1. INTRODUCTION

Diabetes mellitus (DM), especially type 2 diabetes (T2D), represents one of the most important health problems worldwide and according to recent estimations it is likely to worsen to critical levels in the next decades (Vivian, 2006; Abdullah et al., 2014). T2D is a complex and pleomorphic metabolic disorder characterized by chronic hyperglycemia resulting from progressive insulin secretory defect on the background of insulin resistance (American Diabetes Association, 2014). This metabolic dysfunction is pathologically associated with microvascular diseases and various characteristic long-term complications like diabetic neuropathy, retinopathy and nephropathy (Caramori and Mauer, 2003; Mohan et al., 2013; Liebl et al., 2015). Diabetic nephropathy (DN) that progresses to end-stage renal disease (ESRD) is perhaps the most complex microvascular complication of T2D and has become a major human health problem worldwide (Wolf and Ritz, 2003; Evans and Forsyth, 2004; Vinod, 2012). DN affects 20% to 30% of all patients with T2D and profoundly contributes to patient morbidity and mortality (Wu et al., 2010; Ahmad, 2015). DN is manifested clinically by morphological and ultrastructural changes in the kidney, persistent proteinuria, hypertension and progressive decline in glomerular filtration rate (GFR) (Parchwani and Upadhyah, 2012; Jha, 2014).

T2D has become an epidemic in 21st century where India leads the world with largest number of diabetic subjects (Zimmet et al., 2003; Singh, 2011). Over the past 25 years the prevalence of T2D has almost doubled, with three- to five-fold increase in India (Yoon et al., 2006; Chen et al., 2012). Asia is the major site of a rapidly emerging diabetes epidemic and estimates show that India and China will remain the two countries with the highest numbers of people with diabetes by 2030 (Wild et al., 2004; Chan et al., 2009). The national prevalence of diabetes in India is estimated to be 8.3%, with significant differences across geographic areas and socioeconomic classes (International Diabetes Federation, 2011). T2D is now the major cause of ESRD throughout the world in both developed and emerging nations (Reutens et al., 2008). Asian Indians have been identified as one of the ethnic groups with a high prevalence of
DN (30.3%) among T2D cases (Mani, 1998; Ayodele et al., 2004). In a study conducted by Prabahar et al. (2008), the prevalence of chronic renal failure in Chennai and Delhi was reported to be 0.86% and 0.79%, respectively. Due to lack of registries, exact data on the incidence of ESRD in India or other South Asian countries is not available. However, estimates from other studies suggest a figure of 151-232 per million population (pmp) per year in India (Sakhuja and Sud, 2003; Modi and Jha, 2006).

T2D is a complex polygenic disorder in which common genetic variants interact with environmental factors to unmask the disease (Poulsen et al., 1999; Tuomi et al., 2014). Genetic factors are known to play an important role in the development of T2D, as already explained by rare monogenic subtypes, high prevalence in particular ethnic groups and the difference in concordance rates between monozygotic and dizygotic twins (Kaprio et al., 1992; Blackett and Sanghera, 2013). There is also a growing evidence for a genetic component in DN because of the fact that some diabetic patients despite of good glycemic control and a short duration of diabetes develop DN (Fava and Hattersley, 2002; Brennan et al., 2013). Insulin resistance and relative insulin deficiency play key roles in the development of T2D (Stumvoll et al., 2005). Multiple mechanisms contribute to the progression of DN, such as an interaction between hyperglycemia induced metabolic and hemodynamic changes and genetic predisposition, which sets the stage for kidney injury (Ziyadeh, 2004; Vinod, 2012). Hemodynamic factors include the activation of various vasoactive systems, such as the rennin angiotensin system (RAS), endothelin system and increased systemic and intraglomerular pressure. Metabolic pathway leads to nonenzymatic glycosylation, increased protein kinase C (PKC) activity, abnormal polyol metabolism and oxidative stress (Raptis and Viberti, 2001; Ichinose et al., 2007). The activation of these factors in diabetes is responsible for increased glomerular pressure and hyperfiltration resulting in stress related glomerular damage, loss of podocytes, hypertrophy and glomerular changes in diabetic kidney which eventually leads to ESRD (Moriya et al., 2000; Li et al., 2007).

