PUBLICATIONS & PRESENTATIONS

List of Publications:

1. **Rathwa, Nirali**, Roma Patel, Sayantani Pramanik Palit, Nishant Parmar, Sneha Rana, Mohammad Ismail Ansari, A. V. Ramachandran, and Rasheedunnisa Begum. "β-cell replenishment: Possible curative approaches for diabetes mellitus." Nutrition, Metabolism and Cardiovascular Diseases 30, no. 11 (2020): 1870-1881.
2. **Rathwa, Nirali**, Nishant Parmar, Sayantani Pramanik Palit, Roma Patel, A. V. Ramachandran, and Rasheedunnisa Begum. "Intron specific polymorphic site of vaspin gene along with vaspin circulatory levels can influence pathophysiology of type 2 diabetes." Life sciences 243 (2020): 117285.
3. Palit, Sayantani Pramanik, Roma Patel, Shahnawaz D. Jadeja, **Nirali Rathwa**, Ankit Mahajan, A. V. Ramachandran, Manoj K. Dhar, Swarkar Sharma, and Rasheedunnisa Begum. "Publisher Correction: A genetic analysis identifies a haplotype at adiponectin locus: Association with obesity and type 2 diabetes." Scientific reports 10, no. 1 (2020): 1-1.
4. **Rathwa, Nirali**, Roma Patel, Sayantani Pramanik Palit, Shahnawaz D. Jadeja, Mahendra Narwaria, A. V. Ramachandran, and Rasheedunnisa Begum. "Circulatory Omentin-1 levels but not genetic variants influence the pathophysiology of Type 2 diabetes." Cytokine 119 (2019): 144-151.
5. Patel, Roma, Sayantani Pramanik Palit, **Nirali Rathwa**, A. V. Ramachandran, and Rasheedunnisa Begum. "Genetic variants of tumor necrosis factor-α and its levels: A correlation with dyslipidemia and type 2 diabetes susceptibility." Clinical nutrition 38, no. 3 (2019): 1414-1422.
6. **Rathwa, Nirali**, Roma Patel, Sayantani Pramanik Palit, A. V. Ramachandran, and Rasheedunnisa Begum. "Genetic variants of resistin and its plasma levels: Association with obesity and dyslipidemia related to type 2 diabetes susceptibility." Genomics 111, no. 4 (2019): 980-985.
7. Patel, Roma, **Nirali Rathwa**, Sayantani Pramanik Palit, A. V. Ramachandran, and Rasheedunnisa Begum. "Association of melatonin & MTNR1B variants with type 2 diabetes in Gujarat population." Biomedicine & Pharmacotherapy 103 (2018): 429-434.
8. Pramanik, Sayantani, **Nirali Rathwa**, Roma Patel, A. V. Ramachandran, and Rasheedunnisa Begum. "Treatment avenues for type 2 diabetes and current perspectives on adipokines." Current diabetes reviews 14, no. 3 (2018): 201-221.

Manuscripts under communication:

1. Calorie Restriction potentiates the therapeutic potential of GABA in managing type 2 diabetes in mouse model
2. Melatonin as a diabetic therapeutant: A boon or a bane?
3. Combined treatment of melatonin and sitagliptin ameliorates T2D manifestations: Studies on experimental diabetic models
4. Repurposing statin and L-glutamine: replenishing β-cells in hyperlipidemic T2D mouse model

Poster presentations:

1. **Rathwa N**, Patel R, Pramanik S, Parmar N, Ramachandran AV, Begum R. "GABA in combination with CR as possible therapeutic approach for ameliorating insulin resistance and favoring β-cell regeneration in Type 2 Diabetes" at 9th International Conference on 'Nextgen genomics, biology, bioinformatics and technologies (NGBT) held at Mumbai, India on 30th September- 2nd October, 2019.
2. Patel R, Pramanik S, **Rathwa N**, Parmar N, Dhimmar H, Pancholi D, Ramachandran AV, Begum R. "Melatonin and DPP-IV inhibitor: A novel combinatorial approach for β-cells regeneration" at 9th International Conference on NGBT held at Mumbai, India on 30th September -2nd October, 2019.
3. Pramanik S, Patel R, **Rathwa N**, Parmar N, Dalvi N, Ramachandran AV, Begum R. "L-glutamine and Pitavastatin: resuscitating the dying β-cells" at 9th International Conference on NGBT held at Mumbai, India on 30th September -2nd October, 2019.
4. Parmar N, Patel R, Pramanik S, **Rathwa N**, Shetty S, Patel N, Ramachandran AV, Begum R. "Evaluation of genetic variants of LEPTIN and LEPTIN RECEPTOR as risk factors for T2D in Gujarat population" at 9th International Conference on NGBT held at Mumbai, India on 30th September -2nd October, 2019.
5. **Rathwa NN**, Patel R, Palit SP, Parmar NR, Ramachandran AV, Begum R. "Calorie Restriction in Combination with GABA Ameriolates Type 2 Diabetes" at American Diabetes Association's 79th Scientific Sessions, Moscone Centre, San Francisco, USA (7th- 11th June 2019).
6. Roma Patel, Palit SP, **Rathwa NN**, Parmar NR, Dhimmar H, Pancholi DA, Ramachandran AV, Begum R. "Melatonin and DPP-IV Inhibitor: A novel Combinatorial Approach for β-Cell Regeneration" at American Diabetes Association's 79th Scientific Sessions, Moscone Centre, San Francisco, USA (7th- 11th June 2019).

7. **Rathwa N**, Patel R, Palit SP, Parmar Nishant, Ansarullah, Bhaskaran R, Ramachandran AV, Begum R. "Therapeutic potential of γ-Aminobutyric Acid and Calorie Restriction in Type 2 Diabetic Mouse Model" at International Conference on Reproduction, Endocrinology and Development (ICRED), School of Liberal Studies and Education, Navrachna University, Vadodara, Gujarat, India (18th-21st January, 2019).
8. **Rathwa N**, Parmar N, Palit SP, Patel R, Dhimmar H, Ramachandran AV, Begum R. "Genetic Variants of Omentin-1 and Vaspin: Association with Type 2 Diabetes Susceptibility" at ICRED School of Liberal Studies and Education, Navrachna University, Vadodara, Gujarat, India (18th-21st January, 2019).
9. Patel R, **Rathwa N**, Palit SP, Dhimmar H, Ansarullah, Vasu V, Ramachandran AV, Begum R. "β-cell regenerative potential of melatonin and DPP-IV Inhibitor in amelioration of Type 1 Diabetes" at (ICRED), School of Liberal Studies and Education, Navrachna University, Vadodara, Gujarat, India (18th-21st January, 2019).
10. Palit SP, Patel R, **Rathwa N**, Dalvi N, Ramachandran AV, Begum R. "L-glutamine and Pitavastatin: a therapeutic approach to revive the insulin gold mine" at International Conference on Reproduction, Endocrinology and Development, School of Liberal Studies and Education, Navrachna University, Vadodara, Gujarat, India (18th-21st January, 2019).
11. **Rathwa N**, Palit SP, Patel R, Dhimmar H, Ramachandran AV, Begum R. "Genetic Variants of Omentin-1 and its levels: Association with Type 2 Diabetes Susceptibility in Gujarat population" at International Conference on Proteins, miRNA and Exosomes in Health and Disease, Dept. of Biochemistry, Faculty of Science, The M.S University of Baroda, Gujarat, India, (11th-13th December, 2018).
12. **Rathwa N**, Parmar N, Palit SP, Patel R, Ramachandran AV, Begum R. "Association of Vaspin levels and its Genetic Variants with Type 2 Diabetes Susceptibility" at International Conference on Proteins, miRNA and Exosomes in Health and Disease, Dept. of Biochemistry, Faculty of Science, The M.S University of Baroda, Gujarat, India, (11th-13th December, 2018).
13. Palit SP, Patel R, **Rathwa N**, Ramachandran AV, Begum R. "Haplotype at adiponectin locus and its remarkable association with type 2 diabetes" at International Conference on Proteins, miRNA and Exosomes in Health and Disease, Dept. of Biochemistry, Faculty of Science, The M.S University of Baroda, Gujarat, India, (11th-13th December, 2018).
14. Patel R, **Rathwa N**, Pali SP, Parmar N, Ansarullah, Ramachandran AV, Begum R. "Replenishing β-cells with Melatonin & DPP-IV inhibitor: An in-vivo study" at

International Conference on Proteins, miRNA and Exosomes in Health and Disease, Dept. of Biochemistry, Faculty of Science, The M.S University of Baroda, Gujarat, India, (11th-13th December, 2018).

