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Original article

Genetic variants of tumor necrosis factor-α and its levels: A correlation with dyslipidemia and type 2 diabetes susceptibility

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SUMMARY

Background & aim: Tumor necrosis factor-α (TNF-α) and its genetic variants are implicated in the development of type 2 diabetes (T2D) as a result of systemic inflammation, dyslipidemia, and insulin resistance. The aim of the present study was to investigate i) single nucleotide polymorphisms (SNPs) of *TNF-α* and its association with altered *TNF-α* transcript levels and plasma concentrations ii) free fatty acid (FFA) concentrations as a marker for dyslipidemia and its association with TNF-α and iii) genotype–phenotype correlation analysis in T2D patients.

Methods: Plasma and PBMCs were separated from venous blood of 478 diabetic patients and 502 age-matched non-diabetic individuals. Genomic DNA was isolated from PBMCs and RNA was isolated from PBMCs and adipose tissue samples. PCR-RFLP was used for genotyping and qPCR to estimate *TNF-α* levels. TNF-α and FFA concentrations were estimated from plasma samples by ELISA.

Results: Our study suggests: i) involvement of *TNF-α* −857 C/T in T2D patients ($p < 0.0001$), ii) 2.072 and 6.7 fold elevation in *TNF-α* transcript levels in patients' PBMCs and adipose tissues respectively, increased plasma TNF-α ($p = 0.0122$) particularly in obese patients ($p = 0.0405$), increased plasma FFA ($p = 0.0215$) and, iii) association of *TNF-α* −238 G/A with body mass index (BMI) ($p = 0.0270$) and, −857 C/T with fasting blood glucose (FBG) ($p = 0.0122$) and triglycerides (TG) ($p = 0.0015$). Correlation analysis suggests that TNF-α concentrations are positively correlated with BMI ($r = 0.3$, $p = 0.04$) and negatively correlated with HDL ($r = -0.39$, $p = 0.001$) while the FFA concentrations are positively correlated with BMI ($r = 0.35$, $p = 0.0004$).

Conclusion: It can be concluded that the genetic variant of *TNF-α* along with elevated TNF-α and FFA concentrations play a role in the development of dyslipidemia which could be a potent risk factor towards T2D in Gujarat population.

1. Introduction

Type 2 diabetes (T2D) and insulin resistance have been strongly correlated with increased abdominal obesity, a low-grade inflammatory condition [1]. The mechanism involved in the development of obesity-induced T2D or insulin resistance includes the dysregulated secretion of pro and anti-inflammatory adipokines [2,3]. TNF-α, a pro-inflammatory cytokine/adipokine secreted from infiltrating macrophages, is highly expressed in the adipose tissues of obese animals and human subjects [4]. It impedes insulin-induced phosphorylation of the tyrosine residues in insulin receptor and its substrates which is suggested to affect insulin sensitivity [5]. Feingold et al. have reported that TNF-α increases triglycerides in humans by promoting lipolysis and elevating free fatty acid concentration [6].

It is well-known that T2D is a multifactorial and polygenic metabolic disorder [7]. Substantial variation between different ethnic populations has been reported with regard to the genetic architecture underlying T2D [8,9]. Several single nucleotide polymorphisms (SNPs) in the *TNF-α* promoter region i.e. −238 G/A, −308 G/A, −857 C/T, and −863 C/A (rs361525, rs1800629,

Abbreviations: TNF, Tumor Necrosis Factor-α; FFA, Free Fatty Acid; TC, Total Cholesterol; TG, Triglycerides; LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism.

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rs1799724, and rs1800630 respectively) [10,11] have been considered as potent contributors in the pathogenesis of T2D in different ethnicities [12–15]. Reports suggest that genetic variants in the promoter region of *TNF-α* are associated with differences in its gene expression [14,16,17]. Further, −238G/A and −308G/A polymorphisms of *TNF-α* are documented to alter circulating free fatty acid (FFA) concentrations and insulin resistance in obese subjects with T2D [18]. Several studies have revealed a correlation between *TNF-α* expression and risk factors like Body Mass Index (BMI) and plasma lipids [19,20]. Moreover, TNF-α also plays a role in the pathogenesis of various autoimmune diseases like rheumatoid arthritis, inflammatory bowel disease, psoriasis, ankylosing spondylitis [21], cardiovascular disease [22], cancer [23] and vitiligo [24].

