Chapter 2

To evaluate the association of ADIPOQ polymorphisms with type 2 diabetes (T2D) in Gujarat population and to study the possible genotype-phenotype correlation with plasma adiponectin levels and metabolic parameters

2.1 Introduction

Metabolic Syndrome with obesity and type 2 diabetes (T2D) under its umbrella, is affecting millions of people around the globe. Though many suffer worldwide from obesity and T2D, there exists a prominent pattern of ethnicity-based prevalence. Recently it has been identified that in the backdrop of genetic predisposition, other factors like lifestyle, demographic transitions and nutrition play important role in the rising trend of obesity associated T2D in South Asians (Misra and Srivastava, 2013). Over accumulation of visceral adipose tissue (AT) has been identified specifically as a major driving factor towards T2D. AT, apart from being an energy store house, regulates metabolic homeostasis by secreting bioactive peptides (pro- and anti-inflammatory adipokines). In obese condition, the balance between the pro- and anti-inflammatory adipokines gets altered leading to various metabolic disorders (Pramanik et al., 2018). These bioactive peptides function locally and distally to attune and fine tune various metabolic pathways. One such calibrator, adiponectin is expressed in white adipose tissue and exists in three polymorphic forms, low molecular weight (LMW), moderate molecular weight (MMW) and high molecular weight (HMW) in circulation (Vasseur et al., 2002). Remarkably, the ratio of HMW adiponectin to total adiponectin has been found to be significantly correlated with plasma glucose levels than any of three forms alone (Lara-Castro et al., 2006) Adiponectin gene (*ADIPOQ/APM1/GBP28*) locus, 3q27, has been strongly linked with a variety of metabolic disorders which includes obesity, insulin resistance, dyslipidemia and T2D (Kissebah et al., 2000; Vionnet et al., 2000; Mori et al., 2002). Interestingly, certain SNPs of the adiponectin gene (*ADIPOQ*) have been found to be strongly associated with T2D in certain ethnic groups (Vasseur et al., 2002; Gu et al., 2004; Nannipieri et al., 2006; Vimaleswaran et al., 2008; Saxena et al., 2012). However, T2D being a polygenic and multi-factorial metabolic disorder (Hassen, 2002), adiponectin gene SNPs significantly differ in their association with T2D amongst different ethnic populations (Sim et al., 2011; Keaton et al., 2014). The SNPs we aimed to study were selected based on the following criteria: 1) SNPs validated for their frequency in Genome Wide Association Studies (GWAS), 2) SNPs proven for their role in increased or decreased adiponectin protein synthesis. *ADIPOQ* comprises of 2 introns and 3 exons coding for a 30kDa adiponectin protein (Takahashi et al., 2000). We have studied four SNPs: -11377 C/G (rs266729) in promoter, +10211T/G (rs17846866) in intron 1, +45 T/G

(rs2241766) in exon 2 and +276G/T (rs1501299) in intron 2, to explore their association with T2D. Since Indian population is relatively non-homogenous, we conducted our study in the native Gujarat population to exclude the bias due to population stratification. Further, a genotype-phenotype correlation of the above-mentioned SNPs with T2D with plasma adiponectin levels, and fasting blood glucose (FBG), body mass index (BMI) and plasma lipid profile was analysed.

2.2 Materials and Methods

2.2.1 Study subjects

Blood collection camps were conducted to ensure the involvement of individuals from all socio-economic strata. The importance of the study was explained to all the participants and informed consent was obtained from all the subjects. Using a case-control approach, 475 T2D patients (211 males and 264 females) and 493 control subjects (250 males and 243 females) between the age group of 30 to 67 years were recruited with at least five previous generations belonging from Gujarati ethnicity. Autoimmune diseases or cancer patients were excluded from the study. A thorough family history of the patients was documented to collect information on first- and second-degree relatives and their history of T2D. The controls selected were healthy and showed FBG< 110mg/dL with no prior history of T2D while the T2D patients recruited for the study displayed FBG> 125 mg/dL (ADA, 2014). The study subjects comprised of both obese and lean individuals and their BMI (weight in kg/ height in m^2) was calculated by recording height and weight. The study was carried out in agreement with the declaration of Helsinki and was approved by the institutional ethical committee for human research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2016-9).

2.2.2 Blood collection and DNA extraction

FBG levels were measured by finger-prick method using glucometer (TRUEresult® - Nipro). Three ml venous blood was drawn from T2D and ethnically matched controls and collected in K_3EDTA coated tubes (Greiner Bio-One, North America Inc., North Carolina, and USA). Plasma was separated and stored at -20^{o}C for lipid profiling, assay of plasma HMW and total adiponectin levels. DNA was extracted from PBMCs by phenol-chloroform method (Sambrook and Russell, 2006). The DNA content and purity were determined spectrophotometrically by using the 260/280 absorbance ratio. The integrity of

DNA was checked electrophoretically on 0.8% agarose gel. The DNA was normalized and stored at 4^{o}C until further analysis.

