Chapter 4

To assess genotype-phenotype correlation of leptin (LEP) and its receptor (LEPR) in Gujarat T2D patients and controls

4.1 Introduction

Leptin (LEP), a pro-inflammatory adipokine, encoded by the *ob* gene located on chromosome 7q31.3, is associated with food intake and appetite, energy homeostasis, basal metabolism, and insulin secretion (Mantzoros, 1995; Abella et al., 2017; Chan et al., 2003; Yannakoulia et al., 2003). Leptin levels are elevated in obesity and positively correlated with total body fat (Mantzoros et al., 1999; Brabant et al., 2000; Zhang et al., 2005; Lonnqvist et al., 1995). High leptin levels in obesity indicate a state of leptin resistance, implicating impaired leptin receptor sensitivity and action (Meyers et al., 2008). Apart from its central and peripheral action/effects on different tissues, leptin can also exert multiple actions on peripheral blood mononuclear cells (PBMCs) (Sanchez-Margalet et al., 2003). *In vitro* studies have shown that leptin can directly induce the expression of its receptors in PBMCs and thus inflammatory response (Zarkesh-Esfahani et al., 2001). Leptin exerts its important physiological effect by binding to leptin receptor (LEPR), which is a single transmembrane protein that belongs to the class I cytokine receptor family; its gene is located on chromosome 1p31, and LEPR is distributed in a variety of tissues including the brain, adipose tissue, skeletal muscle, liver, pancreatic islets, and immune cells. Studies on LEPR have advanced the understanding of the mechanism of body weight regulation, energy homeostasis and it has also shown to influence the onset of obesity, type 2 diabetes (T2D), and metabolic syndromes (MetS) (Klok et al., 2007; Meister et al., 2000; Muroya et al., 2004). Leptin circulates in a free form or it remains bound to its soluble receptor (sOb-R) (Sinha et al., 1996). The sOb-R is cleaved product of the extracellular domain of a membrane-bound leptin receptor, and its elevated concentrations indicate leptin signalling regulation (Brabant et al., 2000). In obese adults, high serum leptin levels could reduce the sOb-R levels and are associated with leptin resistance, although the molecular mechanism is not yet fully understood (Chan et al., 2002). Leptin resistance is associated with insulin resistance and it plays a crucial role in T2D development (Owecki et al., 2010; Martin et al., 2008; Myers et al., 2010).

Several *LEP* and *LEPR* gene polymorphisms were studied in different populations for their potential association with serum leptin levels, obesity, T2D, and MetS (Dasgupta et al., 2015; Sahin et al., 2013; Ghalandari et al., 2015). Among these variants, the *LEP* (5'UTR *rs2167270* G/A; G2548A *rs7799039* G/A) and *LEPR* (exon 6 Q223R *rs1137101* A/G; exon 14 K656N *rs1805094* G/C) single nucleotide polymorphisms (SNPs) have been studied in different populations (Ghalandari et al., 2015). However, there are only a few studies on these

polymorphisms in Indian population (Bains et al., 2020; Dar et al., 2019; Dasgupta et al., 2015; Murugesan et al., 2010) showing their association with obesity, BMI and T2D. By 2030, there will be 74.9 million T2D cases in India, with Gujarat being the second-highest state (Wild et al., 2004; Joshi et al., 2014). We have previously reported an association of adiponectin, vaspin, omentin, resistin, *IL1B*, and *TNFA* genes and their polymorphisms with T2D in Gujarat population (Palit et al., 2020; Rathwa et al., 2019a,b; 2020; Patel et al., 2016, 2019). In this study, we aimed to investigate *LEP* and *LEPR* genetic variants and their transcript levels in PBMCs, protein levels in plasma, and genotype-phenotype correlation of these polymorphisms with various metabolic parameters as well as T2D in Gujarat population.

4.2 Materials and methods

4.2.1 Study subjects:

The study was performed according to the Helsinki Declaration and was approved by the Institutional Ethical Committee for Human Research (IECHR: FS/IECHR/2016-9). In this study, 439 T2D patients (males/females) and 451 (males/females) controls from Gujarat population participated. The total sample size of 890 was determined using G*Power software (Faul et al., 2007) considering the effect size of 0.2 that achieved 91.9% statistical power for 439 T2D patients and 92.7% statistical power for 451 controls to detect the association of *LEP* and *LEPR* genes polymorphisms in the present study.

4.2.2 Anthropometric parameters, lipid profiling, and DNA extraction:

Body Mass Index (BMI) was calculated by measuring all the participants' height and body weight. 3 ml of venous blood was withdrawn from the participants after 12 h of overnight fasting in K_3EDTA coated tubes (J. K. Diagnostics, Rajkot, India) to estimate the Fasting Blood Glucose (FBG), Total Cholesterol (TC), Triglycerides (TG), Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL) by commercially available kits (Reckon Diagnostics P. Ltd, Vadodara, India). LDL was calculated using Friedewald's (1972) formula (LDL = Total cholesterol – (HDL + VLDL). Genomic DNA was extracted by the phenol-chloroform method (Sambrook and Russell, 2006). The DNA content and purity were determined spectrophotometrically by 260/280 absorbance ratio i.e. ~1.8. The integrity of DNA was checked by 0.8 % agarose gel electrophoresis. The DNA was normalized (50ng/µl) and stored at 4^{o}C until further analysis.

4.2.3 Genotyping of *LEP* and *LEPR* polymorphisms by PCR-RFLP:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype the *LEP* (*rs7799039* G/A; *rs2167270* G/A) and *LEPR* (*rs1137101* A/G; *rs1805094* G/C) polymorphisms. The primers used for PCR are shown in Table 4.1. The reaction mixture (20µl) consisted of 3 µL (150 ng) of genomic DNA, 11µL nuclease-free H_2O, 2 µL 10X PCR buffer, 2 µL 25 mM dNTPs (Puregene, Genetix Biotech), 1 µL of 10 mM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.2 µL (5U/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 4.1) for 30 sec., 72°C for 30 sec, and final annealing at 72°C for 10 min. Details of the restriction enzymes (Fermentas, Thermo Fisher Scientific Inc., USA) and digested products are mentioned in Table 4.1. The amplified products (15µl) were digested with 1U of the corresponding restriction enzyme (RE) in a total reaction volume of 20µl as per the manufacturer's instruction. The amplified PCR products (5µl) and digested products (20µl) were assessed by electrophoresis on a 3.5% agarose gel stained with ethidium bromide (EtBr) along with a 50bp/100bp DNA ladder (Genei Bangalore, India). All the gels were visualized under a UV transilluminator using Gel Doc EZ System (Bio-Rad Laboratories, California, USA).

