Summary

Type 2 diabetes (T2D) is a complex and pleomorphic metabolic disorder arising from a complex interaction between genes and the environment (Tuomi et al., 2014). T2D induced hyperglycemia is considered as the key contributor to the pathogenesis of diabetic nephropathy (DN) which is the most common cause of end-stage renal disease (ESRD) and is increasingly considered as an inflammatory disease (Evans and Forsyth, 2004; Wada and Makino, 2013). In various studies, chemokines, endothelial regulators and profibrotic growth factors such as monocyte chemoattractant protein-1 (MCP-1), endothelial nitric oxide synthase (eNOS) and transforming growth factor-beta1 (TGF-β1), have been implicated in the pathogenesis of DN via increased chemotaxis, endothelial dysfunction and fibrosis (Li and Takahashi, 2012; El-Sherbini et al., 2013; Bagci et al., 2015).

India had 65.1 million people with diabetes in 2013 and these numbers are projected to increase to 109.0 million by 2035 (International Diabetes Federation, 2013). Similar steep rise in the prevalence of diabetes and its complications has been observed in the populations of Punjab and Jammu & Kashmir (Bhatti et al., 2007; Reshi et al., 2008; Mahajan et al., 2013). The two populations have distinct geographical and ethnical origins influencing the genetic susceptibility to lifestyle diseases like T2D and DN. Genetic variations in MCP-1, eNOS and TGF-β1 gene are believed to play a critical role in the induction of inflammation (Ahluwalia et al., 2009; El-Sherbini et al., 2013; Dellamea et al., 2014). The present study was intended to investigate the association of MCP-1 (rs1024611 and rs3917887), eNOS (rs2070744, 27 VNTR 4a/b and rs1799983) and TGF-β1 (rs1800470 and rs1800469) gene polymorphisms with T2D, DN and also to analyze the association of various confounding factors in the development of T2D and DN in the populations of Punjab and Jammu & Kashmir.

To accomplish the proposed objectives the present study enrolled 1313 subjects, out of which 776 samples were collected from Punjab comprising of 461 T2D cases (204 with ESRD and 257 without ESRD) and 315 healthy controls which were age (above the age of 40 years), gender and ethnicity matched with T2D without ESRD cases. A total of
537 samples comprising of 337 T2D cases (150 with ESRD and 187 without ESRD) and 200 age (above the age of 40 years), gender and ethnicity matched healthy controls were enrolled from Jammu & Kashmir. A written informed consent as per Indian Council of Medical Research (ICMR) guidelines was obtained prior to sample collection. The study design of this case-control study has been approved by the ethics committee of Guru Nanak Dev University, Amritsar. DNA isolation was done by inorganic method and was quantified by UV spectrophotometer. Genotyping was done by ARMS-PCR and PCR-RFLP method followed by agarose gel electrophoresis. 10% of the samples were randomly chosen and re-analyzed to assess reliability of the genotyping. Statistical analyses were performed using statistical package for social science program (SPSS version 16.0). Power of study was calculated using the CaTS power calculator and was found to be more than 80% for both studied populations.

Genotypes and allele frequencies (represented as percentages) were calculated by gene counting method. Genotypes were tested for the Hardy Weinberg Equilibrium (HWE). The distribution of genotype and allele frequencies in cases and controls were compared by using chi-square analysis. The extent of association was determined by Odd’s ratio (OR) at 95% confidence interval (CI). Binary Logistic regression analysis was used for correction of confounding variables such as age, sex, body mass index (BMI) and waist to hip ratio (WHR). The continuous data was compared using Student’s t-test. One-way ANOVA was used to compare the effect of genotypes of selected genes on the baseline parameters. Haplotype frequencies and pairwise linkage disequilibrium (LD) for the selected polymorphisms among both studied populations were estimated using Haploview software. The p and OR for diplotype analysis were calculated by MedCalc software. SNP-SNP interaction was analysed using Multifactor Dimensionality Reduction (MDR) software. All results were considered significant at p<0.05.

Comparison of baseline parameters within and between the two populations revealed that higher percentages of males [66.7% (Punjab) and 70.7% (Jammu & Kashmir)] were affected than females [33.3% (Punjab) and 29.3% (Jammu & Kashmir)] among T2D with ESRD group in both populations. BMI of control group was higher than cases (p=1.33x10^{-15}) among population of Punjab while in the population of Jammu &
Kashmir the BMI was higher in T2D without ESRD cases (p=6.35x10^{-04}). BMI was higher among females [26.1 Kg/m^2 (Punjab) and 25.0 Kg/m^2 (Jammu & Kashmir)] in comparison to males [25.5 Kg/m^2 (Punjab) and 23.4 Kg/m^2 (Jammu & Kashmir)] in both the populations. The mean values of WHR, systolic blood pressure (SBP), diastolic blood pressure (DBP), random blood sugar (RBS), fasting blood sugar (FBS), urea and creatinine were higher among cases in both the populations. The mean values of cholesterol, triglyceride and very high density lipoproteins (VLDL) were higher among ESRD cases in both the populations. The mean values of all baseline parameters except RBS and high density lipoproteins (HDL) were higher in the control group from population of Punjab as compared to population of Jammu & Kashmir.