2.2.3 Screening of *ADIPOQ* single nucleotide polymorphisms

Samples from Gujarat population were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for -11377C/G, +10211T/G and +276G/T polymorphisms in the *ADIPOQ* gene. The reaction mixture of the total volume of 20 µL included 3 µL (150ng) of genomic DNA, 11µL nuclease-free H_2O, 2.0 µL of 10X PCR buffer, 2µL of 25mM dNTPs (Puregene, Genetix Biotech), 1µL of 10mM respective forward and reverse primers (Eurofins, Bangalore, India), and 0.2µL (5 U/µL) taq polymerase (Puregene, Genetix Biotech). DNA amplification was performed using an Eppendorf Mastercycler gradient (USA Scientific, Inc., Florida, USA) according to the protocol: 95°C for 10 min., followed by 39 cycles of 95°C for 30 sec., primer dependent annealing (Table 2.2) for 30 sec., 72°C for 30 sec and final annealing at 72°C for 10 min with primer dependent annealing temperatures (Table 2.1). Amplification was confirmed by electrophoresis on an ethidium bromide stained 2.0% agarose gel. Details of the restriction enzymes (Fermentas, Thermo Fisher Scientific Inc., USA) and digested products are mentioned in Table 2.1. 1U of the respective restriction enzyme in a total reaction volume of 20µl as per the manufacturer's instruction was used to digest 15µl of the amplified products. The digested products were resolved on ethidium bromide stained 3.5% agarose gel with 50 base pair DNA ladder (HiMedia, India) and visualized under UV transilluminator i.e., E-Gel Imager Life Technologies (Figure 2.1 A, B & C). >10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently) and further confirmed by sequencing. *ADIPOQ* +45T/ G SNP was genotyped by TaqMan real time PCR using the pre-designed assay ID c__26426077_10 for allelic discrimination, containing specific probes for each allele marked with VIC and FAM fluorescent dyes (Thermo Fisher Scientific, USA). Real-time PCR was performed in 10 µl volume using LightCycler®480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. A no-template control (NTC) was used with the SNP genotyping assay. Samples with each genotype were analyzed together as an internal control.

Table 2.1. Details of primers and restriction enzymes

Gene/SNP	Primer sequence	Annealing Temperature (^{0}C)	Amplicon Size (bp)	Restriction Enzyme	Digested products (bp)
(rs266729) -11377 C/G FP	5'-GCTCTGTGTGGACTGT GGAG-3'	61.3	303	*Hha*I	181bp +122bp
-11377 C/G RP	5'-TAGAAGCAGCCTGGA GAAC T-3'				
(rs17846866) +10211T/G FP	5'-GCTAAGTATTACAGA TTTCAGGGCAG-3'	62	293	*Hinf*I	132bp + 107bp + 54bp
+10211T/G RP	5'-CAGCCATGGAGAGA CAGACCC-3'				
(rs1501299) +276 G/T FP	5'-GATGCAGCAAAGCCA AAGTC-3'	61	196	*Bsm*I	148bp + 48bp
+276 G/T RP	5'-TGGCCTCTTTCATCAC AGACC-3'				