Beyond traditional metabolic and hemodynamic risk factors, T2D and DN are now increasingly considered as an inflammatory disease (Wada and Makino, 2013). The
notion that chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of T2D and its complications, has substantially changed our vision of this disease in the past few years (Navarro and Mora, 2006; Donath, 2014; Esser et al., 2014). Inflammatory cells, cytokines, chemokines, vasoactive agents and growth factors have been implicated in the pathogenesis of DN via increased vascular inflammation and fibrosis (Kanasaki et al., 2013; Elmarakby and Sullivan, 2010). Increased glomerular and interstitial infiltration of macrophages/monocytes has been confirmed in diabetic rodents as well as human renal biopsies (Sassy-Prigent et al., 2000; Ninichuk et al., 2007). The studies where immunosuppressive strategies reduce renal macrophage accumulation and attenuate the development of DN, further support that inflammation contributes to diabetes (Wu et al., 2008; Wittmann et al., 2009). The endothelial dysfuctioning, chemotaxis and fibrosis are often associated with chronic phases of inflammatory diseases and genetic variations among these processes may reflect or control the severity and progression of various immunological phenomena associated with the disease (Pohlers et al., 2009; El-Sherbini et al., 2013).

1.1 Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 is a member of the C-C chemokine family and one of the key factors involved in the initiation of inflammation (Cochran et al., 1983; Yadav et al., 2010). MCP-1 triggers chemotaxis and transendothelial migration of monocytes to inflammatory lesions by interacting with the membrane C-C chemokine receptor 2 (CCR2) in monocytes (O’Hayre et al., 2008; Ji et al., 2014). Human MCP-1 is the first discovered human C-C chemokine mapped to chromosome 17q11.2 with a putative molecular weight of 8685 Da (Van Coillie et al., 1999; Panee, 2012). It is secreted by fibroblasts, endothelial cells, monocytes, T cells and other cell types that mediate the influx of cells to sites of inflammation (Conti and DiGioacchino, 2001; Deshmane et al., 2009). Hyperglycemia stimulates MCP-1 production by kidney mesengial cells and glomerular podocytes resulting in tubular macrophage and accumulation of myofibroblast, which further leads to tubular injury and renal fibrosis (Morii et al., 2003; Tesch, 2008; Jing et
Evidences from human and animal studies have demonstrated that MCP-1 production play a critical role in the development of diabetic renal inflammation which leads to progression to DN (Tesch, 2008; Melgarejo et al., 2009). Genetic variants of MCP-1 gene have been reported to influence the serum levels of MCP-1 among T2D and DN cases (Maeda, 2008). Mutational analysis of MCP-1 has resulted in the identification of two regions of the primary structure that are critical for its biological activity (Beall et al., 1996). It has been demonstrated that the MCP-1 -2518 A>G variant in the promoter region may modulate the levels of MCP-1 expression (Rovin et al., 1999). Moreover, MCP-1 expression in isolated, cytokine-stimulated human peripheral blood mononuclear cells and hepatic cells, and plasma MCP-1 levels in patients with lupus nephritis support the assumption that this gene promoter polymorphism regulates MCP-1 expression at the transcriptional level (Rovin et al., 1999; Kim et al., 2002). The -2518 G allele of MCP-1 gene was linked with increased production of both MCP-1 transcript and protein in comparison to -2518 A allele (Rovin et al., 1999; Fenoglio et al., 2004). The promoter polymorphism (-2518 A>G) at the distal regulatory region and insertion/deletion (I/D) sequence located in intron 1 (14 base-pair deletion, int1del554-567) were found to be associated with T2D and DN (Simeoni et al., 2004; Kouyama et al., 2008; Ahluwalia et al., 2009). These MCP-1 genetic variants are believed to regulate the MCP-1 gene expression in response to inflammatory stimuli (Rovin et al., 1999; Del Guerra et al., 2010). Many population based studies have reported positive association of -2518 A>G polymorphism with T2D and different stages of DN (Simeoni et al., 2004; Ahluwalia et al., 2009; Jing et al., 2011; Raina et al., 2014; Bagci et al., 2015). However in case of MCP-1 (I/D) polymorphism very few studies have been done and only one study by Ahluwalia et al. (2009) have reported the association of MCP-1 I/D polymorphism with T2D and DN.

