SUMMARY

Essential (primary, idiopathic) hypertension (systolic blood pressure $\geq 140$mmHg and/or diastolic blood pressure $\geq 90$mmHg) is a chronic condition requiring life-long therapy because of its co-morbid ramifications in terms of cardiovascular diseases, diabetes, neurological disorders, glomerulopathies and hyperthyroidism. Hypertension is a complex disease with genetic and environmental etiology often exacerbating into and caused by oxidative stress thereby having a cause-effect relationship with oxidative stress. As many as 537 candidate genes and 282 single nucleotide polymorphisms (SNPs) have shown association with hypertension. The prevalence of the disease also shows variation across population-groups from 35.00% to 46.00% (with 32.30% in India) which may be because of genetic predisposition and the behavioural risk factors of insulin resistance, alcohol consumption, salt intake, smoking, sedentary lifestyle, dyslipidemia, obesity and stress. The state of oxidative stress associated with hypertension and the life-long therapy for the disease can cause damage to nucleic acids and other cellular biomolecules with adverse consequences as unrepaird genetic damage has the tendency to manifest into malignancy and cause precocious ageing and age-related diseases. However genetic damage may be modulated by genetic polymorphisms of metabolic genotypes. In literature such studies have not came to attention from this region. Therefore in the present case-control study, as a first study of its kind, genetic damage and oxidative-DNA damage were assessed in peripheral blood leukocytes of local essential hypertensive patients on monodrug therapy and were stratified by cytochrome P450 ($CYP2D6$) and glutathione-S-transferase ($GST$) genotypes to discern whether genetic damage was modulated by these metabolic genotypes. Since dyslipidemia and oxidative stress are associated with hypertension, lipid levels (using standard kits) and some markers of oxidative stress were also determined (spectrophotometrically using standard kits) in serum samples of the study participants.

Primary DNA damage using the single cell gel electrophoresis (SCGE) assay and genetic polymorphisms of some selected alleles of $CYP2D6$ ($*2, *4, *10$) and $GST$ ($T1, M1, P1$) genes were assessed in essential hypertensive patients and in healthy controls
belonging to the *Arora* population sub-group of Amritsar district (Punjab). The study was carried out after ethical clearance from the Institutional Ethics Committee and after obtaining voluntary written informed consent from the participants. Essential hypertensive (unrelated) patients (n=200) as diagnosed by the attending physician and on monodrug (Atenolol- beta blocker) therapy, belonging to the *Arora* sub-group, were contacted from local hospitals. Hypertensive patients with any associated systemic diseases such as diabetes mellitus, cardiovascular, renal and hepatic diseases were not included in the study. The control group (n=200) comprised age-, sex-, sub-group- and socioeconomic status-matched, unrelated healthy normotensive individuals from the general population. On a pre-designed proforma, general and demographic information of all participants was recorded. Standard anthropometric variables (height, weight, waist circumference and hip circumference) were taken of each participant for assessment of general obesity and central adiposity. Blood pressure measurements [systolic blood pressure (SBP) and diastolic blood pressure (DBP)] were recorded using a mercury sphygmomanometer. Peripheral blood samples (~5ml) were taken for assessment of genetic damage, biochemical analysis and molecular work. The data obtained were subjected to analysis using the SPSS programme.

Hypertensive patients (average SBP/DBP 145.05/91.62 mmHg, mean age 53.92±0.72y) comprised 51.50% males (mean age 54.78±1.95y) and 48.50% females (mean age 53.00±0.98y) with an average disease-duration of 2.24±0.06y and average age at onset-of-disease as 51.68±0.72y. The group was on monodrug therapy (50mg/d Atenolol, CAS 29122-68-7; a beta-blocker drug) for an average time of 2.24±0.06y since the diagnosis of the disease. The preferred diet was vegetarian (81.50%) with more use of unsaturated cooking oil (74.50%). Smoking (11.00%) and alcohol drinking (27.00%) were not prominent habits. None of the participants had any type of occupational/accidental exposure; they belonged to upper or upper-middle socioeconomic status, and 63% had a sedentary lifestyle. The females were stratified as 21.65% premenopausal, 42.27% menopausal and 36.08% postmenopausal. The control healthy normotensive group was matched with the patient group for age, sex, dietary preferences, life-styles, area of residence and socioeconomic status.
Despite treatment, 92.00% patients were in Stage I hypertension and 8.00% in Stage II hypertension. About 30.00% had positive family history of the disease with 10.50% having affected first-degree relatives (parent-offspring), 17.50% with second-degree relatives (aunts and uncles, grandparents) and 1.50% with third-degree affected relatives (cousins). From anthropometric measurements, general and abdominal obesity were determined. All patients were abdominally obese on the basis of cut-offs of waist circumference (WC), waist-hip-ratio (WHR) and waist-height-ratio (WHtR) while general obesity, determined on the basis of body mass index (BMI), was present in 68.50%.

