RESULTS

Essential hypertensive patients (n=200) and normotensive individuals (n=200) belonging to the Arora population sub-group in the present case-control study as a first of its kind, were assessed for differences in genetic damage and six genetic polymorphisms. The patients were on monodrug therapy (50mg/day of the beta-blocker, Atenolol). The control group comprised healthy subjects with no previous history of hypertension or any other disease or exposure and were not taking any prescribed/non-prescribed medication. Demographic information and physiometric and anthropometric data were recorded for each participant. Genomic damage in peripheral blood leucocytes was assessed by the alkaline single cell gel electrophoresis (SCGE/comet) assay (Singh et al., 1988) and oxidative DNA damage using the enzymatically-modified comet assay (Collins et al., 2008). In order to discern the effect of genetic composition on disease-occurrence and DNA damage, the participants were genotyped for six variants of two genes viz. cytochrome P450 2D6 (CYP2D6*2, rs16947; CYP2D6*4, rs3892097; CYP2D6*10, rs1065852) and glutathione-S-transferases, GSTs (GSTT1; GSTM1; GSTP1, rs1695) by polymerase chain reaction-fragment length polymorphism (PCR-RFLP) and multiplex-PCR method following standard methodology (Theophilus et al., 2006; Vedyakov and Tonevitskii, 2006). Oxidative stress (OS) levels in blood sera samples were also determined as OS is known to cause genetic damage (Uttara et al., 2009; Klaung et al., 2010).The total oxidant status (TOS) and total antioxidant capacity (TAC) were obtained and oxidative stress index (OSI) was then calculated. Lipid peroxidation levels (malondialdehyde, MDA) as well as the lipid profile were also determined by standard protocols since dyslipidemia and elevated MDA levels have been observed in studies on hypertensive patients (Jeeyar et al., 2011; Manohar et al., 2013).

4.1 Demographic and Clinical Characteristics of the Study Group

The study group included Arora Punjabis following the Hindu or Sikh belief systems. There could be demographic as well as clinical differences because of varying cultural and traditional factors influencing dietary patterns, lifestyles, etc. besides differences in
genetic make-up. On stratification for these (separately in parents and controls), no statistical differences for these variables were there. Hence their data were pooled within the patient and control groups. A comparison of the categorical demographic and clinical variables for observations on patients and controls as made to find whether the groups matched for demographic details and differed with respect to the assessed clinical characteristics.

4.1.1 Demographic Characteristics

The study participants (Table 4) comprised the hypertensive patients (n=200; average age 53.92±0.72y) and healthy controls (n=200; average age 54.30±0.72y) with 51.50% males and 48.50% females in each group with more belonging to the urban areas (78.50% patients and 75.50% controls) which is expected since this is an urban-based study; all the participants were either in the upper or upper-middle socioeconomic classes. Vegetarianism was more common in both groups while use of cooking medium as polyunsaturated fatty acids (PUFA) was more frequent among patients (74.50% vs. 25.50%). Smoking and alcohol drinking were not common though use of mobile phones prevailed. Physical exercise of high level was more predominant in patients. The patients were also characterized for obesity- on the basis of body mass index (BMI) for general obesity as per Misra et al. (2009) and WHO (2004) for Asian Indians and for abdominal obesity using the gender-specific cut-offs for waist-hip ratio (WHR), waist circumference (WC; Snehalatha et al., 2003) and waist-height ratio (WHtR; Hsieh and Muto, 2005) since these are known risk factors prevalent in patients with hypertension (Mungreiphy et al., 2011; Warren-Findlow and Seymour, 2011). According to BMI, 68.50% patients and 51.50% controls were obese whereas on the bases of WC (100% patients, 90% controls), and WHR (100% patients, 99.50% controls) almost all subjects were obese in both groups. The prevalence of obesity is probably due to the rich Punjabi dietary patterns, sedentary habit and the affluent life-styles adopted by the upper and upper-middle socioeconomic to which classes these participants belonged.

4.1.2 Clinical Characteristics

The patient group comprised 200 clinically-diagnosed essential hypertensive individuals by the physicians. The patients were categorized into hypertension stages according to
the JNC 7 (Chaubanian et al., 2003) and IGH-III (2013) guidelines. There were 92% patients in stage I hypertension (140-159 mmHg systolic and/or 90-99 mmHg diastolic blood pressure) and 8% in stage II hypertension (≥160mmHg systolic and/or ≥100 mmHg diastolic blood pressure) despite being on blood-pressure lowering beta-blocker (Atenolol) treatment. The average age-of-disease onset was 51.68±0.72y (range 29.50-87.00y) with duration of disease varying from six months to four years (average 2.24±0.06y) coinciding with the average treatment duration of 2.24±0.06y (range 0.5-4.00y) implying that treatment was initiated immediately on disease-diagnosis. Positive family history of the disease was observed in 29.50% of patients which included parent-offspring (7.50%), siblings (3.00%), second degree relatives (17.50%) and 1.5% third degree relatives.

4.2 Principal Component Analysis for Prevalent Risk Factors in Hypertensive Patients

Essential hypertension is a complex and multifactorial disorder, resulting from the interaction of genetic and environmental factors (Kunes and Zicha, 2009). In the patients of the present study, a number of factors were queried about/which necessitated carrying out Principal Component Analysis (PCA) i.e. identification of those variables (risk factors) which were having a role in the causation of essential hypertension (Table 5a, b).

PCA is a variable reduction technique used to reduce the highly correlated variables to a smaller number of principal components which account for most of the variance of the observed variable. Eigen values indicate the amount of variance explained by each principal component or each factor, and communality is the variance in observed variables accounted by common factors. Results of the PCA showed that 17 factors accounted for about 78.41% of the variance in the data-distribution (Table 5a). Each of the 17 components was a unique combination of the 55 variables including demography, anthropometric measurements, blood pressure measurements, lipid and lipoprotein levels, oxidative stress biomarkers, genetic damage and genetic polymorphism. These 17 factors were then subjected to factor analysis. The resulting rotated-factor matrix ($V_{max}$) showed the correlation of various parameters with essential
hypertension. Only variables with factor loading greater than or equal to 0.4 were considered as it is considered analogous to 0.05 cut-off for defining significance (Sterne and Davey Smith, 2001; c.f. Lawlor et al., 2004). Blood pressure measurements, oxidative stress biomarkers, lipid and lipoprotein levels, genetic damage contributed 21.98% variance in Factor 1 (Eigen value 12.31). In Factor 2, DNA damage end-points, oxidative stress index (OSI) and total antioxidant capacity (TAC) contributed 6.63% variance (Eigen value 3.71). Obesity measures and oxidative stress biomarkers were loaded in Factor 3 (Eigen value 3.57; 6.37% variance). Factor 4 was loaded with total cholesterol (TC), very low-density lipoprotein cholesterol (VLDL-C) and total cholesterol to high-density lipoprotein ratio (TC/HDL-C) with 5.49% variance (Eigen value 3.08). Factor 5 was loaded with obesity measures (Eigen value 2.43; 4.35% variance). Factors 6-17, were loaded with homozygous wild and variant genotypes of CYP2D6*10 (CC/TT), GSTT1 (present), GSTM1 (present), GSTM1 (null), genotypes of CYP2D6*2 and CYP2D6*4 with cumulative variance of 18.83%.

