Chapter - 3

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The present study in the population of Mysore district, Karnataka was planned to identify the novel variants in conserved and coding regions of *ADIPOQ, HHEX* and *KCNJ11* genes which were reported in literature to be associated with type 2 diabetes. The results of the study are presented in the following.

3.1. Results of covariates

Table 3.1 lists the results of covariate analysis of the present study. The mean age of the controls (51.51±14.0 yr) was less than the type 2 diabetes cases (56.30±11.3 yr); mean age of onset of the disease in the cases was 51.2±15.8 yr. The table further shows that BMI of diabetic cases (24.2±4.1) was higher than non-diabetic controls (22.2±4.4) and this difference was found to be statistically significant (p<1.8E-06).

The mean waist circumference in the cases (90.6±9.8 cm) was higher than the controls (80.6±10.7 cm)(Table 3.1) and the difference was statistically significant (p=5.4E-21). Similarly, the hip circumference in diabetic cases (95.3±8.5 cm) was higher than non-diabetic controls (89.6±8.8 cm) (t = -5.67, p = 6.1E-11). The WHR in diabetic cases (0.95±0.1) was also significantly higher than the controls (0.90±0.1) (t = -6.36, p= 5.2E-10). The mean systolic blood pressure was almost equal in the cases and controls, whereas mean diastolic blood pressure was low in diabetic cases (81.6±10.5 mmHg) than controls (87.2±12.2 mmHg) and the difference was statistically significant (t= 5.65, p = 5.7E-07).

The mean fasting plasma glucose level of the controls was 99.7±11.4 mg/dl which was significantly lower than cases (212.1±72 mg/dl) (t= -
21.82, \( p < 5.6 \times 10^{-71} \)). Similarly, 2 hours plasma glucose level in the diabetic cases (329.4±99.3 mg/dl) was significantly higher (\( p < 4.2 \times 10^{-103} \)) than that of non-diabetic controls (122.9±19.0 mg/dl) (Table 3.1).

### 3.2. Life style and medication

Table 3.2 shows that the proportion of the vegetarians was high in the controls (69.26%) compared to type 2 diabetic cases (46.72%) and the difference was statistically significant (\( p < 0.00003 \)). The percentage of non-smokers was similar in the cases and controls while in the cases percentage of subjects who quit smoking was higher (12.62%) than the controls (1.95%) and the difference was statistically significant (\( p < 0.001 \)). Similar trend was observed in other forms of tobacco consumption; 2.8% case subjects quit tobacco consumption whereas no one quit tobacco consumption in the controls. Percentage of subjects who were consuming tobacco in other forms was slightly but significantly higher in the controls (8.29%) compared to the cases (6.54%) (\( \chi^2 = 6.194, p < 0.045 \)). No significant difference was observed in percentage of subjects consuming alcohol, whereas percentage of subjects who quit alcohol consumption was more in type 2 diabetes subjects (5.67%) than the controls (1.46%). Percentage of diabetic cases under hypertension medication (22.34%) was significantly higher than the controls (3.41%) (\( p < 0.00001 \)).

### 3.3. Genetic variation

In the sequenced region of \textit{ADIPOQ} gene, a total of 34 variants were observed, of which 16 were the novel. Seven were in the 5' flanking region, four in intron 1, two in exon 2, two in intron 2 and 19 variants were found in 3' UTR of the gene. Since most of the novel variants were located in non-coding regions and 5' flanking regions of the gene, the coordination system numbering was used as their identification. Of 34 SNPs, 21 were located in exons including 10 novel variants. Two variants
were observed in exon 2 of which one i.e. rs2241766 was synonymous mutation and the other i.e. 186570944 was nonsense mutation which caused change in codon 33 (AAG) to stop (amber) codon and was only found in a single case subject.

In HHEX, no variants were observed in the exonic region of the gene. Sequencing of 1173 base pairs coding region of KCNJ11 gene revealed one novel synonymous variant g. 309C>T (S103S) and seven previously reported variants viz., rs5219, g. 602T>G (R201L), rs5218, rs5216, rs1800467, rs5215 and rs41282930 in the studied population.

**Allele frequencies**

A total of 44 variants were observed in the sequenced regions of the three genes, of which 17 were the novel. In the cases, 28 variants showed minor allele frequency greater than 0.01 whereas in the controls 27 variants showed such minor allele frequency. The Hardy-Weinberg Equilibrium (HWE) test results showed that barring SNPs rs2241766, 186573952, rs1063537, rs2082940 and rs4686803 of ADIPOQ, all remaining variants were in genetic equilibrium (Table 3.3.).

