# Chapter 2

# Investigation of genotype-phenotype correlation of *TNF-α* and *MTNR1B* polymorphisms with T2D susceptibility in Gujarat population

## 2.1 Introduction

Insulin resistance and type 2 diabetes (T2D) have been strongly correlated with increased abdominal obesity which is a low-grade inflammatory stage (Kahn and Flier, 2000). The pathophysiological mechanism involved in the development of insulin resistance and obesity induced T2D includes the dysregulated secretion of pro- and anti-inflammatory adipokines from the adipose tissue (Jung et al., 2014). Tumor Necrosis Factor- alpha (TNF-α), a pro-inflammatory cytokine/adipokine secreted from infiltrating macrophages, is highly expressed in adipose tissue of obese animals and human subjects (Hotamisligil et al., 1993). TNF-α suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates which may affect insulin sensitivity (Hotamisligil et al., 1996). Feingold et al., (1990) have reported that TNF-*α* increases triglycerides in humans by promoting lipolysis and elevating free fatty acid (FFA) levels. Along with increased adiposity, a disturbed circadian rhythm is also strongly related with insulin resistance and T2D (Gale et al., 2011; Qian and Scheer, 2016). Melatonin, a pineal hormone, is known to regulate circadian rhythm and sleep (Dubocovich, 2007). Melatonin mediates its action through its two receptors; MT1 (MTNR1A) and MT2 (MTNR1B), that are present in various tissues including pancreatic islets (Dubocovich et al., 2005; Ramracheya et al., 2008). There might be an association between melatonin and T2D based on the findings that insulin secretion is inversely proportional to plasma melatonin levels (Peschke et al., 2013).

It is well known that T2D is a multifactorial and polygenic metabolic disorder (Hansen, 2001). Significant variations between different ethnic populations have been reported with regards to the genetic architecture underlying T2D (Keaton et al., 2014; Sim et al., 2001). Several single nucleotide polymorphisms (SNPs) in the *TNF-α* promoter region [-238 (rs361525), -308 (rs1800629), -857 (rs1799724), and -863 (rs1800630)] (Higuchi et al., 1998; Wilson et al., 1994) have been found to be associated with T2D in different ethnicities (Jamil et al., 2016; Kallel et al., 2013; Schulz et al., 2004; Yamashina et al., 2007). *TNF-α* expression is also modulated by the genetic variants as polymorphic sites are present in the promoter region of *TNF-α* (González et al., 2003; Kroeger et al., 1997; Schulz et al., 2004). Further, -238G/A and -308G/A polymorphisms of *TNF-α* are documented to alter circulating FFAs and insulin resistance in obese subjects with T2D (Fontaine-Bisson et al., 2007). Several studies have shown correlation between *TNF-α* expression and risk factors like Body Mass Index (BMI) and plasma lipids (Bruun et al., 2002; Mizia-Stec et al., 2003). Moreover, TNF-α is involved in the pathogenesis of various autoimmune diseases like vitiligo (Laddha et al., 2012; Singh et al., 2021), rheumatoid arthritis, inflammatory bowel disease, psoriasis, ankylosing spondylitis (Kalliolias et al., 2016), cardiovascular disease (Haudek et al., 2007) and cancer (Elinav et al., 2013). Furthermore, several studies have identified common genetic variants within *MTNR1B* [-1193 C/T (rs4753426), 5'UTR G/C (rs10830962), and intron C/G (rs10830963)] are associated with higher fasting blood glucose (FBG) levels, impaired insulin secretion, increased risk of T2D and gestational diabetes in different ethnicities (Staiger et al., 2008; Tarnowski et al., 2017). Reduced melatonin levels are found to be associated with T2D (Abdolsamadi et al., 2014). Melatonin being an anti-inflammatory molecule is also known to reduce TNF-*α* expression (Haddadi and Fardid, 2015).

The aim of this study was to examine whether i) *TNF-α* polymorphisms (-238 G/A, -308 G/A, -857 C/T, -863 C/A) and *MTNR1B* polymorphisms (-1193 C/T, 5' UTR G/C and intron G/C) are associated with T2D in Gujarat population, ii) plasma melatonin, FFA, *TNF-α* protein levels and *TNF-α* transcript levels in PBMCs are associated with T2D and iii) the genotype-phenotype correlation of *TNF-α* and *MTNR1B* SNPs with T2D; plasma melatonin, FFA, TNF-α protein levels; *TNF-α* transcript levels in PBMCs; and the metabolic profile.

## 2.2 Materials and Methods

This study 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/2013/1). Importance of the study was explained to all participants and written consent was obtained from all patient and control subjects. The study group included 478 T2D patients (213 males and 265 females) and 502 non-diabetic subjects (251 males and 251 females) as shown in Table 1. The T2D subjects recruited for the study displayed (FBG) levels >125 mg/dl and BMI ≥ 25 kg/m$^2$ (weight kg/height m$^2$ was calculated by using the measured height and weight).

### 2.2.1 Blood Collection, DNA Extraction, and Lipid Profiling

Three ml venous blood was drawn from diabetic and ethnically matched non-diabetic individuals and collected in $K_3$EDTA coated tubes (Greiner Bio-One, North America Inc., North Carolina, USA). Plasma was separated and stored at -20$^o$C for lipid profiling and assay of melatonin, FFA and TNF-α. FBG, total cholesterol (TC), triglycerides (TG) and high-density lipoprotein (HDL) were estimated by using the commercial kits (Reckon Diagnostics P. Ltd, Vadodara, India). Low-density lipoprotein (LDL) was calculated by using

Friedewald's (1972) formula. DNA was extracted by the phenol-chloroform method. The DNA content and purity were determined spectrophotometrically by 260/280 absorbance ratio ~1.8. The integrity of DNA was checked electrophoretically on 0.8% agarose gel. The DNA was normalized and stored at 4°C until further analysis.

### 2.2.2 Genotyping of *MTNR1B* and *TNF-α* SNPs by PCR-RFLP

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used to genotype five *TNF-α* promoter polymorphisms and three *MTNR1B* polymorphisms the primers used for genotyping are shown in Table 2.1. The reaction mixture of the total volume of 20 µL included 3 µL (150 ng) of genomic DNA, 11µL nuclease-free H2O, 2.0 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 2.1) for 30 sec., 72°C for 30 sec and final annealing at 72°C for 10 min. The amplified products were checked by electrophoresis on a 2% agarose gel stained with ethidium bromide. Details of the restriction enzymes (Fermentas, Thermo Fisher Scientific Inc., USA) and digested products are mentioned in Table 2.1. 5 µl of the amplified products were digested with 1U of the corresponding restriction enzyme in a total reaction volume of 20 µl as per the manufacturer's instructions. The digestion products with 50 base pair DNA ladder (Genei Bangalore, India) were resolved on 3.5% agarose gel or 15% polyacrylamide gel stained with ethidium bromide and visualized under UV transilluminator. More than 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.

### 2.2.3 Estimation of *TNF-α* Transcript Levels

**2.2.3.1 RNA Extraction and cDNA Synthesis.** Total RNA from whole blood was extracted by Trizol method. RNA integrity was verified by 1.5% agarose gel electrophoresis/ethidium bromide staining and O.D. 260/280 absorbance ratio ~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).

**2.2.3.2 Real-Time PCR.** Transcripts levels of *TNF-α* and *GAPDH* were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2.1. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed as described previously (Patel et al., 2016).