FP-Forward primer, RP- Reverse primer, bp- base pairs

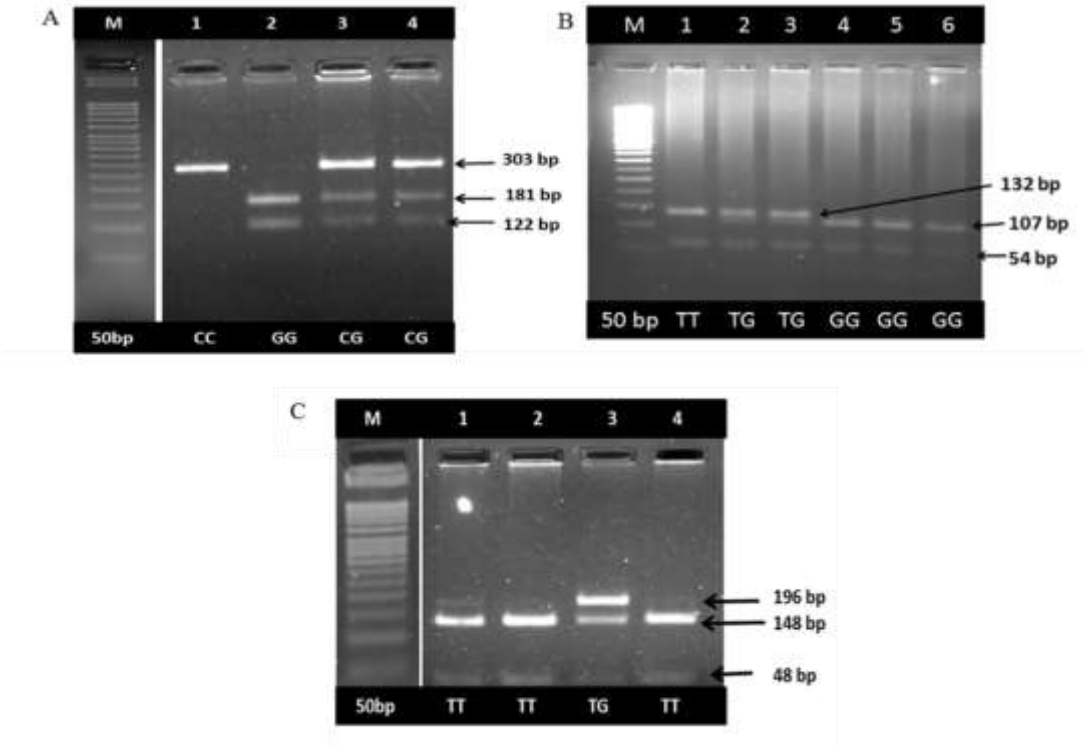

Figure 2.1. PCR-RFLP analysis of *ADIPOQ* -11377 (rs266729) **C/G, +10211 T/G** (rs17846866), and **+276 G/T SNPs**(rs1501299)**: A)** PCR-RFLP analysis of -11377C/G (rs266729) SNP on 3.5% agarose gel electrophoresis; lane 1: shows homozygous (CC) genotype, lane 2: shows homozygous (GG) genotype; lane 3 & 4: show heterozygous (CG) genotype. **B)** PCR-RFLP analysis of +10211 T/G (rs17846866) on 3.5% agarose gel electrophoresis; lane 1: shows homozygous (TT) genotype, lanes 2 & 3: show homozygous (TG) genotype; lane 4, 5 & 6: show homozygous (GG) genotype. **C)** PCR-RFLP analysis of +276 T/G (rs1501299) on 3.5% agarose gel electrophoresis; lane 1, 2 & 4: show homozygous (TT) genotype, lanes 3: shows heterozygous (TG) genotype.

2.2.4 Plasma parameters

Plasma total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL) levels were measured using commercial kits (Reckon Diagnostics P. Ltd, Vadodara, India). Low-density lipoprotein (LDL) was calculated using Friedewald's (1972) formula (Knopfholz *et al*., 2014). Human total adiponectin and HMW adiponectin ELISA kits (Elabioscience Biotechnology Inc., USA) with a sensitivity of 0.47ng/mL and 3.75ng/mL respectively were used to estimate the levels of total adiponectin and HMW adiponectin in patients and controls. The plasma samples used were freeze-thawed only once. All the

estimations from plasma estimations were carried out in duplicates with % coefficient of variation within 10%.

2. 3 Statistical Analyses

The baseline parameter data were checked for normal distribution and were analyzed by unpaired t-test while Mann-Whitney test was used for data not following normal distribution. Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all the SNPs in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The genotypes and allelic frequencies distribution of *ADIPOQ* SNPs for patients and control subjects were compared using the chi-square test with 2x2 contingency tables using GraphPad Prism 5 software. The genotypes were analyzed in an additive, dominant and recessive model as there were low genotype frequencies of the homozygous minor alleles (< 10% frequency). *p* values less than 0.0125 was considered as statistically significant as per Bonferroni's correction for multiple testing for genotypes and allelic distribution. The association strength of the *ADIPOQ* SNPs with risk for T2D was assessed by odds ratio (OR) at a confidence interval (CI) of 95%. Haplotypes and linkage disequilibrium (LD) coefficients ($D' = D/D_{max}$) and r^2 values for the pair of the most common alleles at each site were obtained using http://analysis.bio-x.cn/myAnalysis.php (Li *et al*., 2009). Association studies of SNPs with other parameters were performed using analysis of variance (ANOVA) and Kruskal Wallis test. Adjustments were made to eliminate possible confounding effects of age, sex, and BMI. All the analyses were carried out in GraphPad Prism 5 software. *p* values less than 0.05 was considered significant for all the other association studies. Functional impact of non-coding polymorphisms was predicted using ENCODE prediction tool (https://www.encodeproject.org/) [ENCODE].

2.4 Results

The baseline clinical parameters were significantly different between controls and patients (Table 2.2). A significantly higher FBG ($p<0.0001$) was present in patients as compared to controls. Further, BMI, TC, TG and LDL were significantly elevated ($p<0.0001$, $p=0.0360$ and $p=0.001$, respectively) while HDL was significantly reduced ($p<0.0001$) in patients as compared to controls.

Table 2.2. Baseline characteristics of controls and patients from Gujarat population

	Controls	**Patients**	***p* value**
	Mean ± SD	Mean ± SD	
	(n =493)	(n =475)	
Age (years)	49.64 ± 16.35	55.99 ± 10.42	-
Sex: Male	250 (52.6%)	211 (44.5%)	-
Female	243 (51.2%)	264 (55.5%)	-
FBG (mg/dL)	100.1 ± 7.32	155.3 ± 32.09	<0.0001
BMI (Kg/m^2)	24.24 ± 5.2	27.04 ± 5.1	<0.0001
TC (mg/dL)	160.9 ± 22.2	166.2 ± 19.68	0.036
TG (mg/dL)	111.7 ± 25.90	164.5 ± 11.1	0.001
HDL (mg/dL)	42.79 ± 15.94	38.2 ± 12.6	<0.0001
LDL (mg/dL)	84.69 ± 28.06	110.7 ± 29.2	<0.0001
Onset age (years)	NA	50.65 ± 10.10	-
Duration of disease (years)	NA	8.06 ± 7.3	-