15. **Rathwa N**, Palit SP, Patel R, Dhimmar H, Parmar N, Bhati H, Ramachandran AV, Begum R. "Genetic Variants of Omentin-1 and Vaspin and Their Plasma Levels: Association with Obesity and Dyslipidemia Related to Type 2 Diabetes" at International Conference on Reproductive Biology and Comparative Endocrinology Birla Institute of Technology and Science Pilani, KK Birla Goa Campus 403726, India, (20th-22th January, 2018).
16. Patel R, **Rathwa N**, Palit SP, Dhimmar H, Parmar N, Ramachandran AV, Begum R. "Assessment of Therapeutic Potential of Melatonin and DPP-IV Inhibitor on β-cell Regeneration in Diabetic Mouse Model" at International Conference on Reproductive Biology and Comparative Endocrinology Birla Institute of Technology and Science Pilani, KK Birla Goa Campus 403726, India, (20th-22th January, 2018).
17. Palit SP, Patel R, **Rathwa N**, Patel N, Rana S, Ramachandran AV, Begum R. "Adiponectin: a watchdog in inflammation induced metabolic disorder" at Immunocon-2017. 44th Annual Conference of the Indian Immunology Society (IIS) held at Institute of Science, Nirma University, Ahmedabad, Gujarat-382481, India, (14th-16th December 2017).
18. **Rathwa N**, Patel N, Ramachandran AV, Begum R. "Association of Resistin genetic variants with Type II Diabetes" at "International Conference on Reproductive Biology and Comparative Endocrinology (ICRBCE) and The 35th Annual Meeting of the Society for Reproductive Biology and Comparative Endocrinology (SRBCE-XXXV)" held at The University of Hyderabad, Hyderabad, Telangana, India, 9th-11th February 2017. (Organizers' Best Poster Award)
19. Palit S*, **Rathwa N***, Patel R, Rana S, Patel N, Ramachandran AV, Begum R. "Association of Adiponectin and Resistin genetic variants with Type 2 Diabetes" at National Symposium on "Omics…to Structural Basis of Diseases" held at The M. S. University of Baroda, Vadodara, Gujarat, India on 30th Sept. and 1st Oct. 2016. (*These authors contributed equally to this work).

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Review

Genetic variants of resistin and its plasma levels: Association with obesity and dyslipidemia related to type 2 diabetes susceptibility

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ABSTRACT

Resistin, an adipokine, is involved in obesity and Type 2 Diabetes (T2D). The current study evaluates the association between *RETN* polymorphisms (−638 G/A, −420C/G & −358 G/A) and the risk towards T2D. Controls and T2D patients were enrolled from Gujarat, India. Polymorphisms of *RETN* were genotyped by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism. For the genotype-phenotype correlation analysis Fasting Blood Glucose (FBG), Body Mass Index (BMI) and plasma lipid profile were used. Plasma levels of resistin were assayed by ELISA. Our study suggests an association of *RETN* −420C/G polymorphism with T2D risk. The CC genotype of *RETN* −420C/G polymorphism was found to be associated with FBG, BMI, and total cholesterol. Plasma resistin levels were found to be significantly increased in diabetic patients as compared to controls. Our findings suggest −420C/G polymorphism of *RETN* as an important factor which could pose a powerful risk towards T2D susceptibility.

1. Introduction

Type 2 diabetes (T2D) is characterized by impaired insulin secretion and peripheral insulin resistance in liver, muscle, and adipose tissue [1]. Obesity is one of the most important factors found to be associated with T2D. Adipose tissue, besides being an energy depository, also secretes bioactive molecules called adipokines. They play important roles in the regulation of appetite and satiety, lipid metabolism, insulin sensitivity and glucose metabolism. They also influence other biological processes like cell adhesion, angiogenesis, hypertension, adipogenesis and bone morphogenesis [2, 3].

Both pro-inflammatory and anti-inflammatory adipokines are in a state of equilibrium and involved in glucose homeostasis in the healthy condition. Resistin (*RETN*), a pro-inflammatory adipokine, located on chromosome 19p13.2 is secreted by macrophages infiltrated adipose tissue [3]. Resistin levels are reported to be significantly increased in both genetic and diet-induced models of obesity [4]. Increased resistin levels are reported to inhibit the insulin signaling pathway. This is essentially brought about by the activation of Suppressor of Cytokine Signaling-3 (SOCS-3) leading to Insulin Receptor Substrate 1/2 (IRS1/2) degradation and induction of insulin resistance [5].

The putative role of resistin in the pathogenesis of human obesity and diabetes led to genetic investigations in different populations [6–9]. Several *RETN* polymorphism studies, however, showed contradictory results. Promoter Single Nucleotide Polymorphisms (SNPs) of *RETN* have been shown to increase T2D susceptibility by elevating circulating resistin levels [6, 9]. Moreover, high circulating levels of resistin have been associated with increased risk of obesity, insulin resistance, and T2D [10–13]. Beside T2D, promoter polymorphisms of *RETN* are associated with nonalcoholic fatty liver disease [14], chronic kidney disease [15], coronary artery disease (CAD) [16], polycystic ovary syndrome [17] and hypertrophic cardiomyopathy [18].

Hence, in the present study, we have investigated *RETN* genetic variants (−638 G/A rs34861192, −420C/G rs1862513 and −358 G/A rs3219175) and correlated them with various metabolic parameters such as Fasting Blood Glucose (FBG), Body Mass Index (BMI) and lipid profile.

Abbreviations: RETN, Resistin; BMI, Body Mass Index; T2D, Type 2 Diabetes.; FBG, Fasting Blood Glucose; TC, Total Cholesterol; TG, Triglycerides; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; FFA, Free Fatty Acid; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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Table 1
The clinical characteristics of the studied populations from Gujarat.

	Controls (Mean ± SD)	Patients (Mean ± SD)	*p* value
	(*n* = 382)	(*n* = 469)	
Age (years)	48.63 ± 10.42	55.92 ± 10.42	–
Sex			
Male	189 (49.47%)	253 (53.94%)	
Female	193 (50.53%)	216 (46.06%)	-
Fasting blood glucose (mg/dL)	109.6 ± 17.38	167.8 ± 72.9	< **0.0001**
BMI (Kg/m^2)	25.40 ± 4.893	27.24 ± 5.810	< **0.0001**
TC (mg/dL)	166.6 ± 41.9	162.1 ± 35.55	ns
TG (mg/dL)	121.8 ± 61.24	153.3 ± 96.43	< **0.0197**
HDL (mg/dL)			
Male	35.15 ± 9.432	34.00 ± 8.465	**0.0037**
Female	42.68 ± 9.359	38.46 ± 9.587	< **0.0001**
LDL (mg/dL)	99.69 ± 36.83	95.10 ± 36.83	0.9322
LDL/HDL			
Male	3.231 ± 1.382	3.223 ± 1.376	< **0.0001**
Female	2.947 ± 1.399	2.939 ± 1.398	**0.0007**
TC/HDL			
Male	5.050 ± 1.880	5.045 ± 1.878	**0.0250**
Female	4.637 ± 1.807	4.631 ± 1.804	< **0.0001**
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 $p < .05$. Bold signifies p-value for association.

2. Materials and methods

2.1. Study subjects

The study was carried out in accordance with the principles of Helsinki Declaration and approved by Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2016-9). The importance of the study was explained to all participants and written consent was obtained from each individual. We recruited age, sex and ethnically matched 382 controls (189 males and 193 females) and 469 T2D patients (253 males and 216 females) as shown in Table 1. The patients showing FBG > 125 mg/dL and suffering from no other diseases were recruited from diabetes awareness camps. Ethnically and geographically matched controls were randomly selected from Gujarat community through community screening program over the same period. Controls exhibited FBG < 110 mg/dL with no prior history of T2D.

2.2. Anthropometric measurements, DNA extraction, and lipid profiling

BMI was calculated by measuring height and weight of all the subjects. Venous blood samples (3 ml) for biochemical assessments were obtained from the subjects after 12 h of overnight fasting in K3EDTA coated tubes (J. K. Diagnostics, Rajkot, India). Plasma was separated and stored at −20 °C for assessing lipid profile. FBG, TC (Total Cholesterol), TG and HDL (High-Density Lipoprotein) were assayed by commercially available kits (Reckon Diagnostics P. Ltd., Vadodara, India). LDL (Low-Density Lipoprotein) was calculated using Friedewald's (1972) formula. The plasma levels of resistin were measured by an enzyme-linked immunosorbent assay (ELISA) kit for human resistin (RayBio, Norcross, GA, USA) with the sensitivity of 2 ng/ml. All the plasma estimations were carried out in duplicates to ensure % CV below 10%. Genomic DNA was extracted from the whole blood using a QIAamp DNA Blood Mini Kit (Qiagen, Germany). DNA purity was measured by calculating the ratio of absorbance at 260/280 nm by Cary 60 UV–Vis spectrophotometer (Agilent, California, USA). The integrity of DNA was checked by 0.8% agarose gel electrophoresis. The DNA was stored at −20 °C until further analysis.

