DISCUSSION
CHAPTER 6

DISCUSSION

Foot ulcer is a multifactorial disorder wherein both genetic and environmental factors play important role in its pathogenesis. However the pathophysiology of the disease is still a subject of research. Cytokines play a vital role in the inflammatory phase of wound healing and any defect of cytokine secretion or action can lead to extended inflammatory phase leading to impaired wound healing. Chemokines and HSPs on the other hand promote migration and maturation of immune cells at the site of wound. SNPs in the cytokine genes are of direct functional significance in terms of regulating cytokine production. Towards this end, we investigated the role played by SNPs in four different cytokine/chemokine genes and correlated it with serum levels of these cytokines and other inflammatory/diabetogenic markers in DFU subjects.

In the present study with respect to IL-6 -174 SNP, we observed that the C allele under homozygous (CC) condition conferred significant protection against Pre-DM, NDDM and KDM but not against DFU (both DN and PVD). Our study also showed that the heterozygotic (GC) genotype conferred significant protection for T2DM only. The NGT, Pre-DM and NDDM subjects did not exhibit the GC genotype. Association between -174G/C and T2DM was first reported in U.S. Pima Indians and Spanish Caucasians (Vozarova et al., 2003) where the C-allele was significantly associated with a decreased risk of T2DM. One subsequent study replicated these initial findings (Stephens et al., 2004). The -174 G/C SNP has previously been reported to be linked with obesity, insulin resistance and T2DM with conflicting results (Di Renzo et al., 2008); (Fishman et al.,1998); (Hamid et al., 2005); (Henningsson et al., 2006). Vozarova et al.,2003 has reported on the genetic association of the GG genotype with T2DM in the high risk Pima Indian population. However no association between this SNP and T2DM was found in the Taiwanese population (Chang et al., 2004). In fact, in their study cohort (both DM and non-DM) none of the participants carried a C allele ( Chang et al., 2004). It is
important to note that the C allele is a minor allele and might not be present in certain ethnic populations. In the present study, the frequency of CC genotype was found to be low in T2DM subjects. No individual positive for CC genotype in Filipino population was observed (Medenilla et al., 2014). It has been reported that in the study of inflammatory markers in 232 Han Chinese, only one individual carried the GC genotype (Pan et al., 2011). Investigation done on 259 Southern Chinese coal miners showed one (1) GC genotype and no CC genotype (Zhai et al., 2011). Ethnic variation in IL-6 (-174) G/C SNP in the Malaysian population showed 4%, 19% and 0% of C allele frequencies in Malay, Indian and Chinese ethnic groups, respectively (Gan et al., 2013). Similarly GC genotype frequencies of 0.2% for Eastern Asian, 0.0% for Japanese, 0.6% for Korean and 0.2% for Southern Chinese population have been reported (Jung-Hwa et al., 2012). In a study of osteoarthritis on Thai subjects, IL-6 (-174) polymorphism was found to be 77%, 23%, 0% for GG, GC and CC, respectively (Honsawek et al., 2011). Therefore, it appears that the CC genotype is minimal or not found and the GC genotype ranges from 0% to 23%. In the KORA survey, -174G allele was significantly associated with T2DM but not with IGT (Illig et al., 2004). In a Finnish population based study, conducted on healthy normoglycemic subjects, those with the CC genotype had significantly lower rates of whole-body glucose uptake and energy expenditure both in fasting and during the euglycemic-hyperinsulinemic clamp (Kubaszek et al., 2003). Libra et al., 2006 showed a strong association of the GG genotype with peripheral arterial disease among T2DM subjects. Our results are in agreement with the reports mentioned above and that of Huth et al., 2006 who performed a meta-analysis of 21 association studies which involved 5,601 T2DM cases and 17,019 control subjects. In this study the GC and CC genotypes were found to be strongly associated with a decreased risk of T2DM. Also individuals carrying the C allele have 9% lower odds of suffering from T2DM compared with individuals with the GG genotype. It is plausible that the association of -174G/C with T2DM reflects a true modulating effect of -174G/C or another variant in linkage disequilibrium with this SNP. To the best of our knowledge this is the first study to address the association of this SNP with DFU and we found no genotype or allelic association with DFU-DN and DFU-PVD.
Compared to T2DM, the information on the impact of IL-6 -174G/C gene loci as to the risk for Pre-DM, NDDM and DFU-DN are limited and this work has addressed this issue. Thus, the present work represents a crucial first step toward elucidating the extent to which the IL-6 -174G/C plays a role in T2DM susceptibility and provides additional evidence supporting a direct relationship between chronic subclinical inflammation and T2DM etiology.

