**Alkaline \((pH≥13)\) Treatment** - For the next step, the slides were kept in an electrophoresis buffer (0.3MNaOH, 1mM EDTA; pH≥13; 4°C; 25-40 minutes) for unwinding and to release alkali-labile sites (ALS) and single-strand breaks (SSB).

**Electrophoresis** - After alkaline treatment, the slides were electrophoresed at 300mA, 25V for 25minutes, current was adjusted to 300mA and 25 Volts (0.70 V/cm) by adjusting the level of buffer in the chamber.
Neutralization - After electrophoresis, the slides were taken out from the electrophoretic chamber, were placed on a flat surface and then treated with the neutralization buffer (0.4M Tris; pH 7.5) for ~10 minutes to remove alkali salts and detergents. Then the slides were kept overnight for air-drying.

Silver Staining - Staining was performed as per the protocol given by Nadin et al. (2001). For this, slides were treated with solution ‘A’ (15% w/v tricholoroacetic acid, 5% w/v zinc sulphate, 5% glycerol) for ten minutes and then were washed three times with distilled water and were kept for air-drying. After drying, solution ‘D’ was prepared by mixing 34 ml of solution ‘B’ (0.5% w/v sodium carbonate) and 66 ml of solution ‘C’ (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5%w/v tungstosilicic acid, 0.15% v/v formaldehyde) and was poured over the slides placed in a staining tray. The tray was gently and constantly shaken with more solution added till the development of color. After staining, slides were given washing with distilled water and then placed in the stopping solution ‘E’ (1% glacial acetic acid) for ten minutes followed by again washing with distilled water and then kept for air-drying. After drying, the slides were coded and kept in a moist and dust-free chamber till scoring.


The modified comet assay was performed according to the method given by Collins et al. (1993) for the detection of oxidized purine and pyrimidine bases by using bacterial enzymes (formamidopyrimidine DNA glycosylase, ‘Fpg’ and endonuclease III ‘Endo III’). Fpg recognizes oxidized purines while Endo III recognizes oxidized pyrimidines. For each individual, six slides were prepared (two each to be treated with buffer, Endo III and Fpg). After the lysis step of the standard assay, slides were given washings with the enzyme reaction buffer (40mM HEPES buffer, 100mM KCl, 0.5mM EDTA and 0.2mg/ml bovine serum albumin) at room temperature and two slides preparations treated with 50 µl Endo III (1:1000; one part enzyme and 1000 parts of buffer), two slide preparations treated with 50 µl FPG (1:3000) and two slides treated with the enzyme reaction buffer (controls for enzyme-treated preparations) were incubated at 37°C for 45min. After enzyme treatment, the slides were given three washings with
distilled water. Then the remaining steps (alkaline treatment, electrophoresis, neutralization, and staining) were carried out similarly as for the standard SCGE assay.

3.14.1. Genetic Damage End-points Scored

Both visual scoring and image analysis software were used to score for genetic damage.

3.14.1.1. Scoring of Slide Preparations- Coded slides were scored blind both by manual scoring and using an image analysis system. For each individual, manual scoring was carried out for DNA migration length and the calculation of damage index and damage frequency. Per cent DNA in tail, Olive tail moment and tail moment were recorded using image analysis software. For the standard SCGE assay, per individual 100 nucleoids (50/slide) were scored, by the manual method and by image analysis. For the modified SCGE assay, six slides were scored per individual (2 per treatment) with 50 nucleoids scored per slides both by the manual and image analysis methods.

3.14.1.2. Visual/Manual Scoring- Visual scoring was performed as per the method of Collins (2004). Nucleoids were scored as they came into view and the DNA migration length was measured for 50 nucleoids per slide (100/sample). Visual grading of nucleoids into various classes (0-IV) was done depending on the intensity (amount of DNA) and migration length of the tail as measured with the help of an ocular-micrometer placed inside the eyepiece of a binocular microscope which had been calibrated with the stage micrometer. Nucleoids were categorized into five classes (Collins, 2004), class 0 (nucleoids with no tails), class I (tail with 1.5 times the diameter of the nucleoids), class II (1.5 to 2.0 times the diameter of the nucleus), class III (2.0 to 2.5 times the diameter of the nucleus) and class IV (≥2.5 times the diameter of the nucleus). Damage Index (DI) was calculated (Collins et al., 1995) as:

\[ DI = (0 \times \text{cells in class 0} + 1 \times \text{cells in Class I} + 2 \times \text{cells in class II} + 3 \times \text{cells in class III} + 4 \times \text{cells in class IV}) \]

DI is presented as arbitrary units (AU) which can vary from 0-400 for each sample depending on number of nucleoids in different categories (Plate Ia,b).