2.3. Genotyping of RETN SNPs

Genotyping of RETN polymorphisms was carried out by PCR-RFLP. The primers used for genotyping of these polymorphisms are mentioned in Table S1. 20 µl of the reaction mixture included 3 µl (50 ng) of genomic DNA, 11 µl of nuclease-free water, 2.0 µl of 10× PCR buffer, 2.0 µl of 2.5 mM dNTPs (Sigma Chemical Co,St.Louis, Missouri, USA), 1.0 µl each of 10 µM forward and reverse primers (MWG Biotech, India) and 0.2 µl of 5 U/µlTaq Polymerase (Bangalore Genei, India). Amplification was performed using Applied Biosystems 96 well Thermal cycler (California, USA) as per the protocol of initial denaturation at 95 °C for 5 min followed by 39 cycles each at 95 °C for 30 s, 59–67 °C for 30 s (primer specific; Table S1), and 72 °C for 30s, followed by final extension at 72 °C for 10 min. 5 µl of the amplified products were analyzed by electrophoresis on a 2.0% agarose gel stained with ethidium bromide along with a 50 bp DNA ladder (MBI Fermentas, St.Leon-Rot, Germany) and photographed. Details of the restriction enzymes (Thermo Fisher Scientific, Wilmington, DE, USA) and digested products are mentioned in Table S1. 15 µl of the amplified products were digested with 1 U of the corresponding restriction enzyme in a total reaction volume of 20 µl as per the manufacturer's instruction. A 50 bp DNA ladder (MBI Fermentas, St.Leon-Rot, Germany) was used as a marker. All the gels were visualized under UV transilluminator using Gel Doc EZ System (Bio-Rad Laboratories, California, USA). > 10% of the samples were randomly selected for genotype confirmation and the results showed 100% concordance (analysis of the chosen samples by two researchers independently) and further confirmed by DNA sequencing.

2.4. Statistical analyses

The clinical parameters were checked for the normality test before their comparison. The clinical characteristics (age, sex, FBG, BMI, and lipid profile) were compared using the *t*-test or Mann Whitney test. 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 *RETN* polymorphisms for patients and control subjects were compared using the chi-square test with 2 × 2 and 2 × 2 contingency tables respectively. *P* values < 0.025were considered as statistically significant for the genotype and allele distribution, and genotype-phenotype association analysis 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^2 values for the pair of the most common alleles at each site were obtained using http://shesisplus.bio-x.cn/SHEsis.html [19]. For the genotype-phenotype association analysis, primarily all the parameters were checked for the normality test and accordingly further analyses were carried out. For the normally distributed data, unpaired *t*-test or One-way ANOVA was performed while for the skewed data, Mann Whitney or Kruskal Wallis test was performed for two or more than two groups respectively. Correlation analysis was performed by Spearman's correlation test. All the analyses were carried out in GraphPad Prism 5 software. *P* values < .05 were considered significant for all statistical analyses. Genotype-phenotype association analysis was carried out in all the subjects after adjusting for the disease susceptibility.

3. Results

3.1. Baseline characteristics

Clinical parameters differed significantly between controls and patients (Table 1). Patients had significantly higher FBG levels ($p < .0001$) compared to controls. Moreover, obesity-associated parameters like BMI and TG were significantly elevated ($p < .0001$, $p < .05$, $p < .0001$ respectively) while, HDL was significantly decreased in patients (males: $p = .0037$, females: $p < .0001$) as compared to controls. LDL/HDL ratio, a marker for Cardiovascular Disease (CVD) was significantly high in both male and female patients compared to controls ($p = .0007$) to control ($p < .0001$ [male] and $p < .0001$ [female]). On the other hand, TC/HDL ratio was significantly high in both male and female T2D patients as compared to controls ($p = .0260$ [male] and $p < .0001$ [female]).

3.2. Association of RETN polymorphisms

The genotype and allele frequencies of the explored *RETN* promoter polymorphisms (−638 G/A, −420C/G and −358 G/A) are summarized in Table 2. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations in both patient and control groups ($p > .025$). −420C/G was found to be significantly associated with T2D individuals (genotype and allele frequencies, $p < .0001$). *RETN* −638 G/A was found to be monomorphic in controls as well as T2D patients and was hence discontinued for further analysis. *RETN* −358G/A was not found to be associated with T2D and was thus discontinued after the initial assessment.

3.3. Haplotype analysis of RETN polymorphisms

A haplotype evaluation of the two polymorphisms of *RETN* (−420C/G and −358 G/A) was performed and the estimated frequencies of the haplotypes did not differ significantly between patients and controls (global $p = .049$) (Table 3).

3.4. Linkage disequilibrium analysis of RETN polymorphisms

The LD analysis revealed that the two polymorphisms of *RETN* gene were in moderate linkage (Fig. S2), with respect to −420C/G: −358 G/A ($D' = 0.24$, $r^2 = 0.02$).

3.5. Association of RETN polymorphisms with FBG, BMI and plasma lipids

RETN polymorphisms showed −420 CC genotype to be associated with increased FBG ($p = .0035$), BMI ($p = .0004$) and TC levels ($p = .0190$) as shown in Table 4. However, it was not associated with TG, LDL and HDL levels ($p > .05$). Further, −358 G/A did not show any association with FBG, BMI and plasma lipids ($p > .05$).

3.6. Plasma resistin levels and its correlation with FBG, BMI and plasma lipids

Plasma resistin levels were monitored in 40 controls and patients each and a significant increase ($p = .0129$) was observed in T2D patients (Fig. 1). Also, the levels of resistin were significantly high in obese patients compared to lean controls ($p = .0155$). Spearman's correlation analysis revealed no correlation between resistin levels and BMI, FBG and plasma lipids ($r = 0$, $p > .05$) (Table 5).

3.7. Association of RETN polymorphisms with plasma resistin levels

Our results showed no association of *RETN* polymorphisms with plasma resistin levels. ($p > .05$) (Fig. 2.).

4. Discussion

In 2000, India (31.7 million) had the highest number of people with diabetes in the world. There is a prediction that by 2030 diabetes mellitus may affect up to 79.4 million beings in India. More specifically in the studied cohort, the prevalence of T2D is second highest in India [20]. The Indian diet comprises largely of carbohydrate (around 60–70%) and fat (14–16%) which predisposes the individual towards obesity and T2D [21].

Table 2
Genotype and allele frequencies distribution of *RETN* polymorphisms in T2D patients.

SNP	Genotype or allele	Controls (Frequency)	Patients (Frequency)	*p* for HWE	*p* for Association	Odds ratio	(95% CI)
(rs34861192)	GG	(*n* = 100)	(n = 100)	(C)	NA	NA	NA
RETN -638 G/A	GA	100 (100.00)	100 (100.00)	NA			
	AA	0 (0.00)	0 (0.00)				
		0 (0.00)	0 (0.00)	(P)			
	G			NA			
	A	200 (1.00)	200 (1.00)				
		0 (0.00)	0 (0.00)				
	CC	(n = 382)	(n = 469)				
	CG	63 (16.89)	172 (34.13)	(C)	R	-	-
(rs1862513)	GG	188 (48.42)	204 (48.58)	0.0682	**0.0001**[a]	0.3975	0.2800 to 0.5641
RETN -420C/G		131 (34.69)	93 (17.29)		**0.0001**[a]	0.2600	0.1756 to 0.3850
	C			(P)			
	G	314 (0.41)	573 (0.58)	0.074	**0.0001**[b]	0.5175	0.4275 to 0.6266
		450 (0.59)	425 (0.42)				
	GG	(*n* = 235)	(*n* = 230)				
(rs3219175)	GA	220 (93.72)	209 (91.08)	(C)	R	-	-
RETN -358 G/A	AA	15 (6.18)	21 (8.71)	0.5779	0.3448[a]	1.377	0.7073 to 2.681
		0 (0.10)	0 (0.21)		-	-	-
	G			(P)			
	A	455 (0.97)	439 (0.95)	0.6133	0.3456[b]	1.343	0.7067 to 2.599
		15 (0.03)	21(0.05)				

'n' represents the number of Patients/Controls. 'R' represents reference group. HWE refers to Hardy-Weinberg Equilibrium. CI refers to Confidence Interval. Odds ratio is based on allele frequency distribution. (P) refers to Patients and (C) refers to Controls.

[a] Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table.

[b] Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table. Statistical significance was considered at $p < .025$ as per Bonferroni's correction.