4.2.4 Estimation of *LEP* & *LEPR* transcript levels:

Total RNA from whole blood was extracted by Trizol method. RNA integrity was verified by 1.5% agarose gel electrophoresis/ethidium bromide staining and 260/280 absorbance ratio i.e. ~1.95. RNA was treated with DNase I (Puregene, Genetix Biotech) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions in the Eppendorf Mastercycler gradient (USA Scientific, Inc., Florida, USA). The transcript levels of *LEP*, *LEPR*, and *GAPDH* (reference gene) were measured by LightCycler®480 Real-time PCR (Roche Diagnostics GmbH, Manneheim, Germany) using gene-specific primers (Eurofins, Bangalore, India) as shown in Table 4.1. The thermal cycling conditions comprised an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation (95°C for 10 sec.), annealing (60-69°C for 10 sec; shown in Table 4.1), and amplification (72°C for 10 sec.) steps. The fluorescence data collection was accomplished during the extension step. At the end of the amplification phase, a melt curve analysis was carried out to validate the specificity of the

products formed. The PCR cycle at which PCR amplification begins its exponential phase was considered as crossing point (Cp) or cycle threshold (Ct). The ΔCt or ΔCp value was obtained as a difference between the Ct of *LEP & LEPR* gene and Ct of *GAPDH* gene. The difference among the two ΔCt values (ΔCt Controls and ΔCt patients) was considered as ΔΔCt to obtain the value of fold expression ($2^{-\Delta\Delta Ct}$).

Table 4.1. Primers and restriction enzymes used for genotyping for *LEP* and *LEPR* polymorphisms and expression.

Gene/SNP	Primer Sequence (5'-3')	AT (°C)	Amplicon Size (bp)	R.E.	Digested products size (bp)
LEP -2548 G/A (rs7799039)	**FP:** GTGTGTTCCCTGGTTCAAGG **RP:** GATCTCTCTGTTCGGGGTC	65	313	*HhaI*	241 + 72
LEP 5'UTR G/A (rs2167270)	**FP:** GATCGGGCCGCTATAAGAG **RP:** CCGGTAACCTTCTATCTGGC	62	245	*HpyC H4III*	183 + 62
LEPR Q223R A/G (rs1137101)	**FP:** GTGAATGTCTTGTGCCTGTGC **RP:** AGAAGCCACTCTTAATACCCCC	69	277	*MspI*	189 + 88
LEPR K656N G/C (rs1805094)	**FP:** GAAAGTGCATAAGTGTGTGCTTC **RP:** CCAAAGTAAAGTGACATTTTTCGC	61	159	*BstUI*	136 + 23
Leptin	**FP:** TCACACACGCAGTCAGTCTC **RP:** GGATCACGTTTCTGGAAGGCA	68	151bp	-	-
Leptin receptor	**FP:** TGTTCTGCCTGAAGTGTTAG **RP:** AGAGTGTCGTTGAGTTTGG	61	138bp	-	-
GAPDH	**FP:** CATCACCATCTTCCAGGAGCGAG **RP:** CCTGCAAATGAGCCCCAGCCT	69	122bp	-	-

[Abbreviations: FP: Forward Primer; RP: Reverse Primer; bp: base pair; AT: annealing temperature; RE: restriction enzyme]

4.2.5 Estimation of *LEP* & *LEPR* protein levels:

Plasma protein levels of leptin and sOb-R in patients and age and sex-matched controls were estimated by human leptin ELISA Kit (Ray Biotech., GA, USA) and sOb-R ELISA Kit (Elabscience, WH, China), respectively as per the manufacturers' protocols with a sensitivity of 2 pg/mL and 0.19 ng/mL, respectively. All the plasma estimations were carried out in duplicates to ensure % coefficient of variation below 10%.

4.2.6 Statistical analyses:

Biochemical parameters were compared using unpaired t-test using Prism 6 software (GraphPad Software Inc; San Diego CA, USA). The Hardy-Weinberg Equilibrium (HWE) was evaluated for all the polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *LEP* and *LEPR* polymorphisms for patient and control subjects were compared using the chi-square test with 2×2 contingency tables. *P* values less than 0.025 for genotype and allele distribution were considered statistically significant, as per Bonferroni's correction for multiple testing. Odds Ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Haplotypes and linkage disequilibrium (LD) coefficients D' ¼ D/Dmax 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). Relative gene expression and fold change ($2^{-\Delta\Delta Ct}$) of *LEP* and *LEPR*, plasma protein levels of leptin and sOb-R in patient and control groups were plotted and analysed using unpaired t-test. Association studies of polymorphisms with anthropometric parameters were performed using analysis of variance (ANOVA) and Kruskal Wallis test. In contrast, correlation analysis was performed using multiple linear regression and Spearman's correlation analysis. *P* values less than 0.05 were considered significant for all the association analyses.

4.2.7 Bioinformatics analysis:

In silico prediction tools PANTHER (Thomas et al., 2003), MUPRO (Cheng et al., 2006), and I-MUTANT SUITE (Capriotti et al., 2008) were employed to speculate the sequence-based impact on the protein due to a single amino acid variation.

4.3 Results

4.3.1 Baseline characteristics:

The clinical parameters differed significantly between controls and T2D patients (Table 4.2). The

patients had a significantly higher FBG levels (p<0.0001). Moreover, obesity factors like BMI, total cholesterol, and triglycerides were significantly elevated (p<0.0001, p=0.0420, p=0.001 respectively), while HDL was significantly decreased (p<0.0001) in T2D patients as compared to controls. However, LDL did not differ between patient and control groups (p=0.9322).

Table 4.2. Baseline characteristics of diabetic and non-diabetic individuals from Gujarat population.

	Controls (Mean±SD)	**Patients (Mean±SD)**	***p* value**
	(n =502)	(n =478)	
Age (years)	39.64 ± 16.35	55.99 ± 10.42	-
Sex: Male	251 (50%)	213 (44.5%)	-
Female	251 (50%)	265 (55.5%)	-
Fasting blood glucose (mg/dL)	100.1 ± 7.32	155.3 ± 62.09	**<0.0001**
BMI (Kg/m^2)	24.24 ± 5.2	27.04 ± 5.1	**<0.0001**
Total Cholesterol (mg/dL)	160.9 ± 42.2	166.2 ± 39.68	**0.0420**
Triglycerides (mg/dL)	111.7 ± 60.90	164.5 ± 111.1	**<0.001**
HDL (mg/dL)	42.79 ± 15.94	38.2 ± 12.6	**<0.0001**
LDL (mg/dL)	95.32 ± 41.79	95.10 ± 37.52	0.9322
Age of onset (Years)	NA	50.65 ± 10.10	-
Duration of disease (Years)	NA	8.06 ± 7.3	-
Family history	NA	64 (14%)	-

[Data are presented as Mean±SD. Statistical significance was considered at p<0.05.]

4.3.2 Association of *LEP* and *LEPR* polymorphisms with T2D:

The genotype and allele frequencies of the studied *LEP* and *LEPR* polymorphisms are summarised in Table 4.3, while the representative gel images for PCR-RFLP analyses are shown in Fig. 4.1. Further, the confirmation of genotyping results by Sanger's sequencing of PCR products for respective polymorphisms are shown in Fig. 4.2. The distribution of genotype frequencies for all the investigated polymorphisms was consistent with Hardy-Weinberg (HWE) expectations in both patient and control groups (p>0.05).