DNA migration length (µm)- This is the extent of the damaged DNA migrated from nucleus towards the anode under alkaline condition thus indicating the level of DNA breakage in the cell and is calculated as mean length of 100 nucleoids for each sample.
Damage Frequency (DF)- It is the per cent measure of nucleoids with tails (Lovell and Omori, 2008).

3.14.1.3. **Image Analysis**- For each slide, nucleoids were digitally-captured on a camera (Jenoptik, Progress capture pro CT5) mounted on a binocular microscope (Olympus BX43, Japan) and were scored with the help of CASP (Comet Assay Software Programme; Version 1.2.3beta1; www.casplab.com). The genetic damage end-points recorded were per cent DNA in tail, tail moment and Olive tail moment (Kumaravel and Jha, 2006). Tail intensity (per cent DNA in tail) is Percent of DNA in the comet tail. Tail moment (TM) is tail per cent DNA in tail x tail length. Olive tail moment (OTM) is per cent DNA in tail x (Tail Mean X – Head Mean X) ([per cent DNA in tail] x [distance between the centre of gravity of DNA in the tail and of centre of gravity of DNA in the head in X-direction]).

3.15. **Biochemical Analysis**

This comprised assessing oxidative stress biomarkers (levels of malondialdehyde, total oxidant status and total antioxidant status) and lipid profiling of blood sera samples. Serum was separated from clotted blood samples (not treated with any anticoagulant) and which had been stored at -80°C till analysis (which was within two months of blood sample collection).

3.15.1. **Assessment of Lipid Peroxidation and Oxidative Stress Parameters**

*Estimation of Serum Malondialdehyde*- Malondialdehyde (MDA) levels were determined by the protocol of Buege and Aust (1978). Malondialdehyde results from lipid peroxidation as free radicals reacts with thio-barbituric acid (TBA) and TBA develops a pink-coloured product, the absorbance of which is measured at 532nm.

TBA-TCA-HCl solution was prepared on volume by volume basis (15% trichloro acetic acid, 0.375% thio-barbituric acid and 0.25 N hydrochloric acid) and 2ml of working reagent was added each into two pre-labeled 15ml centrifuge tubes followed by the addition of distilled water (1ml) into a blank tube and 1ml of serum sample into the test-labeled tube. Careful mixing of content was followed by heating at 100°C for 15 min. After cooling, centrifugation was done at 3000 rpm for 10 min and the absorbance of
the coloured supernatant was measured at 532nm against a blank (Perkin Elmer Spectrophotometer Lambda 25). The malondialdehyde concentration was calculated by using its extinction coefficient (1.56*10^5 M^-1 cm^-1).

Estimation of Total Oxidant Status (TOS)- In 225µl of reagent I (xylene orange 150 µM, NaCl 140mM and glycerol 1.35M in 25mM H_2SO_4 solution, pH 1.75) was added in wells of a flat-bottom microplate followed by addition of 5µl of serum sample. The first absorbance was taken at 570 nm against blank (reagent I without serum) and incubation at 37°C for 10 min. Then 11µl of reagent II (Ferrous ammonium sulphate 5mM and O-dianisidine 10mM in 25mM H_2SO_4 solution) was added and the contents were kept at 37°C for 10 min. The second absorbance was then taken against blank (reagent I +reagent II without serum) at 570nm. The absorbance of the standard (reagents I, II and 35µl of 20mmol/L hydrogen peroxide) was also taken on microplate reader (Erel, 2005).

Estimation of Total Antioxidant Status (TAS)- As per the protocol given by Erel (2004), to 200µl of reagent I was mixed 5µl of serum sample in a microplate which was incubated at 37°C for 10 min. Absorbance was taken at 660nm against blank (reagent I without the serum sample). Then 20µl of reagent II was added and it was kept at 37°C for 10 min. Then absorbance was again taken at 660nm for the sample and for the standard (reagents I, II and 5µl of Trolox).

TOS levels were calculated from the absorbance and concentration was calculated (Gupta et al., 2009) and expressed as micromolar H_2O_2 equivalent per liter (µmol H_2O_2 equivalent/l).