2.4.1. Association of *ADIPOQ* SNPs with T2D

The distribution of genotypes and allelic frequencies of *ADIPOQ* SNPs is summarized in Table 2.3. The genotype frequency distribution for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations (HWE) ($p>0.05$). Analysis of the genotype frequencies of +10211T/G (rs17846866) and +276G/T (rs1501299) SNPs in an additive model revealed them to be significantly associated ($p<0.0001$) while the promoter -11377 (rs266729) and exonic +45T/G (rs2241766) SNPs were not associated with T2D (Table 2.3). Further, in the recessive model a significant association was detected for the intron 1 +10211T/G $p<0.0001$ (OR = 1.797, 95% CI = 1.369-2.359) with T2D. Likewise, the intron 2 +276G/T SNP was also found to be significantly associated (OR= 2.05, 95% CI, 1.57-

2.65, $p< 0.0001$) in the recessive model as shown in Table 2.3. The frequency of mutant alleles for +10211T/G and +276G/T was noted to be significantly higher in diabetic patients as compared to controls (OR= 2.33 and 1.726 respectively).

Table 2.3. Genotype and allele frequencies distribution of *ADIPOQ* SNPs in controls and T2D patients in Gujarat population

SNP	N	Genotype		Allele		Odds Ratio [95% CI] (p-value)			
						Allelic	Additive	Dominant	Recessive
Gujarat Population									
rs266729		**CC**	**CG+GG**	**C**	**G**	1.23		1.46	1.28
Controls	286	155	131	427	145	[0.95-	0.2644	[0.72-	[0.92-
T2D Patients	285	137	148	402	168	1.59] (0.118)		2.95] (0.1443)	1.77] (0.1432)
rs17846866		**TT**	**TG+GG**	**T**	**G**	2.33		1.46	1.79
Controls	493	363	130	847	139	[1.85-	<0.0001	[0.15-	[1.36-
T2D Patients	475	289	186	687	236	2.93] (<0.0001)		2.02] (<0.0001)	2.35] (<0.0001)
rs2241766		**TT**	**TG+GG**	**T**	**G**	0.86		0.74	0.86
Controls	467	362	105	822	112	[0.64-	0.6704	[0.22-	[0.61-
T2D Patients	359	287	72	642	76	1.18] (0.3722)		2.55] (0.6325)	1.21] (0.3954)
rs1501299		**GG**	**GT+TT**	**G**	**T**	1.72	<0.0001	1.99	2.05
Controls	489	255	216	692	250	[1.42-		[1.28–	[1.57-

T2D Patients	464	172	298	579	361	2.09] (<0.0001)	3.08] (0.0018)	2.65] (<0.0001)

2.4.2. Haplotype and Linkage Disequilibrium Analysis of *ADIPOQ* SNPs

Haplotype evaluation of the four polymorphic sites of *ADIPOQ* was performed. The estimated frequencies of the haplotypes differed significantly between patients and controls (global $p=7.76 \times 10^{-12}$) as shown in Table 2.4. CGTG ($p=0.0003$), CGTT ($p=6.32 \times 10^{-5}$), GGTT ($p=0.0207$) and GGTG ($p=0.0030$) were found to be the disease susceptible haplotypes (Table 2.4). Furthermore, the GGTG ($p=3.87 \times 10^{-5}$) haplotype was found to be significantly higher especially in obese patients as shown in Table 2.5. The LD analysis revealed that the four SNPs investigated were in low to moderate LD association (Figure 2.2).

Table 2.4. Haplotype frequencies in T2D patients and controls in Gujarat population

Haplotype rs266729, rs17846866, rs2241766, rs1501299	**Patients (Frequency %) (n=494)**	**Controls (Frequency %) (n=458)**	***p* for Association**	***p* (global)**	**Odds Ratio [95%CI]**
C G T G*	61.86(0.12)	26.32(0.05)	0.0003	7.76×10^{-12}	2.37 [1.476~3.829]
C G T T*	32.98(0.06)	6.90(0.015)	6.32×10^{-5}		4.72 [2.058~10.849]
C T G G	18.31(0.03)	26.38(0.05)	0.1398		0.63 [0.344~1.167]
C T T G	135.16(0.27)	200.65(0.43)	1.03×10^{-7}		0.47 [0.361~0.626]
C T T T	88.43(0.17)	66.34(0.14)	0.1390		1.30 [0.917~1.846]
G G T G*	21.95(0.04)	5.66(0.01)	0.0030		3.74 [1.474~9.534]
G G T T*	16.72(0.03)	5.25(0.01)	0.0207		3.04 [1.132~8.188]
G T T G	51.96(0.10)	71.78(0.15)	0.0195		0.63 [0.432~0.931]
G T T T	34.95(0.07)	22.09(0.04)	0.1362		1.51 [0.874~2.625]

*Indicates haplotypes significantly associated with T2D. Frequency<0.03 were ignored in the analysis.