By Fisher's exact test, none of the SNPs showed significant difference in allele frequency between the cases and controls in any of the studied genes (Table 3.4). By Chi square test, variant 186573888 of ADIPOQ showed marginal significance (p=0.045), but the variant was observed in only four control samples. SNP rs2241766 (+ 45 T>G) was said to be associated with type 2 diabetes in European and the East Asian populations, whereas in the present study the minor allele frequency was higher in cases (0.14) than controls (0.13) but the difference was statistically insignificant (Table 3.4).
In *KCNJ 11*, minor allele frequency of rs5219 was higher in the cases (0.36) than controls (0.32) (Table 3.4) but the deference was statistically insignificant. Similarly minor allele frequency of rs5215, which is in strong LD with rs5219 (D’> 0.9), was found to be higher in the cases (0.36) than controls (0.32) but the difference was not statistically significant. SNP rs5128 showed comparatively higher minor allele frequency in the controls (0.20) than cases (0.16) and the difference was found to be statistically non-significant.

In *HHEX* exon regions no variants were found indicating strong conservation of the gene region. The SNPs rs1111875 and rs5015480 which are located near the gene showed slightly higher minor allele frequencies in the cases (0.409 and 0.425, respectively) than the controls (0.376 and 0.371, respectively) (Table 3.4).

### 3.4. Genotype association

Setting a cutoff value for minor allele frequency at <0.01 for their exclusion in this analysis in both the cases and controls, 30 SNPs were considered for genotype association tests. None of the SNPs from the studied three genes showed significant association with type 2 diabetes under additive and dominant genetic models (Table 3.5); under recessive genetic model SNPs rs1111875 and rs5015480, located near *HHEX* gene, showed nominal significance (OR=1.91, 95% CI 1.08±3.39, p=0.027 and OR= 2.18 95% CI 1.23±3.88, p= 0.008, respectively). However, after correcting for multiple tests the association was found to be non-significant.

Similar trend was observed in genotype association with fasting plasma glucose level (Table 3.6). None of the SNPs showed significant association with fasting plasma glucose level under additive and dominant genetic models. SNPs rs1111875 and rs5015480 were found to be nominally
associated with fasting plasma glucose level under recessive genetic model (beta=18.84, 95% CI -0.2±37.9, p=0.054 and beta 22.80, 95% CI 4.0±41.6, p=0.018, respectively). After correcting for multiple tests, the association was found to be non-significant.

An ADIPOQ SNP rs1501299 showed nominal association with 2 h plasma glucose level under additive and dominant models (beta=21.28, 95% CI -39.4±3.2, p=0.022 and beta -24.08, 95% CI -47.2±1.0, p= 0.042, respectively) (Table 3.7). In addition to this, SNP rs5015480 also showed nominal significance with 2 h plasma glucose level under dominant and recessive genetic models (beta= 17.26, 95% CI 1.0±33.5, p= 0.038 and beta= 39.79, 95% CI 9.3±70.2, p= 0.011, respectively). The association of these two SNPs with 2 h plasma glucose level was found to be non-significant after adjusting for multiple tests.

Table 3.8 shows that SNP rs2241766, which is located in exon 3 of ADIPOQ gene, was nominally associated with systolic blood pressure under recessive genetic model (beta= 8.87, 95% CI 0.1±17.6, p= 0.047), but the association was not significant after adjusting p value for multiple testing. In addition to rs2241766, rs5219, a KCNJ11 non-synonymous SNP also showed nominal significant association with systolic blood pressure under dominant model (beta= 3.12, 95% CI 0.2±6.0, p= 0.037. However, the association of these SNPs with systolic blood pressure was not significant after adjusting for multiple tests.

SNP rs1063538, located at 3'UTR of ADIPOQ gene, showed nominal significance with diastolic blood pressure under dominant genetic model (beta= 2.58, 95% CI 0.3±4.9, p= 0.030) but the association was found to be non-significant after correcting for multiple testing (Table 3.9).

None of the SNPs in the studied three genes showed association with BMI (Table 3.10).
Table 3.11 shows that SNP 186559808, located at 5' flanking region of *ADIPOQ* gene, was nominally associated with the age of onset of diabetes under dominant model (beta= -5.58, 95% CI -11.0±-0.2, p= 0.043). But the association was found not significant after adjusting for multiple tests.

### 3.5. Linkage disequilibrium and haplotype analysis

All the SNPs of the three genes were used to generate linkage disequilibrium (LD) plot to identify LD blocks (Figure 3.1). SNPs within the gene were observed to be in linkage disequilibrium. In *ADIPOQ*, four LD blocks were observed, one each in intron 1 and in both exon and intron 2 and the remaining two in 3'UTR region of the gene. Two SNPs near *HHEX* gene loci were in strong LD ($r^2=0.99$). Similarly, *KCNJ11* SNPs rs5219 and rs5215 were in strong LD ($r^2=0.96$).