The levels of TAS (mmol trolox equivalent/l) and TOS (µmol H_2O_2 equivalent/l) were obtained by using the following formula given by Gupta et al. (2009).

\[ \text{Difference in absorbance of blank (ΔA blank)} = \text{Final absorbance of blank} - \text{Initial absorbance of blank.} \]

\[ \text{Difference in absorbance of standard (ΔA standard)} = \text{Final absorbance of standard} - \text{Initial absorbance of standard.} \]

\[ \text{Difference in absorbance of sample (ΔA sample)} = \text{Final absorbance of sample} - \text{Initial absorbance of sample.} \]
Factor = Concentration of the standard/(ΔA blank - ΔA standard)

Levels of TAS and TOS = Factor* (ΔA blank - ΔA sample)

Oxidative Stress Index (OSI) - The ratio of TOS to TAS (both in μmol H₂O₂ equivalent/l) indicates the oxidative stress index and it has arbitrary units (Aycicek and Erel, 2007).

3.15.2. Lipid Profiling

Lipid and lipoprotein levels were determined for each participant from blood serum preparations to ascertain for dyslipidemia. This comprised determining levels of total cholesterol (TC), triglycerides (TG) and high density lipoproteins (HDL-C) and the derived variables of low density (LDL-C) and very low density lipoprotein (VLDL). Atherogenic indices of TC/HDL-C, LDL/HDL-C and TG/HDL-C were also determined.

Determination of Total Cholesterol Levels - The use of standard kits was made and the given reagent was reconstituted by adding 20ml of deionized water to dissolve it. To 500μl of reagent in a tube, 10μl of serum sample was added, mixed well and incubated at 37°C for 10 min. The standard (10μl) was also taken and mixed with 500μl of reagent while a blank was also prepared (10μl of distilled water was added in to 500μl of reagent) and was similarly incubated. The concentration of TC (mg/dl) was obtained as mg/dl on a semi automated analyzer (Erba-Chem7, Germany) at 505nm.

Determination of Triglycerides- The reagent given with the kit was reconstituted by addition of 20ml of deionized water, and to 500μl of reagent, 5μl of serum sample was added followed by its incubation at 37°C for 10 min. For the standard, 5μl was taken, along with 500 μl of reagent and it was also kept for incubation at 37°C for 10 min. In the blank, distilled water (5 μl) was added to the reagent (500 μl). TG concentration was obtained as mg/dl at 505nm after 10min incubation (37°C.).

Determination of HDL-Cholesterol Levels– For this according to the kits instructions, to 500μl of precipitating reagent was added 250μl of serum sample and the mixture was
kept at 15-30°C for 15 min followed by its centrifugation (4000 rpm for 10 min) to obtain a clear supernatant. In 25µl of supernatant, 500µl of total cholesterol reagent was added, mixed gently and kept at 37°C for 10 min. The standard and blank were prepared as for TC and TG determination. The absorbance was taken on the semi-automated analyzer at 505-670 nm. HDL-C concentration in mg/dl was determined/obtained on the analyzer.

**Determination of Other Lipoproteins and the Atherogenic Indices** – LDL-C and VLDL were calculated using Friedewald equation (Friedewald et al., 1972). LDL-C was determined as LDL-C= TC - HDL - TG/5.0 and VLDL = TG/5. Atherogenic indices are ratios of TG/HDL-C, LDL-C/HDL-C and TC/HDL-C (Ugwuja et al., 2013). These were determined for each sample as they provide better risk estimates of CVD (Dobiasova, 2006).

### 3.16. Molecular Genetic Analysis

Genotyping of phase I (*CYP2D6*2, *4 and *10) and phase II (*GSTM1, GSTM1, GSTP1* (A313G) and *GSTP1* (C341T) genes was carried out and the steps included genomic DNA isolation and its quantification followed by DNA amplification and restriction digestion and analysis of restriction fragment length polymorphism.