Table 2.5. Haplotype frequencies in lean and obese T2D patients in Gujarat population

Haplotype rs266729, rs17846866, rs2241766, rs1501299	**Obese Patients (Frequency %) (n=330)**	**Lean Patients (Frequency %) (n=150)**	***p* for Association**	***p* (global)**	**Odds Ratio [95%CI]**
C G T G*	24.49(0.129)	61.62(0.081)	0.0397	$2.26x10^{-8}$	1.68 [1.020~2.780]
C G T T*	15.12(0.080)	25.66(0.034)	0.0053		2.48 [1.285~4.799]
C T G G	12.57(0.066)	35.80(0.047)	0.2851		1.43 [0.738~2.791]
C T T G*	53.25(0.280)	273.96(0.361)	0.0317		0.67 [0.474~0.968]
C T T T*	17.77(0.094)	133.56(0.176)	0.0051		0.47 [0.283~0.809]
G G T G*	15.34(0.081)	16.02(0.021)	3.87×10^{-5}		4.10 [1.993~8.434]
G T T G*	14.89(0.078)	106.21(0.140)	0.0219		0.51 [0.293~0.917]
G T T T*	19.89(0.105)	39.53(0.052)	0.0072		2.14 [1.215~3.774]

*Indicates haplotypes significantly associated with obesity induced T2D. Frequency<0.03 were ignored in the analysis.

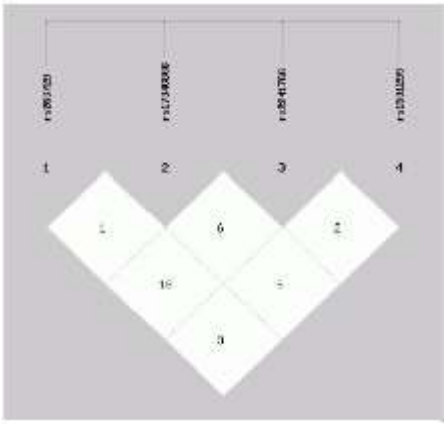

Figure 2.2: Linkage disequilibrium analysis of *ADIPOQ* SNPs in Gujarat population. LD block of *ADIPOQ* -11377C/G (rs266729), +10211T/G (rs17846866), +45T/G (rs2241766) and +276G/T (rs1501299) SNPs show low to moderate LD association.

2.4.3. Plasma HMW adiponectin/total adiponectin ratio in T2D patients and controls

Plasma HMW adiponectin and total adiponectin levels, and their ratio were monitored in 37 controls and 45 patients. They were significantly decreased ($p<0.001$) in T2D patients as compared to controls (Figure 2.3 A). A higher HMW adiponectin/total adiponectin ratio was observed in healthy females than in healthy males ($p<0.001$) and a significant decrease in the ratio was observed in males and with T2D when compared with their healthy counterparts ($p<0.05$ & $p<0.01$ respectively) (Figure 2.3 B). Healthy lean and obese individuals did not show any significant reduction in the HMW adiponectin/total adiponectin ratio. However, a significant decrease was seen in obese patients when compared to lean patients ($p<0.05$) (Figure 2.3 C). Lean and obese diabetic individuals showed reduced HMW adiponectin/total adiponectin ratio as compared to their respective controls ($p<0.05$, $p<0.01$). The decrease in the plasma adiponectin ratio was also seen in obese diabetic patients ($p<0.001$) (Figure 2.3 C).

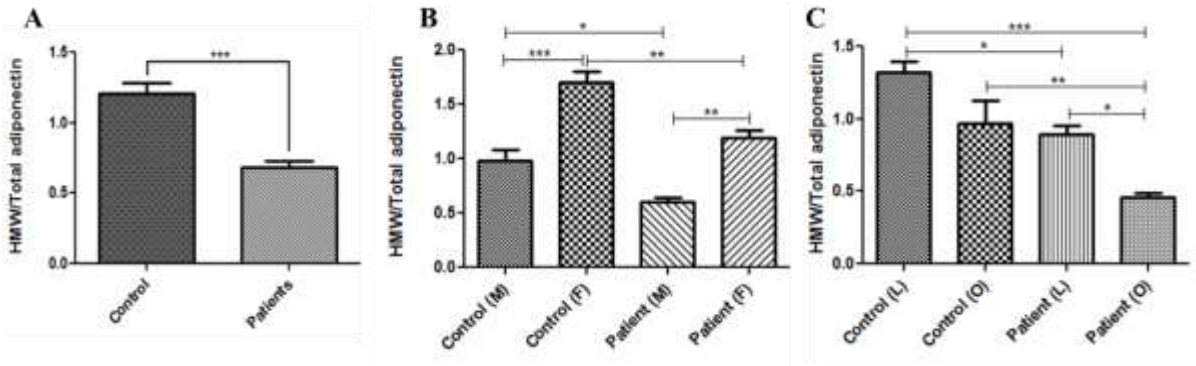

Figure 2.3. HMW adiponectin/ total adiponectin ratio in A) controls versus patients. Plasma HMW adiponectin / total adiponectin ratio in patients were significantly lower than in controls, **B) control and diabetic males and females.** HMW adiponectin /total adiponectin ratio in control and female patients were significantly higher than in control and male patients and **C) lean (L) and obese (O) control and diabetic subjects.** Obese patients showed significantly reduced HMW adiponectin / total adiponectin ratio (*$p<0.05$, ** $p<0.01$, *** $p<0.001$). (Controls n=37; T2D patients n=45).