The aim of this study was to examine whether i) promoter polymorphisms in *TNF-α* (−238 G/A, −308 G/A, −857 C/T, and −863 C/A) are associated with its altered transcript levels and plasma concentrations, and T2D in Gujarat population, ii) plasma FFA as a marker for dyslipidemia is associated with TNF-α, iii) the genotype–phenotype correlation of the above-mentioned SNPs, plasma FFA and TNF-α with the metabolic profile. This is the first genetic association study of *TNF-α* variants and its association with altered gene expression and protein concentration, and FFA concentrations serving as a potent risk factor for dyslipidemia and T2D in Gujarat population.

2. Materials and methods

2.1. Study subjects

This study was conducted according to 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/2013/1). A written consent was obtained from all participants after explaining the importance of the study. The study group included 478 T2D patients (213 males and 265 females) and 502 control subjects (251 males and 251 females) as shown in Table 1. The study size was decided based on the previous literature so as to obtain a significant proportion of the less frequent genotypes. Additionally, tissue samples from abdominal region were taken from 22 obese subjects (10 T2D patients and 12 controls) having BMI > 30 kg/m^2 for the gene expression studies. Visceral (omental) adipose tissue was collected by a single surgeon at the time of elective laproscopic surgery. Clinical parameters of all the study subjects were taken in fasted state. Anthropometric and biochemical parameters are as shown in Table S1. Further, fasting blood glucose (FBG) levels >125 mg/dL were considered for the recruitment of T2D subjects. Height and weight were measured to calculate BMI (weight kg/height m^2).

Table 1
Baseline characteristics of diabetic and non-diabetic individuals from Gujarat population.

	Controls (Mean ± SD) (n = 502)	Patients (Mean ± SD) (n = 478)	P value
Age	39.64 ± 16.35 yr	55.99 ± 10.42 yr	–
Sex	251 (50%)	213 (44.5%)	–
Male			
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 $p < 0.05$. Bold signifies *p* values.

2.2. Blood collection, DNA extraction, and lipid profiling

Three ml venous blood was drawn from diabetic patients and ethnically matched non-diabetic individuals and collected in K3EDTA coated tubes (Greiner Bio-One, North America Inc., North Carolina, USA). Plasma was separated and stored at −20 °C for lipid profile and assay of FFA and TNF-α. FBG, total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL) were estimated by using appropriate commercial kits (Reckon Diagnostics P. Ltd, Vadodara, India). Low-density lipoprotein (LDL) was calculated by using Friedewald's (1972) formula. DNA was extracted by phenol-chloroform method and the DNA content and purity were determined spectrophotometrically by 260/280 absorbance ratio. The integrity of DNA was checked electrophoretically on 0.8% agarose gel. The DNA was normalized and stored at 4 °C until further analysis.

2.3. Genotyping of TNF-α SNPs by PCR-RFLP

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used to genotype the four promoter polymorphisms of *TNF-α*. The primers used for genotyping are mentioned in Table S2. The reaction mixture (20 μL) included 3.0 μL (150 ng) of genomic DNA, 11.0 μL nuclease-free water, 2.0 μL 10X PCR buffer, 2.0 μL of 25 mM dNTPs (Puregene, Genetix Biotech), 1.0 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). The protocol followed was: initial denaturation at 95 °C for 10 min followed by 39 cycles of 95 °C for 30 s (denaturation), primer-dependent annealing (Table S2) for 30 s, extension at 72 °C for 30 s and the final extension at 72 °C for 10 min 5 μL of the amplified product was 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 S2 15 μl of the amplified product was digested with 1U of the corresponding restriction enzyme in a total reaction volume of 20 μl as per the manufacturer's instruction. The digested products with 50 base pair DNA ladder (Genei Bangalore, India) were resolved on 3.5% agarose gels or 15% polyacrylamide gels 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.

2.4. Determination of TNF-α transcript

2.4.1. RNA extraction and cDNA synthesis

Total RNA from whole blood and adipose tissue samples was extracted by Trizol method. RNA integrity and purity were verified by 1.5% agarose gel electrophoresis/ethidium bromide staining and O.D. 260/280 absorbance ratio 1.95, respectively. Further, 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 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).

2.4.2. Real-time PCR

The expression of *TNF-α* and *GAPDH* transcript levels were measured by LightCycler®480 Real-time PCR (Roche Diagnostics GmbH, Mannheim, Germany) using gene-specific primers (Eurofins, Bangalore, India) as shown in Table S2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed as described previously [25].