**DNA Isolation from Whole Blood** - Genomic DNA was isolated by inorganic method given by Miller et al. (1988). For this, to 1ml of whole blood was added 3ml of 1X lysis I buffer (pH 8.0) in a 15ml-centrifuge tube, shaken vigorously for 15 min, incubated in ice (15 min) and again shaken vigorously (15 min) followed by centrifugation at 2500 rpm for 15 min. The supernatant was discarded and 3ml of 1X lysis I buffer was added to the pellet and these steps were repeated till a dirty white pellet (WBCs) was obtained. The pellet was shaken and 1ml of 1X lysis II buffer (pH 8.0) and 57µl of 10% SDS (pH 7.2) were added, mixed gently avoiding frothing and incubated at 37°C overnight. The contents in the tube were brought to room temperature and 500µl of ammonium acetate was added and mixed gently for 15 min to make a suspension followed by centrifugation at 4000 rpm for 15 min. To the supernatant along the sides of the tube 2.5ml chilled ethanol was added which precipitated the DNA. The contents were centrifuged (3,000 rpm for 1 min), the supernatant was discarded and the DNA was
washed twice with 70% ethanol. After repeating centrifugation at 3,000 rpm (1 min), the supernatant was decanted and the pellet was air-dried. The dried pellet was then dissolved in 75µl of TE buffer (pH 8.0), and incubated overnight at 37°C for dissolving the DNA completely. DNA samples were then stored at -20°C till further use.

Quantification of DNA Samples - DNA samples were quantified according to the protocol of Sambrook et al. (1989) by equating the fluorescence with λ-DNA of known concentration (250ng/µl) on agarose gel electrophoresis. From the sample stock DNA, 2µl of DNA was taken, mixed with 3µl of 6X gel-loading buffer and loaded in a well on the gel-casting tray. In the first well, 2µl of λ-DNA (250ng/µl) mixed with 3µl of 6X gel-loading buffer was loaded. Electrophoresis was carried out at 100V and 300mA for 30 min followed by removal of the gel for observance under UV trans-illuminator. The gel was digitally captured and concentration of each DNA sample was assessed by relating its fluorescence with that of the λ-DNA of known concentration (250ng/µl) loaded in the first well (Plate II).

Dilution of DNA Samples - Each DNA sample of known concentration was diluted with sterile water to make a final concentration of 50ng/µl; the samples were stored at 4°C till further use.

3.17. DNA Amplification

The DNA samples were amplified using specific set of primers for amplification of each allelic variant. This required perusal of literature followed by conformation of primer sequences by blast as well as by in silico PCR analysis. Other steps included dilution of primers, setting-up of polymerase chain reaction (PCR) and restriction digestion. The products at each step were resolved under agarose gel electrophoresis. The glutathione S-transferase (GST) four gene variants (GSTT1, GSTM1, GSTP1 A313G and GSTP1 C341T) and the Cytochrome P450 (CYP2D6) three variants (CYP2D6*2, CYP2D6*4 and CYP2D6*10) were genotyped.
## Details of the Primer Sequences and other Relevant Information

<table>
<thead>
<tr>
<th>Gene</th>
<th>5' Primer sequences 3'</th>
<th>Product Size (bp)</th>
<th>Restriction Enzyme</th>
<th>Restriction site</th>
<th>Reference</th>
</tr>
</thead>
</table>
| GSTT1    | F-GAA CTC CCT GAA AAG CTA AAG C  
R-GTT GGG CTC AAA TAT ACG GTG A | 459 bp            |                    |                                                      | Girisha et al., 2004              |
| GSTM1    | F-TT CCT TAG TGG TCC TCA CAT CTC  
R-TCA CCG GAT CAT GGC CAG CA | 209 bp            |                    |                                                      |                                   |
| HLADR B1 | F-TGC CAA GTG GAG CAC CCA A  
R-GCA TCT TGC TCT GTG CAG AT | 796 bp            |                    |                                                      | Vetriselvi et al., 2006           |
| GSTP1 (A313G) | F-CAG CAG AAG CAG CGT GTG TGC  
R-CCC ACA ATG AAG GTC TTG CCT CC | 539 bp            | BsmA1              | 5'…GTCTCN/NNNN…3'  
3'…CAGAGNNNNNN/…5' | Vedyakov and Tonevistski, (2006) |
| GSTP1 (C341T) | F-ACC CCA GGG CTC TAT GGG AA  
R-GCA TCT CAC AAG AAG CCC CT | 176 bp            | Acil               | 5'…C/C/GGC…3'  
3'…GGC/G…5' |                                   |
| CYP2D6*2 | F-GCT GGG GCC TGA GAC TT  
R-GGT TAT CAC CAG GTG CTG GTG CT | 1029 bp           | Hha1               | 5'…GCG/C…3'  
3'…C/GCG…5' | Theophilus et al., 2006           |
| CYP2D6*4 | F-TGC CGC CTT CGC CAA CCA CT  
R-TCGCCCTGCAGAGACTCCTC | 309 bp            | BstN1              | 5'…CC/WGG…3'  
3'…GGW/CC…5' |                                   |
| CYP2D6*10 | F-GTGCTGAGAGTGTCCTGCC  
R-CAACCACCATCCATGTGTT GC | 345 bp            | Hph1               | 5'…GGTGANNNNNNNN/…3'  
3'…CCACTNNNNNNN/N…5' |                                   |
For the \textit{GSTT1} and \textit{GSTM1} genotypes, multiplex PCR (Girisha \textit{et al.}, 2004) was performed using \textit{HLADRB1} as an internal control (Vettriselvi \textit{et al.}, 2006). The GSTP1 (\textit{GSTP1 A313G} and \textit{GSTP1 C341T}) and CYP2D6 (\textit{CYP2D6*2}, \textit{CYP2D6*4} and \textit{CYP2D6*10}) genotyping was carried out using PCR-RFLP as per Vedyakov and Tonevitskii, (2006) and Theophilus \textit{et al.} (2006), respectively.