Table 3
Distribution of haplotype frequencies of *RETN* polymorphisms in T2D patients and controls.

Haplotype (*RETN* −420C/G, −358 G/A)	Patients (Freq. %) (n = 382)	Controls (Freq. %) (n = 502)	p for association	$p_{(global)}$	Odds ratio [95%CI]
CA	12.64 (0.022)	6.73 (0.021)	–	0.049	–
CG	267.36 (0.485)	132.27 (0.408)	0.030		1.359 [1.029–1.796]
GA	6.36 (0.011)	6.27 (0.019)	–		–
GG	282.64 (0.482)	178.73(0.552)	0.030		0.736 [0.557–0.972]

CI represents Confidence Interval. (Frequency < 0.03 in both control & case has been dropped and was ignored in the analysis).

Table 4
Genotype-phenotype association analyses of *RETN* polymorphisms with FBG, BMI and plasma lipid profile.

Genotype	FBG (mg/dL)	BMI (kg/m^2)	TG (mg/dL)	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL) Male	HDL (mg/dL) Female
RETN -420C/G (rs1862513)							
CC	140.8(58.1)	27.2(6.3)	169.8(35.6)	174.9(97.3)	105.7(28.7)	34.9(9.1)	39.7(10.4)
CG	97.7(8.6)	25.0(5.2)	168.6(38.7)	164.2(22.1)	108.2(32.9)	36.2(9.8)	40.1(10.0)
GG	100.1(8.1)	25.3(4.9)	171.4(44.5)	152.2(77.4)	111.1(34.0)	34.1(9.7)	38.1(10.0)
P value	**0.0035**	**0.0004**	0.9430	**0.0190**	0.4219	0.0882	0.2165
RETN -358 G/A (rs3219175)							
GG	119.9(46.2)	25.4(5.2)	155.5(89.1)	156.6(35.6)	105.6(30.9)	36.6(9.2)	36.5(11.3)
GA	131.2(65.7)	25.9(6.1)	151.2(87.9)	155.1(37.8)	106.9(34.9)	34.1(8.2)	39.9(11.1)
AA	–	–	–	–	–	–	–
P value	0.9678	0.4321	0.5678	0.6567	0.8977	0.3619	0.6888

Data are presented as Mean ± SE. Statistical significance was considered at p < .025. Bold signifies p-value for association.

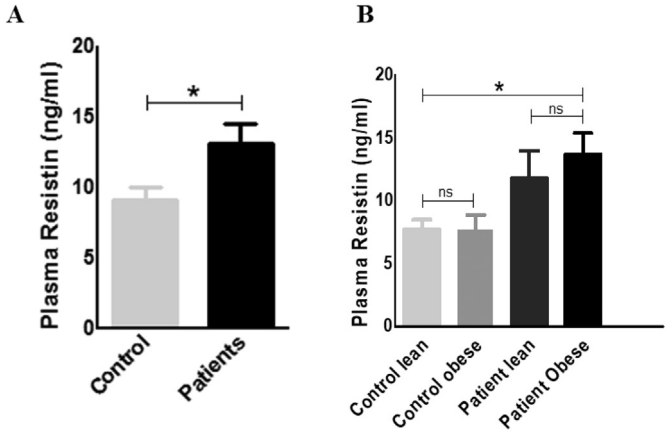

Fig. 1. Plasma resistin levels in a) controls vs. patients, b) control (lean vs. obese) and patients (lean vs. obese). Our results showed a significant increase in plasma resistin levels in T2D patients (p = .0129) compared to controls; obese T2D patients showed a significant increase compared to lean controls (p = .0155).

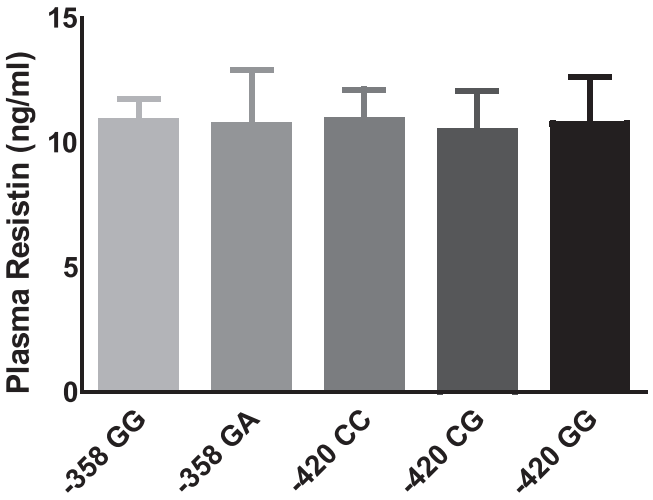

Fig. 2. Association of *RETN* polymorphisms with plasma resistin levels. No association was observed between *RETN* polymorphisms and plasma resistin levels (p > .025).

Table 5
Correlation analysis of plasma resistin with BMI, FBG, and plasma lipids.

Parameters	r	p
BMI (Kg/m^2)	0.0107	0.9314
FBG (mg/dL)	0.0200	0.8986
Triglycerides (mg/dL)	−0.1306	0.3656
Total Cholesterol (mg/dL)	0.0000	0.9998
HDL (mg/dL)		
Male	0.2527	0.2335
Female	0.0914	0.6372
LDL (mg/dL)	0.0624	0.6908

r = correlation coefficient; p > .05, non-significant.

Investigation of genetic variants for T2D in Indian population is essential due to its high-risk for T2D and related metabolic traits. However, studies are scarce in this direction, often restricted to replication of Genome-Wide Association Studies. (GWAS). Though previous GWAS did not identify *RETN* -420 as a T2D susceptible locus, it has been shown that the gene holds much importance in conferring T2D risk in a number of population studies. This may be attributed to factors such as epigenetic effects and ethnic differences.

The genetic variants of *RETN* and resistin levels in T2D patients show *RETN* −420CC genotype and higher plasma resistin levels to be associated with T2D [22]. *RETN* -638G/A polymorphism was found to be nonexistent in our population, as only GG genotype was present in all the samples. Neither we found any association of *RETN* -358 G/A with T2D nor with any other parameters. Similar results are reported with reference to Malaysian population [23]. A previous study in Japanese subjects reported increased resistin levels to be associated with *RETN* −420 C/G polymorphism [9, 24, 25]. Such an association is also reported in Korean subjects [26] and Iranian women [27] but not in Caucasian subjects [28, 29].

Association of *RETN* genetic variants and correlation of resistin levels with BMI, FBG, and plasma lipid profile was also assessed. We have found CC of *RETN*-420C/G SNP to be significantly associated with metabolic risk factors marked by higher FBG, BMI, and TC in T2D

Table 6
Association of *RETN* -420 CC genotype with various disorders.

Genotype	Associated with	Population	References
CC	Insulin resistance, hypertriglyceridemia and hyperglycemia	Finnish	[30]
CC	Increased FPG and HbA1c levels, T2D	Iranian	[31]
CC	CAD, T2D	Iranian	[32]

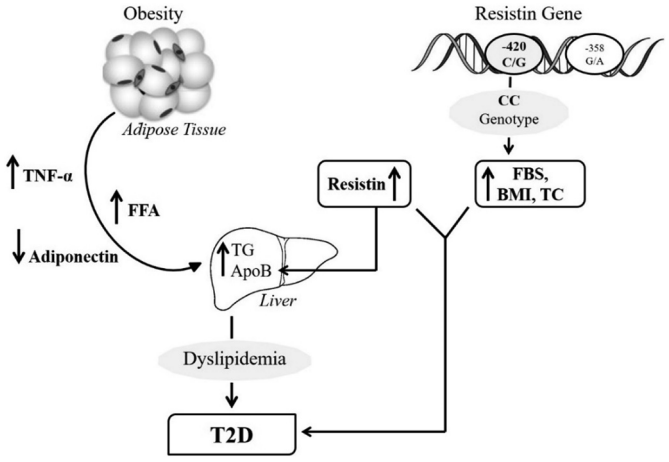

Fig. 3. Role of resistin in T2D: Proposed mechanism of resistin-induced dyslipidemia in T2D.

patients. The CC genotype is associated with other factors as observed in various population (Table 6).

The increased resistin levels due to *RETN* −420C/G polymorphism can be related to specific binding of transcription factors Sp1 and Sp3 to this promoter element leading to enhanced promoter activity as inferred by Osawa et al. [9]. Apparently, −420C/G seems to be an important decisive polymorphic site for *RETN* gene transcription. Furthermore, DNA methylation (DNAm), an epigenetic modification, plays an important role in the regulation of gene expression. −420C/G *RETN* is CpG SNP that affects CpG sequence and thereby regulating resistin levels [33]. Thus DNAm along with binding of transcription factor explains increased resistin levels. Accordingly, increased resistin levels were observed in obesity-induced T2D individuals in conformity with other reports [11, 34, 35].