2.5. Estimation of plasma TNF-α and FFA concentrations

Plasma concentrations of TNF-α and FFA in patients and controls were measured using 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 manufacturer's protocol.

2.6. Statistical analyses

Biochemical parameters were compared using unpaired t-test using Prism 5 software (GraphPad software Inc; San Diego CA, USA). 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-squared analysis. The distribution of the genotypes and allele frequencies of *TNF-α* promoter polymorphisms for patients and control subjects were compared using the chi-squared test with 2 × 2 contingency tables respectively using Prism 5 software. P values less than 0.0125 for genotype and allele distribution were considered as 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' = D/Dmax and r^2values for the pair of the most common alleles at each site were obtained using http://analysis.bio-x.cn/myAnalysis.php [26]. Relative gene expression of *TNF-α*, plasma TNF-α, and FFA concentrations in patient and control groups was plotted and analyzed by unpaired t-test using Prism 5 software. $2^{-\Delta\Delta Ct}$ values (fold change) for *TNF-α* expression levels were compared using t-test between the 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 analysis.

3. Results

Clinical parameters differed significantly between controls and patients (Table 1). Patients had a significantly higher FBG ($p < 0.0001$). Moreover, obesity factors like BMI, TC, and TG 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 the controls. However, LDL did not differ in the study groups ($p = 0.9322$).

3.1. Association of TNF-α 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) are summarized in Table 2 while the representative gel images for PCR-RFLP analysis are shown in Fig. S1. 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 found to be statistically indifferent ($p > 0.0125$) with Bonferroni's correction for multiple testing as shown in Table 2. However, −857 C/T was found to be significantly associated with T2D (genotype and allele frequencies, $p < 0.0001$). The CT genotype increased the risk for the disease with an odds ratio (OR) of 1.907 while the mutant homozygous TT genotype increased the risk by 7.585 fold as suggested by OR.

3.2. Haplotype analyses of TNF-α 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 ($p = 3.11 \times 10^{-5}$) and the disease susceptible haplotypes were GGCA ($p = 0.035$) and GGTC ($p = 1 \times 10^{-4}$) (Table 3).

3.3. Linkage disequilibrium analyses of TNF-α polymorphisms

The LD analysis revealed that the four polymorphic sites of *TNF-α* were found to be in low to high LD association (Fig. S2). 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 C/T, −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, $r^2 = 0.0$).

3.4. Correlation of TNF-α polymorphisms with FBG, BMI and plasma lipids

Correlation analysis of *TNF-α* polymorphisms (Table 4) revealed that −238 GA + AA genotype was 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 lipids ($p > 0.05$).

3.5. Relative gene expression of TNF-α

Comparison of the findings showed significantly increased expression of *TNF-α* transcript levels in PBMCs of 150 patients compared to 152 controls after normalization with GAPDH expression as suggested by Mean ΔCp values ($p < 0.0001$) (Fig. 1a). Moreover, a $2^{-\Delta\Delta Cp}$ analysis showed approximately 2.072 fold change in the expression of *TNF-α* transcript in patients as compared to controls (Fig. 1b).

Further, *TNF-α* transcript levels were also found to be significantly increased in the adipose tissue of 10 patients compared to 12 controls as suggested by Mean ΔCp values ($p = 0.0381$) (Fig. 2a). $2^{-\Delta\Delta Cp}$ analysis showed approximately 6.7 fold change in the expression of *TNF-α* transcript in patients as compared to controls (Fig. 2b).

3.6. Correlation of TNF-α transcript levels with its promoter polymorphisms, BMI, FBG and plasma lipids

The AA genotype of −238 G/A and −308 G/A and, TT genotype of −857 C/T was assessed along with the heterozygous genotype of

Table 2
Genotype and allele frequency distribution of *TNF-α* promoter polymorphisms in T2D patients.