2.4.4. Association of *ADIPOQ* SNPs and their genotypes with metabolic parameters and HMW adiponectin/total adiponectin ratio

The GG genotype of -11377C/G was significantly associated with increased levels of TG, LDL and HDL (females) as shown in Table 2.6. Also, the GG genotype of +10211T/G showed significant association with FBG, BMI, TG, TC, HDL and HMW adiponectin/total

adiponectin ratio while the TT genotype of +276G/T showed a significant association with increased FBG, BMI, TG, TC and LDL and, decreased HDL (p>0.05). However, +45T/G was not found to be associated with any of the parameters in Gujarat population (Table 2.6).

Table 2.6. Genotype-phenotype association analyses of *ADIPOQ* SNPs with metabolic parameters in Gujarat population.

Genotype / Allele	FBG (mg/dL)	BMI (Kg/m^2)	TG (mg/dL)	TC (mg/dL)	HDL (mg/dL)		LDL (mg/dL)	HMW adiponectin: total adiponectin (µg/mL)
					Male	Female		
***ADIPOQ* -11377 C/G (rs266729)**								
CC	124.50 (50.02)	25.37 (5.28)	123.00 (79.00)	161.70 (39.47)	36.81 (10.73)	45.17 (14.02)	93.83 (37.5)	0.97 (0.48)
CG	124.70 (51.02)	25.57 (5.95)	150.00 (102.00)	162.70 (39.52)	37.59 (9.30)	34.63 (9.96)	101.90 (39.36)	1.00 (0.54)
GG	124.10 (30.64)	26.36 (5.51)	166.00 (84.00)	156.40 (37.13)	39.75 (13.25)	26.56 (1.51)	101.40 (32.03)	0.64 (0.24)
***p* value**	0.6241	0.4906	**<0.0001**	0.8671	0.7369	**<0.0001**	**0.0087**	0.2055
***ADIPOQ* +10211 T/G (rs17846866)**								
TT	130.00 (56.13)	25.60 (5.90)	135.80 (92.00)	151.60 (27.89)	42.79 (14.38)	43.18 (14.57)	96.86 (37.5)	1.50 (0.61)
TG	132.20 (55.11)	25.33 (5.20)	138.90 (78.00)	162.20 (38.97)	41.62 (21.49)	44.16 (13.51)	96.64 (46.54)	0.86 (0.39)
GG	148.10	27.82	166.40	175.60	37.76	34.22	99.20	0.82

	(56.86)	(5.60)	(85.60)	(39.02)	(12.92)	(8.07)	(37.57)	(0.36)
***p* value**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0141**	**<0.0001**	0.6024	**0.0001**
***ADIPOQ* +45 T/G (rs2241766)**								
TT	155.40 (4.26)	26.82 (5.20)	164.00 (14.8)	163.80 (37.00)	36.62 (11.85)	40.53 (12.36)	95.79 (39.5)	0.98 (1.20)
TG	171.50 (12.96)	27.16 (5.29)	172.80 (20.3)	164.50 (44.91)	36.51 (11.00)	40.42 (14.46)	96.75 (39.26)	0.83 (0.38)
GG	122.50 (8.50)	30.05 (3.748)	103.90 (15.28)	185.70 (27.61)	34.57 (6.734)	41.27 (11.80)	94.87 (37.83)	0.82 (0.30)
***p* value**	0.3293	0.2619	0.6088	0.4735	0.9708	0.9936	0.9396	0.9284
***ADIPOQ* +276 G/T (rs1501299)**								
GG	151.00 (53.88)	24.98 (4.53)	143.30 (78.00)	153.20 (29.34)	37.87 (12.34)	40.64 (12.52)	70.36 (27.13)	1.36 (0.63)
GT	166.90 (69.67)	27.69 (5.53)	165.20 (89.00)	154.70 (32.12)	35.78 (10.48)	39.25 (12.56)	92.99 (36.33)	0.93 (0.44)
TT	303.80 (94.54)	29.75 (4.23)	266.60 (90.00)	189.00 (25.96)	33.28 (11.93)	37.34 (6.34)	90.62 (34.1)	0.75 (0.33)
***p* value**	**<0.0001**	**0.0001**	**<0.0001**	**0.0001**	**<0.0001**	0.0831	**0.005**	**0.0006**

Data represented as Mean (SD).