1.2 Endothelial nitric oxide synthase (eNOS)

eNOS enzyme is responsible for the production of nitric oxide (NO), by conversion of L-arginine to L-citrulline (Bredt and Snyder, 1994; Komers and Anderson, 2003). The eNOS gene is mapped to chromosome 7q36, has 26 exons and 25 introns and is
approximately 23.5kb in length (Marsden et al., 1993; Shim et al., 2010). Endothelium derived NO is a homeostatic regulator of wide spectrum of physiological actions, including the control of vascular tone, antithrombotic actions, cell cycle regulation, neurotransmission, signal transduction, and inflammation (Marletta, 1989; Tso et al., 2006; Lowry et al., 2013). Some studies suggest that dysfunctional eNOS may play a critical role in the pathogenic pathway leading to diabetic vascular complications including DN (Prabhakar, 2004; Nakagawa et al., 2007). This state of progressive NO deficiency is associated with advanced nephropathy, leading to severe proteinuria, declining renal function and hypertension (Prabhakar, 2004). Genetic variants in the eNOS gene may lead to decreased eNOS expression and may play role in the NO abnormalities that contribute to development and progression of DN (Ahluwalia et al., 2008; Li and Takahashi, 2012). Three eNOS gene variants in particular -786T>C, in the promoter region; 4b/a, a 27 bp-repeat VNTR (variable number of tandem repeats) in intron 4 and 894G>T, which causes a substitution of 298Asp for 298Glu in exon 7 in eNOS have been found to be potentially associated with T2D and different stages of DN ranging from microalbuminuria to ESRD (Noiri et al., 2002; Mehrab-Mohseni et al., 2011; Santos et al., 2011; Dellamea et al., 2014; Narne et al., 2014a; Huo et al., 2015).

The variant in the promoter region at position -786 represented by a base substitution from T to C was shown to effect the rate of transcription, individuals with -786 C allele had a reduced activity of the eNOS gene resulting in nearly 50% reduced eNOS transcription leading to decrease in both protein expression and serum NO levels. This decrease is explained by the fact that a DNA binding protein, the replication protein A1, has the ability to bind only to the -786 C allele isoform (Taverna et al., 2005). Intron 4 (4a/4b) polymorphism is based on a variable 27-base pair tandem repeat; consisting of four (allele 4a) and five (allele 4b) repeats. The deletion of one of the five nucleotide repeats in intron 4 polymorphism could affect the eNOS transcription and processing rates, resulting in modulation of eNOS enzymatic activity and further affecting plasma NO concentrations (Zanchi et al., 2000; Mamoulakis et al., 2009). Carriers of the 4a allele were found exhibiting 20% lower NO levels than 4b/4b homozygous subjects (Zintzaras et al., 2009). The polymorphism at position 894 (G to T) in exon 7 was
reported to change the eNOS protein sequence and leading to an alteration of eNOS enzyme activity (Costacou et al., 2006). This exonic polymorphism was also suggested to control the eNOS intracellular distribution and its interaction with proteins that mediate its degradation (Brouet et al., 2001)