Gene/SNP	Genotype or allele	Controls (Frequency)	Patients (Frequency)	*p* for Association	Odds ratio	(95% CI)
		(n = 295)	(n = 320)			
TNF-α	GG	257	292	R	–	–
−238 G/A	GA	37	27	0.0955[a]	0.6423	0.3804 to 1.084
(rs361525)	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)			
		(n = 493)	(n = 388)			
TNF-α	GG	449	351	R	–	–
−308 G/A	GA	42	34	0.8850[a]	1.036	0.6451 to 1.662
(rs1800629)	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)			
		(n = 478)	(n = 408)			
TNF-α	CC	384	270	R	–	-
−857 C/T	CT	91	122	< **0.0001**[a]	1.907	1.394 to 2.608
(rs1799724)	TT	3	16	**0.0002**[b]	7.585	2.188 to 26.30
	C	859 (0.90)	662 (0.81) 154 (0.19)	< **0.0001**[c]	2.060	1.567 to 2.708
	T	97 (0.10)				
		(n = 489)	(n = 464)			
TNF-α	CC	292	256	R	–	-
−863 C/A	CA	130	141	0.1522[a]	1.237	0.9243 to 1.656
(rs1800630)	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 2 × 2 contingency table, [c] Patients vs controls (allele) using chi-square test with 2 × 2 contingency table, Values are significant at p < 0.0125 due to Bonferroni's correction.
Bold signifies *p* values.

Table 3
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	$p_{(global)}$	Odd Ratio [95%CI]
GGCC	243 (0.461)	317 (0.584)	0.003	3.11×10^{-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)	$\mathbf{1 \times 10^{-4}}$		2.178 [1.459–3.253]

'CI' represents confidence interval (Frequency <0.03 in both case and control has been dropped and was ignored in the analysis).
Bold signifies *p* values.

their respective polymorphisms, due to their lesser frequency. Fold change of *TNF-α* transcript with respect to its promoter polymorphisms revealed that −857 CT + TT genotypes cumulatively increased the expression of *TNF-α* by 3.6 fold (Fig. 3).

Furthermore, none of the other polymorphisms or their genotypes showed increased expression of *TNF-α*. There was no difference in the expression of *TNF-α* between individuals with −238 GG and GA + AA genotype ($p = 0.543$), −308 GG and GA + AA genotype ($p = 0.6412$), −857 CC and CT + TT genotype ($p = 0.6329$) and, −863 CC, CA and AA genotypes ($p = 0.3149$) as suggested by Mean ΔCp values (Fig. S3a). Moreover, ANOVA's trend test was used to see the change in Mean ΔCp values across the different genotypes in controls and patients. The analysis revealed no significant difference in the Mean ΔCp values for patients ($p = 0.7483$) (Fig. S3b) and controls ($p = 0.9517$) (Fig. S3c). However, overall difference across the genotypes between controls and patients was significant ($p < 0.0001$). When, *TNF-α* transcript levels were correlated with FBG, BMI and plasma lipids, it showed weak correlation with TC & LDL ($R^2 = 0.04$, $p = 0.02$ and $R^2 = 0.04$, $p = 0.02$ respectively) (Table 5). 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. S4).

3.7. Plasma TNF-α concentrations and its correlation with FBG, BMI and plasma lipids

Plasma TNF-α concentrations were estimated in 44 controls and 43 patients and it was significantly increased in T2D patients as compared to controls ($p = 0.0122$) as shown in Fig. 4a. Moreover, we found significant elevation in *TNF-α* concentrations in obese patients as compared to lean controls ($p = 0.0405$) (Fig. 4b). Further, correlation analysis was performed for TNF-α plasma concentration with the anthropometric parameters. We found a significant correlation between BMI ($r = 0.3039$, $p = 0.0475$) and HDL ($r = -0.3907$, $p = 0.0096$) (Table 5) whereas, no significant difference was found between TNF-α and its haplotypes ($p > 0.05$) (Fig. S5).

Table 4
Genotype-phenotype correlation analyses of *TNF-α* polymorphisms with BMI, FBG and plasma lipid profile.

Genotype	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 significant was considered at $p < 0.05$.
Bold signifies *p* values.