Genetic damage has not been considered as a direct genetic factor for inducing hypertension. Therefore PCA on 50 factors which are significantly increased/prevalent risk factors for the hypertensive state in patients (Table 5b), revealed variance attributes to Factor 1 was 20.70% contributed by blood pressure measurements, oxidative stress biomarkers, lipid and lipoprotein levels with maximum Eigen value of 10.56. Factor 2 showed correlation with triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and atherogenic indices (Eigen value 3.69; 7.24% variance) and factor 3 correlated with homozygous wild genotypes of CYP2D6*4 (GG) and CYP2D6*10 (CC) along with heterozygous genotype of CYP2D6*10 (CT) with Eigen value 3.54 (6.94% variance). Factor 4 was correlated with TC, VLDL-C and TC/HDL-C with 2.44% variance. Factor 5 was correlated with obesity measures (Eigen value 2.38; 4.66% variance). The factors 6 was correlated with alcohol, smoking, dietary habits and residence (Eigen value 2.13; 4.18% variance) 7-17 showed correlation with homozygous variant genotype of CYP2D6*4 (GG), GSTT1 (present), GSTT1 (null), homozygous and heterozygous genotypes of GSTP1 (AA/AG) as well as some of the demographic variables with Eigen values ranging from 2.04-1.00 and per cent cumulative variance of 30.49%. These observations therefore imply that
among 50 variables as (prevalent/causal factors) risk factors for hypertension in the patient group, 17 factors accounted for maximum variance. Blood pressure measurements, oxidative stress biomarkers and dyslipidemia and genetic variants of \textit{CYP2D6} and \textit{GST} contributing maximum variance as being prevalent in hypertensive patients.

4.3 Gender Differences within Patients and Controls

Since the study group comprised males and females in both patient and control groups, these groups (female vs. male patients; female vs. male healthy controls) were analyzed for any differences in their demographic and clinical characteristics. The (Chi-squared test was performed for categorical variables; Students’t-test for continuous variables.

4.3.1.1 Demographic and Clinical Characteristics- The Chi-squared test for categorical data (Table 6a) revealed that the average ages of males and females were matched in both, patient (54.78±1.95y males vs. 53.00±0.98y females) and in control (54.23±1.11y males vs. 54.37±0.89y females) groups. Probably the religious and cultural restraints caused females to be purely vegetarian, non-smokers and non-alcoholic, while 35.92% male patients and 40.78% male controls were non-vegetarian and approximate 50% were taking alcohol among patients and controls. The study group comprised 50% Sikh followers with no smoking history while there were 21.36% Hindu patients and 16.50% Hindu controls who smoked. Within the patient group, there were no significant gender differences of clinical characteristics for age-of-onset, treatment time and duration of disease, and for the blood-pressure categories.

4.3.1.2 Continuous Variables- Continuous variables of age, BMI, WC and WHR were compared between patient and control groups (Table 6b). The results revealed that patients were significantly obese with respect to BMI (p≤0.01) and WHR (p≤0.001) than control group; however the control group had significantly increased WC (p≤0.001) than the patient group.

The results revealed no significant differences within control group for the various continuous variables, on the basis of gender. In patient group, males were more obese with respect to WHR, BMI and WC (p≤0.001) as compared to female hypertensive patients.
Male patients compared to controls had significantly increased BMI (p≤0.01) and WHR (p≤0.001) while female patients were significantly more obese only with respect to WHR (p≤0.01) than control females. Rather, WC was significantly increased in normotensive female controls as compared to female patient group.

4.3.2 Gender Stratification for Genetic Damage, Lipid Levels and Oxidative Stress

The mean values (continuous variables) of genetic damage indices (Table 7), biochemical (Table 9) and oxidative stress (Table 11) were also compared for gender effects besides for the comparison between patient and control groups.

4.3.2.1 Genetic Damage- The alkaline SCGE assay quantifies for single-strand and double-strand DNA breaks, alkali-labile sites and DNA-DNA and DNA-protein cross-links in the form of migration of DNA under electrophoresis. Using image analysis software, the amount of DNA in tail (per cent tail DNA), tail moment (percentage of DNA in the tail multiplied by the length between the centers of the head and tail) and Olive tail moment (product of the tail length and the fraction of total DNA in the tail) were recorded. The Damage Frequency (DF) which reflects nucleoids with tails and Damage Index (DI; summation of nucleoids in different categories) were also calculated. DNA damage indices were highly significant (p=0.000) in essential hypertensive patients as compared to values in healthy individuals (Table 7). The increase was ~ 9 folds for per cent DNA in tail (18.36±0.46 vs. 2.75 ±0.17), ~ 24 folds for tail moment (73.14±3.01 vs. 3.65 ±0.38) and the Olive tail moment (52.29±1.64 vs. 4.71 ±0.37) was 13 times elevated in the patient group in comparison to the control values. Damage frequency (91.72±0.59 vs. 57.75±1.23) and damage index (117.95±2.08AU vs. 59.69± 1.33AU) were each approximately two times elevated in patient group as compared to respective values in the control group.

As genetic damage can differ between genders (Manikantan et al., 2009), hence the indices of DNA damage were analyzed for gender effects. In males, per cent tail DNA, tail moment, Olive tail moment, damage frequency and damage index were higher though not significantly from values in females. In controls on the other hand, though DNA damage indices were higher in females, yet these were not statistically significant
(Table 7, Fig. 1). These observations imply that there were no differences between genders for genetic damage in patients and controls.

**Principal Component Analysis for Genetic Damage End-points** - On performing the Principal Component Analysis (PCA) with the five DNA damage end-points scored (per cent tail DNA, tail moment, Olive tail moment, damage frequency and damage index) to find which genetic end-point best explained or contribute to genetic damage in the patient and control groups separately as well as in the combined study group (Table 8). Only one factor got extracted in separate and combined analyses. The common greater communality estimate and factor-loading were observed for per cent tail DNA and the least for damage frequency

**4.3.2.2 Dyslipidemia** - Dyslipidemia has also been observed in hypertensive patients (Osuji et al., 2012; Thukral et al., 2012) and with gender differences being more acute in females compared to males (Nakhjavani et al., 2006; Gilani et al., 2010). Therefore, in the present study, lipid profiling was carried out. The atherogenic index (LDL-C/HDL-C), coronary risk index (TG/HDL-C) and ratio of total cholesterol to high density lipoprotein-cholesterol (TC/HDL-C) were also calculated, as these are independent risk factors for coronary artery disease (Ugwuja et al., 2013). Compared with hypertension, the risk for vascular problems is probably proportionately increased with dyslipidemia (Vernooji et al., 2012).

The results of the lipid and lipoprotein levels (Table 9, Fig. 2) revealed elevated levels of TC (1.41 times, 260.72 ±3.86 mg/dl), TG (1.7 times, 226.97±4.33mg/dl), LDL-C (1.68 times, 196.80±3.57 mg/dl), VLDL-C (1.7 times,45.39±0.87mg/dl) and decreased levels of HDL-C (1.29 times, 35.54±0.77 mg/dl) in patients in comparison to the respective controls values (184.34 ±2.49 mg/dl, 133.62 ±2.27 mg/dl, 117.36±2.71 mg/dl, 26.72±0.45 mg/dl, 45.89 ±0.69 mg/dl).

In patients, TC levels were significantly (p≤0.01) elevated in males whereas TG levels were elevated in females (p≤0.001). LDL-C levels were also elevated but not significantly in females and the reverse was observed for HDL-C. In patients, the TG/HDL-C and LDL-C/HDL-C ratios were significantly (p≤0.001) higher in females while TC/HDL-C ratio was higher in males (Table 9). In controls, all indices as well as
TG and LDL-C levels were higher in females. The elevated lipid and lipoproteins levels in hypertensive patients were indicative of dyslipidemia.

4.3.2.2.1 Metabolic Syndrome- Another pertinent yet incidental observation which emerged from the data of the study participants was the presence of other conditions which are established risk factors/predictors of the metabolic syndrome. Therefore, the study data were also perused for the prevalence of metabolic syndrome in both hypertensive (patients) and normotensive (controls) participants. Metabolic syndrome is the medical term for a combination of hyperglycemia, hypertension, high triglyceride levels, low HDL-cholesterol (HDL-C) levels, and abdominal obesity (NCEP ATP-III). The co-occurrence of three out of the five following medical conditions i.e. central obesity, elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides (TG) and low high-density lipoprotein cholesterol (HDL-C) levels indicates the metabolic syndrome (Huang, 2009). For the present study, central obesity was assessed according to Snehalatha et al. (2003), levels of TG and HDL-C were considered risk levels as recommended by the kits used as well as NCEP ATP-III (2001)/International Diabetic Federation (2005) and blood pressure categorization according to ATP III/IDF guidelines. None of the patients or controls were diabetic (excluding criteria of the study) thereby ruling-out this conditions for consideration of metabolic syndrome.