A number of studies have established an association between resistin levels and lipid profile [36–39]. It is evident that elevated resistin levels contribute towards progression/development of T2D. Our unpublished results also reveal increased plasma Free Fatty Acid (FFA) and Tumor necrosis factor-alpha (TNF-α) levels in T2D patients. Interestingly, resistin is shown to elevate TNF-α levels [40, 41]. Reports have suggested a mechanism for FFA mediated TG and Very Low-Density Lipoprotein (VLDL) production in relation to diabetic dyslipidemia. It is shown that TNF-α increases hepatocellular TG levels. Further, it is also shown that the influence of FFA on hepatic TG production leads to an increased hepatic release of VLDL, which encloses apolipoprotein B (apoB) [42, 43]. ApoB is a crucial protein component of VLDL and any irregularities related to the metabolism of ApoB are accountable for the development of dyslipidemia and the associated increased risk of CVD [44]. In fact, a direct involvement of resistin in the synthesis of apoB has been shown to influence hepatic lipid and lipoprotein levels [45]. In addition, resistin is also known to decrease the expression and secretion of other adipokines such as adiponectin and leptin which affect hepatic lipoprotein regulation [46]. Our unpublished data on TNF-α and adiponectin conform to these results [47, 48]. To our knowledge, this is the only study that ascribes an association between *RETN* -420C/G SNP and plasma resistin and lipid levels in Indian population. Thus our results contribute towards the understanding of resistin's role in dyslipidemia, obesity, and T2D.

Hence, the current data suggest that lifestyle, diet, and ethnic differences play a major role in the pathogenesis of T2D. An overall mechanism involving *RETN* genetic polymorphisms and its levels in obesity and dyslipidemia associated with T2D is shown schematically (Fig. 3).

5. Conclusion

Our findings suggest *RETN* -420C/G polymorphism to be strongly associated with elevated resistin levels and increased BMI, FBG and TC, a generalized metabolic profile leading towards T2D susceptibility.

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Competing interests

The authors declare that no competing interests exist.

Author contributions

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2018.06.005.

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Circulatory Omentin-1 levels but not genetic variants influence the pathophysiology of Type 2 diabetes

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ABSTRACT

Objective: Omentin-1, an anti-inflammatory protein, is secreted by the visceral adipose tissue. Altered levels of Omentin-1 are associated with obesity and Type 2 Diabetes (T2D). Although Omentin-1 is implicated in the insulin signaling pathway, the relationship between the genetic variants of *Omentin-1* and T2D is not yet explored. The current study evaluates the association of *Omentin-1* polymorphisms (rs2274907 A/T and rs1333062 G/T), its transcript and protein levels, and genotype-phenotype correlation with metabolic parameters and T2D susceptibility.

Methods: Plasma and Peripheral Blood Mononuclear Cells (PBMCs) were separated from venous blood taken from 250 controls and 250 T2D patients recruited from Gujarat, India. Genomic DNA was isolated from PBMCs and genotyping of *Omentin-1* variants was performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). RNA was isolated from Visceral Adipose Tissue (VAT) samples of 12 controls and 10 patients, and transcript levels of *Omentin-1* were assessed by qPCR. Plasma Omentin-1 levels were estimated by ELISA. Fasting Blood Glucose, Body Mass Index (BMI) and plasma lipid profile were considered for the genotype-phenotype correlation analysis.

Results: Our study revealed no association of *Omentin-1* genetic variants with T2D risk ($p > 0.05$). However, the AT genotype of *Omentin-1* rs2274907 A/T polymorphism was associated with increased BMI ($p = 0.0247$). Plasma Omentin-1 levels were significantly decreased ($p < 0.0001$) however, increased VAT *Omentin-1* transcript levels ($p = 0.0127$) were observed in T2D patients.

Conclusion: Our findings suggest that decreased circulatory Omentin-1 levels could pose a risk towards T2D susceptibility.

1. Introduction

Insulin resistance at the level of the liver, muscle, and adipose tissue along with impaired insulin secretion are the hallmarks of Type 2 Diabetes (T2D) [1]. In the past few decades, obesity has been identified as one of the prime factors that lead to T2D. Adipose tissue (AT) serves not only as an energy depository but also as an organ that secretes bioactive molecules called adipokines (pro- and anti-inflammatory). The pro-inflammatory and anti-inflammatory adipokines are in a state of equilibrium and they play an important role in regulating lipid metabolism, insulin sensitivity, glucose metabolism, appetite and satiety [2]. *Omentin-1*, the anti-inflammatory adipokine gene, is located on chromosome 1q22-q23 and is secreted by visceral adipose tissue (VAT) [3]. Circulating Omentin-1 levels were reported to be reduced in obese subjects and have been negatively correlated with markers of obesity, such as Body Mass Index (BMI), waist circumference, and circulating leptin [4]. Omentin-1 has been implicated in insulin signaling pathway by Akt activation and consequently increased insulin sensitivity [5]. Reports suggest that reduced *Omentin-1* gene expression and circulating plasma Omentin-1 concentrations are associated with impaired glucose tolerance in T2D patients [6,7]. Moreover, fasting serum Omentin-1 levels have been negatively correlated with fasting insulin and

Abbreviations: T2D, Type 2 Diabetes; FBG, Fasting Blood Glucose; TC, Total Cholesterol; HDL, High Density Lipoprotein; TG, Triglycerides; LDL, Low Density Lipoprotein; BMI, Body Mass Index; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) [7].

There are a few studies on the genetic variants of *Omentin-1* where Val109Asp rs2274907 has been exclusively studied Non-alcoholic Fatty Liver Disease (NAFLD) [8], Coronary Artery Disease (CAD) [9,10], psoriasis [11], high calorie-diet intake [12], breast cancer [13] and rheumatoid arthritis [14]. There is only one report on *Omentin-1* 3′ UTR rs1333062 in Indian population showing an association with diabetes [15]. Hence, we aimed to investigate *Omentin-1* genetic variants (Exon 4 Val109Asp rs2274907 and 3′ UTR rs1333062), *Omentin-1* transcript levels in VAT along with its plasma levels, and genotype-phenotype correlation with various metabolic parameters.

2. Materials and methods

2.1. Study subjects

The study was carried out in agreement with the principles of Helsinki Declaration and approved by Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2016-9). The importance of the study was explained to all the participants and written consent was taken from each individual. We recruited age, sex and ethnically matched 250 controls (142 males and 108 females) and 250 T2D patients (123 males and 127 females) for the study (Table S1). Samples of visceral (omental) adipose tissue were taken from the individuals undergoing bariatric surgery and fasting clinical parameters of all the study subjects are as described previously [16]. The patients showing Fasting Blood Glucose (FBG) > 125 mg/dL and suffering from no other diseases were recruited from diabetes awareness camps. Ethnically and geographically matched controls were randomly chosen from the Gujarati community by community screening program over the same period. Controls showed FBG < 110 mg/dL with no prior history of T2D.

2.2. Anthropometric measurements, DNA isolation, and lipid profiling

BMI was estimated by measuring the height and weight of all the subjects. Venous blood samples (3 ml) for biochemical assessments were acquired from the subjects after 12 h of overnight fasting in K_3EDTA coated tubes (J. K. Diagnostics, Rajkot, India). Plasma was separated and stored at −20 °C for estimating lipid profile parameters. FBG, Total Cholesterol (TC), Triglycerides (TG) and High-Density Lipoprotein (HDL) were assayed by commercially available kits (Reckon Diagnostics P. Ltd, Vadodara, India). Low Density Lipoprotein (LDL) was calculated using Friedewald's (1972) formula. Genomic DNA was extracted from the whole blood using QIAamp DNA Blood Mini Kit (Qiagen, Germany). DNA purity was assessed by calculating the ratio of absorbance at 260/280 nm by Cary 60 UV–Vis spectrophotometer (Agilent, California, USA). The integrity of genomic DNA was assessed by 0.8% agarose gel electrophoresis. The DNA was stored at −20 °C until further analysis.