\textit{Dilution of Primers}–The vial containing lyopholysed primers supplied by the manufacturers was centrifuged for 5-10 sec allowing the contents to settle at the bottom. Stock solutions (100pmol/µl) were prepared in deionized water as per the given instructions and stored at -20°C. Working primers (10pmol/µl) were prepared using deionized water and kept at 4°C for further use.

\textbf{3.17.1. Screening for \textit{GSTT1} and \textit{GSTM1} Polymorphisms}

\textit{Setting-up of Multiplex PCR} – For each reaction, a 15 µl reaction mixture was prepared. The reaction mixture contained 1.5 µl of 10X reaction buffer (15 mM MgCl$_2$), 0.3 µl of dNTPs mixture (2.5 mM of each dNTP), 0.5 µl (10 pM/µl) of each of the three forward and reverse primer sets (\textit{GSTT1}, \textit{GSTM1} and Intron 3 of Major Histocompatibility Complex, Class II, DR Beta 1 (\textit{HLADRB1}) which was used as an internal positive control), 0.25 µl (3U/µl) of \textit{Taq} DNA polymerase, 0.5 µl (50 ng) of genomic DNA and 9.45µl deionized water. These contents were thoroughly mixed and the tubes were centrifuged for 10-15 sec and placed in thermal-cycler for carrying out amplification. The standardized amplification conditions were initial denaturation at 95°C for 5 min followed by 33 cycles of denaturation at 95°C for 45 sec, annealing at 61°C for 30 sec, extension at 72°C for 45 sec, and final extension at 72°C for 10 min. The reaction was carried out for 1h 21 min.

\textit{Loading and Analysis of Amplified PCR Products} - Using 2% agarose gel stained with ethidium bromide (0.5%), the PCR products were subjected to electrophoresis (100V, 45 min). The 100bp ladder was used as a marker. The amplified products were visualized under UV trans-illuminator and photographed. Fragments of 796bp, 459bp and 209bp mark the presence of Intron 3 of \textit{HLADRB1}, \textit{GSTT1} and \textit{GSTM1}, respectively. In the case of null mutations of \textit{GSTT1} and \textit{GSTM1}, the respective products of 459bp and 209bp are not obtained (Plate III).
3.17.2. Screening for GSTP1 (A313G; rs1695, Ile105Val) Polymorphism

Setting of PCR- The reagents of the reaction mixture (15 µl) included 1.5 µl of 10X reaction buffer (15 mM MgCl₂), 0.4 µl of dNTPs mixture (2.5 mM of each dNTP), 0.4 µl (10 pM/µl) of each of the two primers (forward and reverse), 0.3 µl (3U/ µl) of Taq DNA polymerase, 0.6 µl (50 ng) of genomic DNA and 11.4µl deionized water. A negative control was prepared by adding only PCR reagents without DNA sample. The tubes were centrifuged for 10-15 sec and placed in a thermal-cycler with the standard amplification conditions of: initial denaturation at 95°C for 5 min followed by 33 cycles of denaturation at 95°C for 45 sec, annealing at 61°C for 30 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min.