In patients, prevalence of metabolic syndrome with three risk factors was observed to be maximum of the 89.00% (Table 10) with overlapping risk factors (WC+TG+HDL-59.50%; WC+TG+BP-89.00%; WC+ HDL-C +BP-63.00%) as per the recommended risk levels of lipid parameters on the kits used whereas according to ATP III/IDF the prevalence was 96.00% (WC+TG+ HDL-C -73.00; WC+TG+BP-96.00%; WC+HDL-C+BP-74.00%) among patients. There were more female patients (97.94% vs. 80.58% as per kits used; 97.94% vs. 94.17% as per ATP III/IDF) than male patients with metabolic syndrome due to more percentage of female patients with higher TG levels. Besides this, 59.50% (Kits used) or 73.00% (ATP III/IDF) patients had all the four risk factors making them more prone to cardiovascular diseases (Prasad et al., 2012).

In controls, 12% with the recommended risk levels of lipid profile indicated on the kits and 13.50% as per ATP III/IDF had three risk factors (1.00% with >3 risk factors)
indicative of the metabolic syndrome. In fact, prevalence of metabolic syndrome was also higher in normotensive female patients as compared to males.

4.3.2.3 Oxidative Stress- Oxidative stress has been observed in patients with essential hypertension, either due to decrease in antioxidants or increase in oxidants or both (Maharjan et al., 2008; Subash et al., 2010b). When compared with the normotensive control group (Table 11, Fig. 3), hypertensive patients had significant (p=0.000) increase in serum total oxidant status, oxidative stress index and malondialdehyde levels and a significant (p=0.000) decrease in total antioxidant capacity. In patients, 1.67-fold elevated TOS levels (5.78± 0.09 µmol H$_2$O$_2$ equivalent/l) and 2.68-fold increased OSI (0.59± 0.04) were observed as compared to the respective control levels (3.46 ± 0.09 µmol H$_2$O$_2$ equivalent/l; 0.22 ± 0.01). The levels of TAC as expected were 1.14-fold decreased in patients (1.47± 0.05 mmol Trolox equivalent/l) as compared to the control value (1.68 ± 0.03 mmol Trolox equivalent/l). MDA, (biomarker for lipid peroxidation) levels were also 1.90-fold elevated in patients (2.76± 0.16 µmol/l) as compared to in controls (1.45 ± 0.08 µmol/l).

However, no gender differences in patients and controls were observed for oxidative stress levels. Among patients, antioxidant levels were lower in females which may be influenced by various factors including menopausal stage (Shukla et al., 2013), dietary pattern (Lopez-Lagarrea et al., 2013) and physical activity (de Lemos et al., 2012), whereas TOS levels were higher in males probably from excessive workload, smoking, alcohol drinking and non-vegetarian dietary habits. MDA levels were elevated in female patients probably from dietary patterns causing increased lipid and lipoprotein levels in females which are substrates for lipid peroxidation. However, no gender differences were seen with respect to oxidative stress index. In controls also though TOS and OSI levels were higher in females, yet these were non-significant with TAC levels almost equal in both. Lipid peroxidation was elevated in male controls which could be due to increases oxidative stress because of work stress, dietary habits, smoking and alcohol consumption inducing release of reactive oxygen species.
4.4 Assessed Biomarker Levels as a Function of Menopausal Status (in females), Age, Disease Stages, Disease Duration/treatment of Disease and Obesity

As non-significant gender differences within patients and control groups were seen for demographic variables, clinical characteristics as well as for genetic damage, dyslipidemia and oxidative stress, therefore data for these parameters were pooled for further statistical analysis whereby these parameters were analyzed as a function of age/age-of-onset, hypertension categories, disease duration/treatment of disease and general as well as abdominal obesity (Tables 13-17). However first the analysis was carried out stratified for menopausal status among females (Table 12).

4.4.1 Biomarker Levels as a Function of Menopausal Status in Females- Increased lipid levels and hormonal changes, independent of body mass index during menopause, can contribute to the pathogenesis of hypertension with age in females (Coylewright et al., 2008; Olszanecka et al., 2010). The four stages of menstrual cycle are premenopausal, perimenopausal, menopausal and postmenopausal (WHO, 1996). A woman was defined as postmenopausal if she has not menstruated during the previous 12 months; as premenopausal if she was still menstruating regularly and as perimenopausal or menopausal if menstruation has become irregular but has occurred during the previous 12 months. In the present study, the perimenopausal ages of 45-56 years of 70 females overlapped with menopausal ages of 45-57 years of 82 females and so these two groups were combined as menopausal status (45-57y). In patients and controls therefore, the females were stratified only into three stages viz. premenopausal (35-44 years, mean 41.60 ± 0.71y in patients and 41.41±0.76y in controls), menopausal (45-57 years, mean 51.66±0.59y in patients and 52.07±0.48y in controls) and postmenopausal (≥58 years, mean 63.97±1.01y in patients and 62.44±0.90y in controls). Thereafter, the comparison for values were made for blood pressure measurements, lipid peroxidation, lipid profiles, oxidative stress and DNA damage as a function of menopausal status. Non-significant differences were observed within the different patient sub-groups for all these parameters (Table 12, Fig. 4a-d).

On comparing the levels of biomarkers between patients and controls in different menopausal states, highly significant (p≤0.001) results were obtained for all parameters
being increased in the patients. However, total antioxidant capacity levels were found lower in the patient group as compared to the control group values in all menopausal stages while a significant difference \( (p \leq 0.001) \) was observed only between the postmenopausal groups.

Within the control sub-groups, the DNA damage indices were significantly increased in premenopausal females as compared to menopausal and postmenopausal females. However, there were no significant differences between these values in menopausal and postmenopausal control groups.

4.4.2 Biomarker Levels as a Function of Age- With increase in age, there is a risk of hypertension due to structural changes in arteries which cause stiffness of arteries (Pinho, 2007). In order to assess if there were changes in blood pressure, lipid levels, oxidative stress and DNA damage as a function of age, the study participants were appropriated into three categories (35-44 years, 45-57 years and ≥58 years). On comparing various parameters in patient groups with those in the parallel control groups (Table 13, Fig. 5a-d), there were highly significant increases for all of them except for levels of high-density lipoprotein and total antioxidant capacity which were significantly \( (p \leq 0.001) \) depleted in the patient group. None of the parameters showed any significant differences within the patient sub-groups, while in the control sub-groups, significant \( (p \leq 0.001) \) genetic damage and oxidative stress were surprisingly observed in the younger age-range (35-44 years).

4.4.3 Biomarker Levels as a Function of Blood Pressure (Disease)

Participants and controls were categorized as normotensive, prehypertensive or in hypertension stages I and II and DNA damage levels were compared within and between groups. A gradual increase was observed for all parameters being maximum in patients in the hypertension stage II category.

In hypertension stage II patients, DF was 1.06 fold, DI 1.33 fold, per cent tail DNA 1.51 fold, TM 1.86 fold and OTM 1.60 fold higher as compared to the respective values in patients in hypertensive stage I. Therefore highly significant \( (p=0.000) \) increased genetic damage in patients in hypertension stage II was apparent (Table 14, Fig. 6). It
can be hypothesized that this increase is because of higher blood pressure in patients in the Stage II disease-category.

In controls, the levels of DNA damage viz. per cent tail DNA (5.42±0.24%), OTM (11.29±0.61), DF (59.96±1.55) and DI (61.87±1.67 AU) were significantly (p≤0.01) increased in prehypertensive individuals compared to respective levels in normotensive individuals (4.27±0.32%, 8.54±0.91, 52.94±1.83, 54.94±2.04) implying that increase in DNA damage was associated with higher blood pressure levels (prehypertensive category). Tail moment was also increased in prehypertensive group but this was not statistically significant.

**Stage I Hypertensive Patients vs. Prehypertensive Controls**- DNA damage was significantly (p=0.000) increased in stage I hypertensive patients compared to prehypertensive controls.