**Table 2.1 Primers and restriction enzymes used for *TNF-α* and *MTNR1B* SNPs genotyping, and *TNF-α* gene expression analysis**

| Gene/SNP Primer | Sequence (5'–3') | Anneal-ing Tempe-rature | Amplicon Size (bp) | Restriction Enzyme | Digested products (bp) |
|---|---|---|---|---|---|
| ***TNF-α* (rs361525)**<br>-238 G/A FP<br>-238 G/A RP | AGAAGACCCCCCTCGGAA**C**C<br>ATCTGGAGGAAGCGGTAGTG | 61 | 152 | MspI | 133 bp + 19 bp |
| ***TNF-α* (rs1800629)**<br>-308 G/A FP<br>-308 G/A RP | GAGGCAATAGGTTTTGAGGG**C**CAT<br>TCTGCTGTCCTTGCTGAGGGA | 57 | 360 | NcoI | 339 bp + 21 bp |
| ***TNF-α* (rs1799724)**<br>-857 C/T FP<br>-857 C/T RP | GCATCTGCACCCTCGATGAAG<br>CCTCTACATGGCCCTGTCTAC | 63 | 325 | TaiI | 306 bp + 19 bp |
| ***TNF-α* (rs1800630)**<br>-863 C/A FP<br>-863 C/A RP | GCTCAAAGGGAGCAAGAGCTG<br>CTACATGGCCCTGTCTTCGTTACG | 65 | 323 | TaiI | 302 bp + 21 bp |
| ***MTNR1B* (rs4753426)**<br>-1193 C/T FP<br>-1193 C/T RP | AACATATTTGTGATTAATCCA**G**GC<br>CTGATTATCCTATATTTGTGGAGG | 55 | 361 | BsuRI | 338bp +23 bp |
| ***MTNR1B* (rs10830962)**<br>5' UTR G/C FP<br>5' UTR G/C RP | ACAAAAGAATGCAGATGTCCCC<br>ATTAGCAGGCACGATGGC | 64 | 374 | TaaI | 300 bp +74 bp |
| ***MTNR1B* (rs10830963)**<br>Intron C/G FP<br>Intron C/G RP | GCTAAGAATTCACACCAGCT<br>AGT GCAGACTGTTTTCTAATC | 60 | 120 | PvuII | 100 bp + 20 bp |
| *TNF*-α Exp. FP<br>*TNF*-α Exp. RP | GCCCCCAGAGGGAAGAGTTCCCCA<br>GCT TGAGGGTTTGCTACAACATGGGC | 69 | 124 | - | - |

| | | | | | |
|---|---|---|---|---|---|
| *GAPDH* Exp. FP<br>*GAPDH* Exp. RP | CATCACCATCTTCCAGGAGCGAG<br>CCTGCAAATGAGCCCCAGCCT | 69 | 122 | - | - |

### 2.2.4 Estimation of Plasma Melatonin, TNF-α, and FFA Levels

Plasma levels of Melatonin, TNF-α and FFA in patients and controls were measured by using the human melatonin ELISA Kit (Glory Science Co., Ltd, TX, USA), human TNF- α ELISA Kit (Ray Biotech., GA, USA) and Free Fatty Acid Quantification Colorimetric/Fluorometric Kit (BioVision, Inc., CA, USA) respectively as per the manufacturers' protocols.

### 2.2.5 Statistical Analyses

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed 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 *TNF-α* promoter polymorphisms and *MTNR1B* polymorphisms for patients and control subjects were compared using the chi-square test with 2x2 contingency tables respectively using Prism 5 software (Graphpad software Inc; San Diego CA, USA). *p* values less than 0.0125 and 0.017 for genotype and allele distribution of *TNF-α* and *MTNR1B* SNPs, respectively, were considered as statistically significant due to 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'= 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 of *TNF-α;* plasma melatonin, TNF-α, and FFA levels in patients and controls were plotted and analyzed by unpaired t-test using Prism 5 software (Graphpad software Inc; San Diego CA, USA). $2^{-\Delta\Delta Ct}$ values (fold change) for *TNF-α* expression levels were compared using t-test between different study groups. Association studies of polymorphisms with other parameters were performed using analysis of variance (ANOVA) and Kruskal-Wallis test while correlation analysis was performed using multiple linear regression and spearman's correlation analysis in GraphPad Prism ver. 5 software. *p* values less than 0.05 were considered significant for all the association studies.

## 2.3 Results

Clinical parameters differed significantly between controls and patients (Table 2.2). Patients had a significantly higher FBG ($p$<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 patients as compared to controls. However, LDL did not differ in the study groups ($p$=0.9322).

**Table 2.2 Baseline characteristics of diabetic and non-diabetic individuals from Gujarat population**

| | **Controls (Mean±SD)** | **Patients (Mean±SD)** | ***p* value** |
|---|---|---|---|
| | (n =502) | (n =478) | |
| Age | 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 |
| Onset age (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.

### 2.3.1 Association of *TNF-α* and *MTNR1B* Polymorphisms with T2D

The genotype and allele frequencies of the investigated *TNF-α* promoter polymorphisms (-238 G/A, -308 G/A, -857 C/T and -863 C/A) in 464 patients and 493 controls are summarized in Table 2.3, and the representative gel images for PCR-RFLP analysis are shown in Fig. 2.1. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations in both patient and control groups ($p$>0.05).

The genotype and allelic frequencies of *TNF-α* promoter polymorphisms (-238 G/A, -308 G/A, -863 C/A) were not found to be statistically significant ($p$>0.0125) with Bonferroni's correction for multiple testing as shown in Table 2.3. However, -857 C/T was found to be significantly associated with T2D (genotype and allele frequencies, $p$<0.0001). As compared to the reference CC genotype, CT genotype increased the risk for the disease with an Odds Ratio (OR) of 1.907. Moreover, the mutant homozygous TT genotype increased the risk by 7.585-fold as suggested by OR.