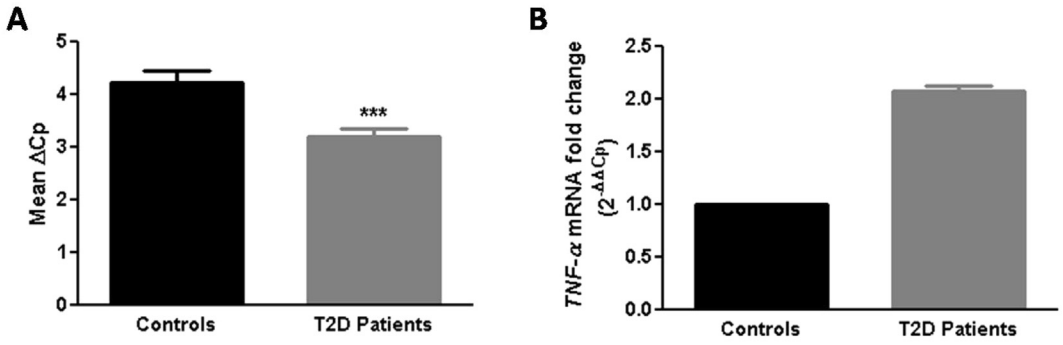

Fig. 1. a) Relative gene expression of *TNF-α* in PBMCs of controls and patients: Significant increase in *TNF-α* mRNA transcript was observed patients (Mean ΔCp ± SEM: 4.24 ± 0.21 vs 3.63 ± 0.13; $p < 0.0001$). **b) Relative fold change of *TNF-α* expression in controls and patients.** Diabetic patients showed 2.072 fold increase in *TNF-α* mRNA expression as determined by $2^{-\Delta\Delta Cp}$ method.

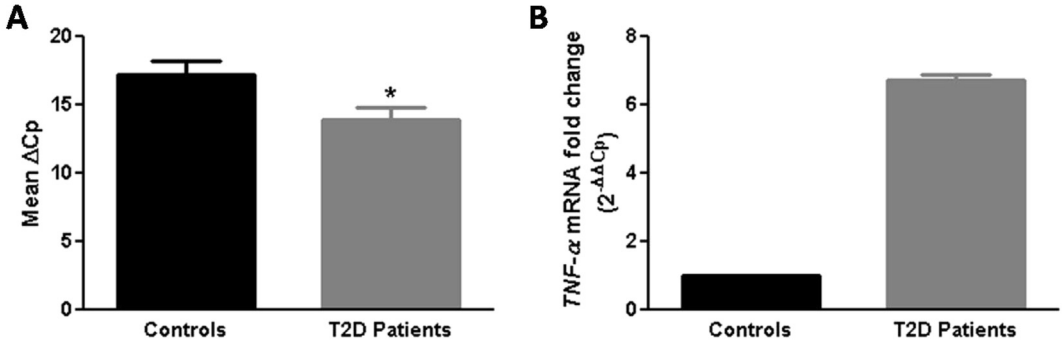

Fig. 2. a) Relative gene expression of *TNF-α* in adipose tissues of controls and patients: Significant increase in *TNF-α* mRNA transcript was observed patients (Mean ΔCp ± SEM: 17.23 ± 0.99 vs 13.87 ± 0.98; $p = 0.0381$). **b) Relative fold change of *TNF-α* expression in controls and patients.** Diabetic patients showed 6.7 fold increase in *TNF-α* mRNA expression as determined by $2^{-\Delta\Delta Cp}$ method.

3.8. Plasma free fatty acid and its correlation with plasma TNF-α, and anthropometric parameters

Plasma free fatty acid concentrations were monitored in 150 controls and 100 patients. FFA concentrations were found to be significantly elevated in patients as compared to controls ($p = 0.0215$) (Fig. 5a). Subjects were further classified into lean and obese. FFA was found to be significantly higher in obese control than lean controls ($p < 0.0001$) (Fig. 5b). However, there was no difference between lean and obese patients ($p > 0.05$) (Fig. 5c). Further, FFA concentrations were correlated with *TNF-α* promoter polymorphisms which were indifferent ($p > 0.05$) (Fig. S6).

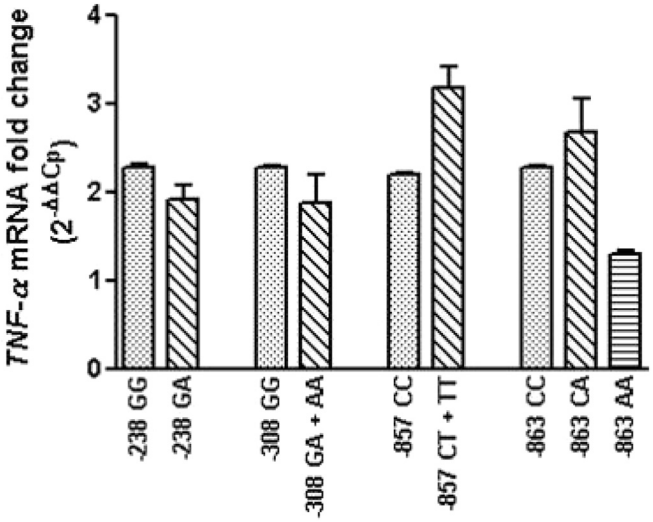

Fig. 3. Genotype-Phenotype correlation of *TNF-α* polymorphisms with its mRNA fold change. Individuals with −857 CT + TT showed 3.6 fold increase in its expression as compared to CC genotype. No difference was observed with respect to other polymorphisms and respective genotypes.