**Treated vs. Untreated Hypertensive Patients**- The drug treatment could also be contributing to genetic damage given that some studies on genotoxicity of Atenolol have been documented in literature (Telez et al., 2000; 2010). The diagnosis of the patients by the attending physicians was based on symptomatic history and the status of blood pressure measurements. Given the seriousness of the problems in the patients, antihypertensive therapy was initiated straight away. Therefore newly diagnosed, untreated hypertensive patients were not available. Hence, the genetic damage in treated hypertensive patients was compared with that in prehypertensive (untreated) controls, as well as with historic data from the same laboratory (Gandhi and Jyoti, 2010). Comparison with prehypertensive controls revealed that genetic damage was significantly increased in treated patient group.

DNA damage as a function of DI (117.95±2.08AU) in the treated hypertensive patients when compared with DI (75.43±0.94AU) of (historic data) untreated hypertensive patients and significantly (p≤0.01) increased in treated group.

These results imply that the severity of disease and/or treatment could be contributing to the observed genetic damage in the patients.
4.4.4 Biomarker Levels as a Function of Disease Duration/treatment of Disease

Patients were categorized into two groups based on duration of disease and treatment time of disease i.e ≤ 2y and > 2y (Table 15). On comparing the two groups for DNA damage indices no significant differences were observed implying that in the present study group duration and treatment of disease did not influence the DNA damage.

4.4.5 Biomarker Levels as a Function of Obesity

As overweight and obesity can increase genetic damage (Andreassi et al., 2011), genetic damage was analyzed as a function of obesity in hypertensive patients and healthy controls. For this, genetic damage was assessed, both as a function of general obesity (BMI basis) and abdominal obesity (on the bases of WC, WHR, WHtR).

4.4.5.1 General Obesity and Biomarker Levels- On the basis of body mass index, patients and controls classified as normal, overweight and obese were compared for DNA damage. Damage indices were found significantly higher (p≤0.001) in hypertensive patients in all sub-groups compared to values in controls. Increase in percent tail DNA was four times, TM six times, OTM five times and DF and DI were each twice as high compared to values in parallel control groups (Table 16).

No significant differences were observed for damage parameters within patient and within control BMI sub-groups indicating that genetic damage did not differ as a function of BMI.

4.4.5.2 Genetic Damage as a Function of Abdominal Obesity- Abdominal obesity is the excessive deposition of fat around the stomach and abdomen which is also known to increase genetic damage (Savini et al., 2013). WC, WHR and WHtR are commonly used measures of abdominal obesity and in the present study the comparison with cut-off values revealed that all patients (100%) were obese while most healthy controls were also obese viz. 90% as per WC and 98% as per WHR and per WHtR. The per cent tail DNA and OTM were each five folds increased, TM was seven folds and DF, DI were each two folds increased in patients as compared to observed non-obese controls. Within the patient group no significant differences were observed for DNA damage parameters. Within control group significantly increased TM (1.53 fold) and OTM (1.35
fold) were observed in non-obese individuals as compared to obese individuals as per WC may be due to difference in number of individuals (Table 17).

4.5 Predictors of Genetic Damage and Oxidative Stress

In order to assess if there was any association of DNA damage indices with any of the confounding factors (independent variables) of demography, obesity, blood pressure, lipid levels, lipid peroxidation and oxidative stress biomarkers, correlation and univariate regression analyses and analysis of variance (ANOVA) were performed (Table 18a). Multiple linear regression analysis was then carried out on those variables which significantly correlated with genetic damage and oxidative stress parameters on univariate regression. Multivariate regression analysis of combined variables was also performed to find the simultaneous effect of various confounding factors on genetic damage and on oxidative stress (Tables 18b-e).

4.5.1 Patients

Correlation and Univariate Regression Analyses- Among the demography and obesity variables, only waist-hip-ratio and waist circumference showed association with genetic damage (Table 18a). Waist-hip ratio (p≤0.05) was significantly positively correlated with all scored DNA damage indices, i.e., per cent tail DNA (r=0.19), TM (r=0.18), OTM (r=0.18), DI (r=0.14) and DF (r=0.21). Waist circumference, however, only significantly (p≤0.05) correlated with TM (r=0.16) and OTM (r=0.19).

On analysis of blood pressure measurements, association with genetic damage, systolic and diastolic as well as the derived parameters of mean arterial pressure and pulse pressure all significantly (p≤0.001) correlated with all the DNA damage indices.

Lipid and lipoprotein levels as well as lipid peroxidation (malondialdehyde), oxidative stress biomarkers, were significantly (p≤0.001) positively correlated with DNA damage indices. However, high density lipoprotein-cholesterol levels negatively correlated (p=0.000) with DNA damage.

Univariate Regression Analysis followed by Multivariate Regression Analysis- On multiple linear regression analyses of all the significantly correlated variables (Table 18
b,c), DNA damage parameters independently correlated (p=0.000) with WHR, TAC and OSI whereas the DF correlated only with TAC (p=0.000). Malondialdehyde showed independent correlation with MAP (p=0.023), PP (p=0.015) and TC/HDL-C (p=0.036). The total antioxidant capacity correlated only with TG (p=0.049) and VLDL-C (p=0.049). All DNA damage parameters showed maximum variance (ANOVA) with OSI (F=110.86-176.22) whereas maximum variance of SBP was observed with all the oxidative stress biomarkers (F= 95.62-371.41).

**Combined Multivariate Regression Analysis and Step-wise Regression Analysis**

Multiple linear regression analysis was also performed on combined variables (Table 18d,e) since genetic damage can be triggered by food habits, lifestyle, obesity measures as well as by oxidative stress, either independently or jointly. DNA damage end-points of per cent tail DNA, TM, OTM, DF and DI were significantly correlated respectively with WHR (p=0.003, 0.020, 0.035, 0.003, 0.001) and OSI (p=0.000). Total antioxidant capacity significantly correlated with per cent tail DNA (p=0.001), OTM (p=0.011) and DF (p=0.000). There was also significant association of MDA levels with PP (p=0.037) and TG/HDL-C ratio (p=0.019). There were also correlation of TAC with residence (p=0.032) and with HDL-C (p=0.023); of TOS with residence (p=0.053), and WC (p=0.008). On step-wise regression analysis, the decreased TAC significantly (p=0.000) contributed the most with 42.90% per cent tail DNA and 19.50% DF while OSI contributed 46.90% TM, 44.70% OTM and 35.90% DI. Pulse pressure was associated with lipid peroxidation (56.90%, p=0.000) and TOS with WC (10.50%, p=0.000).

Overall, in correlation and regression analysis DNA damage in essential hypertensive patients was significantly associated with WHR, TAC and OSI.

**4.5.2 Controls**

**Correlation and Univariate Regression Analyses**- In normotensive healthy controls, except for age, none of the demographic, obesity measures, lipid profile and lipid peroxidation variables were significantly associated with DNA damage indices as observed in Pearson correlation analysis (Table 19a). Age (p≤0.001) was negatively correlated with all scored DNA damage parameters i.e. per cent tail DNA (r = -0.25),
TM (r = -0.25), OTM (r = -0.27), DI (r = -0.24) and DF (r = -0.22). The individuals in higher age group were hence having improved dietary habits containing more antioxidants compared to that of lower age group. Blood pressure measurements of SBP, DBP and MAP were significantly positively correlated with the scored DNA damage indices. Oxidative stress biomarkers of TOS and OSI showed a positive correlation with all DNA damage indices whereas TAC was negatively associated with DNA damage parameters.

**Univariate Regression Analysis followed by Multivariate Regression Analysis**- On multiple linear regression analyses of these significantly correlated variables (Table 19b, c), DNA damage parameters were independently correlated with age (p<0.01) whereas the SBP was correlated with per cent tail DNA (p = 0.038), TM (p = 0.004), OTM (p = 0.034), MDA (p = 0.013). Damage index was also associated with TAC (p = 0.044) and TOS (p = 0.036). Maximum variance of OSI was observed with DNA damage parameters (F=21.39-31.09). Malondialdehyde showed independent correlation with MAP (p=0.001) whereas TOS was correlated only with MDA.