Reports on the relationship between -174G/C polymorphism and circulating IL-6 level are contradictory. Several studies indicate that -174G/C SNP is associated with plasma levels of IL-6 in control subjects. In the present study no such association has been found between the IL-6 SNP and serum IL-6 levels in control subjects which is in accordance with the report of Huang et al., 2013. Further in an in vitro model, no association was seen between -174 SNP and IL-6 secretion in HUVEC cells following immune stimulation (Kiszel et al., 2007). However in subjects with KDM and DFU-DN significantly elevated levels of serum IL-6 were noted in the GG genotype and higher levels of serum IL-6 in the GG genotype has been reported (Bennermo et al., 2004). Di Renzo et al., have also reported increased IL-6 levels in obese subjects with the GG genotype (Di Renzo et al., 2008). Libra et al., 2006 had shown an association between the GG genotype and increased susceptibility to peripheral arterial disease (PAD) which was associated with elevated levels of IL-6, fibrinogen and CRP in diabetic subjects. In certain populations genotype specific differences in circulating levels of IL-6 has been documented. Although the present study showed association for -174G/C SNP among Pre-DM and NDDM subjects, the circulating levels of serum IL-6 was not associated in these subjects. However adiponectin alone in the Pre-DM subjects were decreased in GG genotype, which is indicating an indirect effect of this SNP on other serum biomarkers. The levels of IL-6 were higher among DFU-DN in the GG genotype than other study subjects. Subjects with DFU have been shown to have higher IL-6 and lower adiponectin plasma levels (Tuttolomondo et al., 2010). Further in the present study we observed a significant negative correlation between IL-6 levels and some cardiovascular risk factors such as HDL-c, adiponectin and leptin among DFU-DN subjects. Recently Zietz et al., 2008 reported that low levels
of adiponectin was associated with low levels of HDL-c and might represent an independent cardiovascular risk factor, whereas high levels of adiponectin is associated with high levels of HDL-c indicating protective action.

It has been well documented that the reference genotype GG was associated with higher triglycerides, very low density lipoprotein, plasma glucose and lower high density lipoprotein concentrations than C allele carriers (Fernandez-Real et al., 2000). In the present study, we found a significant increase in GG genotype for FPG levels in the Pre-DM subjects and an increase in serum TGL of KDM subjects under GG genotype. Joint analysis, based on data from 17 studies suggested an association of IL-6 G/C genotype with FPG levels independent of the BMI (Riikola et al., 2009). Cross sectional studies, conducted in several populations had already reported a fluctuating lipid profile in the reference genotype individuals compared with variant carriers. In Caucasian population, significantly higher TGL, total cholesterol and LDL-c and slightly lower HDL-c were observed in individuals with the reference genotype than in C variant allele carriers (Fernandez-Real et al., 2001). In contrast, a study from Finnish Caucasian population showed lower FPG in the GG genotype than CG or CC genotypes (Riikola et al., 2009). For IL-6, the present study showed that only Pre-DM subjects had significantly lower levels of adiponectin in GG genotype. An earlier report by Zubair et al., 2012 had reported lower plasma levels of adiponectin and higher levels of CRP, IL-6 and TNF-α in subject with DFU. In the present study, at baseline, none of the clinical parameters and biomarkers showed any significant difference between genotypes. However in the presence of diabetes, these effects were nullified. In DFU-DN the GC+CC genotype had significantly elevated levels of urea which was not seen at the baseline. Based on these results, it would be interesting to study the association of this SNP with diabetic nephropathy. Henningsson et al., 2006 has reported lower levels of cholesterol and LDL in women with CC genotype. Previously, few studies have documented an association of the CC genotype with high plasma levels of leptin (Wernstedt et al., 2004). But in the present study no such association was evident. Further, lack of association between -174 SNP and serum CRP levels as seen in this study is in accordance
with previous reports (Altarescu et al., 2008); (Sainz et al., 2008). The -174 SNP has recently been linked to insulin resistance (Hamid et al., 2005). However no such association was evident in the present study.