Table 5
Correlation analysis of *TNF-α* transcript levels, plasma TNF-α, and FFA concentrations with BMI, FBG and plasma lipids.

	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

FPG = Fasting plasma glucose, BMI = Body mass index, HDL = High density lipids, LDL = Low density lipids, R^2 = Coefficient of correlation, r = Spearman's correlation coefficient [$p < 0.05$, significant; $p > 0.05$, non significant].
Bold signifies *p* values.

Correlation of FFAs with anthropometric parameters revealed that it was significantly and positively correlated with BMI ($r = 0.3515$, $p = 0.0004$) (Table 5).

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 [27]. Such a body composition of Indians is partly responsible for predisposition to obesity and insulin resistance [28]. Several studies have associated alterations in cytokine gene expression with obesity, changes in insulin sensitivity, and risk of T2D [29] and, reports suggest that SNPs in the regulatory region of cytokines [30] alter their expression profile.

Of the four *TNF-α* promoter polymorphisms studied, only −857 C/T is seen to be significantly associated with T2D [31]. 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. also showed an association of −857 C/T polymorphism with T2D and LDL in the Japanese population [15]. Further, Ohara et al. demonstrated *TNF-α* −857 T allele to be linked with insulin resistance and fatty liver in the Japanese population [32]. Our genotype–phenotype correlation analysis reveals a 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 concentration. Additionally, plasma TNF-α is particularly increased in obese patients. Gupta et al. have reported TNF-α −863 C/A to increase plasma concentrations of TNF-α [33]. This result confirms that genetic variation in part plays a role in altered expression pattern. Interestingly, reports suggest that *TNF-α* −857 C/T and −863 C/A affects the binding of the transcription factors octamer binding transcription factor (OCT-1) and nuclear factor-κB (NF-κB) respectively, to its putative consensus binding sites, thus regulating the expression of the *TNF- α* indirectly [34]. Our adipose tissue gene expression analysis in obese subjects shows a 6.7 fold increase in *TNF-α* transcript levels in T2D patients. This suggests heightened expression of *TNF-α*, a pro-inflammatory adipokine in the visceral adipose tissue of T2D individuals. The reports of Samaras et al. [35] and Winkler et al. [36] of elevated levels of expression of pro-inflammatory adipokines in the visceral adipose tissue and their possible linkage with visceral obesity and insulin resistance in T2D susceptibility provide support to our findings.

TNF-α −238 G/A and −308 G/A are not associated with T2D in our population, an inference supported by a large-scale study by Zeggini et al. [37]. Studies of Kolla et al. [38] and Dabhi et al. [39] on the southern and western Indian populations also provide a similar conclusion. However, −238 A allele seems to be associated with increased BMI ($p = 0.027$) in Gujarat Indian population but not found in Caucasian and African American population [40]. In addition, −863 C/A did not show any risk towards T2D in our