The genotype and allelic frequencies of *LEP rs7799039* G/A and *rs2167270* G/A polymorphisms and *LEPR rs1805094* G/C polymorphism were did not differ significantly

(*p*>0.025), hence were discontinued after an initial assessment. Interestingly, *LEPR rs1137101* A/G was found to be associated T2D patients, as the genotype and allele frequencies differed significantly between patients and controls (*p*=0.009 and *p*=0.02 respectively). In particular, GG genotype was prevalent in patients as compared to controls (23% vs. 16%). The susceptible GG genotype and G allele showed an increased risk for T2D with an Odds Ratio (OR) of 1.66 and 1.24, respectively (Table 4.3).

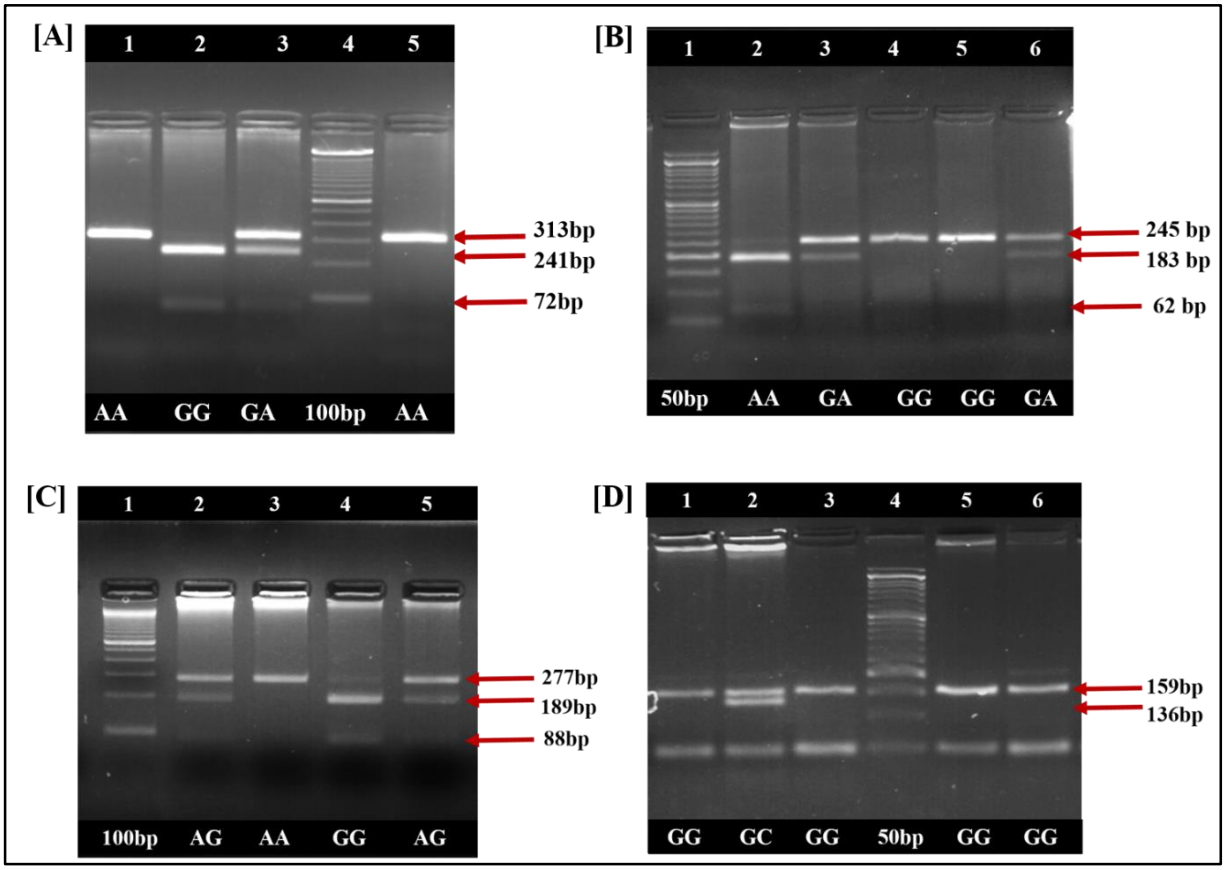

Figure 4.1. PCR-RFLP analyses of *LEP* and *LEPR* polymorphisms. [A] PCR-RFLP analysis of *LEP* -2548 G/A polymorphism on 3.5% agarose gel: PCR product was digested by restriction enzyme *Hha1*. Digestion resulted in a 313-bp fragment for the A allele and 241 and 72 bp fragments for the G allele. **[B]** PCR-RFLP analysis of LEP 5'UTR G/A polymorphism on 3.5% agarose gel: PCR product was digested by restriction enzyme *HpyCH4III*. Digestion resulted in a 245bp fragment for the G allele and 183 and 62 bp fragments for the A allele. **[C]** PCR-RFLP analysis of LEPR Q223R Intron 6 A/G polymorphism on 3.5% agarose gel: PCR product was digested by restriction enzyme *MSPI*. Digestion resulted in a 277-bp fragment for the A allele and 189 and 88 bp fragments for the G allele. **[D]** PCR-RFLP analysis of LEPR K656N Intron 6 G/C polymorphism on 3.5% agarose gel: PCR product was digested by restriction enzyme *BstUI*. Digestion resulted in a 159-bp fragment for the G allele and 136 and 23 bp fragments for the C allele.