**Combined Multivariate Regression Analysis followed by Step-wise Regression Analysis**- On multiple regression analysis of combined variables, all the DNA damage indices significantly correlated with age, MAP and MDA (p≤ 0.01). PP and TOS also showed significant (p≤ 0.05) association with damage indices except with TM. Alcohol (p=0.048) and smoking (p=0.015) significantly correlated with TAC. There was also significant association of MDA with MAP (p=0.09), PP (0.000) and the oxidative stress biomarkers were significantly (p=0.000) correlated with each other (Table 19d, e).

**4.6 Oxidative DNA Damage**

Oxidative DNA damage was assessed by the modified SCGE assay as per the method of Collins (2009) by which oxidative damage to purines and pyrimidines can be assessed by treating the lysed cell preparations with specific enzymes (Endonuclease III; Endo III and Formamidopyrimidine-N-glycosylase; Fpg) which create nicks in DNA by removing the oxidized nitrogenous bases. In the present study, oxidative DNA damage was assessed in a total of 100 representative participants: 50 essential hypertensive Stage I patients, and 50 normotensive controls. The demographic and clinical details of
this sub-group are presented in Table 20. The participants were selected on the basis of their blood pressure measurements. There were 50 patients in hypertension stage I (25 females, 25 males; mean age 53.26±1.56y; average SBP 143.36mmHg and DBP 90.76mmHg) on treatment with Atenolol and 50 controls (24 females, 26 males; mean age 58.72±1.56y; average SBP 122.31mmHg and DBP 81.06mmHg) with optimal blood pressure without any previous history of hypertension or taking any antihypertensive medication. Demographic features of age, gender, dietary preferences, residence and smoking habits did not differ significantly between the two groups. However, alcohol drinking was more common in hypertensive patients who all belonged to upper and upper-middle socio-economic status while 80.50% controls were of the upper socio-economic status. Mobile usage was also more predominant in the hypertensive group (84%). Abdominal obesity was however (100% prevalence) present in both groups. Family history of disease was positive in 48% of the patients. The results of the oxidative DNA damage assessment (Table 21; Fig. 7) using Endo III (oxidised pyrimidines) and Fpg (oxidized purines) revealed elevated per cent DNA in tail (5.23±0.60),TM (30.59±4.25),OTM (16.84±2.07) of oxidised pyrimidines in comparison to the respective controls values (1.70±1.04, 3.96±2.67, 3.72±2.51). Oxidized purines in the patients were also elevated viz. per cent DNA in tail (7.36±0.75), TM (51.61±6.41) and OTM (27.53±3.24) of in comparison to their respective controls values (4.55±1.65, 32.19±4.20, 16.66±3.83). Oxidative damage to purines was significantly more as compared to pyrimidines viz. per cent tail DNA – 1.41 fold, TM – 1.69 fold and OTM – 1.63 fold. Also, in patients total oxidative damage (purines+pyrimidines) was significantly (p=0.000) as compared to controls.

In hypertensive stage I patient group, higher oxidative damage was found in females probably due to decreased TAC than in males. However in individuals with optimal blood pressure, the increased TOS as well as OSI was higher in females. On analysis for gender differences as no significant results were observed within both groups. The data on male and female patients were pooled and as also for the control group. Further analysis was carried out on pooled data to study the effects of menopausal status (Table 22, Fig. 8) and age (Table 23, Fig. 9) on oxidative DNA damage.
4.6.1 Oxidative DNA Damage as a Function of Menopausal Status

The total female participants (26 hypertensive and 24 normotensive) were in three menopausal stages, i.e. premenopausal (mean age 40.14±0.99 years, n=7 in patients and mean age 39.667±0.882 years, n=3 in control group), menopausal (mean age 50.36±1.11 years, n=11 in patient group and mean age 53.38±0.87 years, n=8 in control group) and postmenopausal (mean age 64.57±3.10 years, n=3 in patient group and mean age 65.69±1.85 years, n=13 in control group). On comparing patient groups with parallel control groups, significant differences were noticed for oxidative DNA damage indices, although per cent tail DNA showed almost two times, TM four times and OTM three times increase in the patient group (Table 22, Fig. 8).

4.6.1.1 Endonuclease III (Endo III) sites-Levels of Oxidized Pyrimidines- The menopausal patient group revealed a significant increase in TM (p≤0.001), OTM (p≤0.01) and per cent tail DNA (p≤0.01) as compared to control group while, on comparing postmenopausal patient and control participants, significant increases were observed for TM and OTM (p≤0.001) but not for per cent tail DNA despite 2.5 times increase in the patient group (2.82±0.60 vs. 0.94±0.28). There were no significant differences observed for different menopausal stages within the patient group. However within the control group, maximum damage was seen in premenopausal females followed by that in menopausal and then in postmenopausal females.

4.6.1.2 Formamidopyrimidine-DNA Glycosylase (Fpg) Sites- Levels of Oxidized Purines- There were no significant differences for damage indices within the patient sub-groups, while in control sub-groups, almost five times increase (p≤0.01) in per cent tail DNA was seen in premenopausal controls as compared to values in menopausal and postmenopausal control groups. Although both, TM and OTM were six times increased in premenopausal control group, yet these indices were not significantly higher compared to values in the menopausal group and postmenopausal groups. On comparing the menopausal and postmenopausal patient groups with parallel control groups significant differences were observed for all damage indices.
4.6.2 Oxidative DNA Damage as a Function of Age

Oxidative DNA damage (purines and pyrimidines) observed was increased significantly for per cent tail DNA (two times), TM (six times) and OTM (three times) in 35-44 years, patient group compared with that in age-matched control group.

No significant within the group differences were observed in the both the groups, for oxidative DNA damage. The results revealed almost equal damage to purines (Fpg sites) and pyrimidines (Endo III sites) in patients of age range 35-44 years, whereas, in other two age ranges, somewhat increased damage to purines was noticed (Table 23, Fig. 9).

4.6.3 Oxidative DNA Damage as a Function of Disease Duration/treatment of Disease

The patients were stratified on the basis of duration of disease/ treatment of disease into two groups (≤2 and >2). No significant differences were observed for Oxidative DNA damage indices for oxidized purines and pyrimidines between the two groups (Table 24).

4.7 Predictors of Oxidative DNA Damage

The association of oxidative DNA damage to both pyrimidines (Endo III sites) and purines (Fpg sites) with various confounder factors was studied on correlation, univariate regression and multiple regression analyses in both, patients and controls (Tables 25a-e, 26a-e).

4.7.1 Patients

4.7.1.1 Endo III sites (Oxidized pyrimidines)- Correlation Analysis- On performing correlation analysis, association was neither found between demographic variables and DNA damage nor between obesity variables and DNA damage parameters (Tables 25a). However, significant positive correlation was observed between all damage indices (per cent tail DNA, TM, OTM) and SBP, MAP, PP, MDA, TG/HDL-C ratio, LDL-C/HDL-C ratio and oxidative stress biomarkers (TAC, TOS, OSI). TC and LDL-C positively associated with per cent tail DNA and TM. VLDL-C and DBP positively correlated
with per cent tail DNA and OTM. The ratio of TC/HDL-C also positively correlated with per cent tail DNA.

**Univariate Regression Analysis followed by Multivariate Regression Analyses**- On multivariate regression analysis of significant variables (Table 25b), significant association was retained of oxidative stress index with per cent tail DNA (p=0.038), with TM (p=0.024) and with OTM (p=0.025). Olive tail moment also significantly associated with VLDL-C (p=0.003), TC/HDL-C ratio (p=0.013) and LDL-C/HDL-C ratio (p=0.004).

**Combined Multivariate Regression Analysis followed by Step-wise Regression Analyses**- However, on combining all variables in multiple regression analysis no association was observed for these parameters with any of the variables (Table 25c).