The patients were also dyslipidemic with significantly higher levels of total cholesterol (TC -1.41 fold), triglycerides (TG-1.70 fold), low-density lipoprotein-cholesterol (LDL-C - 1.68 fold) and of very low-density lipoprotein-cholesterol (VLDL-C- 1.70 fold) in comparison to control values while patients had significantly decreased levels of high-density lipoprotein-cholesterol (HDL-C- 1.29 fold). The derived lipid and lipoprotein index TC/HDL-C and the coronary risk index of TG/HDL-C and the atherogenic index of LDL-C/HDL-C were also significantly (p=0.000) higher in patients. The higher lipid levels and lower HDL-C levels indicate the dyslipidemic state and imply a higher risk for cardiovascular diseases in these patients. In fact prevalence of metabolic syndrome was 96.00% in patients and 13.50% in controls on the bases of WC, TG, HDL-C.

Oxidative stress was also significantly elevated as revealed by increased levels of total oxidant status (TOS, 1.67-fold), oxidative stress index (OSI, 2.68-fold) and malondialdehyde (MDA, 1.90-fold) and decreased total antioxidant capacity (TAC, 1.14 fold) in patients compared to levels in controls. Lipid peroxidation, elevated oxidant status with decreased antioxidant capacity are indicative of oxidative stress in patients.

Primary DNA damage and oxidative DNA damage were assessed by the standard alkaline and modified SCGE assays, respectively in peripheral blood leukocytes. For the standard assay, percent tail DNA (9-fold), tail moment (TM, 24-fold), Olive tail moment (OTM, 13-fold), damage index (DI) and damage frequency (DF; 2-fold each) showed very highly significant (p<0.001) increase in patients as compared to values in
controls. Oxidative DNA damage in a sub-group of Stage-I hypertensive patients was also significantly elevated compared to that in 50 normotensive controls as assessed by the enzymatically-modified SCGE assay (using endonuclease III for detection of oxidized pyrimidines and formamidopyrimidine DNA glycosylase for oxidized purines). The total oxidative DNA damage end-points of per cent tail DNA (~3-fold), tail moment (~8-fold) and Olive tail moment (~5-fold) were significantly higher (p=0.000) in patients. The results indicate that the state of hypertension has potential to cause genetic damage and oxidative modifications of nucleic acids.

On comparing the different genotypes of the six alleles singly for genetic damage, both within patients and controls, no significant differences were observed except for CYP2D6*2 (A2850G). In this, more genetic damage was observed in individuals with heterozygous CYP2D6*2 (AG) genotypes than damage in those with homozygous (AA) wild and with variant (GG) genotypes in the control group.

As the study group had an almost equal representation of gender (51.50% males; 48.50% females) and of religion (50.00% Hindus and 50.00% Sikhs), which could be confounders for genetic damage by virtue of physiological and any genetic differences and dietary as well as life-style preferences, it was thought appropriate to rule-out the effects of these factors. For this, the data were and stratified separately by gender and religion within patients and controls subjected to statistical analysis. The analyses did not reveal statistically significant differences within the patient and control groups for the assessed biomarkers of genetic damage, oxidative stress, lipid and lipoprotein levels and genetic polymorphisms. Hence the data on these biomarkers were pooled for further statistical analysis on patients and controls.

For finding out whether there was differential genetic damage and oxidative stress as a function of demographic and clinical variables, data in patients and controls were stratified. For age, three sub-groups (35-44y, 45-57y, ≥58y) were stratified coinciding also with those of the menopausal status and age-of-onset of disease. For clinical characteristics, data were stratified on the bases of blood pressure categories (normotensive, prehypertensive, stage I hypertensive and stage II hypertensive), disease duration / treatment of disease (<2y and ≥2y) and obesity. As a function of general and
abdominal obesity, genetic damage did not differ in patients though non-obese healthy group had significant increase in genetic damage compared to levels in obese healthy individuals. In females, data were also stratified for menopausal status into three sub-groups.

The analysis revealed that in the patient group, increases in age, disease duration/treatment of disease, general and abdominal obesity as well as menopausal status did not exhibit increase in genetic damage and oxidative DNA damage.

However when stratified on the basis of blood pressure categories, genetic damage was significantly (p=0.000) increased in patients with stage II hypertension (SBP ≥180mmHg and/or DBP ≥110mmHg) as compared to genetic damage in patients with stage I hypertension (SBP ≥140mmHg and/or DBP ≥90mmHg). Within control group, also, significant (p=0.000) increased genetic damage was observed in prehypertensive (SBP 120-139mmHg and/or DBP 80-89mmHg) individuals than in normotensives (SBP <120mmHg and/or DBP <80mmHg).