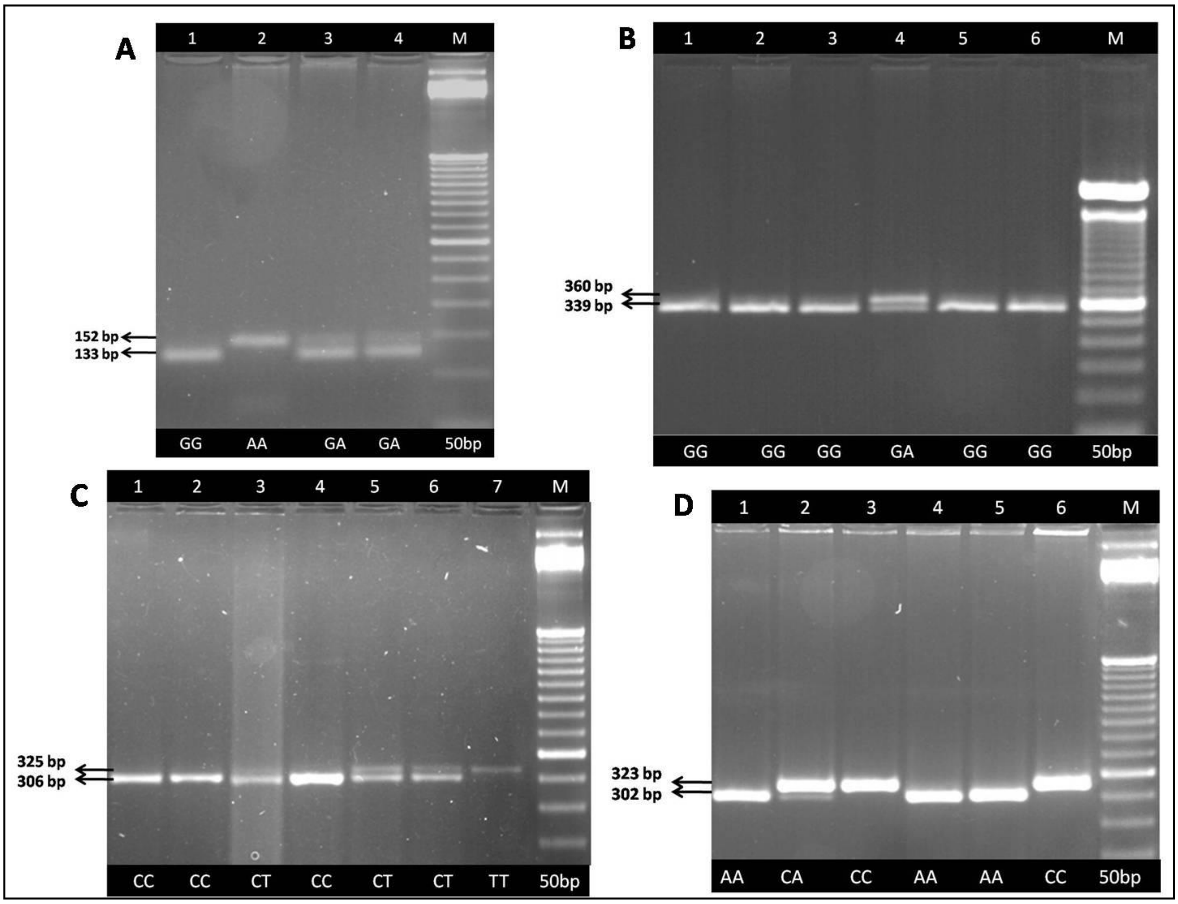


**Figure 2.1 PCR-RFLP analysis of *TNF-α* -238 G/A, -308 G/A, -857 C/T and -863 C/A polymorphisms. A)** PCR-RFLP analysis of *TNF-α* -238 G/A polymorphism on 3.5% agarose gel: lane 1 shows homozygous (GG) genotype; lane 2 shows homozygous (AA) genotype; lanes 3 and 4 show heterozygous (GA) genotype. **B)** PCR-RFLP analysis of *TNF-α* -308 G/A polymorphism on 3.5% agarose gel: lanes 1, 2, 3, 5, and 6 show homozygous (GG) genotypes; lane 4 shows heterozygous (GA) genotypes. **C)** PCR-RFLP analysis of *TNF-α* -857 C/T polymorphism on 3.5% agarose gel: lanes 1, 2 and 4 show homozygous (CC) genotypes; lanes 3, 5 and 6 show heterozygous (CT) genotypes; lane 7 shows homozygous (TT) genotype. **D)** PCR-RFLP analysis of *TNF-α* -863 C/A polymorphism on 3.5% agarose gel: lanes 1, 4, and 5 show homozygous (AA) genotypes; lane 2 shows heterozygous (CA) genotype; lanes 3 and 6 show homozygous (CC) genotype.

The genotype and allele frequencies of the investigated *MTNR1B* polymorphisms (-1193 C/T, 5’ UTR G/C and intron C/G) in 434 patients and 489 controls are summarized in Table 2.4, and the representative gel images for PCR-RFLP analysis are shown in Fig. 2.2. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations in both patient and control groups ($p$>0.05).

The genotype and allelic frequencies of *MTNR1B* studied polymorphisms were not found to be statistically significant ($p$>0.017) with Bonferroni’s correction for multiple testing as shown in Table 2.4.

**Table 2.3 Genotype and allele frequency distribution of *TNF-α* promoter polymorphisms in T2D patients and controls**

| Gene/ SNP | Genotype or allele | Controls (Frequency) | Patients (Frequency) | *p* for Association | Odds Ratio | (95% CI) |
|---|---|---|---|---|---|---|
| *TNF-α* -238 G/A (rs361525) | | (n = 295) | (n = 320) | | | |
| | GG | 257 | 292 | R | - | - |
| | GA | 37 | 27 | 0.0955[a] | 0.6423 | 0.3804 to 1.084 |
| | AA | 1 | 1 | 0.9288[b] | 0.8801 | 0.05474 to 14.15 |
| | G | 551 (0.93) | 611 (0.95) | 0.1110[c] | 0.6706 | 0.4090 to 1.099 |
| | A | 39 (0.07) | 29 (0.05) | | | |
| *TNF-α* -308 G/A (rs1800629) | | (n = 493) | (n = 388) | | | |
| | GG | 449 | 351 | R | - | - |
| | GA | 42 | 34 | 0.8850[a] | 1.036 | 0.6451 to 1.662 |
| | AA | 2 | 3 | 0.4690[b] | 1.919 | 0.3187 to 11.55 |
| | G | 940 (0.95) | 736 (0.95) | 0.6360[c] | 1.111 | 0.7191 to 1.715 |
| | A | 46 (0.05) | 40 (0.05) | | | |
| *TNF-α* -857 C/T (rs1799724) | | (n = 478) | (n = 408) | | | |
| | CC | 384 | 270 | **R** | - | **-** |
| | CT | 91 | 122 | **<0.0001[a]** | 1.907 | 1.394 to 2.608 |
| | TT | 3 | 16 | **0.0002[b]** | 7.585 | 2.188 to 26.30 |
| | C | 859 (0.90) | 662 (0.81) | **<0.0001[c]** | 2.060 | 1.567 to 2.708 |
| | T | 97 (0.10) | 154 (0.19) | | | |
| *TNF-α* -863 C/A (rs1800630) | | (n = 489) | (n = 464) | | | |
| | CC | 292 | 256 | R | - | **-** |
| | CA | 130 | 141 | 0.1522[a] | 1.237 | 0.9243 to 1.656 |
| | AA | 67 | 67 | 0.4948[b] | 1.141 | 0.7816 to 1.665 |
| | C | 764 (0.71) | 653 (0.81) | 0.8042[c] | 1.025 | 0.8451 to 1.242 |
| | A | 314 (0.29) | 154 (0.19) | | | |

'n' represents number of samples, 'R' represents reference group, CI refers to confidence interval, [a, b] Patients vs controls (genotype) with respect to Reference using chi-square test with 2x2 contingency table, [c] Patients vs controls (allele) using chi-square test with 2x2 contingency table, Values are significant at $p$<0.0125 due to Bonferroni's correction.