**4.7.1.2 Fpg sites (Oxidized Purines)-Correlation and Univariate Regression Analyses**- In case of oxidative damage to purines (Table 25a), the results of correlation and linear regression analyses showed significant positive association of SBP (p=0.009), MAP (p=0.019), PP (p=0.010), MDA (p=0.007), TAC (p=0.000), TOS (p=0.010) and OSI (p=0.000) with per cent tail DNA. SBP (p=0.051), PP (p=0.041), TAC (p=0.001), TOS (p=0.044) and OSI (p=0.001) also showed association with TM. PP (p=0.052), TAC (p=0.004) and OSI (p=0.002) were significantly associated with OTM.

**Univariate Regression Analysis followed by Multivariate Regression Analyses of Combined Variables**- None of the variables showed any association with DNA damage parameters on multiple regression analysis of significant variables as well as on combined analysis (Tables 25d,e).

**4.7.2 Controls**

**4.7.2.1 Endo III sites (Oxidized Purines levels)-Correlation and Univariate Regression Analyses**- In the control group the results of correlation and linear regression analyses revealed positively significant association of TOS and OSI with pyrimidine oxidized damage parameters of per cent tail DNA (p=0.000), TM (p=0.002, 0.005) and OTM (p=0.000, 0.001) and showed significant association of cooking
medium with DNA damage parameters (Tables 26a, b). DBP and VLDL-C were also showed significantly associated with TM (p=0.035, 0.023) and OTM (p=0.038, 0.050).

**Univariate Regression Analysis followed by Multivariate Regression Analyses**- The multiple regression analysis of significant variables revealed significant association of TM with cooking medium (p=0.030) and VLDL-C (0.016) and OTM with VLDL-C level (p=0.026) and TOS (p=0.017).

**Combined Multivariate Regression Analysis**- On multiple regression analysis (Table 26c) of combined variables no significant association of any of these variables on oxidative DNA damage was observed.

**4.7.2.2 Fpg sites (Oxidized purines levels)- Correlation and Univariate Regression Analyses**- In the normotensive control group, WHR and PP associated with all scored oxidative DNA damage parameters viz. per cent tail DNA (p=0.009, 0.049), TM (p=0.026) and OTM (p=0.014, 0.039). The ratios of TC/HDL-C, LDL-C/HDL-C and OSI also positively correlated with damage parameters (Table 26a, d). Positive association was also observed of per cent tail DNA with LDL-C (p=0.046), TM with TC (p=0.002) and with LDL-C (p=0.000), and of OTM with TC (p=0.042).

**Univariate Regression Analysis followed by Multivariate Regression Analyses**- On multiple regression analysis of significant variables, only LDL-C/HDL-C ratio significantly associated with OTM (p=0.036).

**Combined Multivariate Regression Analysis**

On multiple regression analysis (Table 26e) of combined variables no significant association of any of these variables on oxidative DNA damage was observed.

**4.8 Molecular Genetic Analysis**

Molecular genetic analysis in patients and controls comprised genotyping of six alleles-three of *CYP2D6* (*CYP2D6*2; rs16947, *CYP2D6*4; rs3892097, *CYP2D6*10; rs1065852) and three of *GSTs* (*GSTT1, GSTM1, GSTP1; rs1695) genes. These genes respectively encode for debrisoquine hydroxylase (*CYP2D6*) and glutathione S-transferases (*GSTs*) which take part in a two-stage detoxification process of a wide
range of environmental toxins and carcinogens (Parveen et al., 2010). The genotypic frequencies were in accordance with Hardy-Weinberg equilibrium in both groups for CYP2D6*2, CYP2D6*4, CYP2D6*10 and GSTP1 allelic variants. The GSTM1 and GSTT1 were genotyped using multiplex PCR method, homozygous wild type and heterozygous were considered together within present genotype, therefore not evaluated for Hardy-Weinberg equilibrium (Table 27).

4.8.1 Analysis of CYP2D6 Gene Polymorphisms

CYP2D6 gene is localized on chromosome 22q13.1. It has nine exons and encodes a polypeptide of 497 amino acids. Most of the known variant alleles of CYP2D6 gene are inactive and produce the PM phenotype, which however is rare in most Asian populations (Bernard et al., 2006). Frequency distributions of the CYP2D6*2, CYP2D6*4 and CYP2D6*10 genotypes among the patient and control groups are presented in Table 24.

4.8.1.1 CYP2D6*2 (A2850G; rs16947) - The allelic frequencies of patients and controls were matched ($\chi^2=3.826$, $p=0.060$) with minor allele (G) frequency of 0.35 in patients and 0.29 in controls. The genotypic frequencies of AA (42.00%), AG (45.50%) and GG (12.50%) genotypes also matched ($\chi^2=4.589$, $p=1.008$) with frequencies of genotypes in controls (48.00%, 45.50%, 6.50%).

4.8.1.2 CYP2D6*4 (G1934A; rs3892097) - The CYP2D6*4 allelic frequencies also matched ($\chi^2=0.275$, $p=0.600$) in patients and controls with minor allele (A) was 0.21 in patient group and 0.20 in control group. The genotypic frequencies of GG (62.00%), GA (33.00%) and AA (5.00%) in patients were matched ($\chi^2=1.11$, $p=0.575$) with frequencies of genotypes in controls (66.00%, 28.50%, and 5.50%).

4.8.1.3 CYP2D6*10 (C100T; rs1065852) - No significant differences were seen in genotypic ($\chi^2=0.380$, $p=0.827$) and allelic ($\chi^2=0.313$, $p=0.576$) distribution in patient and control groups for this gene. The minor allele (T) frequency was 0.17 in patients and 0.20 in controls. The respective genotypic frequencies of CC genotype (71.50% and 68.00%), CT genotype (24.00% and 22.50%) and TT genotype (4.50% and 9.50%) were not significantly different between patient and control groups.
4.8.2 Analysis of GST Polymorphisms

The genotypic and allelic distributions of the \textit{GSTM1}, \textit{GSTT1} and \textit{GSTP1} alleles among the patient and control groups are presented in Table 27.

4.8.2.1 \textit{GSTP1} (\textit{A313G}; ile105val; rs1695)- Chi square analysis did not reveal any significant differences in genotypic ($\chi^2$-1.957, p=0.378) and allelic frequencies ($\chi^2$-1.520, p=0.218) between patients and controls, with frequency of variant allele (G) of 0.28 in patient group and 0.32 in control group. The frequencies in patients and controls of AA (49.50% and 43.00%), AG (45.00% and 49.50%) and GG (5.50% and 7.50%) genotypes were matched implying no disease-associated differences.

4.8.2.2 \textit{GSTT1} and \textit{GSTM1}- The \textit{GSTT1} (theta) is located on chromosomes 22q11.23 and \textit{GSTM1} (mu) on 1p13.3 (Vojtková et al., 2013). The present include homozygous wild and heterozygous genotypes while the null is homozygous variant. The genotypic distribution of present and null genotypes of \textit{GSTT1} ($\chi^2$-0.350, p= 0.555) and \textit{GSTM1} ($\chi^2$-0.890, p=0.345) were matched in patient and control groups. The frequency of \textit{GSTM1} null genotype was higher in patients (62.50%) and controls (67.50%) whereas in case of \textit{GSTT1} polymorphism, the frequency of the present genotypes was higher in both, patients (88.00%) and controls (85.50%).

4.8.3 Factor Loading of \textit{CYP2D6} and \textit{GST} Variants

Genetic polymorphisms of six allelic variants of \textit{CYP2D6} and \textit{GST} genes in the studied population were analyzed by PCA (Table 28).

In patient group PCA revealed that there was a cumulative variance of 87.77% for seven factors in which \textit{CYP2D6}*4 (GA) and \textit{CYP2D6}*10 (CT) had 19.11% followed by \textit{GSTM1} null (14.60%), \textit{GSTT1} Present (12.259%), heterozygous genotype of \textit{GSTP1} (11.49%), heterozygous and homozygous variant genotypes of \textit{CYP2D6}*2 (16.95%) and homozygous variant of \textit{CYP2D6}*10 (8.39%).