To find out whether genetic damage was higher in treated hypertensive patients comparisons were made with untreated hypertensive patients for genetic damage levels and also with prehypertensive controls. All the patients were on monodrug treatment and had significantly (p=0.000) higher genetic damage compared to prehypertensive untreated controls. On comparing the treated patients (average SBP/ DBP 145.05/91.62 mmHg)with some untreated hypertensive patients (average SBP/ DBP 150.00/97.50 mmHg), genetic damage (DI) was significantly (p=0.000) higher in treated patients.

Therefore it was observed that genetic damage significantly increased with hypertension stages i.e. from prehypertensive to stage I and then to stage II and in treated compared to untreated patients. These observations imply that severity of the disease and/or drug treatment probably are causative factors of observed genetic damage.

Furthermore on combinational analysis of all the six allelic variants, genetic damage was observed to differ as a function of genotypes. From 102 combinational genotypes of CYP2D6 and GST genetic polymorphisms in patients and controls, some genotypic combinations (in patients n=14, in controls n=11; with ≥5 participants) were compared
pair-wise separately in patients and controls for all the genetic damage end-points scored. For patients with GSTM1 null with all the other five wild type genotypes, the genetic damage was the highest being significantly higher than in the six wild type genotype group. In pair-wise comparisons, the combinational genotypes of GSTM1 null genotype with other wild type genotypes or in combination (≥ singly) with heterozygous variants of CYP2D6 *2, *4, *10 genes had more genetic damage in both, patient and control groups.

On considering the six CYP2D6 and GST (homozygous wild vs. heterozygous +homozygous variant) combinational analysis on logistic regression for the genetic end-point of per cent tail DNA, out of the 57 combinations 13 combinations significantly associated with increased per cent tail DNA compared to the mean per cent tail DNA in controls. The GSTM1 null heterozygous CYP2D6*2(AG) with other wild type combinations or with heterozygous genotypes of other genes, was associated with significantly elevated genetic damage compared to damage in those with the homozygous wild type genotype combination. Even after adjustment for confounding factors (age, gender, BMI, diet preference, alcohol intake, levels of TG, TC, HDL-C), two combinations were retained wherein GSTM1 null, GSTP1 (AG/GG), CYP2D6*2 (AG/GG), GSTT1 present, CYP2D6*4 (GG) and CYP2D6*10 (CC) combination and the GSTM1 null, GSTP1 (AG/GG), CYP2D6*2 (AG/GG), CYP2D6*10 (CT/TT), GSTT1 present combination associated with significantly increased genetic damage compared to genetic damage in those with all wild type genotypes. These observations demonstrate that the GSTM1null and CYP2D6*2 (AG/GG) genotypes on crude odds ratio analysis, and after adjustment also the GSTP1 (AG/GG) and CYP2D6*10 (CT/TT) genotypes when considered together had increased genetic damage.

On performing correlation analysis, DNA damage in patients showed association with abdominal obesity (WC, WHR), blood pressure measurements, MDA, OSI and HDL-C levels with most variance contributed by OSI. Oxidative DNA damage also correlated with all these except MDA and HDL-C and further correlated with TOS and all the atherogenic indices. On univariate regression analysis, genetic damage and oxidative DNA damage coorelated with all these variables which on multivariate analysis were
limited to WHR, TAC and OSI. Combined multivariate analysis revealed that WHR, TAC, OSI were predictors of primary DNA damage. Therefore, predictors of genetic damage (on association and correlation analyses) included WHR, TAC and OSI.

Genotyping results of the six allelic variants [\textit{CYP2D6}*2-rs16947 (A2850G), \textit{CYP2D6}*4-rs3892097 (G1934A), \textit{CYP2D6}*10-rs1065852 (C100T), \textit{GSTT1}-rs17856199, \textit{GSTM1}-rs366631, \textit{GSTP1}-rs1695 (A313G)] in patients and controls revealed that the distribution of allelic and genotypic frequencies were matched. Genotypic distributions of all variants were in accordance with Hardy-Weinberg equilibrium. In patients, the frequency of homozygous (mutant/recessive) variant genotype of \textit{CYP2D6}*2 was double (12.50%) whereas of \textit{CYP2D6}*4 (5.00%), \textit{CYP2D6}*10 (4.50%) and \textit{GSTP1} (4.50%) were slightly lower than in controls (6.50%, 5.50%, 5.50% and 5.50% respectively). For the \textit{GSTM1} genotypic distribution in patients and controls, the frequency of null genotype was twice as compared to that of the present genotype; the reverse trend was seen for distribution of \textit{GSTT1} genotypes. The frequency of wild type alleles of \textit{CYP2D6}*2, \textit{CYP2D6}*4, \textit{CYP2D6}*10, \textit{GSTT1}, \textit{GSTP1} were higher and of \textit{GSTM1} was low in this population sub-group, irrespective of the disease-state. Despite the genotypic (and allelic) differences in patients and controls, these did not differ significantly. The frequencies of predicted phenotypes EM, IM and PM matched in both patients and controls and therefore in the combined study group, the frequencies of 83.17% EM, 15.08% IM and 1.75% PM. On combination of \textit{CYP2D6}*2, *4, *10 genotypes, predicted phenotypes were extensive 21.00% and intermediate 79.00% only.