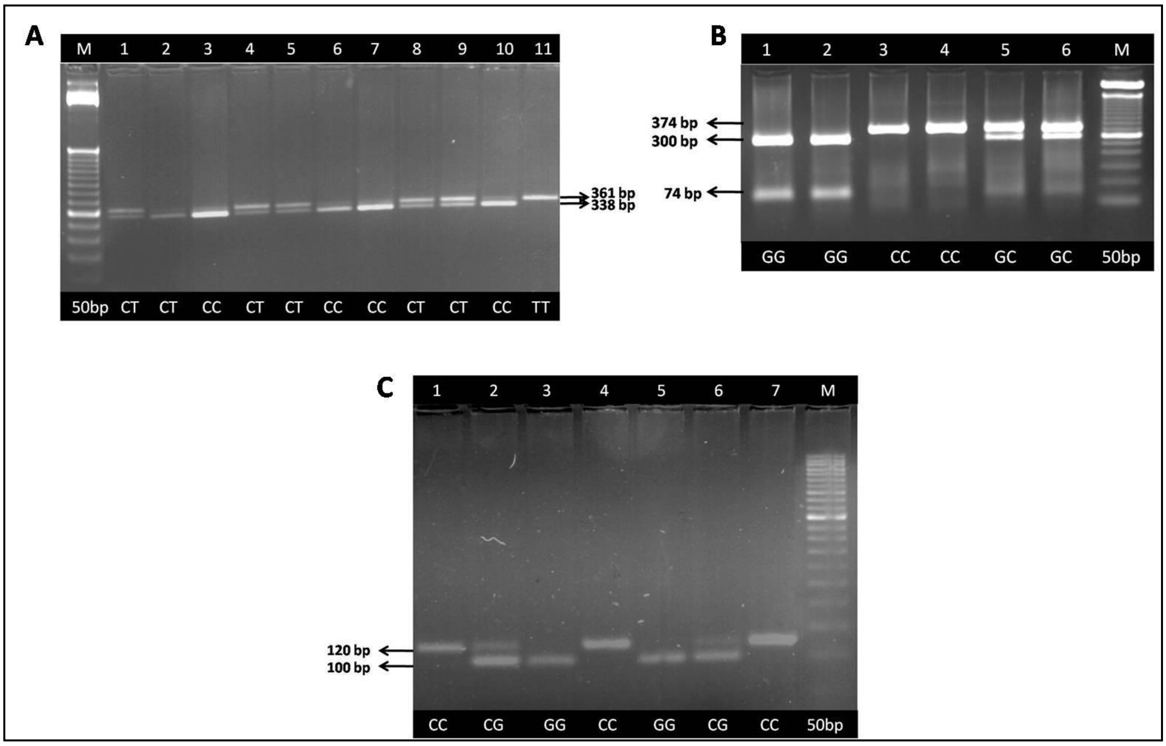


**Figure 2.2 PCR-RFLP analysis of *MTNR1B* -1193 C/T, 5' UTR G/C and intron C/G polymorphisms. A)** PCR-RFLP analysis of *MTNR1B* -1193 C/T polymorphism on 3.5% agarose gel: lanes 1, 2, 4, 5, 8, and 9 show heterozygous (CT) genotypes; lanes 3, 6, 7, and 10 show homozygous (CC) genotypes; lane 11 shows homozygous (TT) genotype. **B)** PCR-RFLP analysis of *MTNR1B* 5' UTR G/C polymorphism on 3.5% agarose gel: lanes 1 and 2 show homozygous (GG) genotypes; lanes 3 and 4 show homozygous (CC) genotypes; lanes 5 and 6 show heterozygous (GC) genotype. **C)** PCR-RFLP analysis of *MTNR1B* intron C/G polymorphism on 3.5% agarose gel: lanes 1, 4 and 7 show homozygous (CC) genotypes; lanes 2 and 6 show heterozygous (CG) genotypes; lanes 3 and 5 show homozygous (GG) genotypes.

**Table 2.4 Genotype and allele frequency distribution of *MTNR1B* polymorphisms in T2D patients and controls**

| Gene/ SNP | Genotype or allele | Controls (Frequency) | Patients (Frequency) | *p* for Association | Odds Ratio | (95% CI) |
|---|---|---|---|---|---|---|
| *MTNR1B* rs4753426 (-1193 C/T) | | (n = 481) | (n = 426) | | | |
| | CC | 95 (0.20) | 102 (0.24) | R | - | - |
| | CT | 252 (0.52) | 201 (0.47) | 0.0819[a] | 0.7429 | 0.5312 to 1.039 |
| | TT | 134 (0.28) | 123 (0.29) | 0.4081[b] | 0.8549 | 0.5896 to 1.240 |
| | C | 442 (0.46) | 405 (0.48) | 0.4983[c] | 0.9381 | 0.7798 to 1.129 |
| | T | 520 (0.54) | 447 (0.52) | | | |
| | GG | (n = 470) | (n = 417) | R | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | GC | 122 (0.26) | 114 (0.27) | 0.8546[a] | 0.9707 | 0.7065 to 1.334 |
| *MTNR1B* | CC | 226 (0.48) | 205 (0.49) | 0.4212[b] | 0.8596 | 0.5946 to 1.243 |
| rs10830962 | | 122 (0.26) | 98 (0.24) | | | |
| (5' UTR G/C) | G | | | 0.4198[c] | 0.9261 | 0.7685 to 1.116 |
| | C | 470 (0.50) | 433 (0.52) | | | |
| | | 470 (0.50) | 401(0.48) | | | |
| | | (n = 489) | (n = 434) | | | |
| *MTNR1B* | CC | 169 (0.35) | 133 (0.31) | R | - | - |
| rs10830963 | CG | 259 (0.53) | 266 (0.61) | 0.0663[a] | 1.305 | 0.9819 to 1.734 |
| (intron C/G) | GG | 61 (0.12) | 35 (0.08) | 0.1902[b] | 0.7291 | 0.4540 to 1.171 |
| | C | 597 (0.61) | 532 (0.61) | 0.9133[c] | 0.9896 | 0.8204 to 1.194 |
| | G | 381 (0.39) | 336 (0.39) | | | |

'n' represents number of samples, 'R' represents reference group, CI refers to confidence interval, [a, b] Patients vs controls (genotype) with respect to Reference using chi-square test with 2x2 contingency table, [c] Patients vs controls (allele) using chi-square test with 2x2 contingency table, Values are significant at $p<0.017$ due to Bonferroni's correction.

### 2.3.2 Haplotype Analyses of *TNF-α* and *MTNR1B* Polymorphisms

A haplotype evaluation of the four polymorphic sites of *TNF-α* (-238 G/A, -308 G/A, -857 C/T, -863 C/A) revealed that the haplotypes differed significantly between patients and controls ($p$=3.11x10$^{-5}$) and the susceptible haplotypes were GGCA ($p$=0.035) and GGTC ($p$=1x10$^{-4}$) as shown in Table 2.5. Further, evaluation of three polymorphic sites of *MTNR1B* (-1193 C/T, 5'UTR G/C, intron C/G) was performed and the estimated frequencies of the haplotypes did not differ significantly between patients and controls (global $p$=0.681) as shown in Table 2.6.

**Table 2.5 Haplotype frequencies of *TNF-α* polymorphisms in T2D patients and controls**

| Haplotype (*TNF-α* -238G/A, -308 G/A, -857C/T, -863C/A) | Patients (Freq. %) (n=475) | Controls (Freq. %) (n=502) | *p* for Association | $p_{(global)}$ | Odds Ratio [95%CI] |
|---|---|---|---|---|---|
| GGCC | 243(0.461) | 317(0.584) | 0.003 | 3.11x10$^{-5}$ | 0.744 [0.611~0.907] |
| GACC | 29(0.055) | 28(0.051) | 0.729 | | 1.097 [0.647~1.859] |
| **GGCA** | 133(0.252) | 109(0.201) | **0.035** | | 1.336 [1.019~1.751] |
| AGCC | 18(0.034) | 30(0.055) | 0.118 | | 0.627 [0.347~1.132] |
| GGTA | 22(0.041) | 15(0.027) | 0.182 | | 1.563 [0.805~3.031] |
| **GGTC** | 75(0.142) | 38(0.07) | **1x10$^{-4}$** | | 2.178 [1.459~3.253] |

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

**Table 2.6 Haplotype frequencies of *MTNR1B* polymorphisms in T2D patients and controls**

| Haplotype (*MTNR1B* -1193 C/T, 5' UTR G/C and intron C/G) | Patients (Freq. %) (n=459) | Controls (Freq. %) (n=501) | *p* for Association | *p* (global) | Odds Ratio [95%CI] |
|---|---|---|---|---|---|
| CGG | 242(0.31) | 290(0.325) | 0.206 | 0.681 | 0.878 [0.719~1.074] |
| CGC | 54(0.069) | 47(0.052) | 0.242 | | 1.269 [0.849~1.897] |
| CCC | 110(0.141) | 130(0.145) | 0.511 | | 0.913 [0.696~1.197] |
| TGC | 69(0.088) | 71(0.079) | 0.717 | | 1.065 [0.755~1.503] |
| TGG | 40(0.051) | 39(0.043) | 0.608 | | 1.124 [0.716~1.765] |
| TCC | 251(0.321) | 291(0.326) | 0.408 | | 0.919 [0.753~1.122] |

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

### 2.3.3 Linkage Disequilibrium Analyses of *TNF-α* and *MTNR1B* Polymorphisms

The LD analysis of four polymorphic sites of *TNF-α* was also found to be in low to high LD association (Fig. 2.3). Specifically, -238G/A: -308G/A, -857C/T, -863C/A were in high and low LD association respectively (D'=0.99, $r^2$=0.00; D'=0.88, $r^2$=0.00; D'=0.44, $r^2$=0.00). -308G/A: -857, -863C/T showed complete linkage and moderate LD association respectively. (D'=1, $r^2$=0.00 and D'=0.91, $r^2$=0.01). Further, -857C/T: -863C/A were in low LD association (D'=0.26, $r^2$=0.0).