Restriction Digestion Reaction- The amplified PCR product of the expected size (176bp) was checked on 2% agarose gel electrophoresis. Restriction digestion was carried out with 3U of the BsmA1 on the amplified PCR product by incubating at 55°C for overnight. Restricted products were resolved on 3% agarose gel stained with ethidium bromide (Plate IV). The absence of restriction site at rs1695 position indicated wild type genotype (AA; 176bp), while three fragments indicated heterozygous genotype (AG; 176bp + 95bp+ 81bp) and two fragments which indicated the homozygous mutant genotype (GG; 95bp + 81bp). The substituted amino acid (Val) acts by abolishing enzyme activity.

3.17.3. Screening for GSTP1 (C341T; rs1138272 Ala114Val) Polymorphism

Setting of PCR- In 15 µl of the reaction mixture, the ingredients were 1.5 µl of 10X reaction buffer (15 mM MgCl₂), 0.4 µl of dNTPs mixture (2.5 mM of each dNTP), 0.4 µl (10 pM/µl) of each of the two primers (forward and reverse), 0.3 µl (3U/ µl) of Taq DNA polymerase, 0.6 µl (50 ng) of genomic DNA and 11.4 µl deionized water. These were mixed and centrifuged for 10-15 sec (3000 rpm) before placing in the thermal-cycler. The amplification conditions had been initially standardized and included initial denaturation at 95°C for 5 min followed by 33 cycles of denaturation at 95°C for 45 sec, annealing at 63°C for 30 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min and the complete duration of the cycle was 1h 21min.
Restriction Digestion Reaction- On 2.5% agarose gel, the products were checked for amplification followed by restriction digestion (3U of Acii) overnight at 55°C. Restricted products were resolved on 2.5% agarose gel stained with ethidium bromide (Plate V). The presence of restrictions sites resulted in three fragments indicating wild type genotype (CC; 365bp+120bp+54bp), while four fragments indicated heterozygous genotype (CT; 485bp+365bp+120bp+54bp), and with only two fragments indicating the homozygous mutant genotype (TT; 485bp+54bp). In the mutant genotype, the restriction sites are abolished and so restriction digestion leads to only two fragments of sizes 485bp and 54bp.

3.17.4. Genotyping of CYP2D6 Polymorphisms

For genotyping of CYP2D6 gene variants, the PCR-RFLP methods as given by Theophilis et al. (2006) for the CYP2D6*2, CYP2D6*4 and CYP2D6*10 allelic variants were followed.

3.17.5. Screening for CYP2D6*2 (A2850G; rs16947) Polymorphism

PCR and Restriction Digestion - For amplification of CYP2D6*2, the reaction mixture containing, 1.5 µl of 10X reaction buffer (15 mM MgCl2), 0.3 µl of dNTPs mixture (2.5 mM of each dNTP), 0.4 µl (10 pM/µl) of each of the two primers (forward and reverse), 1.5 µl DMSO (10%), 0.2 µl (3U/ µl) of Taq DNA polymerase, 0.3 µl (50 ng)of genomic DNA and 10.4µl deionized water, was centrifuged for 10-15 sec and then placed in a thermal-cycler. The previously standardized amplification conditions included initial denaturation at 95°C for 5 min followed by 33 cycles of denaturation at 95°C for 45 sec, annealing at 69°C for 30 sec, extension at 72°C for 45 sec and the final extension at 72°C for 10 min. The reaction took 2h 20 min to complete.

The presence of the amplified product (1029bp) was checked on 2% agarose gel under electrophoresis. Restriction digestion was carried out with 3U of restriction enzyme (HhaI) at 37°C for overnight and the restricted products were resolved under electrophoresis on agarose gel (2.7%) stained with ethidium bromide (Plate VI). The presence of five restriction fragments indicated wild type genotype (AA; 414bp+ 372bp+111bp+91bp+41bp), while six fragments indicated the heterozygous genotype...
(AG; 786bp+414bp+372bp+111bp+91bp+41bp) and the presence of four fragments was indicative of the homozygous mutant genotype (GG; 786bp+111bp+91bp+41bp).

The Hha1 restriction enzyme recognizes four restriction sites in the wild type genotype of the exon 5 region of the CYP2D6*2 allele. This region on amplification comprises 1029bp, which produces the five fragments. In the homozygous mutant, one site at position 414 is abolished and so 786bp fragment is cleaved-off; restriction digestion caused formation of four fragments.