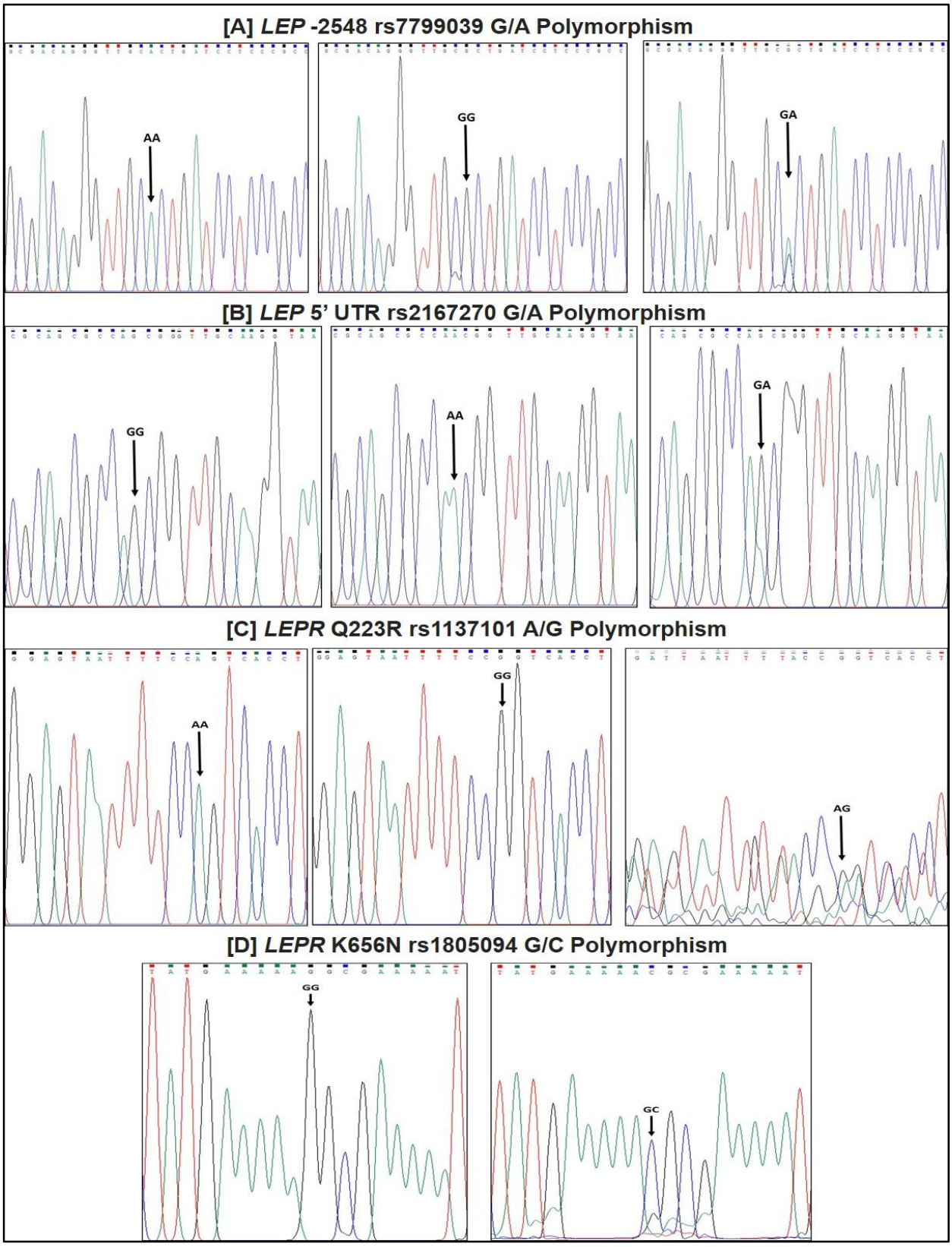

Figure 4.2. Confirmation of genotyping results by Sanger's sequencing of PCR products. [A] *LEP* -2548 rs7799039 G/A Polymorphism. [B] *LEP* 5' UTR rs2167270 G/A Polymorphism. [C] *LEPR* Q223R rs1137101 A/G Polymorphism. [D] *LEPR* K656N rs1805094 G/C Polymorphism.

Table 4.3. Distribution of genotype and allele frequencies of *LEP* and *LEPR* polymorphisms in T2D patients and controls.

SNP	Genotype/ allele	Controls (Freq.)	Patients (Freq.)	*p* value for HWE	*p* value	Odds Ratio	CI (95%)
	GG	n=297	n=297				
***LEP* -2548**	GA	77 (0.26)	78 (0.26)	(C)	R	-	-
rs7799039	AA	139 (0.47)	140 (0.47)	0.27	0.97[a]	0.99	0.67-1.47
(G/A)		81 (0.27)	79 (0.26)		0.86[b]	0.96	0.61-1.49
	G			(P)			
	A	293 (0.49)	296 (0.50)	0.32	R	-	-
		301 (0.51)	298 (0.50)		0.86[c]	0.98	0.78-1.23
		n=269	n=268				
***LEP* 5'UTR**	GG	140 (0.52)	138 (0.51)	(C)	R	-	-
rs2167270	GA	106 (0.39)	116 (0.43)	0.64	0.56[a]	1.11	0.79-1.58
(G/A)	AA	23 (0.08)	14 (0.05)		0.17[b]	0.61	0.30-1.25
				(P)			
	G	386 (0.72)	392 (0.73)	0.09	R	-	-
	A	152 (0.28)	144 (0.27)		0.61[c]	0.93	0.71-1.21
		n=451	n=439				
***LEPR* Exon**	AA	143 (0.31)	125 (0.28)	(C)	R	-	-
6 Q223R	AG	238 (0.53)	212 (0.48)	0.07	0.9[a]	1.01	0.75-1.38
rs1137101	GG	70 (0.16)	102 (0.23)		**0.009[b]**	1.66	1.13-2.45
(A/G)				(P)			
	A	524 (0.58)	462 (0.53)	0.51	R	-	-
	G	378 (0.42)	416 (0.47)		**0.02[c]**	1.24	1.03-1.5
		n=302	n=308				
***LEPR* Exon**	GG	260 (0.86)	250 (0.81)	(C)	R	-	-
14 K656N	GC	42 (0.14)	58 (0.19)	0.19	0.1[a]	1.43	0.93-2.21
rs1805094	CC	0	0		-	-	-
(G/C)				(P)			
	G	562 (0.93)	558 (0.91)	0.07	R	-	-
	C	42 (0.07)	58 (0.09)		0.1[c]	1.39	0.91-2.10

'n' represents the number of samples, 'R' represents reference group, CI refers to the confidence interval, a, b patients vs controls (genotype) with respect to reference using the chi-square test with 2×2 contingency table, c patients vs controls (allele) using the chi-square test with 2×2 contingency table, values are significant at $p < 0.025$ due to Bonferroni's correction.

4.3.3 Haplotype analysis:

A haplotype evaluation of two polymorphic sites of *LEPR rs1137101* A/G and *rs1805094* G/C revealed that the haplotypes differed significantly between patients and controls (global *p*=0.026), and the susceptible disease haplotype GG was prevalent in patients' group (*p*=0.018) (Table 4.4).

Table 4.4. Distribution of haplotype frequencies of *LEPR* polymorphisms in T2D patients and controls.

Haplotype (*LEPR* rs1137101, rs1805094)	Patients (Freq. %) (n=275)	Controls (Freq. %) (n=300)	*p* for association	*P* (Global)	Odds Ratio	95% CI
A C	43.74 (0.080)	33.91 (0.070)	0.497		1.17	0.73-1.8
A G	240.26 (0.440)	257.09 (0.531)	**0.007**	**0.026**	**0.71**	0.55-0.91
G C	8.26 (0.015)	0.09 (0.000)	-		-	-
G G	253.74 (0.465)	192.91 (0.399)	**0.018**		**1.35**	1.05-1.72

CI' represents confidence interval (frequency < 0.03 in both patients and controls has been dropped and was ignored in the analysis).

4.3.4 Linkage disequilibrium (LD) analysis:

LD analysis revealed that two polymorphic sites of the *LEPR* gene were in low LD association (D'=0.723, r2=0.038), as shown in Fig 4.3.