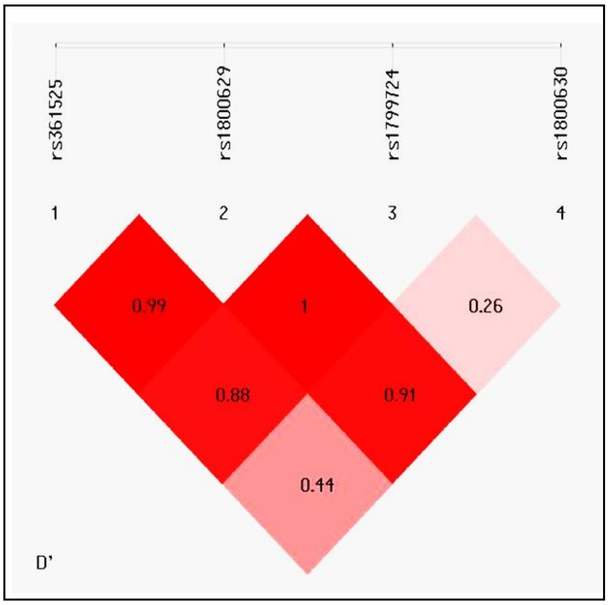


**Figure 2.3 Linkage Disequilibrium (LD) block of *TNF-α* polymorphisms.** LD block with respect to *TNF-α* -238 G/A, -308 G/A, -857 C/T and -863 G/C polymorphisms in Gujarat population.

Further, the LD analysis revealed that the three polymorphisms investigated in the *MTNR1B* gene were in low to high LD association (Fig. 2.4). In particular, -1193 C/T: 5'UTR G/C (D'=0.44, $r^2$= 0.18) and -1193 C/T: intron C/G (D'=0.68, $r^2$=0.24) showed moderate LD association. 5'UTR G/C: intron C/G showed high LD association (D'=0.87, $r^2$=0.47).

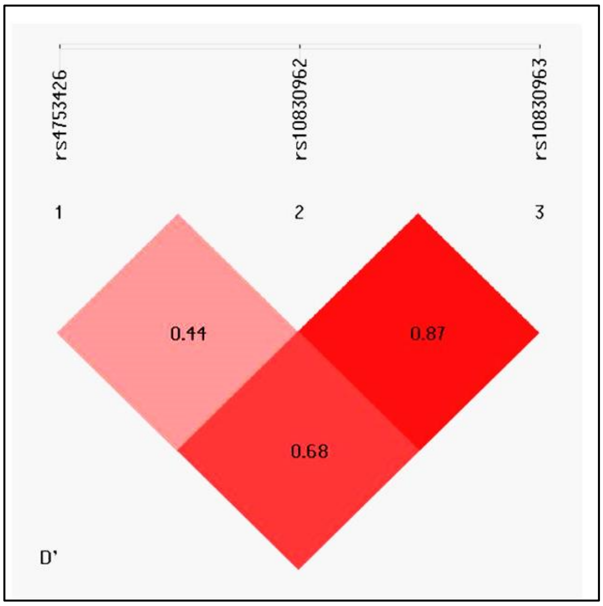


**Figure 2.4 Linkage Disequilibrium (LD) block of *MTNR1B* polymorphisms:** LD block with respect to *MTNR1B* -1193 C/T; 5' UTR G/C and intron C/G polymorphisms in Gujarat population.

### 2.3.4 Correlation of *TNF-α* and *MTNR1B* Polymorphisms with FBG, BMI, and Plasma Lipid Profile

Correlation analysis of *TNF-α* polymorphisms (Table 7) revealed that -238 GA+AA genotypes are found to be associated only with BMI (*p*=0.02) while, *TNF-α* -857 TT genotype with elevated FBG and TG levels (*p*=0.01 and *p*=0.001 respectively). As the frequency was less for AA genotype, it was cumulatively assessed with GA genotype of -238 G/A polymorphism. Further, no association was observed for -308 G/A and -863 C/A SNPs with FBG, BMI, and plasma lipid profile (*p*>0.05).

**Table 2.7 Genotype-phenotype correlation analyses of *TNF-α* polymorphisms with BMI, FBG, and plasma lipid profile**

| Genotype/ Allele | FBG (mg/dL) | BMI (Kg/m2) | TG (mg/dL) | TC (mg/dL) | HDL (mg/dL) | LDL (mg/dL) |
|---|---|---|---|---|---|---|
| ***TNF-α* -238 G/A (rs361525)** | | | | | | |
| GG | 127.2(56.64) | 25.33(5.440) | 135.3(99.37) | 161.5(39.46) | 39.64(12.62) | 94.77(37.42) |
| GA + AA | 118.4(29.64) | 26.92(5.420) | 154.1(106.7) | 167.6(36.96) | 39.15(9.918) | 97.64(35.76) |
| *P* value | 0.5124 | **0.0270** | 0.0983 | 0.1720 | 0.9807 | 0.5507 |
| ***TNF-α* -308 G/A (rs1800629)** | | | | | | |
| GG | 125.8(48.35) | 25.49(5.456) | 137.2(95.31) | 162.9(41.27) | 40.78(13.95) | 94.70(39.38) |
| GA | 113.0(31.56) | 24.67(4.352) | 126.4(75.80) | 162.6(38.91) | 39.97(16.66) | 97.31(40.18) |
| AA | 163.5(87.03) | 22.94(3.136) | 115.2(75.33) | 144.3(20.86) | 38.06(11.14) | 83.16(17.20) |
| *P* value | 0.0634 | 0.2606 | 0.7373 | 0.5438 | 0.6102 | 0.5856 |
| ***TNF-α* -857 C/T (rs1799724)** | | | | | | |
| CC | 129.5(54.65) | 25.51(5.524) | 133.8(91.73) | 162.6(42.03) | 40.80(15.26) | 95.05(40.88) |
| CT | 132.6(57.94) | 25.74(4.897) | 150.8(93.47) | 161.8(37.54) | 39.99(11.61) | 91.62(35.60) |
| TT | 185.0(98.90) | 24.24(5.379) | 162.8(83.93) | 166.3(52.18) | 42.13(21.68) | 91.59(52.69) |
| *P* value | **0.0122** | 0.3069 | **0.0015** | 0.8958 | 0.9246 | 0.4256 |
| ***TNF-α* -863 C/A (rs1800630)** | | | | | | |
| CC | 131.1(54.84) | 25.46(5.696) | 138.5(96.24) | 164.4(40.63) | 40.70(14.45) | 96.03(39.17) |
| CA | 135.4(63.24) | 26.03(5.182) | 140.4(88.26) | 164.4(42.66) | 39.17(13.65) | 97.16(40.96) |
| AA | 134.1(57.10) | 25.69(4.403) | 142.8(91.06) | 164.0(39.25) | 42.13(17.51) | 93.34(40.84) |
| *P* value | 0.7091 | 0.2976 | 0.4698 | 0.9383 | 0.2968 | 0.9413 |

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

Correlation of *MTNR1B* polymorphisms (Table 2.8) showed that intron CG genotype was found to be associated with increased FBG (*p*=0.02). However, it was not associated (*p*>0.05) with BMI and plasma lipid profile (Total Cholesterol, Triglycerides, HDL, LDL). Further, -1193 C/T and 5’ UTR G/C also did not show any association with FBG, BMI, and plasma lipid profile (*p*>0.05).