### 3.17.6. Screening for CYP2D6*4 (G1846A; rs3892097) Polymorphism

**PCR and Restriction Digestion**- The reaction mixture (15 µl) for amplification of CYP2D6*4 comprised 1.5 µl of 10X reaction buffer (15 mM MgCl2), 0.3 µl of dNTPs mixture (2.5 mM of each dNTP), 0.4 µl (10 pM/µl) of each of the two primers (forward and reverse), 0.2 µl (3U/µl) of Taq DNA polymerase, 0.3 µl (50 ng) of genomic DNA and 11.9 µl deionized water. These contents were mixed, centrifuged (10-15 sec) placed in a thermal-cycler which had been set-up with the amplification conditions of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 65°C for 30 sec, extension at 72°C for 45 sec, and with a final extension at 72°C for 10 min. The reaction time duration was 2h.

The PCR products were checked on agarose gel (1.8%) and this was followed by restriction digestion (3U of BstN1) on incubation at 60°C overnight. Restricted products were resolved on 2.4% agarose gel stained with ethidium bromide (Plate VII). The presence of two restriction fragments indicated wild type genotype (GG; 201bp+108bp), three fragments indicated heterozygous genotype (GA; 309bp+201bp+108bp) and the non-restricted fragment indicated homozygous mutant genotype (AA; 309bp).

The BstN1 recognizes the restriction site (rs3892097) containing G nucleotide in the WT at 1934 position which is abolished in the mutant genotype by the replacement of G with A. This causes a transition and leads to splicing defect instead leading to deficient enzyme production and the person has poor metabolizing activity (Topic et al., 1998).

### 3.17.7. Screening for CYP2D6*10 (C100T; rs1065858) Polymorphism

**Setting up of PCR and Analysis of Restriction Product**- The reaction mixture for amplification of CYP2D6*10 included: 1.5 µl of 10X reaction buffer (15 mM MgCl2),
0.3 µl of dNTPs mixture (2.5 mM of each dNTP), 0.4 µl (10 pM/µl) of each of the two primers (forward and reverse), 0.2 µl (3U/ µl) of Taq DNA polymerase, 0.3 µl (50 ng) of genomic DNA and 11.9µl deionized water. After thorough mixing, tubes were centrifuged for 10-15 seconds and placed in a thermal-cycler. The amplification conditions included initial denaturation at 95°C for 5 min followed by 33 cycles of denaturation at 95°C for 45 sec, annealing at 62°C for 30 sec, extension at 72°C for 45 sec, and final extension at 72°C for 10 min. The reaction took 2h 05min.

After the confirmation of the amplified product of the expected size (345bp) on an agarose gel (2%) under electrophoresis, restriction digestion was carried out with 3Uof restriction enzyme Hph1 by incubating at 37°C for overnight (Plate VIII). Restricted bands were resolved on 2.5% agarose gel stained with ethidium bromide. The presence of two restriction fragments indicated wild type genotype (CC; 282bp+63bp), while four fragments indicated heterozygous genotype (CT; 282bp+182bp+100bp+63bp) and three restricted fragments indicated homozygous mutant genotype (TT; 182bp+100bp+63bp).

3.18. Limitations of the Study

The study results would have been more gainful if it were possible to assess genetic damage in atherosclerotic lesions of the patients. Also if specific regions of chromosomes, classes of DNA or specific genes within comet would have been investigated, the study would be more informative. However because of time constraints this would not be done but this should be possible in future studies. A study with a larger sample size will be useful in confirming the results of the present study.