As several cytokines are also produced by adipose tissue, it was postulated that an “adipo-vascular” axis (Matsuda et al., 2002) may contribute to the increased risk of cardiovascular events in patients with T2DM. In patients with DFU, lower plasma levels of adiponectin and higher plasma levels of IL-6 could be linked to foot ulcers pathogenesis by vascular and inflammatory mechanisms. Pinto et al., 2008 had reported the role of DFU is to predict cardiovascular events in T2DM patients, even after adjusting for confounding factors associated with cardiovascular risk. These findings further show the importance of inflammatory and metabolic “milieu” such as cytokines and adipose hormones in foot complications as already reported for other vascular complications of diabetes (Boyko et al., 2006).

TNF-α is involved in the early phases of wound healing. The most extensively investigated SNPs in the TNF-α promoter are the -308G/A and -238G/A. In the present study, we studied the association of these SNPs with the intermediate phenotype (serum TNF-α level) and disease phenotype (DFU) along with other clinical parameters. The present study provides three novel findings: 1) Out of two SNPs studied, only the -308 SNP was associated with T2DM and DFU-DN 2) At the baseline, the GG genotype had significantly lower levels of serum leptin and 3) The GG genotype had significantly higher levels of serum leptin and lower levels of SBP in the Pre-DM subjects, while the GA+AA genotype had significantly higher levels of serum leptin and serum cholesterol in the DFU-DN subjects. 4) Also, the GA+AA genotype showed lower levels of serum CRP in the DFU subjects (both DN and PVD). 5) There was a statistically significant association between the TNF-α -308G/A genotype in the DFU-DN subjects and the severity of diabetic foot ulceration based on Wagner’s grade analysis.

In the present study the -308G/A SNP was found to be strongly associated with both diabetes and diabetic neuropathy but not with PVD. Here we observed a
high frequency of GG homozygous in both patients and control groups, followed by GA and AA. The AA genotype conferred significant risk to both T2DM and diabetic neuropathy (but not PVD) in both homozygous and heterozygous condition. Previously, this SNP was shown to be linked to obesity and insulin resistance in certain populations (Hoffstedt et al., 2000) and not in other populations (Walston et al., 1999). Our results are in agreement with previous reports which showed a strong association between this SNP and foot ulcer in non diabetic subjects (Nagy et al., 2007; Wallace et al., 2006). However absence of association of this SNP with microvascular complication in North West Indian population was reported by Sikka et al., 2014. Our results are in contrast with a recent large scale association analysis of TNF/LTA polymorphisms with T2DM wherein no association was seen between this SNP (along with 11 SNPs in this region) and T2DM (Boraska et al., 2010). This contradiction observed between our results and the other reports mentioned could be due to ethnic differences. With respect to the effect of this SNP on serum cytokine levels, in diabetic subjects, the GA genotype had the lowest levels of TNF-\(\alpha\). Altarescu et al., showed a significant correlation between serum TNF-\(\alpha\) levels and TNF-\(\alpha\) AA genotypes in patients with Type-I Gaucher's disease (Altarescu et al., 2005).