**Table 2.8 Genotype-phenotype correlation analyses of *MTNR1B* polymorphisms with BMI, FBG, and plasma lipid profile**

| Genotype/ Allele | FBG (mg/dL) | BMI (Kg/m$^2$) | Triglycerides (mg/dL) | Total Cholesterol (mg/dL) | HDL (mg/dL) | LDL (mg/dL) |
|---|---|---|---|---|---|---|
| ***MTNR1B* -1193 C/T (rs4753426)** | | | | | | |
| CC | 134.3(59.98) | 25.35(5.46) | 131.5(87.05) | 164.8(42.63) | 40.74(12.77) | 97.76(40.26) |
| CT | 129.1(52.01) | 25.71(5.51) | 138.8(96.98) | 162.9(41.07) | 41.65(16.73) | 93.45(41.17) |
| TT | 133.5(62.24) | 25.72(5.04) | 142.0(85.58) | 161.9(39.62) | 38.96(12.10) | 94.54(36.65) |
| *P* value | 0.3272 | 0.5775 | 0.1710 | 0.8221 | 0.2053 | 0.5263 |
| ***MTNR1B* 5' UTR G/C (rs10830962)** | | | | | | |
| GG | 133.9(61.83) | 25.24(5.46) | 138.6(89.46) | 138.6(89.46) | 39.75(13.10) | 93.32(38.72) |
| GC | 130.9(54.49) | 25.79(5.43) | 140.7(101.40) | 140.7(101.40) | 41.48(16.57) | 94.37(40.69) |
| CC | 131.4(57.66) | 25.41(5.22) | 134.9(83.62) | 134.9(83.62) | 39.91(12.81) | 97.38(40.83) |
| *P* value | 0.5599 | 0.4869 | 0.9440 | 0.9440 | 0.9170 | 0.8120 |
| ***MTNR1B* intron C/G (rs10830963)** | | | | | | |
| CC | 130.7(61.79) | 25.33(5.05) | 134.8(91.39) | 162.5(39.90) | 40.60(13.15) | 94.98(37.13) |
| CG | 133.4(52.74) | 25.82(5.40) | 140.7(95.14) | 163.2(41.56) | 40.64(16.14) | 94.40(41.06) |
| GG | 160.6(65.94) | 24.63(5.99) | 136.6(90.98) | 170.6(43.85) | 40.82(11.98) | 102.5(42.65) |
| *P* value | **0.0029** | 0.0528 | 0.7865 | 0.3515 | 0.4910 | 0.3895 |

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

### 2.3.5 Plasma Melatonin Levels and their Correlation with FBG, BMI, and Plasma Lipid Profile

Plasma melatonin levels monitored in 37 controls and 45 patients showed a significant decrease ($p=0.001$) in T2D patients as compared to controls (Fig. 2.5). Spearman's Correlation analysis revealed that there was no correlation between melatonin levels and BMI, FBG, and plasma lipid profile ($R^2=0$, $p>0.05$) (Table 2.9).

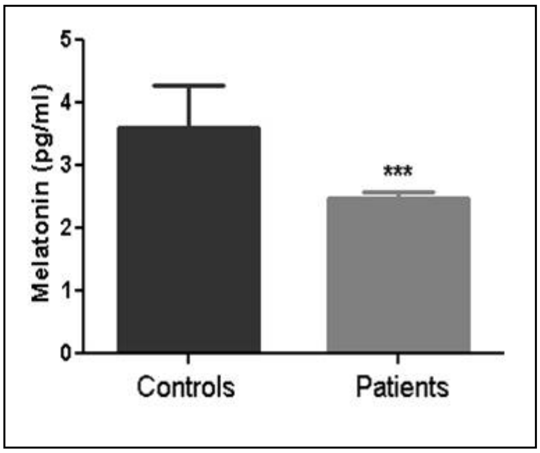

**Figure 2.5 Plasma melatonin levels in controls and patients.** Comparison of findings showed significant decrease in the melatonin levels in patients as compared to controls ($p$<0.001).

**Table 2.9 Correlation analysis of plasma melatonin with BMI, FBG, and plasma lipid profile**

| | **Melatonin** | |
|---|---|---|
| | ***$R^2$*** | ***p*** |
| **BMI (Kg/m²)** | 0.01 | 0.44 |
| **FBG (mg/dL)** | 0.11 | 0.34 |
| **Triglycerides (mg/dL)** | 0.00 | 0.81 |
| **Total Cholesterol (mg/dL)** | 0.03 | 0.20 |
| **HDL (mg/dL)** | 0.01 | 0.45 |
| **LDL (mg/dL)** | 0.06 | 0.10 |

$R^2$= Coefficient of correlation, r= Spearman's correlation coefficient [$p$<0.05, significant; $p$>0.05, non-significant].

### 2.3.6 Relative Gene Expression of *TNF-α* and its Correlation with its Promoter Polymorphisms, BMI, FBG, and Plasma Lipid Profile

Our results showed significant increase in *TNF-α* transcript levels in 150 patients as compared to 152 controls after normalization with *GAPDH* expression as suggested by mean ΔCp values ($p$<0.0001) (Fig. 2.6A). Moreover, $2^{-\Delta\Delta Cp}$ analysis showed approximately 2.1-fold increase in the *TNF-α* transcript levels in patients as compared to controls (Fig. 2.6B).

Fold change of *TNF-α* transcript levels with respect to its promoter polymorphisms revealed that -857 CT+TT genotypes cumulatively increased the expression of *TNF-α* by 3.5-fold (Fig. 2.6C). TT genotype was assessed with CT genotype due to its lesser frequency. Moreover, *TNF-α* transcript levels were also correlated with the haplotypes of *TNF-α* polymorphisms but no significant difference was observed between them ($p$>0.05) (Fig. 2.6D). Also, *TNF-α* transcript levels were correlated with FBG, BMI, and plasma lipid profile, and showed weak correlation with total cholesterol and LDL ($R^2$= 0.04, $p$=0.02 and $R^2$=0.04, $p$=0.02 respectively) (Table 2.10).

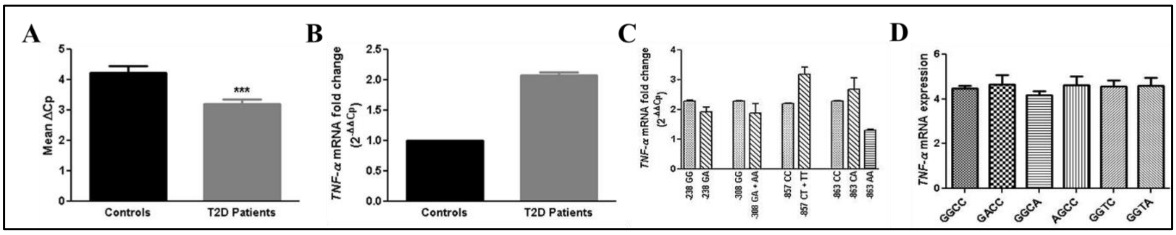


**Figure 2.6 *TNF-α* transcript levels: A. Relative gene expression of *TNF-α* in PBMCs of controls and patients:** Significant increase in *TNF-α* transcript levels was observed patients as compared to

controls (Mean ΔCp ± SEM: 4.24 ± 0.21 vs 3.63 ± 0.13; $p$<0.0001). **B. Relative fold change of *TNF-α* expression in controls and patients.** Diabetic patients showed 2.1-fold increase in *TNF-α* expression compared to controls as determined by $2^{-\Delta\Delta Cp}$ method. **C. Genotype-Phenotype correlation of *TNF-α* polymorphisms with its transcript fold change.** Individuals with -857 CT+TT showed 3.6-fold increase in its expression as compared to CC genotype. No difference was observed with respect to other polymorphisms and respective genotypes. **D. Genotype-Phenotype correlation of *TNF-α* haplotypes with its transcript levels.** No difference was observed between individuals with different haplotypes and their transcript levels ($p$>0.05).

### 2.3.7 Plasma TNF-α Protein Levels and their Correlation with BMI, FBG, and Plasma Lipid Profile

Plasma TNF-*α* protein levels were estimated in 44 controls and 43 patients. Plasma TNF-α protein levels were significantly increased in T2D patients as compared to controls ($p$=0.0122) as shown in Fig. 2.7A. Moreover, we found significant elevation in TNF-α protein levels in obese patients as compared to lean controls ($p$=0.0405) (Fig. 2.7B) whereas no significant difference was found between TNF-α protein levels and their haplotypes ($p$>0.05) (Fig. 2.7C). Further, correlation analysis was performed for TNF-*α* protein levels with the anthropometric parameters. A significant correlation was seen between BMI ($r$=0.3039, $p$=0.0475) and HDL ($r$=-0.3907, $p$=0.0096) (Table 2.10).