1.3 Transforming growth factor-beta 1 (TGF-β1)

TGF-β1 is a ubiquitously expressed fibrogenic cytokine belonging to a large superfamily of activins/bone morphogenetic proteins (Shi and Massague, 2003; Ziyadeh, 2008a). TGF-β1 plays a central role in fibrosis, matrix remodelling and infiltration and activation of inflammatory cells and fibroblast (Massague et al., 2000; Pohlers et al., 2009). It exerts its biological effects via binding to high affinity type I and type II cell surface receptors (Shi and Massague, 2003; Schmierer and Hill, 2007). Both of these TGF-β1 receptors possess tyrosine kinase activity and mediate their cellular actions through interaction and phosphorylation of Smad proteins (Roberts, 1999; Attisano and Wrana, 2002). TGF-β1 is encoded by the gene located on chromosome 19q13.1 and includes 7 exons and 6 introns (Fujii et al., 1986; Bosco et al., 2013). Multiple mediators in the diabetic environment converge to upregulate TGF-β1 in the diabetic kidney (Ban and Twigg, 2008; Ziyadeh, 2008a). TGF-β1 has been studied extensively as a major mediator of hypertrophic and prosclerotic changes in DN, including tubular degeneration, epithelial to mesenchymal transition (EMT) and renal fibrosis (Lan 2011; Lee, 2013). Almost all of the intracellular signaling pathways that have been identified in diabetic injury have also been found to stimulate the renal TGF-β1 activity as an intermediary step (Ziyadeh et al., 1994; Ziyadeh, 2004). Altered TGF-β1 expression due to polymorphisms affects a wide variety of normal cellular and disease processes (El-Sherbini et al., 2013).

Genetic variations of TGF-β1 gene has been linked with an increased likelihood of having DN, among these, -509C>T, 915G>C (Arg25 Pro, codon 25) and 869T>C (Leu10Pro, codon 10) are the most frequently studied polymorphisms (Dixon et al., 2003; Park et al., 2005; Mou et al., 2011; El-Sherbini et al., 2013). The 869T>C polymorphism at exon 1 and promoter -509C>T polymorphism of TGF-β1 gene are
associated with increased circulating levels of TGF-β1 (Sharma and Ziyadeh, 1994; Shah et al., 2006). TGF-β1 gene variant 869 T>C results in the replacement of a leucine with proline at amino acid position 10 in the signal sequence that results in dysfunction of TGF-β1 pre-protein export efficiency (Li et al., 1999; Wood et al., 2000). At the molecular level, there is differential regulation of TGF-β1 expression due to the presence of polymorphisms in the regulatory gene region, which promote defective binding affinity of transcription factors (Shah et al., 2006). TGF-β1 -509 C>T promoter polymorphism results in differential regulation of TGF-β1 expression which promotes defective binding affinity of transcription factors resulting in faulty transcription rate (Shah et al., 2006a). Several studies have reported the association of 869T>C and -509 C>T polymorphism with T2D and DN (Babel et al., 2006; Buraczynska et al., 2007; Kumar et al., 2007; El-Sherbini et al., 2013; Raina et al., 2015).

1.4 Rationale of the study

T2D is rapidly increasing in prevalence and resulting in profound socioeconomic effects in both developed and developing countries (Hu, 2011). Furthermore, the parallel increase in the prevalence of T2D complications, particularly nephropathy, is placing enormous demands on healthcare budgets (Zhuo et al., 2013). India had 65.1 million people with diabetes in 2013 and these numbers are projected to increase to 109.0 million by 2030 with highest prevalence of T2D in North-India, emphasizing the sheer magnitude of the diabetes epidemic in India (Anjana et al., 2011; International Diabetes Federation, 2013; Deepa et al., 2014; Gutch et al., 2014). North Indians are becoming more prone for diabetes and its secondary complications because of rapid westernization of lifestyle, consumption of high fat diet, poor quality of health services and lack of disease awareness (Gutch et al., 2014). Similar steep rise in the prevalence of diabetes and its complications has been observed in the population of Punjab and Jammu & Kashmir (Bhatti et al., 2007; Reshi et al., 2008; Mahajan et al., 2013). The two populations have widely different geographical distribution and environmental conditions influencing the genetic susceptibility to lifestyle diseases like T2D and DN.
There is strong evidence implicating that genetic susceptibility factors are associated to T2D and DN and several single nucleotide polymorphisms (SNPs) have been linked with its increased likelihood (McDonough et al., 2011; Mou et al., 2011; Wheeler and Barroso, 2011; El-Sherbini et al., 2013).