In addition to the study of association between -308 SNP with T2DM and foot ulcer, we further investigated the effect of this SNP on the clinical parameters of the study subjects. The GA+AA genotype of DFU-DN subjects had significantly higher levels of serum leptin and serum cholesterol. At the baseline, the GG genotype had significantly lower levels of serum leptin and in the presence of diabetes the AA genotype had significantly reduced levels of both FBG and PPBG. Thus in addition to serum TNF-\(\alpha\) level, the -308 SNP seems to affect blood glucose and leptin levels. Our results on leptin are in agreement with Canhao et al., who had shown that GG genotype of -308 SNP exhibit low serum levels of leptin (Canhao et al., 2008). Other report by Fernandez-Real et al., showed that the serum leptin concentration was clearly increased in the \textit{TNF}-308 A allele, thereby suggesting that this SNP may exacerbate the alterations in leptin levels normally found among insulin-resistant subjects (Fernandez-Real et al., 1997).
Gonzalez-Sanchez et al., had reported that the -308A allele of the TNF-α gene was associated with decreased circulating adiponectin concentrations and increased sTNFR2 concentrations in T2DM subjects, independent of insulin resistance and BMI (Gonzalez-Sanchez et al., 2006). Also, the same study had showed a higher incidence of IGT and low circulating adiponectin levels may be associated with interaction between the -308G/A SNP of the TNF-α gene and Adipo Q45 T/G SNP in the adiponectin gene.

The GA+AA genotype of DFU subjects (both DN & PVD) showed lower levels of serum CRP. Araujo et al., reported higher CRP serum levels in individuals harboring the TNF-308 A allele (Araujo et al., 2004). Devaraj et al., had shown the frequency of the TNF-308 A allele was unaltered in high and low CRP groups (Devaraj et al., 2005). Compared to -308 SNP, there have been relatively a few studies addressing the association between the -238G/A SNP with diabetes and/or foot ulcer. Zeggini et al., reported no association between this SNP and T2DM (Zeggini et al., 2005). TNF-α -238G/A SNP was not associated with T2DM in Finnish and Danish (Rasmussen et al., 2000) populations. Recently, Feng et al., published a meta-analysis in which they reported a lack of association between -238G/A polymorphism and T2DM (Feng et al., 2009). However in a Chinese population based study, the -238 SNP (and not the -308 SNP) was shown to be strongly associated with insulin resistance (Hu et al., 2009). In HIV+ patients -238 G/G genotype was shown to be associated with a higher risk of developing low HDL-c levels (Marzocchetti et al., 2011). In children, the TNF-α -238G/A SNP was not associated with the degree of obesity and/or insulin resistance (21). Sheu et al., had reported that -238G/A & -308G/A SNPs do not play a major role in the pathogenesis of insulin resistance in Chinese subjects with or without hypertension (Sheu et al., 2001). Earlier report from a larger sample size of French men and women showed that the SNPs in UCP3, FATP1, TNF-α, LEP, and GNB3 genes are not major contributors to the risk of metabolic syndrome (Meirhaeghe et al., 2005). Therefore it appears that the TNF-α -308 SNP (but not -238 SNP) seems to confer genetic susceptibility towards diabetes and DFU in the South Indian population.
SDF-1 is a protein that acts as a pro-inflammatory factor and attracts monocytes and lymphocytes to the site of inflammation (Robak et al., 2007). They play an important role in the etiology and pathogenesis of T2DM and its associated complication like PVD and nephropathy. In the present study, we studied the association of this 801G/A SNP with the intermediate phenotype (serum SDF-1 levels) and disease phenotype (DFU) along with other clinical parameters.

The present study provides the following findings: 1) SDF-1 serum levels were significantly increased in the Pre-DM and DFU-DN subjects. 2) Interestingly, serum SDF-1 levels were below detection limits in DFU-PVD subjects. 3) Leptin levels were found to be increased in GA+AA genotype among DFU-DN subjects. 4) The "AA" allele of SDF-1 showed a protective role against DFU-DN and KDM subjects, when compared to wild "GG" genotype. 5) The "GA" allele of SDF-1 showed a protective role in Pre-DM subjects, when compared to wild "GG" genotype.

While the 801G/A SNP was found to be strongly associated with Pre-DM, KDM and DFU-DN it was not associated with DFU-PVD. Here, we observed a high frequency of GA heterozygous in both patients and control groups, followed by GG and AA. The AA allele conferred significant protection to both KDM and DFU-DN (but not DFU-PVD) in homozygous condition. Though the GG allele is considered as the wild form of SDF-1 gene in the 801 region, our results show that the frequency of this allele is found to be increased in all disease subjects than the NGT subjects. Our results are in agreement with an earlier report by Derakhshan et al., where it has been shown that the frequency of GG genotype is higher in T2DM patients when compared to healthy controls (Derakhshan et al., 2012).