In controls, eight factors got extracted with cumulative variance of 88.62%. \textit{GSTM1} present alone contributed the maximum variance of 17.27% followed by \textit{GSTT1} present (13.75%), heterozygous \textit{GSTP1} (11.64%), homozygous wild genotype of \textit{CYP2D6}*4
(10.35%), homozygous wild genotype of CYP2D6*2 (8.75%) and homozygous variant genotype of CYP2D6*2 (6.68%). The heterozygous genotype of CYP2D6*10 and homozygous variant genotypes of CYP2D6*4 together contribute 12.92% variance whereas homozygous variant genotypes of CYP2D6*4 (AA) and GSTP1 (GG) contribute 7.25% variance.

In the total group, out of 8 factors GSTM1 present genotype in factor 1 contributed 17.04% variance from the total cumulative variance of 87.77%. GSTTI present genotype had 17.04% variance followed by homozygous wild genotype of CYP2D6*4 (12.55%), heterozygous genotype of GSTP1 (11.54%), heterozygous genotype of CYP2D6*2 (8.19%) and homozygous wild genotype of CYP2D6*2 (7.32%). The heterozygous and homozygous variant genotypes of CYP2D6*10 (CT) and CYP2D6*10 (TT) together contribute 10.83% variance, however least variance was contributed by homozygous variant genotypes of CYP2D6*4 and GSTP1 (6.68%).

4.8.4 Combinations of Genotypes in Patients and Controls

In order to find if combinations of genotypes were more likely to be prevalent among the patients and among the controls, the genotypic combinations of CYP2D6 alleles (Table 29) and of GST alleles (Table 30) separately and together (Table 31) were subjected to analysis (Odds ratio; OR) with combination of homozygous wild genotypes as reference.

4.8.4.1 CYP2D6*2, CYP2D6*4 and CYP2D6*10 Combinations- Out of a total 52 possible combinations of CYP2D6*2, CYP2D6*4 and CYP2D6*10 genotypes, 47 were present in patients and 45 were present in controls. On considering the combination of homozygous wild genotypes as reference, analysis revealed that the CYP2D6*2 homozygous variant (GG) and CYP2D6*4 homozygous wild (GG) genotypes combinations had 0.393 fold (95% CI- 0.163 to 0.947, p=0.038) likelihood of being prevalent compared to wild homozygotes. Heterozygous genotypes of CYP2D6*2 (AG), CYP2D6*4 (GA) and CYP2D6*10 (CT) significantly associated with 0.259 fold risk for disease (95% CI-0.089 to 0.759, p=0.014) being significantly higher in patients as compared to controls.
4.8.4.2 GSTT1, GSTP1 and GSTM1 Combinations- GSTT1, GSTM1 and GSTP1 genotypes were combined to form 27 combinations in patients and 28 combinations in controls. No significant difference was observed for relative risk of disease for any combination between patients and controls implying no association of these genotypic combinations with hypertension.

4.8.4.3 CYP2D6*2, CYP2D6*4, CYP2D6*10, GSTT1, GSTP1 and GSTM1 Combinations- There were 102 combinations (6 genotypes) of the six alleles of CYP2D6 and GST genes. Of these, 72 were present in patients and 75 in controls. Odds ratio analysis revealed that no combination was associated with disease-risk in patients.

4.9 Metabolic Phenotypes of CYP2D6 Allelic Variants

CYP2D6 is a gene whose enzymatic product metabolizes xenobiotics in phase I. The gene has >100 variants (Montana Jaime et al., 2013) with different levels of enzyme activity and therefore the metabolic phenotypes may be poor metabolizer (PM), extensive metabolizer (EM), intermediate metabolizer (IM) and ultrarapid metabolizer (UM). In the present study, three allelic variants (CYP2D6*2, CYP2D6*4 and CYP2D6*10) were genotyped. On the basis of their predicted enzymatic activities, these fall under the categories of extensive (CYP2D6*2), poor (CYP2D6*4) and intermediate (CYP2D6*10) metabolizers (Kaiser et al., 2002; Ismail et al., 2003; Ishiguro et al., 2004). The putative phenotypes on the basis of genotyping results of CYP2D6*2, CYP2D6*4 and CYP2D6*10 reveal nine genotypes out of which six (CYP2D6*2 AA/AG; CYP2D6*4 GG; CYP2D6*10 CC/CT) would have extensive metabolizing activity (EM), three with intermediate activity (IM; CYP2D6*2 GG; CYP2D6*4 GA; CYP2D6*10 TT) and one (CYP2D6*4 AA) poor metabolizer.

Considering these variants singly, 90.50% of CYP2D6*2 variants should be phenotypically EM and 9.50% IM among total participants (Table 32). In patients, there would be 12.50% IM and 87.50% EM, and in controls 96.46% EM and 6.50% IM, which on statistical analysis revealed to be matched.

The CYP2D6*4 genotypes may be graded as those being EM (64.00%), IM (30.75%) and PM (5.25%). In patients and controls, frequencies of genotypes with PM activity
were equal in patients (5.00%) and controls (5.50%) and the frequencies of EMs, IMs and PMs in patients and controls showed no significant differences.

On the basis of CYP2D6*10, EMs should be 95.00% and IM in 5.00% in the total group with no differences observed in patients (EM 75.50%; IM 4.50%) and controls (EM 74.50%; IM 5.50%).

Hence overall in the Arora sub-group, the frequency of EM genotype (83.17%) was higher compared to IM genotype (15.08%) while frequency of PM genotype (1.75%) was lowest.

Combinations of allelic variants of CYP2D6 (*2, *4, *10) were considered for the predicted phenotypes and among these, there were 21.00% EMs and 79.00% IMs which exactly matched the values in controls.

4.10 Linkage Disequilibrium and Haplotype Analysis of CYP2D6 Variants

Haplotype analysis was performed using Haplovew programme to look for any haplotype distribution differences of CYP2D6*2 (rs16947), CYP2D6*4 (rs3892097), CYP2D6*10, (rs1065852), GSTP1 (rs1695) in patients and controls (Table 33; Plate. VIII). A p-value of less than 0.05 was considered as suggestive evidence of association. Linkage disequilibrium (LD) occurs when genotypes at the two loci are not independent of another and LD plot gives various measures of LD viz. D’ varies between 0 and 1 and allow to assess the extent of linkage disequilibrium; LOD (≥3.00) is the logarithm of likelihood of disease and r² is the correlation coefficient between the two loci.

The analysis revealed that allele frequencies in patients and controls were in H-W equilibrium. Linkage disequilibrium (LD) plot constructed with all SNPs of CYP2D6 and GST. Showed that among the three possible pairs of variants, only one pair (rs16947, A2850G and rs3892097, G1846A) showed weak LD with a D’ value of 0.341 (LOD – 8.14, r² - 0.094). These findings imply that in the present study group (Arora sub-group), the CYP2D6*2 (rs16947) and CYP2D6*4 (rs3892097) have slight tendency to be inherited together.

The haplotype analysis also showed that out of 15 combinations of the four SNPs CYP2D6*2 (rs16947), CYP2D6*4 (rs3892097), CYP2D6*10, (rs1065852), GSTP1
(rs1695) only two combinations, one with CYP2D6*10 variant allele (T100) in combination with other wild alleles (p=0.051) and second combination having CYP2D6*10 (T100) and GSTP1 (G313) alleles (p=0.050) showed marginal significant association with disease.

4.11 Inheritance Models of CYP2D6 and GST Genes

Four genetic models were analyzed to find which model (dominant, co-dominant, additive and recessive) for each gene showed the best fit for disease-risk (Table 34). In the dominant model, numbers of homozygous recessive and heterozygotes were grouped together and compared to those of the homozygous wild type. In the co-dominant model assuming the distinct effect of heterozygotes, frequency of heterozygotes was compared to homozygous wild. For the additive model, homozygous wild type group was compared with homozygous recessive and in the recessive model, frequency of recessive homozygotes was considered with the homozygous wild type and heterozygous combined. The OR analysis for the CYP2D6 and GST genes revealed that only the additive CYP2D6*2 model showed significance (p=0.035) showing a 2.2 fold risk associated with disease.