The \textit{CYP2D6}*2 and \textit{CYP2D6}*4 were observed in linkage disequilibrium in the present study group. Also, the \textit{CYP2D6}*10 (rs1065852) alone or in combination with \textit{GSTP1} (rs1695) along with other wild type alleles were associated with disease on haplotype analysis.

In disease-prediction, on testing the various inheritance models of the genotypes of the six alleles, only the additive model of \textit{CYP2D6}*2 exhibited best fit with homozygous (AA) wild genotype more frequent as compared to homozygous variant. On double-combinational analysis, the homozygous (GG) variant of \textit{CYP2D6}*2 (A2850G) with homozygous (GG) wild type of \textit{CYP2D6}*4 (G1934A) had 0.393x likelihood of
occurrence in hypertensive patients. The triple gene combinations of heterozygous variants of CYP2D6*2 (AG), CYP2D6*4 (GA) and CYP2D6*10 (CT) showed 0.259-fold likelihood of association with disease. The GSTs singly or in combinations however showed no significant prevalence in patients and this was also not observed on GST-CYP2D6 combinational analysis.

It was also thought of interest to find which of the 50 variables in patients contributed maximally to the total variance using principal component analysis (PCA). A cumulative variance of 78.98% was observed from 17 factors. Systolic blood pressure, mean arterial pressure, pulse pressure, malondialdehyde levels, total antioxidant capacity, lipid and lipoprotein levels in factor 1 contributed 20.70% variance. In factor 2, the 7.24% variance was contributed by HDL-C, LDL-C and atherogenic indices. In other factors, GSTT1 null, GSTM1 null, homozygous wild genotype of CYP2D6*10 (CC), heterozygous genotypes of GSTP1 (AG) and CYP2D6*2 (AG) contributed small variances. On including the genetic damage indices, PCA revealed also 17 factors with an almost similar cumulative variance (78.41%). The factor 1 loaded with the same variables but interestingly included also genetic damage end-points and the variance increased to 21.98%. Factor 2 also loaded with DNA damage parameters and oxidative stress index (6.63% variance) and factor 3 with systolic blood pressure, oxidative stress index and total oxidant status (6.37% 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%.

On summing up it is observed that the levels of genetic damage and oxidative DNA damage were significantly elevated in essential hypertensive patients as were dyslipidemia, oxidative stress and obesity (general and central obesity). With increase in blood pressure, genetic and oxidative damage also increased being significantly higher in patients with stage II hypertension. Genetic damage was also significantly increased in treated patients compared to levels in untreated patients. Though singly none of the genetic variants showed significantly elevated genetic damage from wild types but among the combinations the CYP2D6*2 (AG) and GSTM1 null with other
wild genotypes (compared to combination with all the wild type genotypes), had significantly increased genetic damage. For per cent tail DNA end-point, significantly increased genetic damage was observed in 13 combinations with most damage in *CYP2D6*<sup>2</sup>(AG), *GSTM1* null and other wild type genotypes in combination. The predictors of genetic damage were WHR, TAC and OSI.

These observations highlight significantly increased genetic damage and oxidative DNA damage in hypertensive patients which could be from the disease and/or atenolol therapy. Increased abdominal obesity (WHR) and OSI and decreased TAC most likely increased genetic damage in these patients. Patients with heterozygous *CYP2D6*<sup>2</sup> (AG) and *GSTM1* null genotypes had more genetic damage compared to levels in the wild type genotype combination. Furthermore the homozygous (GG) variant of *CYP2D6*<sup>2</sup> (A2850G) with homozygous (GG) wild type of *CYP2D6*<sup>4</sup> and *CYP2D6*<sup>2</sup> (AG), *CYP2D6*<sup>4</sup> (GA) as well as the *CYP2D6*<sup>10</sup> (CT) genotypic combination were more prevalent among hypertensive patients of the present study group.