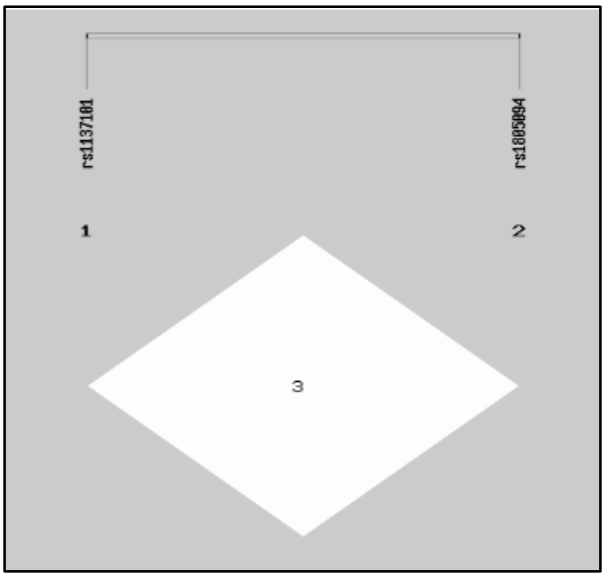

Figure 4.3. Linkage disequilibrium (LD) block. LD block with respect to *LEPR rs1137101*

A/G and *rs1805094* G/C polymorphisms in Gujarat population.

4.3.5 Correlation of *LEP* and *LEPR* polymorphisms with FBG, BMI and plasma lipids:

Correlation analysis revealed that *LEP* (*rs7799039 G/A, rs2167270 G/A*) and *LEPR* (*rs1805094 G/C*) polymorphisms did not show any association with FBG, BMI, or plasma lipids levels (p>0.05). Further, the GG genotype of *LEPR* rs1137101 A/G polymorphism was found to be associated with elevated FBG (p=0.027) and TC (p=0.025) levels. However, it was not associated with BMI and plasma lipid profile, i.e., TG, LDL and HDL (Table 4.5).

Table 4.5. Genotype-phenotype correlation of *LEP* and *LEPR* polymorphisms with BMI, FBG and plasma lipid profile.

Genotype	FBG (mg/dl)	BMI (kg/m^2)	TG (mg/dl)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
***LEP* -2548 G/A (rs7799039)**						
GG	160.5±27.73	28.48±2.59	221.8±64.2	170.1±35.75	98.44±29	37.36±11.47
GA	190±66.75	29.98±3.74	216±56.95	174.6±39.67	106.3±32.52	36.75±12.56
AA	176±75.59	29.52±3.49	210±58.20	170.3±30.26	103.8±29.77	37.81±14.26
***p* value**	0.15	0.16	0.63	0.73	0.4	0.66
***LEP* 5'UTR G/A (rs2167270)**						
GG	151.6±62.32	27.74±5.634	161.6±80.14	173.7±43.25	103.4±39.29	40.49±12.3
GA	144.6±50.76	28.27±5.814	151.8±74.05	171.6±47.71	106.9±34.60	40.54±15.01
AA	128.6±20.35	28.13±5.600	181.9±79.36	170.9±26.02	87.28±31.14	47.19±20.34
***p* value**	0.76	0.74	0.35	0.91	0.23	0.68
***LEPR* Q223R Exon 6 A/G (rs1137101)**						
AA	150.3±38.13	29.85±5.13	207.5±44.3	182.5±19.77	100.6±32.7	37.34±12
AG	158±56.17	29.63±3.6	210.4±66.82	191.7±26.24	107±31.49	38.4±14
GG	**171.7±48.51**	29.39±3.7	199.6±35.64	**194.6±23.96**	106.9±28.03	38.56±10.58
***p* value**	**0.027**	0.59	0.7	**0.025**	0.22	0.71
***LEPR* K656N Exon 14 G/C (rs1805094)**						
GG	151.1±54.78	27.86±6.531	162.7±82.51	166.5±35.71	104.6±37.21	41±14.05
GC	148.1±66.23	27.83±5.595	160.2±71.10	175.0±40.75	111.6±35.81	45.12±17.16
***p* value**	0.38	0.79	0.73	0.29	0.16	0.11

[Data are presented as Mean ± SD. Statistical significance was considered at $p < 0.05$.]

4.3.6 Assessment of *LEP* and *LEPR* transcript levels from PBMCs:

LEP transcript levels were significantly increased in PBMCs of 119 patients as compared to 120

controls after normalization with *GAPDH* expression, as suggested by Mean ΔCp values (p<0.0001) with a fold change of 2.85 (Figs. 4.4a and b).

Further, *LEPR* transcript levels were found to be significantly decreased (36% reduction; p=0.0146) in PBMCs of 168 patients as compared to 179 controls, as suggested by Mean ΔCp values with a fold change of 0.64 (Figs. 4.4c and d).