4.12 Genetic Damage and Genetic Polymorphism

Patients and controls stratified by genotypes (separately for the six variants) were compared for genetic damage (Table 35a-e). In patients, no significant difference was observed for all the genetic damage parameters between homozygous wild, heterozygous and homozygous variant genotypes of CYP2D6*2, CYP2D6*4, CYP2D6*10 and GSTP1. For the GSTTI and GSTMI also no differences were seen for genetic damage between non-null and null genotypes.

In the controls, only significant differences in CYP2D6*2 genotypes viz. homozygous wild, heterozygous and homozygous variant were found for genetic damage parameters of per cent tail DNA (p=0.007), tail moment (p=0.012) and Olive tail moment (p=0.009) (ANOVA) with higher damage in those having G allele. It was observed that in controls with the heterozygous CYP2D6*2 genotype (AG), genetic damage was highest (5.31±0.29 per cent tail DNA), followed by that in homozygous wild, AA
(5.14±0.29% tail DNA) with least in the homozygous variant, GG (2.37±0.29 per cent tail DNA). A similar trend of the heterozygous variant having highest TM and OTM in controls was observed. However, no differences were seen for genetic damage parameters within genotypes of CYP2D6*4, CYP2D6*10, GSTP1, GSTT1 and GSTM1.

4.13 Association of Genetic Polymorphism with DNA Damage Parameters

The association (if any) of genetic polymorphism (homozygous wild vs. heterozygous and homozygous variant) with various parameters of DNA damage and oxidative stress was determined by correlation analysis. No significant association was observed in the patient and control groups and in the total (combined) participants (Table 36a-c).

4.14 Genetic Damage as a Function of Genotype Combinations

The study participants with genotype combinations of CYP2D6 and GST variants were further analyzed for genetic damage by the Student’s t-test (Tables 37). Out of 102 combinations present, combinations with at least five participants were selected for analysis which resulted in 14 combinations in the patient group (given below). Levels of genetic damage were compared pairwise for all combinations.

1. AA (CYP2D6*2)+ GG (CYP2D6*4)+ CC (CYP2D6*10) + Present (GSTT1) + Present (GSTM1) + AA (GSTP1)
2. AA + GG+ CC+ Present+ Present+ AG
3. AA + GG+ CC+ Present + Null +AA
4. AA+ GG+ CC+ Null + Present +AG
5. AG + GG+ CC+ Present + Present +AA
6. AA + GG+ CC+ Present + Present + AG
7. AG + GG+ CC+ Present + Null +AA
8. AG + GG+ CC+ Present + Null + AG
9. GG + GG+ CC+ Present + Null +AA
10. AA + GA+ CC+ Present + Null +AA
11. AA + GG+ CT+ Present + Null +AA
12. AA + GA+ CT+ Present + Null +AA
13. AG+ GA+ CT+ Present + Present + AG
14. AG + GA+ CT+ Present +Null+ AA
The DNA damage parameters as a function of genotypes i.e. 14 combinations in patients were compared. Per cent tail DNA was significantly (p= 0.053) higher in CYP2D6*2 heterozygous of two variants (CYP2D6*4, *10), wild of CYP2D6*2, GSTT1, GSTP1 and null of GSTM1 allele combination (n=7; 20.71±2.48) as compared to value in the participants differing for the GSTM1 (present) and GSTP1 (AG) genotypes (n=7; 14.41±1.57). TM and OTM were significantly (p=0.021; 0.018) higher in homozygous wild combination of six alleles (n=6; TM 83.70±15.57; p=0.032 and OTM 58.43±8.37; p=0.035) and combination with heterozygous genotype of CYP2D6*4 and GSTM1 (null) with other wild genotypes (n=7; TM 86.97±15.64 and OTM 61.74±8.97) as compared to heterozygous CYP2D6*2 genotype in combination with five homozygous wild genotypes (n=14; TM 51.19±6.41 and OTM 40.47±3.78). Damage frequency was higher in combination with heterozygous CYP2D6*4 and GSTM1 (null) (n=7; 97.29±1.19, p=0.010; 0.026; 0.026; 0.051; 0.025; 0.046) as compared to combination with heterozygous GSTP1 (n=9; 87.33±2.76); GSTT1 null and heterozygous GSTP1 (n=10; 91.60±1.95); heterozygotes of CYP2D6*2 and GSTP1 (n=7; 87.00±3.52); heterozygous CYP2D6*2 and GSTM1 null (n=7; 89.29±3.24); heterozygous CYP2D6*2 and GSTP1, GSTM1 null (n=10; 91.60±1.92); heterozygotes of CYP2D6*2, *4, *10 and GSTM1 null genotypes (n=6; 91.17±2.61). Damage index was found significantly higher in combination with CYP2D6*4 heterozygous and GSTM1 null (n=7; 129.14±7.63, p=0.017) and only GSTM1 null genotype with other wild type genotypes (n=8; 142.00±17.20, p=0.052) as compared to heterozygous genotypes of CYP2D6*2 and GSTP1 along with other wild type genotypes (n=7; 100.57±6.98).

Null genotype of GSTM1 was observed to contribute maximally to elevated genetic damage, alone as well as in combination with the heterozygous CYP2D6 variants. Furthermore, this was followed by those with null genotype of GSTM1 along with heterozygous GSTP1 and CYP2D6 genotypes. Least genetic damage was observed in those with genotypic combinations of heterozygous GSTP1 and heterozygous CYP2D6 variants. Also null genotype of GSTT1 in combination with heterozygous GSTP1 had increased genetic damage in comparison to combinations of null genotype of GSTM1 and heterozygous variants of CYP2D6*2 and GSTP1 genotypes.
In controls, 11 gene-gene interactions were selected on the basis of meeting the criterion of at least five individuals per class and compared for genetic damage (Tables 38).

1. GG+ GG+ CC + Present+ Present + AA
2. GG + GG + CC + Present + Present + AG
3. GG + GG + CC + Present +Null+ AA
4. GG + GG + CC + Present + Null + AG
5. GA+ GG + CC + Present + Present + AA
6. GA + GG + CC + Present + Present + AG
7. GA + GG + CC + Present + Null + AA
8. GA + GG + CC + Present + Null + AG
9. GA + GA + CC + Present + Null + AA
10. GA + GA + CC + Present + Null + AG
11. GG + GA + CT+ Present + Null + AG

In controls also the null genotype of GSTM1 contributed to elevated genetic damage, alone as well as in combination with heterozygous GSTP1 and CYP2D6 heterozygous variants, followed by heterozygous CYP2D6*2 genotype alone and along with heterozygous GSTP1 and GSTM0 genotypes. In combination with wild type genotypes of all alleles and the combination only with heterozygous GSTP1 genotype least genetic damage was observed.

4.15 Combinations of Genotypes and DNA Damage (Per cent tail DNA)

To find out the risk of DNA damage (Table 39) considering only one parameter of assessed DNA damage viz. per cent tail DNA which is often regarded as the best predictor of genetic damage in the SCGE assay associated with 57 combinations with respect to six variants in total study sample (n=400) and taking mean of per cent tail DNA (5.06±0.20) of the controls as reference, logistic regression analysis was performed on homozygous wild vs. heterozygous and homozygous variant combinations (crude as well as adjusted for age, gender, BMI, diet preference, alcohol intake, levels of triglycerides, total cholesterol and high-density lipoprotein cholesterol). Out of the 57 combinations, 13 variant combinations on unadjusted (crude) OR analysis were significantly associated with increased risk for DNA damage as
compared to wild forms. The combination of null genotype of *GSTM1* and heterozygous genotype of *CYP2D6*<sup>*</sup>2 alone as well as with heterozygous genotypes of other variants were observed significantly associated with DNA damage. After adjustment for the confounding factors, triple combination with variants of *GSTM1*, *GSTP1*, *CYP2D6*<sup>*</sup>2 (triple combination) and combination of four variant genotypes *GSTM1*, *GSTP1*, *CYP2D6*<sup>*</sup>2, *CYP2D6*<sup>*</sup>10 were associated with increased per cent tail DNA.