Genetic analysis of *MCP-1* -2518 A>G and I/D polymorphism revealed that -2518 AG and GG genotypes were providing 1.5-2.6 fold risk towards ESRD progression while *MCP-1* ID and DD genotype provided 1.5-2.0 fold risk towards both T2D and ESRD development in both the studied populations. In case of *eNOS* -786T>C polymorphism, -786 TC and CC provided 1.5-1.7 fold risk towards both T2D and ESRD cases from Punjab and nearly 2.0 fold risk towards ESRD in the population of Jammu & Kashmir. *eNOS* 4a/b genotypes conferred 1.8-4.2 fold risk towards ESRD in the population of Punjab and Jammu & Kashmir. *eNOS* 894 gene variant provided 1.5 fold risk towards ESRD and 1.6-1.7 fold risk towards both T2D and ESRD in the population of Punjab and Jammu & Kashmir. *TGF-β1* 869 TC and CC genotype provided 1.5-3.0 fold risk towards both T2D and ESRD cases from Punjab and nearly 5 fold towards T2D without ESRD in the population of Jammu & Kashmir. *TGF-β1*-509 CT and TT genotype provided 1.6-5.5 fold risk towards T2D and ESRD cases from Jammu & Kashmir while no association was observed among cases from Punjab.

One way ANOVA analysis, *MCP-1* -2518 and I/D genotypes were observed to be significantly associated with WHtR, cholesterol and LDL among T2D and ESRD cases from Punjab. *eNOS* -786 genotypes were significantly associated with DBP, triglyceride, VLDL, cholesterol, creatinine and duration of ESRD among T2D and ESRD cases from Punjab and Jammu & Kashmir, respectively. While among T2D without ESRD cases, -786 genotypes were significantly associated with FBS. *eNOS* 894
genotypes were significantly associated with triglyceride, VLDL and LDL among ESRD cases from Punjab. *eNOS* 4a/b genotypes were significantly associated with SBP, urea and creatinine among T2D and ESRD cases from both populations. *TGF-β1* 869 genotypes were significantly associated with RBS and FBS among T2D without ESRD cases from Jammu & Kashmir. *TGF-β1* -509 genotypes were significantly associated with RBS among ESRD cases from Jammu & Kashmir.

Haplotype analysis revealed that *MCP-1* G-D haplotype provided increased risk for T2D and ESRD development in both populations. *eNOS* haplotype C-b-T and C-a-T conferred 2.9-3.9 fold risk towards T2D and ESRD in both population groups. *TGF-β1* C-T haplotype provided 1.9-2.8 fold risk towards T2D and ESRD progression in both studied populations. Diplotypes of *MCP-1*, *eNOS* and *TGF-β1* polymorphisms also provided increased towards T2D and ESRD development in both populations. The SNP-SNP interaction revealed that 7-locus interaction between selected SNPs conferred increased risk towards T2D and ESRD development with testing balance accuracy (TBA) more than 0.5 and cross validation consistency (CVC) of 10/10 in both populations.

In conclusion the findings of the present study suggest that the differences observed between the two populations may reflect a differential susceptibility of the two groups to the development of the disease. This may be mediated by gene environment interactions such as exposure of the two populations to different kinds of environments and lifestyle. The present study is the first systematic study which has taken two different populations and three distinct groups for better understanding of selected genetic variants in the background of ethnicity and population diversity. The present study suggested that inflammation might be an important predictor of development of renal complications in T2D subjects. All the selected SNPs were found to be associated with increased risk of ESRD in both populations. The carriers of major alleles of selected genes are more likely to survive the secondary complications and these alleles may be nephroprotective. However, among T2D cases without ESRD, lack of association was observed with *MCP-1* -2518 A>G (both populations), *eNOS* 4a/b polymorphism (both populations), -786 T>C (Jammu & Kashmir), 894 G>T (Punjab).
and -509 C>T polymorphism (Punjab). The present observations indicated that probably these polymorphisms may not directly influence the susceptibility to T2D, however, in the background of diabetic metabolic state, these SNPs increases the risk of progression to ESRD. Haplotype and diplotype combinations of MCP-1, eNOS and TGF-β1 were associated with increased risk of T2D and ESRD. SNP-SNP interaction between various selected genes was also associated with increased risk of disease development. The present study has provided the preliminary data for selected polymorphisms in both studied populations and these genetic variations may serve as a useful genetic marker to identify diabetics at high risk for the development of ESRD. However, larger future prospective studies are required to confirm the functional role of these polymorphisms in T2D and ESRD susceptibility.