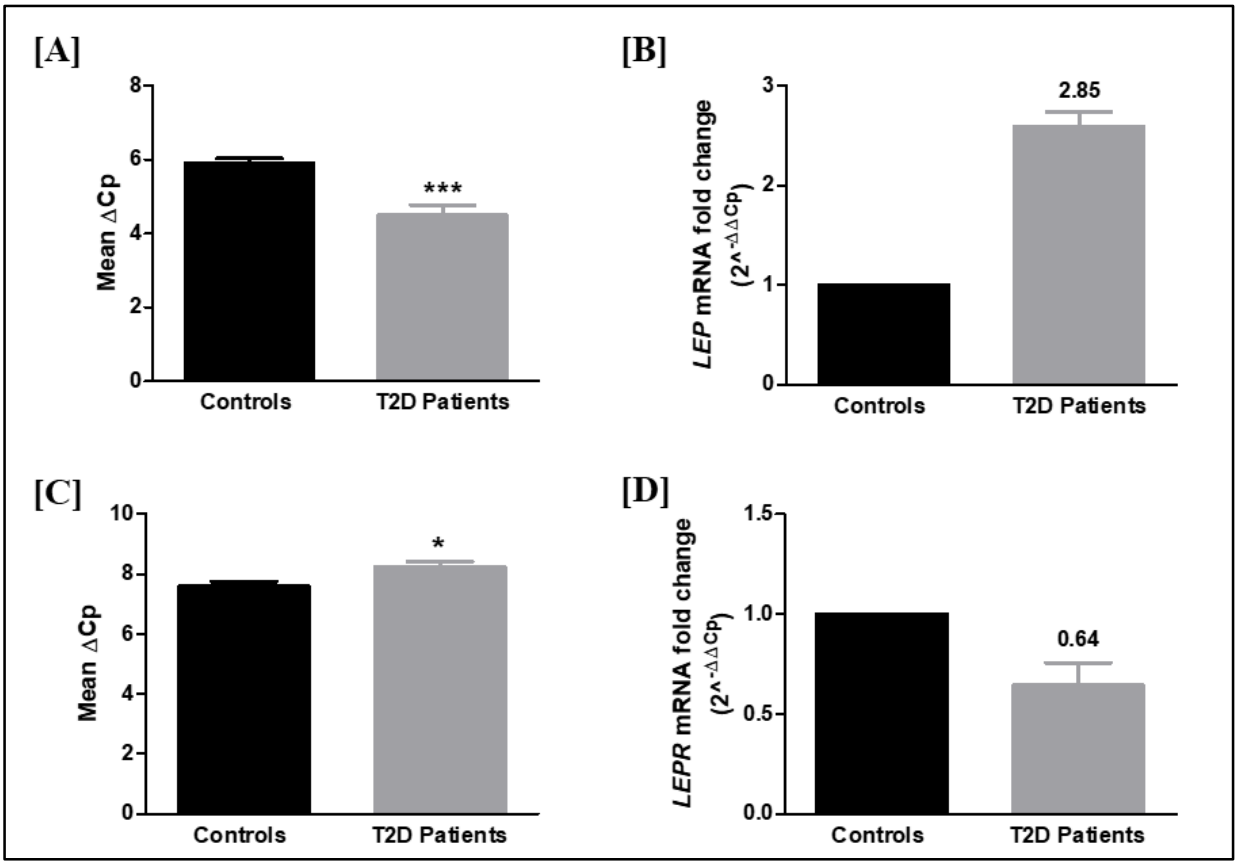

Figure 4.4. *LEP* and *LEPR* transcript levels in PBMCs of T2D patients and controls. [A] Relative gene expression of *LEP* in PBMCs of controls and patients: Significant increase in *LEP* transcript was observed in patients (Mean ΔCp ± SEM: 5.88 ± 0.143 vs 4.5 ± 0.26; p < 0.0001). **[B] Relative fold change of *LEP* expression in controls and patients:** Diabetic patients showed a 2.85 fold increase in *LEP* mRNA expression as determined by the $2^{-\Delta\Delta Cp}$ method (controls n=120; T2D patients n=119). **[C] Relative gene expression of *LEPR* in PBMCs of controls and patients:** Significant decrease in *LEPR* mRNA transcript was observed in patients (Mean ΔCp ± SEM: 7.58 ± 0.17 vs 8.22 ± 0.19; p < 0.05). **[D] Relative fold change of *LEPR* expression in controls and patients:** Diabetic patients showed 36% reduction in *LEPR* mRNA expression as determined by $2^{-\Delta\Delta Cp}$ method (controls n=179; T2D patients n=168).

4.3.7 Estimation of plasma protein levels of leptin and sOb-R:

Plasma leptin and sOb-R protein levels were estimated in 44 controls and 43 patients. Plasma leptin was significantly increased in T2D patients as compared to controls (p=0.0047), and particularly in obese patients as compared to lean patients (p=0.0002) and lean controls

(*p*=0.0019) (Fig. 4.5a and b). In addition, sOb-R levels were significantly reduced in T2D patients as compared to controls (*p*=0.0294), as shown in Fig. 4.5c. Interestingly, we found a significant elevation in sOb-R levels in obese patients as compared to lean patients (*p*=0.0188) and reduced sOb-R levels in lean patients as compared to lean (*p*=0.0193) and obese controls (*p*=0.0019) (Fig. 4.5d).

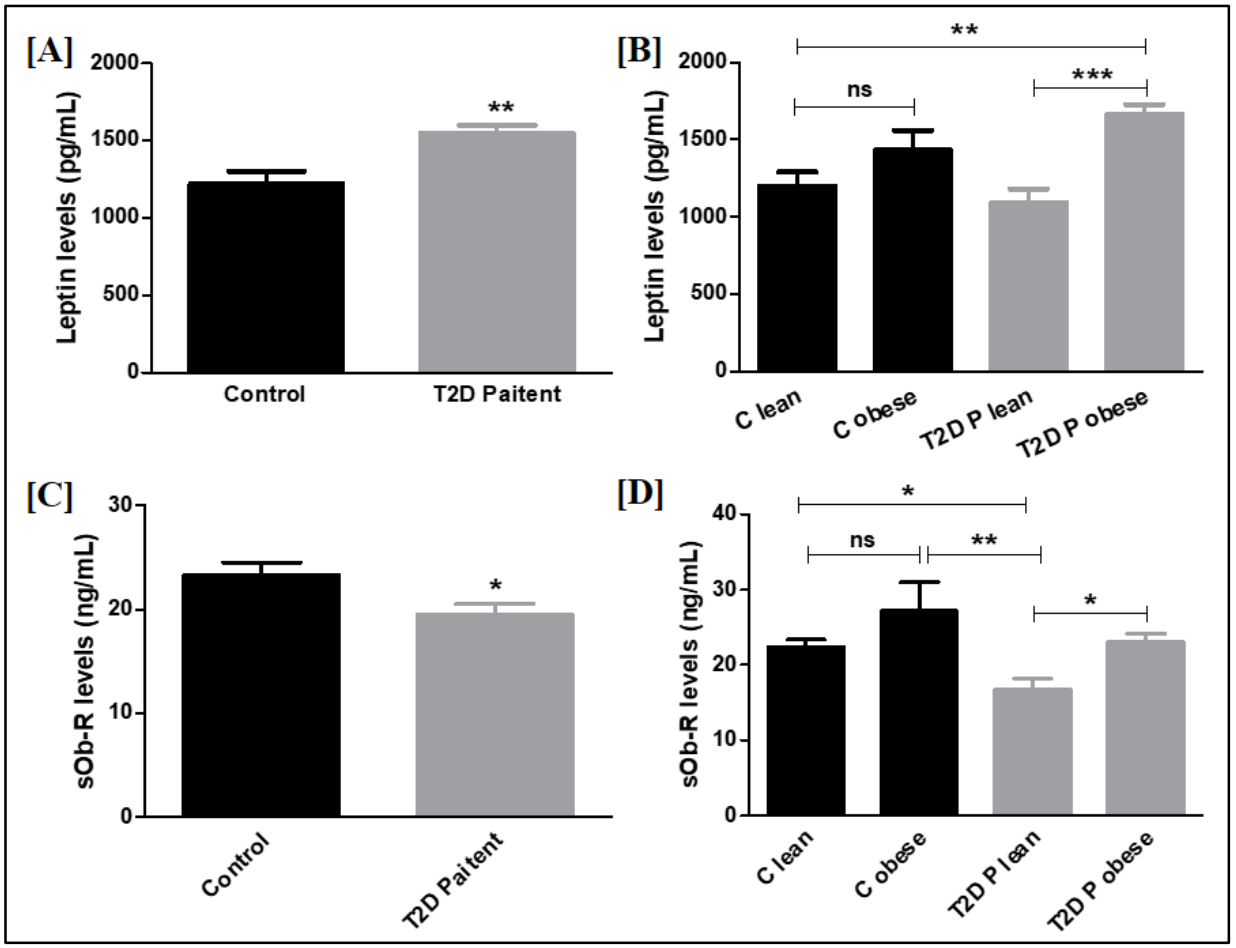

Figure 4.5. Plasma protein levels of leptin and sOb-R in T2D patients and controls. [A] Plasma leptin protein levels in controls *vs* patients: leptin levels were considerably increased in patients (*p*=0.0047). **[B] Control lean *vs* obese and patient lean *vs* obese:** control lean vs patient obese (*p*=0.0019) and patient lean vs obese (*p*=0.0002) showed a considerable differences, while no difference was observed between other groups. **[C] Plasma sOb-R levels in controls *vs* patients:** sOb-R levels were considerably decreased in patients (*p*=0.0294). **[D] Control lean *vs* obese and patient lean *vs* obese:** Control lean vs. obese lean (*p*=0.0193), control obese vs. patient lean (*p*=0.0019) and patient lean vs. obese (*p*=0.0188) showed significant difference (controls n=44; T2D patients n=42).