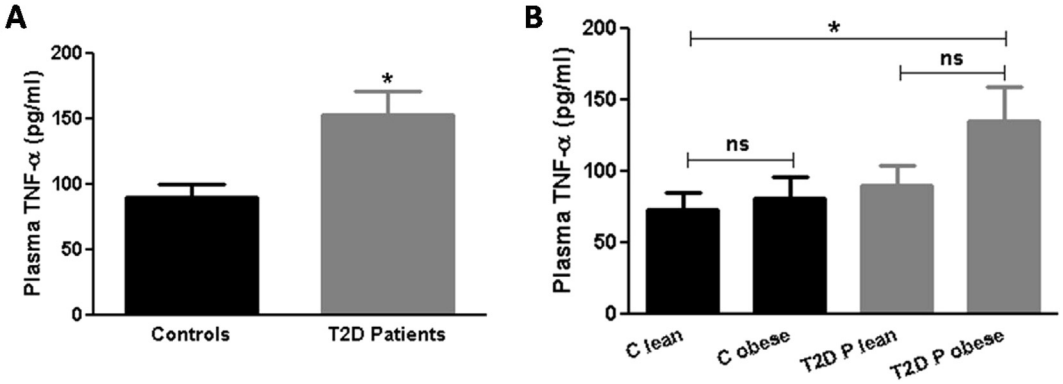

Fig. 4. Plasma TNF-α concentrations in a) controls vs patients Plasma TNF-α concentrations were increased in patients ($p = 0.0122$) and **b) control lean vs obese and patients lean vs obese.** Control lean vs patient obese showed a significant difference ($p = 0.0405$) while no difference was observed between the other groups.

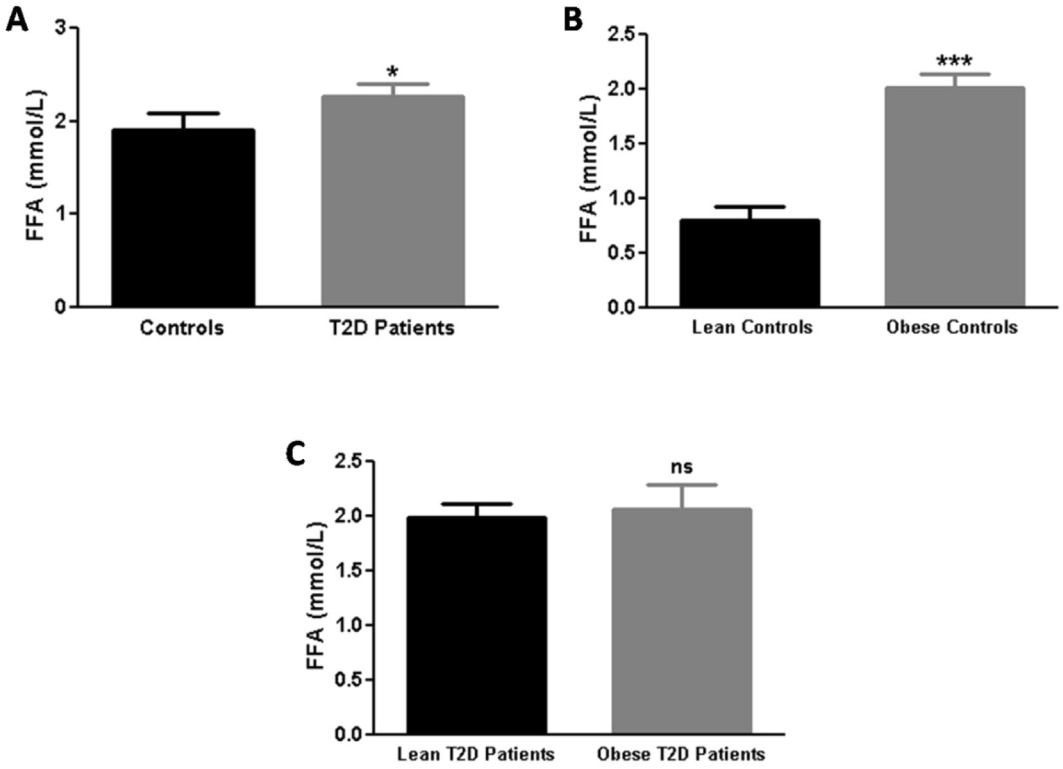

Fig. 5. Plasma free fatty acid concentrations in a) controls vs patients, Plasma FFA in patients showed significant elevation ($p = 0.0215$). **b) lean vs obese controls** Plasma FFA exhibited a significant increase in control obese as compared to control lean ($p < 0.0001$), and **c) lean vs obese patients.** No difference was found between patient lean vs obese ($p > 0.05$).