The result of our study is also found to be similar with a previous report on North Indian population (Chaudhary et al., 2008). The association studies of SDF-1 in high risk seronegative and HIV-1 positive patients has indicated that the frequency of wild GG allele is higher in HIV-1 positive patients when compared to healthy individuals, showing GG genotype to be a risk factor. On the other hand the AA genotype was found to be protective against HIV-1 infection since the
frequency of mutant AA genotype is lower in HIV-1 positive patients. The A allele has been recognized as a susceptibility allele for proliferative diabetic retinopathy (Djuric et al., 2010). To our knowledge, this is the first report on Pre-DM and SDF-1 association.

Significantly higher levels of SDF-1 in DFU-DN and Pre-DM were obtained, when compared to NGT subjects. It has been reported that individuals with SDF-1 A allele have higher levels of the SDF-1 protein due to the up-regulating effect of this allele (Tashiro et al., 1993). SDF-1 A allele has been demonstrated to play a role in the microvascular manifestation in multiple sclerosis (MS) and thus may be one of the susceptibility factors that lead to MS (Manetti et al., 2009). SDF-1 A allele variants have also been shown to play a role in metastasis of breast cancer (Hassan et al., 2008). The expression level of SDF-1 was found to be high in specific organs in breast cancer patients bearing the A allele.

In the present study KDM subjects showed high levels of serum SDF-1, but there was no significant association when compared to NGT subjects. Derakhshan et al., found an increased levels of SDF-1 in KDM subjects (Derakhshan et al., 2012). However no association of SDF-1 801G/A SNP was found. Azin et al., had reported that serum SDF-1 levels are markedly higher in multiple sclerosis patients than healthy controls, but no association between SDF-1 801G/A SNP and its serum levels could be found (Azin et al., 2002). In the present study, serum SDF-1 levels were undetectable in the DFU-PVD subjects. The levels of leptin was also found to be significantly higher in the GA+AA genotype of DFU-DN subjects, which indicates that SDF-1 has an indirect effect on leptin concentration. No earlier reports on serum leptin and SDF-1 genotypes are available in the literature for making comparisons.

The current study on HSPA1B provides these novel findings: 1) With regard to 1538 A/G SNP, the GG genotype conferred significant protection only against Pre-DM, but not against NDDM, KDM and DFU (both DN and PVD subtypes). Also, the GA+GG genotypes of Pre-DM subjects had significantly lower serum
concentrations of TNF-α and IL-6 than those with AA genotype. 2) In the KDM subjects, GA and GG genotype showed significant risk. Also, GA+GG genotypes of KDM subjects had significantly lower serum concentrations of SDF-1 and leptin, than those with AA genotype. The GA+GG genotypes of KDM subjects had significantly elevated levels of glycated hemoglobin and a decreased level of LDL-c than those of AA. 3) The GA genotype showed a significant risk for DFU-DN subjects, but not for DFU-PVD. None of the serum cytokines showed any significant association for DFU-DN. However the GA+GG genotypes of DFU-PVD subjects alone had significantly lower concentrations of HOMA-IR and glycated hemoglobin. 4) Genotype analysis did not show any association for NDDM subjects, but the GA+GG genotypes of NDDM subjects alone had significantly lower serum concentrations of leptin and HOMA-IR, when compared with other serum biomarkers. The levels of glycated hemoglobin were significantly elevated in the GA+GG genotypes of NDDM. 5) There was no statistically significant association between the HSPA1B genotype and the severity of diabetic foot ulceration based on Wagner’s grade analysis.