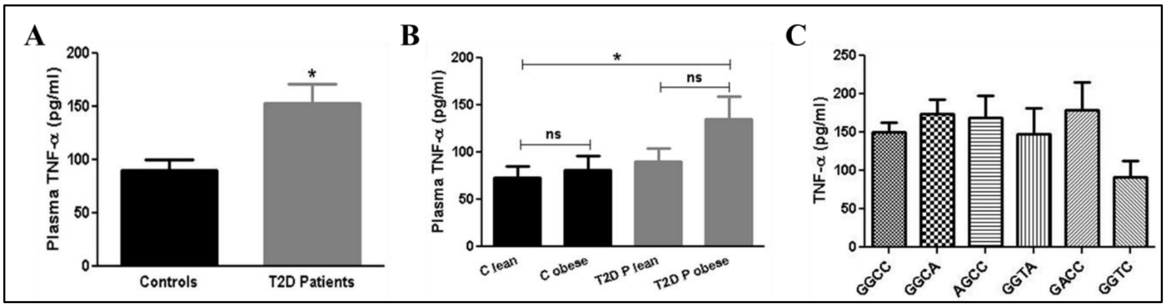


**Figure 2.7 Plasma TNF-α protein levels: A. TNF-α protein concentrations in controls vs patients.** TNF-α protein concentrations were significantly increased in patients ($p$=0.0122). **B. TNF-α protein concentrations in control lean vs obese and patients lean vs obese.** Control lean vs patient obese showed a significant difference ($p$=0.0405) while no difference was observed between the other groups. C. **Genotype-Phenotype correlation of *TNF-α* haplotypes with plasma TNF-α protein concentration.** No difference was observed between individuals with different haplotypes and TNF-α protein levels ($p$>0.05).

### 2.3.8 Plasma Free Fatty Acid Levels and their Correlation with BMI, FBG, and Plasma Lipid Profile

Plasma free fatty acid levels were monitored in 150 controls and 100 patients. FFA levels were found to be significantly elevated in patients as compared to controls ($p$=0.0146) (Fig.

2.8A). Study subjects were further classified into lean and obese. FFA levels were found to be significantly higher in obese controls than lean controls ($p$<0.0001) (Fig. 2.8B). However, there was no difference between lean and obese patients ($p$>0.05) (Fig. 2.8C). Correlation of FFA levels with anthropometric parameters suggests a significant and positive correlation with BMI ($r$= 0.3515, $p$=0.0004) (Table 2.10).

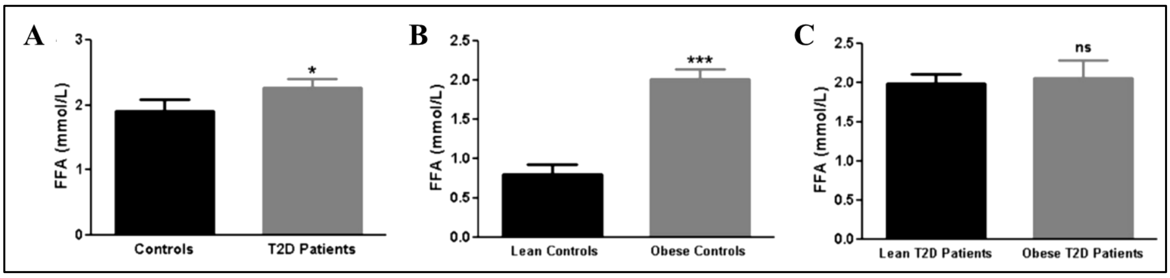


**Figure 2.8 FFA levels: A. Free fatty acid concentrations in controls vs patients,** Plasma FFA levels in patients showed significant elevation ($p$=0.0215). **B. Free fatty acid concentrations in lean vs obese controls** FFA levels exhibited a significant increase in control obese as compared to control lean ($p$<0.0001), and **C. Free fatty acid concentrations in lean vs obese patients.** No difference was found between patients lean and patients obese ($p$>0.05).

**Table 2.10 Correlation analysis of *TNF-α* transcript levels, plasma TNF-α protein levels, and FFA concentrations with BMI, FBG, and plasma lipid profile**

| | ***TNF-α*** | | **TNF-α** | | **FFA** | |
|---|---|---|---|---|---|---|
| | $R^2$ | ***p*** | ***r*** | ***p*** | ***r*** | ***p*** |
| **BMI (Kg/m$^2$)** | 0.00 | 0.82 | 0.3 | **0.04** | 0.35 | **0.0004** |
| **FBG (mg/dL)** | 0.03 | 0.30 | 0.01 | 0.95 | 0.15 | 0.17 |
| **Triglycerides (mg/dL)** | 0.00 | 0.36 | -0.07 | 0.64 | 0.01 | 0.37 |
| **Total Cholesterol (mg/dL)** | 0.04 | **0.02** | 0.08 | 0.61 | -0.02 | 0.87 |
| **HDL (mg/dL)** | 0.01 | 0.14 | -0.39 | **0.001** | 0.12 | 0.22 |
| **LDL (mg/dL)** | 0.03 | **0.02** | 0.28 | 0.07 | 0.01 | 0.89 |

## 2.4 Discussion

Asian Indians have a higher percentage of body fat for a given BMI compared to white Caucasians and African-Americans but have a lower muscle mass. Additionally, they also have an inclination towards ectopic fat deposition (Misra and Shrivastava, 2013). Such a body composition of Indians is partly responsible for predisposition to obesity and insulin resistance (Bhardwaj et al., 2011). Several studies have reported association of changes in cytokine gene expression with obesity, insulin resistance and T2D (Kammoun et al., 2014). Reports also suggest that SNPs that are present in the regulatory regions of cytokine genes (Banerjee and Saxena, 2014) alter their expression profile.

Of the four *TNF-α* promoter polymorphisms studied, only -857 C/T showed significant association with T2D. TT genotype and T allele showed approximately 7-and 2-fold increased risk for T2D, respectively. Moreover, TT genotype shows strong association with elevated FBG and TG levels. Interestingly, Yamashina et al., (2007) also showed an association of -857 C/T polymorphism with T2D and LDL in the Japanese population. Further, Ohara et al., (2012) demonstrated *TNF-α* -857 T allele to be linked with insulin resistance and fatty liver in the Japanese population. Our genotype-phenotype correlation analysis reveals 3.6-fold increase in *TNF-α* transcript levels in the individuals having -857 TT genotype compared to other genotypes and polymorphisms. Overall, T2D patients show 2-fold increase in *TNF-α* transcript levels and elevated plasma TNF-α protein levels. TNF-α protein levels are predominantly increased in obese patients. Gupta et al., (2015) have reported TNF-α -863 C/A to increase TNF-α protein levels. This result confirms that genetic variations in part play a role in *TNF-α* altered expression. Interestingly, reports suggest that *TNF-α* -857 C/T and -863 C/A affect binding of the octamer binding transcription factor (OCT-1) and nuclear factor-κB (NF-κB) respectively, to their putative consensus binding sites, thus regulating the expression of *TNF- α* (van Heel et al., 2002).

*TNF-α* -238 G/A and -308 G/A SNPs are not associated with T2D in our population, an inference supported by a large-scale study by Zeggini et al., (2005). Studies of Kolla et al., (2009) and Dabhi et al., (2015) on the southern and western Indian populations also corroborate our results. However, -238A allele seems to be associated with increased BMI ($p$=0.027) in Gujarat Indian population but not found in Caucasian and African American populations (Walston et al., 1999). In addition, -863 C/A did not show any risk towards T2D in our population, but it was found to be a risk factor in first-degree relatives of T2D in Spain (Costa et al., 2003) and Tunisia (Kallel et al., 2013).

Haplotype analysis of the four polymorphic sites in *TNF-α* reveals that GGCA and GGTC haplotype frequencies are found to be higher in patients, increasing the risk for T2D by 1-fold and 2-fold, respectively, as suggested by Odds Ratio. Furthermore, LD analysis suggests that *TNF-α* -238 G/A, -308 G/A and -857 C/T have strong LD association demonstrating a high linkage between these loci.