Haplotype GT of SNPs rs182052 and rs62292784 of *ADIPOQ* gene showed relatively higher frequency in the cases (0.09) than controls (0.04) (Table 3.12). The Chi square test showed that this difference was nominally significant (p= 0.0067), but after 10,000 permutations the association was found to be non-significant. In fact, none of the 17 haplotypes considered in this study was found to be statistically associated with type 2 diabetes (Table 3.12).

It has been well established that type 2 diabetes is one of the major chronic non-communicable diseases in the world. Being a developing country with population exceeding one billion, India occupies the first place in the world in number of individuals suffering with the disease (Whiting et al., 2011). The Asian Indians are more prone to type 2 diabetes than are the Europeans and other ethnic groups of the world. As defined by Joshi (2003), the Asian Indian phenotype, with less body
mass index and more body fat, was said to be one of the reasons for the high prevalence of diabetes in India.

In the present study mean BMI of diabetic cases (24.2±4.1) was higher than non-diabetic controls (22.2±4.4) but it was within the normal range as per the WHO classification (WHO, 2000). But according to WHO (2004) classification for the Asian populations, the diabetic case group was at increased risk category for diabetes and heart diseases. Further support comes from measures of central obesity, waist circumference and waist to hip ratio (WHR). Mean waist circumference of type 2 diabetic subjects was higher than the control subjects and the same pattern was observed for waist to hip ratio. WHR of diabetic cases (0.95±0.1) was more than the WHO (2011) risk cut-off (>0.9). Alarmingly, WHR of non-diabetic controls (0.90±0.1) was at the borderline of WHO risk cut-off. These results suggest that the Asian Indian phenotype, which was defined as low BMI but with more visceral fat, was evident not only in the present cases but also the control subjects who therefore were at risk for the metabolic complications.

Diastolic blood pressure was low in type 2 diabetic cases than the control subjects, which could be attributed to hypertension medication in diabetic subjects. Relatively higher percentage of type 2 diabetes subjects were found to be under hypertension medication implying that such subjects are at high risk for secondary hypertension in the study population. Low levels of diastolic blood pressure in type 2 diabetes subjects might be due to hypertension medication, but low levels of diastolic blood pressure (<70 mmHg) in elderly was also known for increased risk for cardiovascular complications (Protogerou et al., 2007; Anderson et al., 2011).

In the studied population, the majority of diabetes subjects were non-vegetarians (69.26%) indicating high calorie food intake compared to the
control subjects (30.75%). In addition to the food habits, life style is one of the major factors involved in increasing the risk of type 2 diabetes in the Indian populations. In the studied population, relatively more type 2 diabetic subjects quit consumption of tobacco (12.62%) and alcohol (5.67%) than controls (1.96% and nil, respectively), which might be due to the physician’s prescription to quit smoking or tobacco consumption after the disease was diagnosed. Though the tobacco and alcohol consumption was slightly higher in the controls, but in view of the subjects who quit these habits in type 2 diabetic subjects, it was evident that these two life style habits have significant effect on disease development. In addition to smoking Indian populations are known for consuming tobacco orally. In the studied population other forms of tobacco consumption in the controls (8.29%) and cases (6.54%) imply that there were more such consumers in the controls, but as for smoking, subjects who quit other forms of tobacco consumption was higher in the cases (2.8%) than controls (nil).

Experimental and clinical studies suggest that smoking decreases insulin sensitivity and consequently results in the disorders of glucose and lipid metabolism such as hyperglycemia and dyslipidemia, including low HDL cholesterol and postprandial lipid intolerance (Borggreve et al., 2003). Particularly in diabetic patients, it was clear that cigarette smoking worsens the metabolic control. A larger insulin dose is needed to achieve similar metabolic control in smoking patients as in non-smokers (Madsbad et al., 1980). Furthermore it was demonstrated that the nicotine-induced oxidative stress differentially targeted the mitochondria in the pancreas (Bruin et al., 2008a), resulting in mitochondrial-mediated beta cell apoptosis (Bruin et al., 2008b).

Genome wide association (GWA) studies for common complex diseases have thus far succeeded in explaining only a modest fraction of the
genetic component leaving majority of the component unknown. To explain the extent of genetic component in the complex diseases, identifying the rare variants and their role in disease development is important.

In the present study, more than half (16) of the observed 34 variants in ADIPOQ gene were novel, indicating the diversity within the gene. Out of 16 novel variants, eight were having frequency higher than 0.01 in the study population. Identification of these SNPs revealed the hidden population specific diversity in this gene. This also helps in explaining population specific risk SNPs and alleles. In a previous report by Codner et al. (2005), g. 602T>G (R201L) SNP was observed in a case of early onset of diabetes, but in the present study it was observed in three type 2 diabetic subjects with mean age of onset of the disease as 47.25 yr. This explains population specific functionality of genetic variants.