4.3.8 Plasma protein levels of leptin and sOb-R and their correlation with metabolic profile:

Spearman's correlation analysis showed a positive correlation of leptin with BMI (p = 0.0001) and TG levels (p = 0.0163), and sOb-R protein levels with BMI (p = 0.0001), FBG (p = 0.0434), and TG levels (p = 0.0084) (Table 4.6).

Table 4.6. Correlation analysis of plasma protein levels of leptin and sOb-R with the metabolic profile.

Parameters	Leptin		sOb-R	
	r^2	p	r^2	p
BMI (Kg/m^2)	0.5224	**0.0001**	0.3941	**0.0001**
FBG (mg/dL)	0.0222	0.3923	0.1214	**0.0434**
Triglycerides (mg/dL)	0.1391	**0.0163**	0.1574	**0.0084**
Total Cholesterol (mg/dL)	0.0002	0.9164	0.0126	0.4736
HDL (mg/dL)	0.0014	0.8093	0.0001	0.9472
LDL (mg/dL)	0.0047	0.6599	0.0055	0.6348

[r = Spearman's correlation coefficient ($p < 0.05$, significant; $p > 0.05$, non-significant)].

4.3.9 Bioinformatics analysis:

We further investigated the impact of *LEPR rs1137101* polymorphism on its protein function using bioinformatics tools. The polymorphism results in a Glutamine to Arginine substitution at 223 position (Gln223Arg). PANTHER tool showed that Gln223Arg variation is probably damaging *LEPR* function. I-MUTANT and MUPRO predictions revealed a decreased stability for Gln223Arg *LEPR* variant compared to the native structure of *LEPR* (Table 4.7).

Table 4.7. *In-silico* prediction results for *LEPR* Q223R A/G polymorphism.

Amino acid change	PANTHER	I-MUTANT	MUPRO
Gln223Arg	Probably Damaging	Decreased stability	Decreased stability

4.4 Discussion:

The alarmingly increasing prevalence of obesity poses adverse health problems (Hossain et al., 2007). It is a multifactorial and heterogeneous condition due to genetic and environmental factors (Herbert 2008). It is well known that obesity could lead to T2D, especially characterized by insulin resistance (Bhardwaj et al., 2011). As leptin has long been linked with obesity, and its signalling is an essential factor in metabolic regulation, recent studies have depicted the role of

leptin in T2D and insulin resistance. To our knowledge, our study is the first attempt to evaluate the association between *LEP* and *LEPR* polymorphisms with T2D in Gujarat population.

Our results revealed no association between *LEP* (*rs7799039* G/A & *rs2167270* G/A) and *LEPR* (*rs1805094* G/C) polymorphisms with T2D risk or with any anthropometric parameters. Earlier, similar observations were reported in Egyptian, Turkish, Polish, Iranian, Swiss, Mexican, and North and South Indian populations (Abroon et al., 2016; Angel-Chavez et al., 2012; Bains et al., 2020; Bender et al., 2011; Motawi et al., 2015; Murugesan et al., 2010; Roszkowska-Gancarz et al., 2014; Taghizadeh et al., 2017). We report here a significant association of *LEPR rs1137101* A/G polymorphism with T2D; the susceptible GG genotype and G allele showed 1.66- and 1.24- fold increased risk for T2D, respectively. Moreover, the GG genotype showed a strong association with elevated FBG and TC levels. Similarly, Boumaiza et al. (2012) showed an association of this polymorphism with TC and BMI in the Tunisian population. However, similar to our findings, studies on Turkish (Mergen et al., 2007) and Brazilian (Angeli et al., 2011) populations did not show significant association between *LEPR rs1137101* A/G polymorphism and BMI.

Several case-control studies have shown an association of *LEPR rs1137101* A/G and *rs1805094* G/C polymorphisms with T2D and obesity in different ethnic populations. The findings suggest that *LEPR rs1137101* polymorphism confers T2D risk in Malaysian, Indian (Punjabi, Coimbatore), Korean, Chinese, Malay, Slavonic, and Ukrainian populations (Etemad et al., 2013; Bains et al., 2020; Murugesan et al., 2010; Gan and Yang, 2012; Jiang et al., 2014; Ahmed and Ghali, 2017; Ziablitsev et al., 2018). This polymorphism causes a non-conservative change by converting glutamine to arginine at codon 223 (CAG to CGG) (Gotoda et al., 1997), which leads to a change in the charge, i.e., neutral to positive, and it is located within the leptin-binding region in the extracellular domain (N-terminal CRH1 domain). Thus, this change in amino acids affects all isoforms of the receptors and may be linked to impaired signal transduction, which then leads to increase in the susceptibility of T2D (Yiannakouris et al., 2001). Our *in silico* analyses also reveal that this variation probably damages LEPR function and decreases the stability of the mutant *LEPR* as compared to the native structure.

LEP and *LEPR* expression in PBMCs and plasma sOb-R protein levels of T2D patients and controls were monitored for the first time in Gujarat population. T2D patients showed 2.85- fold increase in *LEP* transcript levels and plasma leptin protein levels. Further, T2D patients showed

a 36% reduction in *LEPR* transcript and reduced plasma sOb-R protein levels. According to several studies, hyperleptinemia and decreased sOb-R protein levels are the key markers of the leptin resistance (Posey et al., 2009; Khan et al., 2016). We found a strong, inverse association between circulating sOb-R protein levels and the risk of T2D. Sun et al. (2010) reported a similar observation in the U.S. population. Our correlation analysis revealed that plasma leptin levels positively correlate with BMI and TG levels, and these results were in line with the above mentioned study. In contrast, plasma sOb-R protein levels are positively correlated with BMI, FBG and TG levels.

In our population, plasma leptin and sOb-R levels were increased in obese T2D patients as compared to lean T2D patients and these results are in line with the previous findings (Medici et al., 2010; Manju et al., 2021). However, a contradictory finding reported reduced protein levels of sOb-R to be associated with obesity. This study mentioned that increased sOb-R protein levels might augment the physiological actions of leptin in lean subjects as compared to obese subjects (Ogier et al., 2002). Devos et al. (1997) suggested that complexes of leptin with sOb-R reflect a molecular ratio of 1:1. The presence of two-fold or higher circulating sOb-R suppresses leptin action *in vitro* and *in vivo* (Posey et al., 2009). This study indicated that increased sOb-R protein levels could be one of the reasons for leptin resistance in obesity.

Chronic low-grade inflammation is a trademark of obesity, resulting from an imbalance of pro- and anti-inflammatory adipokines (Mancuso, 2016). A pro-inflammatory adipokine, resistin was reported to be elevated in T2D patients (Santilli et al., 2016) and it has been suggested to initiate the cAMP-mediated activation of PKA and NF-kB pathways, which promote the transcription of various inflammatory adipokines, i.e., TNF-α and IL-1β (Lee et al., 2014). Moreover, hyperleptinemia can trigger an inflammatory response in PBMCs and promote TNF-α and iNOS synthesis by activating JAK/STAT-3 pathway (Nalini et al., 2019). We have also reported a similar pattern of imbalance in pro- and anti-inflammatory adipokines i.e., increased levels of pro-inflammatory resistin (Rathwa et al., 2018), TNF-α (Patel et al., 2019), and IL-1β (Patel et al., 2016) and reduced levels of anti-inflammatory adiponectin (Palit et al., 2020), vaspin (Rathwa et al., 2020) and omentin-1 (Rathwa et al., 2019) in T2D patients from Gujarat population.