2.3. Genotyping of Omentin-1 polymorphisms

Omentin-1 polymorphisms (rs2274907 and rs1333062) were genotyped by performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The primers used for genotyping of these polymorphisms are as shown in Table S2. 20 µl of the reaction mixture included 3 µl (50 ng) of genomic DNA, 11 µl of nuclease-free water, 2.0 µl of 10X PCR buffer, 2.0 µl of 2.5 mM dNTPs (Sigma Chemical Co, St.Louis, Missouri, USA), 1.0 µl each of 10 µM forward and reverse primers (MWG Biotech, India) and 0.3 µl of 3U/µl Taq Polymerase (Bangalore Genei, India). Amplification was performed using Applied Biosystems 96 well Thermal cycler (California, USA) as per the protocol of initial denaturation at 95 °C for 5 min followed by 39 cycles each at 95 °C for 30 s, 59–67 °C for 30 s and 72 °C for 30 s, followed by final extension at 72 °C for 10 min. 5 µl of the amplified products were analyzed by electrophoresis on a 2.0% agarose gel stained with ethidium bromide along with a 50 bp DNA ladder (MBI Fermentas, St.Leon-Rot, Germany) and photographed. Details of the restriction enzymes (Thermo Fisher Scientific, Wilmington, DE, USA) and digested products are mentioned in Table S2. 15 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 instruction. A 50 bp DNA ladder (MBI Fermentas, St.Leon-Rot, Germany) was used as a marker. All the gels were visualized under UV transilluminator using Gel Doc EZ System (Bio Rad Laboratories, California, USA) (Fig. S1).

2.4. Determination of Omentin-1 transcript levels

RNA isolation and cDNA synthesis: Total RNA was isolated from VAT 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 1.9 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 Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions in the Eppendorf Mastercycler gradient (USA Scientific, Inc., Florida, USA).The expression of *Omentin-1* and *GAPDH* transcripts was monitored by LightCycler®480 Real-time PCR (Roche Diagnostics GmbH, Manneheim, 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 [16].

2.5. Determination of plasma Omentin-1 levels

The plasma levels of Omentin-1 were estimated by the enzyme-linked immunosorbent assay (ELISA) kit for human Omentin-1 (RayBio, Norcross, GA, USA) with the sensitivity of 2 ng/ml. All the plasma estimations were carried out in duplicates to ensure % Coefficient of Variation (CV) below 10%.

2.6. Statistical analyses

The clinical characteristics of the study subjects were compared using the *t*-test. Hardy-Weinberg equilibrium (HWE) was performed for *Omentin-1* polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using the chi-square analysis. The distribution of genotype and allele frequencies of *Omentin-1* polymorphisms for patients and control subjects were compared using the chi-square test with 2x2 contingency tables. *p*-values < 0.025 for genotype and allele distribution were considered as statistically significant as per Bonferroni's corrections. Odds ratio (OR) with respective Confidence Interval (95% CI) for disease susceptibility was calculated. Haplotype and linkage disequilibrium (LD) analysis were carried out using http://shesisplus.bio-x.cn/SHEsis.html [17]. For analyses of the transcript and protein levels, unpaired *t*-test and one-way ANOVA were applied. Post hoc Tukey test was applied for multiple group analysis. All the genotype-phenotype correlation analyses were carried out in T2D patients. All the analyses were carried out in GraphPad Prism 5 software. The statistical power of detection of the association with the disease at the 0.025 level of significance was determined by using the G* Power software

2.7. Bioinformatics analysis

In silico prediction tools PANTHER [18], POLYPHEN [19], I-MUTANT [20], were employed to predict the sequence based impact on the protein due to single amino acid variation and the details are provided

Table 1
Genotype and allele frequencies distribution of *Omentin-1* polymorphisms in T2D patients and controls.

SNP	Genotype	Controls (Frequency)	Patients (Frequency)	*p* for HWE	*p* for Association	Odds ratio	(95% CI)
		(n = 250)	(n = 235)				
(rs2274907)	TT	206	189	(C)	R	-	-
Omentin-1	TA	44	46	0.2285	0.1992[a]	1.378	0.8436 to 2.250
Exon 4 Val109Asp A/T	AA	0	0	(P) 0.1087	-	-	-
	T	430 (0.93)	416 (0.90)		0.2212[c]		
	A	34 (0.07)	44 (0.10)			1.338	0.8381 to 2.135
		(n = 250)	(n = 235)				
	TT	45	35	(C)	R	-	-
(rs1333062)	TG	109	105	0.1541	0.4167[a]	1.239	0.7387 to 2.077
Omentin-1	GG	96	95	(P)	0.3681[a]	1.272	0.7526 to 2.151
3'UTR				0.4993			
G/T	T	199 (0.40)	175 (0.37)		0.4119[b]		
	G	301 (0.60)	291 (0.63)			1.114	0.8602 to 1.444

n:Number of Patients/ Controls, R:Reference group, HWE: Hardy-Weinberg Equilibrium, CI: Confidence Interval, Odds ratio is based on allele frequency distribution. (P) refers to Patients and (C) refers to Controls.

[a] Patients vs. Controls (genotype) using chi-square test with 2 × 2 contingency table.

[b] Patients vs. Controls (allele) using chi-square test with 2 × 2 contingency table. Statistical significance was measured at $p < 0.025$ as per Bonferroni's correction.

in supporting data.

3. Results

3.1. Clinical parameters

The clinical parameters of 250 controls and 250 patients used for genetic association study are as shown in Table S1.

3.2. Association of Omentin-1 polymorphisms

The genotype and allele frequencies of the explored *Omentin-1* polymorphisms (rs2274907 A/T and rs1333062 G/T) are summarized in Table 1. The distribution of genotype frequencies for all the polymorphisms were in agreement with Hardy-Weinberg expectations in both patient and control groups ($p > 0.025$). Our results suggest no difference in genotype as well as allele frequencies of *Omentin-1* SNPs among diabetic patients and controls. None of the polymorphisms of *Omentin-1* were found to be associated with T2D ($p > 0.05$), and were hence discontinued after an initial assessment of 250 samples. This study has 85% statistical power for the effect size 0.1 to detect association of *Omentin-1* polymorphisms at $p < 0.025$ in T2D patients and controls

3.3. Haplotype and linkage disequilibrium (LD) analysis

The estimated frequencies of the haplotypes obtained for rs2274907 A/T and rs1333062 G/T did not differ significantly between patients and controls (global $p = 0.853$) (Table 2). None of the haplotypes were found to be associated with T2D. The LD analysis revealed that the two polymorphisms of *Omentin-1* were in moderate association (D'=0.56, $r^2 = 0.05$) (Fig S2).

3.4. Association of Omentin-1 polymorphisms with FBG, BMI and plasma lipids:

Omentin-1 rs2274907 AT genotype was found to be associated with increased BMI ($p = 0.0247$) (Table 3). However, it was not associated with FBG and plasma lipids ($p > 0.05$). Further, rs1333062 G/T did not show any association with FBG, BMI and plasma lipids ($p > 0.05$).

3.5. Bioinformatics analysis

The positive genotype-phenotype association for *Omentin-1* rs2274907 AT genotype with increased BMI suggests their crucial role in Omentin-1 activity. Therefore, we further investigated the impact of polymorphism on Omentin-1 protein using bioinformatics tools. *Omentin-1* rs2274907 A/T polymorphism results in aspartate to valine substitution at position 109 of Omentin-1 protein [21]. PANTHER and POLYPHEN tools showed that *Omentin-1* rs2274907 is probably benign suggesting that the substitution does not affect the phenotype nor has damaging effects on the function of Omentin-1 protein. I-MUTANT predictions revealed decreased stability of *Omentin-1* rs2274907 variant as compared to its native structure (Table 4).

3.6. Relative gene expression of Omentin-1 and its association with Omentin-1 SNPs, and a correlation with metabolic profile

Significantly increased *Omentin-1* transcript levels were observed in T2D patients as compared to controls after normalization with *GAPDH* expression as suggested by the significant ($p < 0.0127$) mean ΔCt values (Fig. 1A). Moreover, a $2^{-\Delta\Delta Cp}$ analysis showed approximately 4.2 fold change in the expression of *Omentin-1* transcript levels in patients as compared to controls as shown in Fig. 1B. Further, there was no significant difference observed between *Omentin-1* transcript levels and its SNPs ($p > 0.05$) as shown in Fig. 1C. Spearman's correlation analysis

Table 2
Distribution of haplotype frequencies of *Omentin-1* polymorphisms in T2D patients and controls.

Haplotype (*Omentin-1* rs2274907 A/T and rs1333062 G/T)	Patients(Freq. %) (n = 230)	Controls(Freq. %) (n = 250)	*p* for association	$p_{(global)}$	Odds ratio [95%CI]
TT	142(0.307)	135(0.322)	0.064	0.853	1.296 [0.983 ~ 1.707]
TG	276(0.597)	249(0.595)	8.60×10^5		1.641 [1.283 ~ 2.099]
AT	28(0.06)	29(0.069)	0.684		1.116 [0.654 ~ 1.905]

CI represents Confidence Interval. (Frequency < 0.03 in both control & case has been dropped and was ignored in the analysis).

Table 3
Genotype-phenotype association analysis of *Omentin-1* polymorphisms with metabolic profile.