2.4.5. Bioinformatics analyses

ENCODE data base did not show overlap with any cis-response elements (cREs) or display any cREs within 2kb for -11377C/G (rs266729), +10211T/G (rs17846866), +45T/G (rs2241766) and +276G/T (rs1501299). Further, similar to our findings, the eQTL database GTex showed TG and GG genotypes of rs17846866 to have significantly reduced levels of plasma adiponectin. However, the eQTL data for the rest of the SNPs were not

available. Analysis of +45T/G, a synonymous exonic SNP, revealed that the glycine residue at 15th position remains unchanged (SIFT). On calculating the change in codon usage by relative synonymous codon usage (RSCU) approach to comprehend the relevance of ribosomal pause in reduced amount of protein being expressed it was found that the delta relative synonymous codon usage (RSCU) value for the GGT to GGG codon change was -0.31. However, no significant association of the +45T/G polymorphism was found with adiponectin levels.

2.5. Discussion

Our findings collectively suggest that CGTG, CGTT, GGTT and GGTG haplotypes of *ADIPOQ* -11377C/G (rs266729), +10211T/G (rs17846866), +45T/G (rs2241766) and +276G/T (rs1501299) polymorphisms were associated with T2D, with GGTG significantly associated with obesity induced T2D. Further, +10211T/G and +276G/T were strongly associated with obesity induced T2D susceptibility in Gujarat population. The findings were further associated with reduced levels of HMW adiponectin and disease-associated risk factors like FBG, BMI and lipid parameters thereby suggesting their crucial role in metabolic disease susceptibility.

Obesity has been associated with reduced anti-inflammatory and increased pro-inflammatory adipokines. The normal range of total adiponectin in healthy individuals range from 2-20µg/mL (Turer and Scherer, 2012). The typical short stature of South Asians in combination with visceral adiposity results in increased weight per area distribution predisposing them to metabolic diseases (Mohan *et al*., 1986; Bhardwaj *et al*., 2011; Misra and Srivastava, 2013). Though varying from population to population, genome-wide association studies have demonstrated a close association between adiponectin, *ADIPOQ* SNPs, fasting hyperglycemia and various metabolic diseases (Ling *et al*., 2009; Wu *et al*., 2010). Earlier studies have revealed promoter -11377C/G polymorphism to have a positive association with hypoadiponectinemia and risk of developing T2D (Ramya *et al*., 2013). However, we did not find this SNP to be associated with T2D or BMI in Gujarat population. Schaffler *et al.* also reported the absence of any transcription factor binding sites at or around this SNP site (Schäffler *et al*., 1998). However, the GG genotype of -11377 C/G did display an association with increased serum triglycerides and LDL, and reduced HDL in females. In spite of not being associated with

T2D, a possible indirect effect of other SNPs could be the reason for the observed altered association of the -11377C/G with the serum lipid levels.

Adiponectin gene expression is regulated by a 34 bp enhancer located in the first intron (Qiao *et al*., 2005). Thus, the finding of +10211T/G located close to this enhancer and its association with lipid metabolism and adiponectin levels in the present study is of significance. Though there is no overlap of this polymorphism with any cREs or display any cREs within 2kb as per ENCODE data base; eQTL database GTex display TG and GG genotypes of +10211 T/G to have significantly decreased levels of plasma adiponectin which is similar to our findings. Additionally, this SNP is also found to be associated with increased BMI, FBG, TG, TC and reduced HDL. Three independent studies, including ours, have recognized the association of +10211T/G with three different Indian populations from different demographical and geographical regions, further validating the significance of this SNP (Vimaleswaran *et al*., 2008; Saxena *et al*., 2012). +45T/G is a synonymous SNP with a codon change from GGT to GGG. Our results show no association between +45T/G and T2D as reinforced by studies on Italian, French and Swedish populations (Vasseur, *et al*., 2002; Gu *et al*., 2004; Nannipieri *et al*., 2006). However, studies on Chinese Han population found an association between +45T/G and insulin resistance (Tu *et al*., 2014). We also found a significant association of +276G/T with T2D, and serum lipid profile in Gujarat population. Supporting our data, similar results were reported in earlier studies in German (Stumvoll *et al*., 2002), Swedish (Ukkola *et al*., 2003), Italian Caucasian (Menzaghi *et al*., 2002), French Caucasian (Vasseur, *et al*., 2002) and South Indian populations (Ramya *et al*., 2003). The TT genotype conferred approximately twice the risk for developing T2D against the GG genotype in +276G/T. Furthermore, +276 G/T was also found to be linked with increased BMI, FBG, TC, and TG, and reduced HDL in males. These findings also suggest the association of +276G/T with Non-Alcoholic Fatty Liver Disease (NAFLD), a co-morbidity associated with T2D as supported by Wang *et al.* (Wang *et al*., 2016). Additionally, we have also found increased levels of resistin, Free Fatty Acids (FFA) and TNFα in obese patients (Rathwa *et al*., 2018; Patel *et al*., 2019). As TNFα is shown to be an important regulator in the formation of globular adiponectin by multimerization (He *et al*., 2016), our observations in concordance with increased TNFα and reduced HMW adiponectin levels in obese patients are clear. We had also reported an increase in IL1β levels in obese diabetic patients (Patel *et al*., 2016), asserting the rise in