Our analysis of plasma FFA levels shows a significant increase in both lean and obese T2D patients and only in control obese individuals. In this connection, higher plasma FFA levels have been associated with obese individuals in general (Boden, 2008; Reaven et al., 1998) as well as with insulin resistance (Boden et al., 1994). Fontaine-Bisson et al., (2007) have

shown -238 G/A and -308 G/A to alter circulating free fatty acid levels (Boden et al., 1994). However, we do not find any association between FFA levels and *TNF-α* polymorphisms.
Our correlation study suggests that *TNF-α* transcript levels show a weak positive correlation with obesity-related traits i.e., LDL and TC levels. It is well known that cytokines like TNF-α induce hyperlipidemia. When TNF-α is administered exogenously in humans, it increases serum cholesterol concentration (Feingold, 1997; Grunfeld and Feingold, 1991, Grunfeld et al., 1990) demonstrating that it plays a key role in cholesterol and triglyceride metabolism (Popa et al., 2007). Herein, we find a definite positive correlation between plasma TNF-α levels and BMI, and a negative correlation with HDL. Moreover, plasma FFA levels also show a positive correlation with BMI.

Melatonin, a pineal hormone regulates energy metabolism by maintaining circadian rhythms (Tsuneki et al., 2016). Disturbances in sleep have been reported to increase the risk of T2D and diabetic complications (Nedeltcheva and Scheer, 2014). Genome-wide association studies have shown association between *MTNR1B* polymorphisms, increased FBG levels and T2D (Bouatia-Naji et al., 2009). Our study on genetic variants of *MTNR1B* (-1193 C/T, 5' UTR G/C, intron C/G), melatonin levels and their correlation with BMI, FBG and plasma lipid profile in T2D patients show that *MTNR1B* polymorphisms are not associated with T2D and obesity-related traits but intron GG genotype (rs10830963) has a significant association with elevated FBG. Similarly, an association of this genotype with FBG has been shown in both South Indian and Han Chinese populations (Gao et al., 2016; Salman et al., 2015). However, this SNP does not show any correlation with obesity-linked parameters in T2D (Ling et al., 2011). Moreover, several studies in different populations have found no association between *MTNR1B* polymorphisms and T2D (Been et al., 2012; Ling et al., 2011; Semiz et al., 2013). Interestingly, a large-scale meta-analysis when stratified according to the ethnicity revealed that *MTNR1B* rs10830963 is associated with T2D in Caucasians but not in Asians (Wang et al., 2013). Our observation of association of G allele with FBG stands substantiated by a meta-analysis (Sparsø et al., 2009) and GWAS (Bouatia-Naji et al., 2009; Lyssenko et al., 2009). However, they also found an association with increased T2D risk which is not seen in our study. Further, Takeuchi et al., (2010) have shown rs10830963 polymorphism to be associated with elevated FBG in two Asian population, South Asian Sri Lankans and East Asian Japanese. The effect of the G allele on FBG was found in children and adolescents indicating an early impact of this SNP during development (Barker et al., 2011; Kelliny et al., 2009). Our study also shows a clear correlation between lowered melatonin levels and T2D risk, a finding supported by the observations of Peschke et al., (2006) and McMullan et al., (2013). Although the location of the rs10830963 variant is in an intron, an un-conserved genomic region, its association with increased *MTNR1B* expression in islets, FBG and T2D stand well documented as discussed above.

Haplotype analysis of the three polymorphic sites of *MTNR1B* reveals that none of the *MTNR1B* haplotypes have an association with T2D risk. Further, LD analysis suggests that *MTNR1B* variants rs4753426 and rs10830963 have strong LD association demonstrating a high linkage between these loci. We did not find any correlation between melatonin levels and FBG, BMI or plasma lipid profile. Yildiz et al., (2014) have also reported that melatonin did not show any correlation with any of the anthropometric and biochemical parameters.

The *MTNR1B* risk allele genotype (GG) has been associated with insulin resistance (Morris et al., 2012; Tuomi et al., 2016) and an increase in *MTNR1B* expression by 2 to 4-fold in human pancreatic islets (Tuomi et al., 2016). Gaulton et al., (2015) revealed that the G allele of rs10830963 favourably binds to NEUROD1 in islet cells further increasing FOXA2-bound enhancer activity and MTNR1B expression leading to increased risk of T2D. Our observation of association of *MTNR1B* risk genotype with higher FBG could be attributed to reduced insulin levels probably through the MTNR1B mediated reduced cAMP levels in pancreatic beta cells in keeping with the reported effects of MTNR1B induced inhibition of insulin secretion (Mühlbauer et al., 2011; Tuomi et al., 2016).

Thus, obesity, altered lipid metabolism, increased expression of pro-inflammatory *TNF-α* and lowered melatonin levels altogether contribute to the increased risk of T2D in Gujarat population (Fig. 2.9).

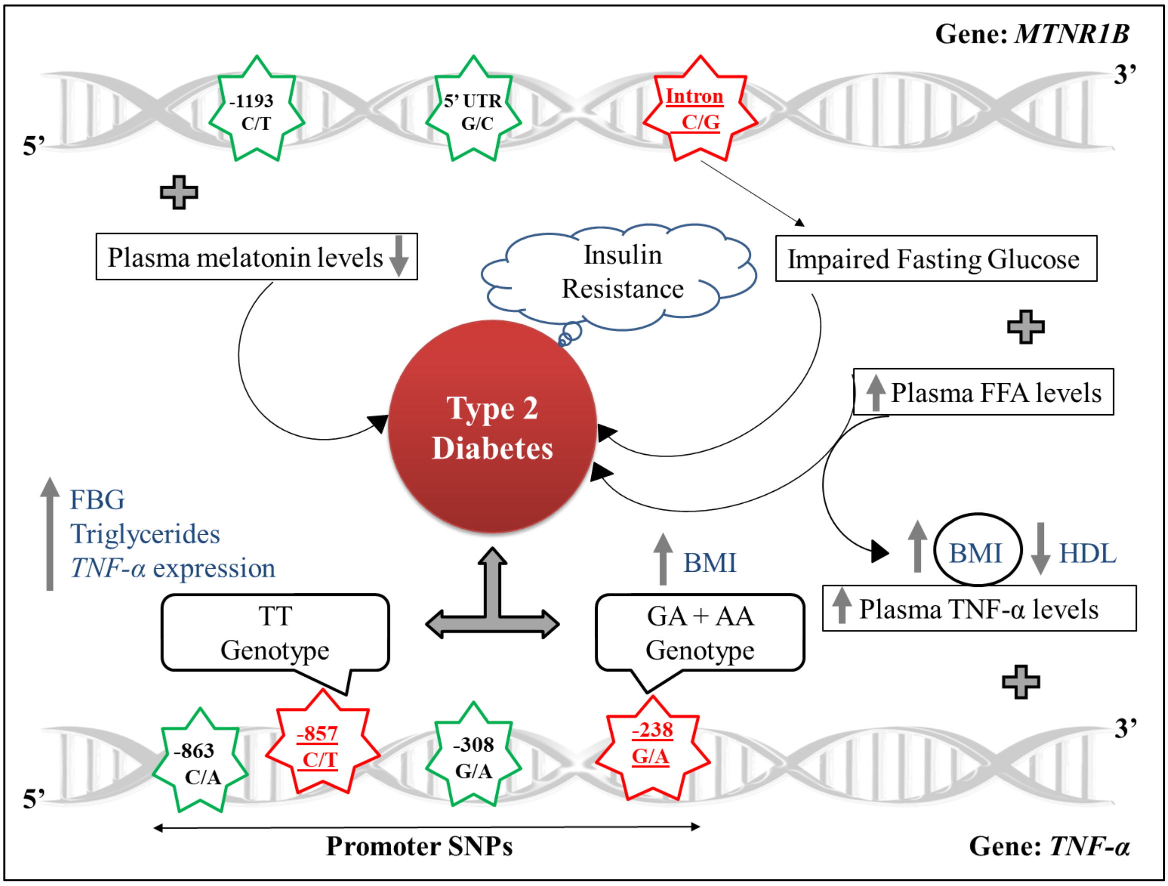


**Figure 2.9 Schematic representation of role of *TNF-α* & *MTNR1B* polymorphisms in T2D pathogenesis in Gujarat population.**

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