Genotype	FBG(mg/dL)	BMI(kg/m^2)	TG(mg/dL)	TC(mg/dL)	LDL(mg/dL)	HDL(mg/dL) Male	HDL (mg/dL) Female
***Omentin-1* rs2274907 A/T**							
TT (n = 189)	118.7(45.53)	25.6(5.42)	157.0(85.52)	161.3(36.86)	100.1(29.86)	36.4(9.92)	41.3(9.63)
AT (n = 46)	127.8(45.15)	27.0(5.55)	168.6(86.17)	166.3(32.72)	106.1(28.89)	33.8(7.62)	41.3(8.60)
AA (n = 0)	–	–	–	–	–	–	–
p value	0.1369	**0.0247**	0.1763	0.2010	0.0825	0.1248	0.8184
***Omentin-1* rs1333062 G/T**							
TT (n = 35)	119.7(54.22)	25.4(6.03)	144.6(74.93)	162.0(37.56)	101.8(25.16)	36.0(9.18)	41.8(11.10)
TG (n = 105)	120.1(43.27)	25.9(5.31)	155.8(88.89)	163.0(34.15)	103.6(32.24)	36.5(10.41)	41.9(9.49)
GG (n = 95)	120.3(41.22)	25.8(5.22)	163.9(88.55)	159.3(37.12)	98.7(29.83)	35.8(8.14)	40.8(8.59)
p value	0.9150	0.4323	0.1852	0.3773	03,678	0.8933	0.5850

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

Table 4
In-silico analysis of *Omentin-1* rs2274907 A/T polymorphism.

Amino acid change	PANTHER	POLYPHEN	I-MUTANT
Asp109Val	probably benign	benign	Decrease

revealed no correlation between *Omentin-1* transcript levels and BMI, FBG or plasma lipids ($r^2 = 0$, $p > 0.05$) (Table 5).

3.7. Plasma Omentin-1 levels and its association with Omentin-1 SNPs, and a correlation with metabolic profile

Plasma Omentin-1 levels showed a significant decrease

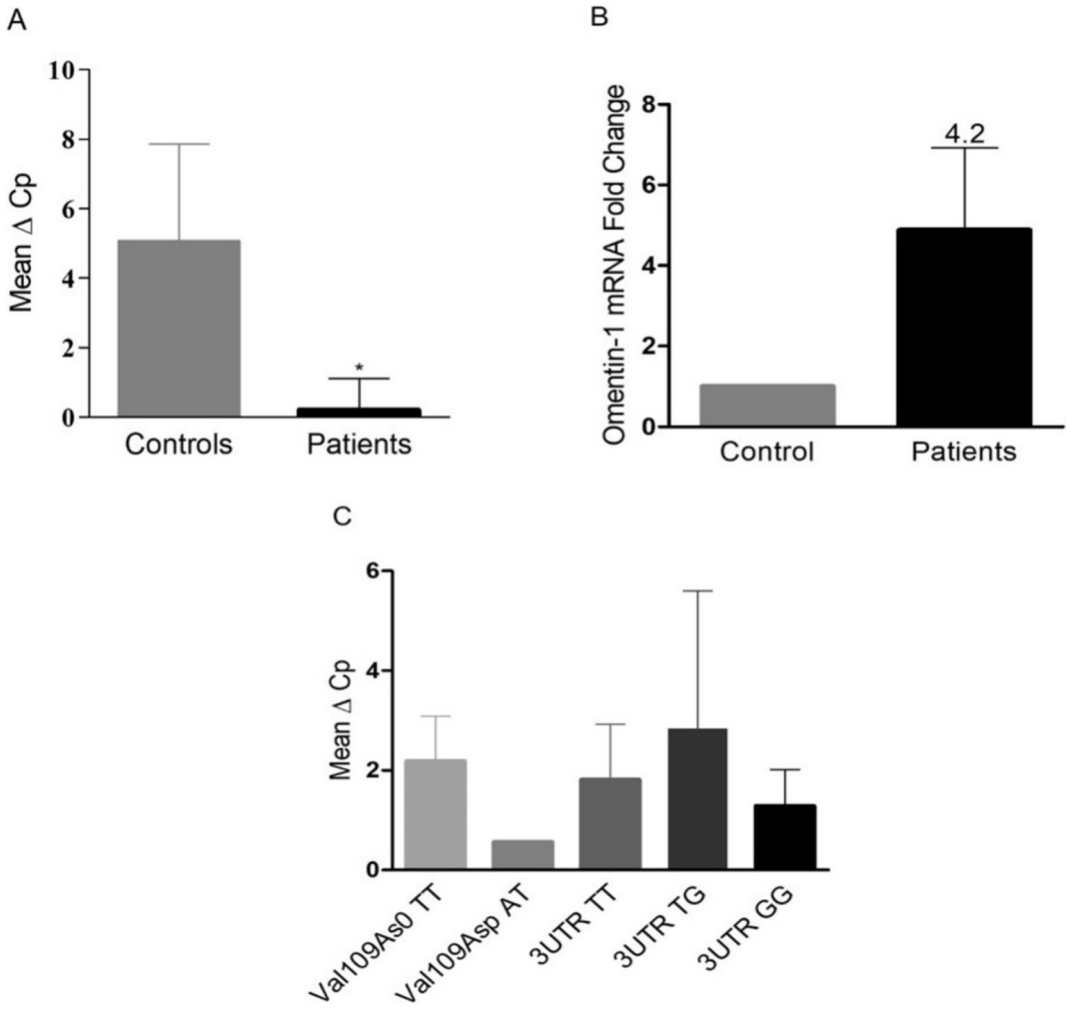

Fig. 1. (A) Relative gene expression of VAT *Omentin-1* in controls and patients: Significant increase in *Omentin-1* transcript levels was observed in patients (Mean ΔCt ± SEM: 5.06 ± 2.79 vs 0.20 ± 0.90; $p = 0.0127$). **(B) Relative fold change of *Omentin-1* expression in controls and patients.** T2D patients showed 4.2 fold increase in *Omentin-1* mRNA expression as determined by the $2^{-\Delta\Delta Cp}$ method (Controls n = 12; T2D patients n = 10). **(C) Association of *Omentin-1* polymorphisms with *Omentin-1* transcript levels.** *Omentin-1* polymorphisms with *Omentin-1* transcript levels showed no association with Omentin-1 transcript levels ($p > 0.05$).

Table 5
Correlation analysis of *Omentin-1* transcripts with metabolic profile.

Parameters	r^2	p
BMI (Kg/m^2)	0.2571	0.6583
FBG (mg/dL)	−0.4000	0.7500
TG (mg/dL)	0.4000	0.7500
TC (mg/dL)	0.3491	0.7568
HDL (mg/dL): Male	0.5678	0.6789
Female	0.9876	0.5678
LDL (mg/dL)	0.4000	0.7500

$p > 0.05$, non-significant. n = 10.

Table 6
Correlation analysis of plasma Omentin-1 with metabolic profile.

Parameters	r^2	p
BMI (Kg/m^2)	−0.0127	0.9020
FBG (mg/dL)	0.2427	0.1538
TG (mg/dL)	0.1728	0.2401
TC (mg/dL)	0.0940	0.4865
HDL (mg/dL): Male	0.1420	0.4541
Female	0.3000	0.1642
LDL (mg/dL)	0.1192	0.4520

$p > 0.05$, non-significant. n = 40.

($p < 0.0001$) in T2D patients (Fig. 2A). Further, the levels of Omentin-1 were significantly low ($p = 0.017$) in obese patients compared to obese controls (Fig. 2B). Further, no association was found between Omentin-1 plasma levels and its SNPs ($p > 0.05$) as shown in Fig. 2C. Spearman's correlation analysis revealed no correlation between Omentin-1 protein levels and BMI, FBG and plasma lipids ($r^2 = 0$, $p > 0.05$) (Table 6).

4. Discussion

There are numerous studies on the association of adipokine genetic variants in T2D but with few being explored in the Indian population. The present study was designed to determine genetic risk factors from one of the strongly linked chromosomal regions 1q21-23 in Gujarat population for T2D.

Our results revealed that the genetic variants of *Omentin-1* (rs2274907 A/T and rs1333062 G/T) are not associated with T2D. Similar observations were reported in the Caucasian population [21,22] though not in Polish and North Indian population [23,14]. Further, our association analysis revealed rs2274907 AT genotype to be significantly associated with increased BMI in T2D patients. In context to this, it is also reported to be associated with the increased risk towards NAFLD [8]. *Omentin-1* rs2274907 polymorphic (A/T) site is present in exon-4 and is reported to result in a change of amino acid from Asp (GAC) to Val (GTC) at position 109 [21]. Our *in silico* analysis revealed the site as benign, having no major structural effect on the protein activity.