In this study, exons of HHEX gene were sequenced in addition to genotyping two previously reported SNPs viz., rs1111875 and rs5015480 near the gene region. No variant was found in the exon regions of HHEX gene, this may be due to high level of conservation of the gene region. In addition to the present work, a study conducted in the European population was able to find only 2 SNPs with very low minor allele frequency in the exonic regions of the gene indicating high level of conservation of the gene region (Minton et al., 2007).

In the present study, none of the SNPs showed significant association with type 2 diabetes. Although minor allele frequencies of SNPs rs5219 and rs5215 in KCNJ11 gene were high in the cases than controls but the difference was found to be statistically no-significant. Similar pattern was observed for two SNPs rs1111875 and rs5015480 which are located near HHEX gene. The present findings are in corroboration with the studies conducted in different endogamous populations of North India.
(Sanghera et al., 2008; Gupta et al., 2010). As in the present investigation, these studies had relatively low sample size, but even in other studies despite sufficient sample size (Chauhan et al., 2010; Chavali et al., 2011), the significance of association between \textit{KCNJ11} and \textit{HHEX} SNPs and type 2 diabetes was none or marginal in the Indian populations.

The findings of the present study regarding \textit{HHEX} gene are interesting because no SNP or mutation was found in the exonic region of the gene while the two SNPs near the gene showed nominal association with type 2 diabetes. This can be explained by two possibilities, one, there might be functionally active SNPs in the promoter or intronic region of the gene which may cause variations in gene expression and in turn cause the disease. Second, the associated SNPs near \textit{HHEX} lie in a 295 kb block of LD that includes three genes viz., \textit{HHEX}, \textit{KIF1} and \textit{IDE}. The association signal of these SNPs might be better explained if we study the other two genes. Promising results were obtained in studies on congenic rats with the \textit{IDE GK} allele which displayed postprandial hyperglycaemia, reduced lipogenesis in fat cells, blunted insulin-stimulated glucose transmembrane uptake and reduced insulin degradation in isolated muscle (Fakhrai-Rad et al., 2000).

\textit{ADIPOQ} SNP rs1501299 showed nominal association with 2 h plasma glucose level under additive and dominant genetic models, suggesting its possible role in insulin resistance. The insulin-sensitizing effect of adiponectin appears to be primarily attributed to its direct actions on skeletal muscle and liver, through the activation of AMPK and peroxisome proliferator-activated receptor (PPAR) (Yamauchi et al., 2002; Tomas et al., 2002).

In liver, stimulation of AMPK by full-length adiponectin leads to decreased expression of gluconeogenic enzymes, such as
phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, which may account for its glucose-lowering effect in vivo (Combs et al., 2004; Yamauchi et al., 2002). In skeletal muscle, activation of AMPK by globular or full-length adiponectin causes increased expression of proteins involved in fatty acid transport (such as CD36), fatty acid oxidation (such as acyl-coenzyme A oxidase) and energy dissipation (such as uncoupling protein-2), resulting in enhanced fatty acid oxidation and energy dissipation, and decreased tissue triglyceride (TG) accumulation. Excessive tissue TG accumulation has been proposed to be a major causative factor of insulin resistance in skeletal muscle (Hegarty et al., 2003). Therefore, reduction of tissue TG contents by adiponectin might be the major contributor to the insulin-sensitizing activity of this adipokine.

Further evidence of the role of adiponectin in type 2 diabetes development came from the results of haplotype analysis. Haplotype GT of SNPs rs182052 and rs62292784 of ADIPOQ gene showed relatively high frequency in the cases (0.09) than controls (0.04). This difference was nominally significant (p = 0.0067). Similar results were reported by Sanghera et al. (2010) in the Indian Sikh population. A study conducted on an endogamous North Indian population reported that a differential association of TCF7L2 SNPs rs7903146, rs4506565 and rs12256372 and ADIPOQ SNPs rs2241766 and rs1501299 with higher BMI, systolic blood pressure and WHR was observed, indicating indirect role of ADIPOQ in type 2 diabetes development (Gupta et al., 2012).

The possible explanation for not observing association between ADIPOQ variants and type 2 diabetes in the present study could be that only the conserved regions along with the promoter regions of the gene were selected to identify the novel variants. In this process the enhancer regions of the gene might have been missed. For instance, a study
conducted in South Indian population reported strong association of +10211T->G SNP of *ADIPOQ* with type 2 diabetes and obesity and hypoadiponectinemia (Vimaleswaran et al., 2008) but the SNP was not included in the present study due to its design limitations. This warrants further studies for re-sequencing of entire *ADIPOQ* gene and its promoter region so that more functional variants can be found for the better explanation of the role of the gene in the development of type 2 diabetes.