Previously, we also reported the significant association of angiotensin converting enzyme (*ACE*) I/D polymorphism with T2D in Gujarat population (Dwivedi et al., 2011). The *ACE* 'D' allele

was found to be associated with elevated angiotensin II levels (Alsafar et al., 2015) and it has been suggested that increased angiotensin II leads to decreased adiponectin levels. Furthermore, the role of circadian rhythm has been implicated in regulating the metabolic processes of adipose tissue and the expression and secretion of adipokines (Gómez-Santos et al., 2009; Johnston, 2012). Furthermore, the metabolic regulation is predicted to be mediated by the action of melatonin (pineal gland hormone) on visceral adipose tissue receptors or via the sympathetic nervous system (de Farias et al., 2015; Vriend and Reiter, 2015). We have previously reported decreased plasma melatonin levels in T2D patients (Patel et al., 2018). Also, we have observed the involvement of neuropeptide Y (NPY) promoter polymorphism regulating NPY levels, which reduces melatonin levels (Patel et al., 2016). A lack of melatonin signalling induces leptin resistance, thus suggesting a vital role for melatonin in leptin signaling (Buonfiglio et al., 2019). Melatonin contributes in normalizing expression as well as secretion patterns of leptin and adiponectin (Favero et al., 2015), thereby providing a broader perspective on the relationship between melatonin and obesity. Hyperleptinemia and increased TNF-α levels play an important role in the imbalance of pro-inflammatory (resistin, IL1-β, TNF-α)/anti-inflammatory (vaspin, adiponectin, and omentin) adipokines and melatonin levels, which could play a major role in developing leptin and insulin resistance governing the risk of obesity-induced T2D in Gujarat population.

A summary illustrating the possible role of *LEP* and *LEPR* polymorphisms and their altered transcript and protein levels along with altered adipokines in obesity-induced T2D is shown in Fig. 4.6.

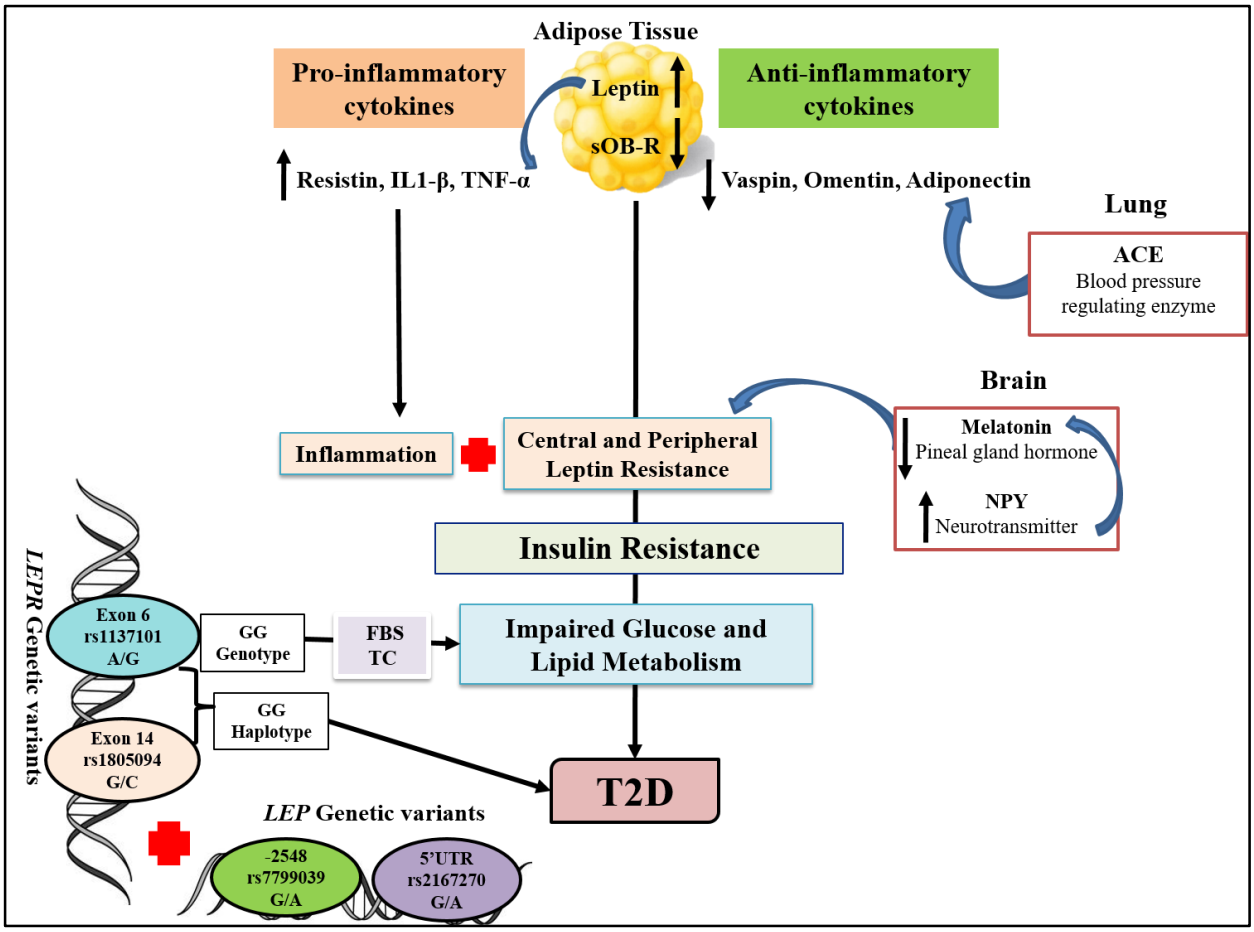

Figure 4.6. Role of leptin (LEP) and leptin receptor (LEPR, sOb-R) and altered adipokine levels in T2D. *LEPR rs1137101* A/G polymorphism is significantly associated with T2D, and the homozygous GG genotype increases the risk of disease by 1.66 fold. Moreover, the GG genotype shows a strong association with elevated FBG and TC levels. A haplotype evaluation of *LEPR rs1137101* A/G and *rs1805094* G/C revealed that the GG haplotype is significantly associated with T2D and increases the risk of diseases by 1.35 fold. The increased leptin and decreased sOb-R levels might be responsible for the leptin resistance. Hyperleptinemia further elevates TNF-α levels, which play an important role in the imbalance of pro-inflammatory (resistin, IL1-β, TNF-α) / anti-inflammatory (vaspin, adiponectin, and omentin) adipokines and melatonin levels. Further, ACE and NPY down regulate the anti-inflammatory adipokine-adiponectin and melatonin, respectively.

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