pro-inflammatory (IL1β) and decrease in anti-inflammatory (adiponectin) adipokines in obesity associated low-grade inflammatory condition. Further, adiponectin levels show sexual dimorphism (Luque-Ramírez *et al*., 2013) and our results further confirm this as females in general demonstrated a higher tendency of HMW adiponectin/total adiponectin ratio than males. Also, a significant decrease in adiponectin ratio of lean T2D individuals was observed which was further marked in obese T2D patients. Moreover, the plasma HMW adiponectin/total adiponectin ratio tends to be lower in subjects with the homozygous mutant allele for +10211T/G and +276G/T. In concordance with our findings, adiponectin levels were reported to be strongly and inversely associated with diabetes risk (de Luis *et al*., 2016; Goto *et al*., 2017). Alongside, we had also reported the incidence of a significantly high number of angiotensin convertase enzyme (ACE) I/D polymorphism in the same population (Dwivedi *et al*., 2011). The ACE D allele in particular has been shown to be associated with increased levels of angiotensin II (Alsafar *et al*., 2015) which may further be adding to the down regulation of adiponectin. Thus, we suggest that the reduced HMW adiponectin in particular is accountable for insulin resistance as the HMW isoform binds to its receptor with maximum affinity leading to a potent stimulation of 5' AMP-activated protein kinase (AMPK) and the lowered HMW adiponectin may be partly responsible for developing T2D (Zhu *et al*., 2010). The increased levels of TG may be due to a decrease in the lipoprotein lipase activity and very low-density lipoprotein receptor (VLDLr) expression levels, which have been proposed to be modulated by adiponectin (Qiao *et al*., 2008). While HDL levels and their particle size are inversely correlated with the catabolic rate of apolipoprotein (ApoA-I), a direct role of reduced adiponectin with increased catabolism of the major ApoA-I present in HDL has been proposed (Verges *et al*., 2008), explaining how hypoadiponectinemia leads to decreased HDL levels. The correlation between hypoadiponectinemia and reduced HDL levels, as observed by us further strengthens this hypothesis. To summarize, +10211 T/G (*rs17846866*) and +276 G/T (*rs1501299*) are significantly associated with increased FBG, BMI, TG, TC and reduced HMW adiponectin/total adiponectin ratio. More importantly, the haplotype analysis reveals that, individuals with GGTG haplotype in particular show an increased tendency towards obesity induced T2D (Figure 2.4). Thus, we may conclude that adiponectin gene is associated with T2D, nonetheless variation in the susceptibility loci within the gene depends on ethnic variation among different populations. However, further

investigations to understand the mechanistic aspects of genetic variants regulating adiponectin levels are warranted in other cohorts.

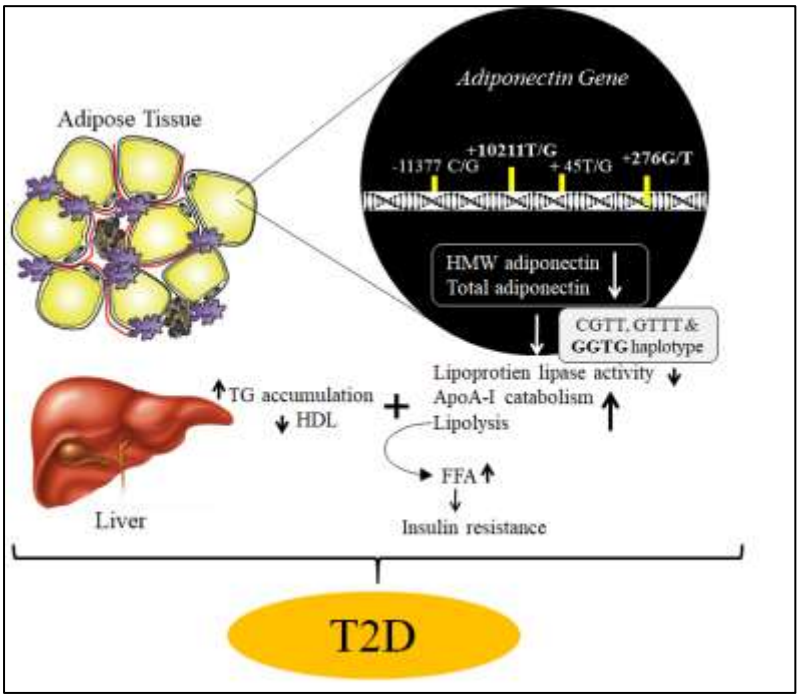

Figure 2.4. Role of *ADIPOQ* SNPs in T2D: The *ADIPOQ* CGTT, GTTT and GGTG haplotypes in presence of *ADIPOQ* +10211 T/G (rs17846866) and +276 G/T (rs1501299) along with decreased plasma HMW adiponectin and total adiponectin leads to altered metabolic profile thereby contributing to insulin resistance and T2D in Gujarat population.

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