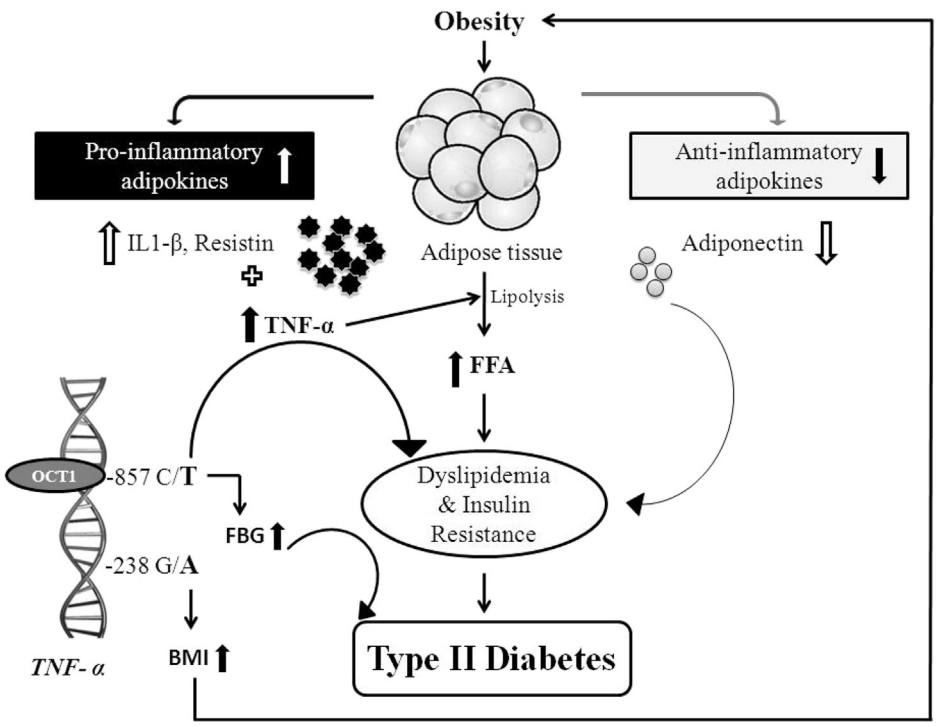

Fig. 6. Mechanism of imbalance in adipokines leading to dyslipidemia and obesity-induced T2D. In obesity, dysregulation of pro and anti-inflammatory adipokines is manifested. Genetic variant of *TNF-α* −857 T allele modulates its expression by strongly binding to the OCT1 transcription factor. Elevated TNF-α concentration promotes lipolysis in adipocytes thereby increasing circulating FFA concentrations. Together, altered adipokines expression and increased FFA partly contributes to dyslipidemia and insulin resistance conferring risk towards obesity-induced T2D in Gujarat population.

population but it was found to be a risk factor in first-degree relatives of T2D in Spain [41] and Tunisia [13].

Haplotype analysis of the four polymorphic sites in *TNF-α* reveals that the haplotypes are significantly associated with the T2D patients. Specifically, GGCA and GGTC haplotype frequencies are found to be higher in patients, increasing the risk for T2D by one fold 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 concentrations shows a significant increase in both lean and obese T2D patients and only in control obese individuals. In this connection, higher plasma FFA concentration has been associated with obese individuals in general [42,43] as well as with insulin resistance [44]. Fontaine-Bisson et al. have shown −238 G/A and −308 G/A to alter circulating free fatty acid concentrations [18]. However, we do not find any association between FFA concentration 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 total cholesterol 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 [45–47] demonstrating that it plays a key role in cholesterol and triglyceride metabolism [48]. Herein, we find a definite positive correlation between plasma TNF-α concentration and BMI, and a negative correlation with HDL. Moreover, plasma FFA concentration also shows a positive correlation with BMI.

Our earlier report suggests that pro-inflammatory cytokine ie. Interleukin 1-β (IL1-β) is also elevated in T2D patients and also contributes to dyslipidemia [25]. Additionally, our unpublished data reveals that adiponectin, an anti-inflammatory adipokine, and resistin, a pro-inflammatory adipokine, exhibit an imbalance in their expression contributing to dyslipidemia in T2D patients [49,50]. Thus, an imbalance in circulating adipokines and elevated FFA concentration is a potent factor for dyslipidemia and obesity-induced T2D in Gujarat population (Fig. 6).

5. Conclusion

Our findings collectively suggest that *TNF-α* −857C/T polymorphism is associated with increased *TNF-α* expression. This taken together with the elevated plasma TNF-α and FFA concentrations, alludes to a strong association with dyslipidemia and obesity, signifying a key role in T2D susceptibility.

Author contributions

RB conceived the idea and designed the experiment. RP performed the experiments. RP, SP, and NR contributed to data acquisition. Data analysis was performed by RP. RB and AVR contributed to the critical revision and approval of the article.

Conflicts of interest

Authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.06.962.

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