Historically T2D and DN were thought to be non-immune diseases; however there is an accumulating evidence supporting that immunologic and inflammatory mechanisms play a significant role in the development and progression of these diseases (Navarro and Mora, 2006; Chen et al., 2013). Recent evidence indicates that innate immunity rather than adaptive immunity is the major driving factor in the inflammatory response in diabetic kidneys (Lim and Tesch, 2012). Diverse inflammatory molecules and pathways, including growth factors (Insulin like growth factor, TGF-β1), chemokines (MCP-1), enzymes (eNOS, cyclooxygenase-2), adhesion molecules (intercellular adhesion molecule-1) and inflammatory cytokines are implicated in processes related to T2D and DN (Chow et al., 2004; Melgarejo et al., 2009; Pohlers et al., 2009; Gatenby and Kearney, 2010; Cheng et al., 2011; Gu et al., 2013; Lowry et al., 2013). The understanding of these molecular pathways of inflammation with an integrative comprehension of this network will help in the development of anti-inflammation therapeutic strategies that can be translated successfully into clinical applications (Wada and Makino, 2013).

MCP-1 being an inflammatory chemokine controls the recruitment of leukocytes in inflammation and tissue injury, resulting in increased production of reactive oxygen species (ROS) and activation of proinflammatory cytokines (Tesch, 2008). Endothelium derived NO acts as an important inflammatory mediator and any impairment in its level activates proinflammatory transcription factors which results in increased production of cytokines (Baylis et al., 2003). TGF-β1, another immunomodulatory cytokine having pleotropic effects acts as an anti-inflammatory cytokine when secreted from certain classes of T cells and as a pro-inflammatory cytokine when secreted from proximal tubule cells of the kidney (Wang et al., 2000a; Matagrano et al., 2003).
It is a well known fact that change in environment and genetic background can significantly change the resulting phenotype. A same variant can have heterogeneous effect due to ethnic and genetic differences (Lin et al., 2007). Moreover, in some cases individual SNPs may not be associated with disease manifestation but may be linked to disease status when linked to other disease influencing SNPs located within a same gene or on the other related genes, emphasizing on the joint effect of variants on disease outcome (Liu et al., 2008). Genetic variations in MCP-1, eNOS and TGF-β1 genes are believed to play a critical role in chemotaxis, endothelial dysfunctioning and fibrosis and in further induction of inflammation (Ahluwalia et al., 2009; El-Sherbini et al., 2013; Dellamea et al., 2014). The role of these gene polymorphisms in T2D and DN cases from North-Indian population have been documented in few studies (Prasad et al., 2007; Ahluwalia et al., 2008; Ahluwalia et al., 2009). Moreover, most of the previously reported studies have compared T2D and DN with each other rather than healthy controls which can give inconclusive association and moreover, DN cases included were of all stages (from glomerular hyperfiltration to ESRD) (Prasad et al., 2007; Valladares-salgado et al., 2010).

Keeping this in mind the present study enrolled only last stage DN cases with ESRD and compared them with T2D cases without any complication and healthy controls to get irrefutable results. The present study was also intended to fill the lacunae in the genetic association studies for the selected genes involved in inflammation (MCP-1, eNOS and TGF-β1) in two populations from North India which have distinct geographical and ethnical origins, and also to compare the two populations in terms of polymorphism and environmental factors. To best of our knowledge the present study is the first to analyze the association of these genes in T2D and ESRD resulting from T2D in both studied populations. The further identification of high risk alleles in the studied populations would also help in introducing potential therapies in susceptible individuals that would reduce the economic burden on the society as well as improve the quality of life.
1.5 Objectives

1. To analyze *MCP-1* (-2518 G>A, I/D), *eNOS* (-786 T>C, 894 G>T, 27VNTR 4a/b) and *TGF-β1* (-509C>T, 869T>C) gene polymorphisms in T2D with ESRD cases, T2D without ESRD cases and age, gender and ethnicity matched healthy controls from the population of Punjab and Jammu & Kashmir.

2. To analyze the effect of anthropometric (BMI, WC, HC, WHR, WHtR), clinical (SBP, DBP, RBS, FBS) and biochemical (Cholesterol, triglycerides, HDL, VLDL, LDL) parameters on T2D and ESRD susceptibility.

3. To determine the association between different genotypes of studied polymorphisms and baseline parameters.

4. To analyze SNP-SNP interaction and haplotype combinations to find a correlation if any, to understand the broader aspect of disease development.