With respect to HSPA1L, this study has provided the following novel findings: 1) Genotype analysis showed that TT genotype conferred significant risk against KDM and DFU-DN, but not against Pre-DM, NDDM and DFU-PVD. 2) There was no statistically significant association between the HSPA1L genotype and the severity of diabetic foot ulceration according to Wagner’s grade analysis. 3) The CT+TT genotypes of KDM subjects alone had significantly lower serum concentrations of CRP, than those with CC genotype, whereas the CT+TT genotypes of DFU-PVD subjects had significantly higher levels of HOMA-IR. 4) In KDM subjects, the CT+TT genotypes had significantly lower levels of serum TGL, BMI and VLDL-c. However, the same CT+TT genotype had significantly elevated levels of VLDL-c in the DFU-PVD subjects.

The present study suggests that the GA genotype of HSPA1B is associated with an increased risk for foot ulceration. Several factors predispose to ulceration, including peripheral neuropathy, peripheral arterial disease and trauma. It is possible that excessive inflammation occurs in a subset of patients of GA genotype,
leading to increased morbidity. Our results are in agreement with an earlier report from Mir et al., 2009, who had investigated HSP-70 polymorphism in South Indian population with DFU and reported that the GA genotype was significantly associated with the severity of foot ulceration. However with respect to the severity of foot ulcer, our results are in contrast to that of Mir et al., who had reported that the GA genotype of \( \text{HSPA1B} \) is associated with the severity of DFU. This contradiction may be due to the reason that both neuropathy and vascular diseases were considered as a single group by Mir et al., while we have considered the two categories DFU-DN (ulcer due to neuropathy) and DFU-PVD (ulcer due to vascular dysfunction) separately.

In the present study, we report that the GG genotype of \( \text{HSPA1B} \) confer significant protection only against Pre-DM subjects. This association should be confirmed in similar populations, and its biological basis needs further investigation since there are no earlier reports available on the SNP of \( \text{HSPA1B} \) in pre-diabetes as well as NDDM for making comparisons. Shibata et al., 2009, had reported a protective role of genetic polymorphism of HSP-70 in gastric cancer. Also, the GA+GG genotypes of Pre-DM subjects had significantly lower serum concentrations of TNF-\( \alpha \) and IL-6 than those with AA genotype indicating that the levels of these inflammatory cytokines also may be an essential factor in conferring protection. A strong association of HSP70-2 SNP with the GG genotype and also an association of the G allele with diabetic nephropathy has been reported (Buraczynska et al., 2009).

In the present study, the levels of LDL-c were found to be significantly lower in the presence of a G allele of \( \text{HSPA1B} \). This observation is different from the earlier report on T2DM and atherosclerosis patients where the total cholesterol and LDL-c levels are found to be higher in the presence of G allele than the A allele (Giacconi et al., 2005). This shows that, although the GA and GG genotypes confer significant risk for KDM subjects, there is no increase in cardiovascular markers such as leptin and LDL-c, which is showing an indirect effect.
The HSP70-hom +2437 C/T polymorphism (Met→Thr amino acid substitution at position 493) was reported to be associated with spondyloarthropathies (Vargas et al., 2002) and sarcoidosis (Bogunia et al., 2006). It was also found to affect T1DM (Pociot et al., 1993). It is thought that this substitution may be associated with variations in the peptide-binding specificity of different HSP70-hom haplotypes. In our present study, this HSP70-hom +2437 C/T polymorphism showed susceptibility to KDM and DFU-DN. This report is in agreement with an earlier report where the TT genotype was associated with DFU. Mir et al., had analyzed the HSP-70 gene association only in patients with DFU and did not compare them with healthy controls. In contrast, we have compared the genotypes of patients with DFU to that of healthy controls and found TT genotype to be significantly associated towards the risk for T2DM and DFU-DN. Though in the present study we have not examined the levels of serum HSP-70, the possibility that the substitution of Met→Thr amino acid (at position 493) may result in variations in the peptide-binding specificity of different HSP70-hom haplotypes. The neutrality of the Thr residue may affect the efficiency of the HSP70-hom protein in acting as a molecular chaperone by lowering the strength of hydrophobic interactions between the chaperone and target protein has been described by Pociot’s theory (Pociot et al., 1993).

One of the limitation of this work is the cross sectional nature of the study. The causal mechanisms of genetic background and clinical phenotypes need to be observed in prospective and longitudinal studies.