3.19. Statistical Analysis

All the statistical analyses were performed using the SPSS (Statistical Package for Social Sciences; SPSS Inc. Chicago, IL), Version 16.0 for windows. The data for continuous variables of demographic study-characteristics and the assessed genetic damage, oxidative stress and lipid profile parameters are expressed as mean±S.E.M. The categorical demographic variables and the genotypic data are presented as numbers and percentages. The Chi-square (χ²) analysis was performed on the categorical study-
variables to check for any differences between the patient and the control groups for demographic details as well as to check the distribution of the genotypic and allelic frequencies in the patients and the controls. The normality of the data was assessed using the Kolmogorov and Smirnov test. The data were observed to be non-parametric but as the number of samples in each of the groups was 200 and also the results both on Mann-Whitney U test and Students’ t-test were performed which showed similar significance levels. As Students’ t-test is more applicable when sample size is >30 (Fagerland, 2012) therefore, Students’ t-test was done to check for differences, if any, for the assessed genetic damage, oxidative stress and lipid profile parameters between the patient and the control groups. The Principal Component Analysis (PCA) was carried out to extract the variables that contribute variance thus reducing the data set complexity. To check the linear association between the confounding variables and the assessed parameters of genetic damage, oxidative stress and lipid profile parameters, Pearson correlation analysis was done. To check the predictors for genetic damage, oxidative stress and lipid levels, both the univariate and the multivariate analyses were done. The Hardy-Weinberg equilibrium (HWE) for genotypic and allelic distribution was checked by the Chi-square ($\chi^2$) analysis. The Odds ratio was performed on genotypic and allelic frequencies to check for risk genotypes for CAD, if any. Pearson correlation and the univariate and the multivariate analyses were also performed to look for any relation between the genotypes and the levels of genetic damage, oxidative stress and lipid profile. The p value $\leq 0.05$ was considered significant.
PLATE Ia

The SCGE assay showing nucleoids without (a) and with (b) tail
The SCGE assay showing varying degrees of comet tail length

PLATE Ib
A representative agarose gel (0.8%) showing Quantification of Human genomic DNA samples
Lane 1: Lambda DNA (250 ng); Lanes 2-15: Genomic DNA

PLATE II
A representative agarose gel (1.2%) showing multiplex PCR products of GSTTI and GSTMI
Lane 1: 100bp Ladder; Lanes 2, 4: *GSTT0M1* genotype (796bp+209bp); Lanes 3, 7: *GSTTIM0* genotype (796bp+459bp); Lane 5: *GSTT0M0* genotype (796bp); Lane 6: *GSTT1M1* genotype (796bp+459bp+209bp); Lane 8: Negative control

PLATE III
A representative agarose gel (2.0%) showing PCR products of \textit{GSTPI} (C341T) 
Lane 1: Ladder (100bp); Lanes 2-7: PCR product (539 bp); Lane 8: Negative control

A representative agarose gel (2.5%) showing PCR-RFLP products of GSTP1 (C341T) 
Lane 1: Ladder (100bp); Lane 3: Heterozygous genotype (CT); Lanes 2,4,5,6,7,8,9: 
Homozygous wild genotype (CC)
Representative agarose gel (2.0%) showing PCR products of GSTP1 (A313G)
Lane 1: Ladder (100bp); Lanes 2-7: PCR product (176 bp); Lane 8: Negative control

Representative agarose gel (3.0%) showing PCR-RFLP products of GSTP1 (A313G)
Lane 1: Ladder (100bp); Lanes 2,3,6: Homozygous wild genotype (AA); Lanes 4,7: Heterozygous genotype (AG); Lane 5: Homozygous mutant genotype (GG); Lane 8: Positive control

PLATE V
Representative agarose gel (2.0%) showing PCR products of CYP2D6*2 (A2850G)
Lane 1: Ladder (100bp); Lanes 2-7: PCR product (1029 bp); Lane 8: Negative control

A representative agarose gel (2.7%) showing PCR-RFLP products of CYP2D6*2 (A2850G)
Lane 1: Ladder (100 bp); Lanes 2,5,7: Homozygous wild genotype (AA); Lanes 3,4: Heterozygous genotype (AG); Lane 6: Homozygous mutant genotype (GG), Lane 8: Positive control (1029 bp)
Representative agarose gel (1.8%) showing PCR products of CYP2D6*4 (G11846A)
Lane 1: Ladder (100bp); Lanes 2-7: PCR product (309 bp); Lane 8: Negative control

Representative agarose gel (2.4%) showing restriction products of CYP2D6*4 (G11846A)
Lane 1: Ladder (100bp); Lane 2: Homozygous mutant genotype (AA); Lane 3,5,7: Homozygous wild genotype (GG); Lane 4, 6: Heterozygous genotype (GA); Lane 8: Positive control

PLATE VII
Representative agarose gel (2.0%) showing PCR products of CYP2D6*10 (C100T)
Lane 1: Ladder (100bp); Lanes 2-7: PCR product (345 bp); Lane 8: Negative control

Representative agarose gel (2.5%) showing restriction products of CYP2D6*10 (C100T)
Lane 1: Ladder (100bp); Lanes 2-6: Homozygous wild genotype (CC); Lane 7: Heterozygous genotype (CT); Lane 8: Positive control

PLATE VIII