The transcript as well as protein levels of Omentin-1 reveal quite an intriguing picture of increased mRNA levels and decreased protein levels in T2D patients. Though studies carried out by other research

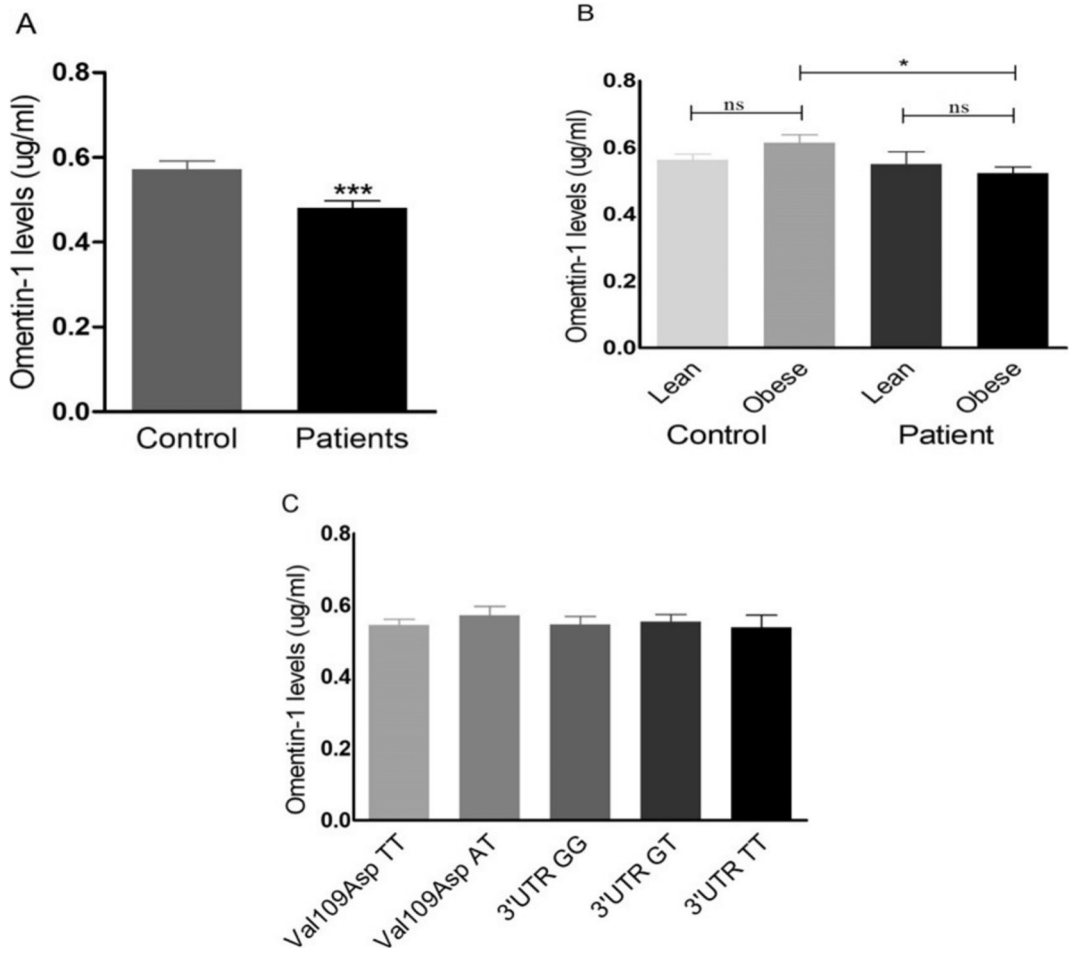

Fig. 2. Plasma Omentin-1 levels in (A) controls vs. patients (B) control (lean vs. obese) and Patients (lean vs. obese). Our results showed a significant decrease in plasma Omentin-1 levels in T2D patients ($p < 0.0001$) compared to controls; obese T2D patients showed a significant decrease compared to obese controls ($p = 0.017$) (Controls n = 40; T2D patients n = 40). **(C) Association of *Omentin-1* polymorphisms with plasma Omentin-1 levels.** *Omentin-1* polymorphisms showed no association ($p > 0.05$) with plasma Omentin-1 levels.

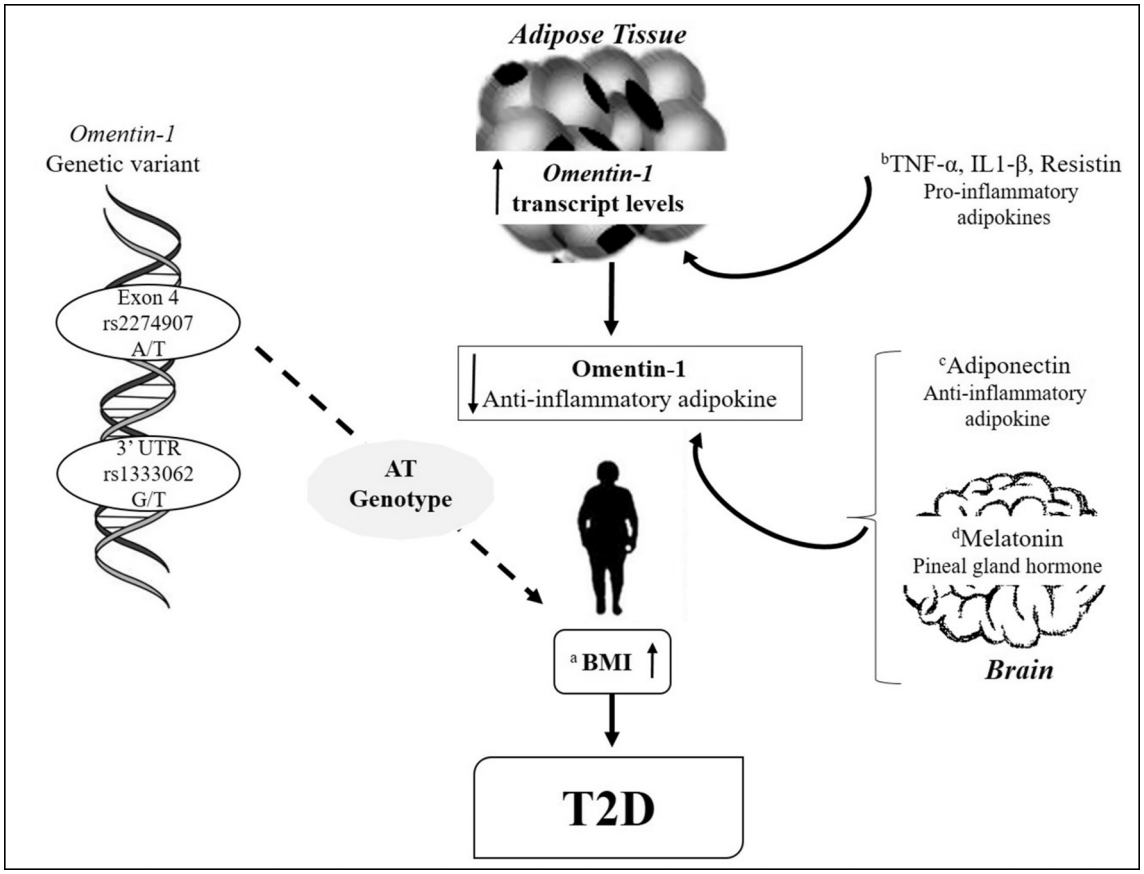

Fig. 3. Role of Omentin-1 in T2D: [a]The genetic variants of *Omentin-1* are not associated with T2D susceptibility, however the AT genotype (rs2274907) is associated with an increased BMI. In obese individuals, Omentin-1 might be regulated by multiple factors at transcriptional as well as translational levels. Our previous studies demonstrate increased [b]pro-inflammatory adipokines, decreased [c]anti-inflammatory adipokines and [d]melatonin levels. Thus, these factors might contribute to *Omentin-1* VAT transcript levels and plasma protein levels, which might play a role in the development of obesity-induced T2D condition.

groups are in discord with our transcript results [4,24,25], it is important to note that these groups have not monitored the protein levels. Our results on the transcript levels are in agreement with the report of Schäffler et al. [5] who showed an increase in *Omentin-1* transcript levels as a response to elevated levels of pro-inflammatory adipokines. It could at best be explained as a defence mechanism elicited under obesity-induced changes in the micro-environment of adipose tissue [5,26]. As an explanation of elevated anti-inflammatory levels, Li et al. have suggested it to be a stimulation induced by various pro-inflammatory cytokines besides differential binding frequencies of NF-κB, a major adipokine regulator [27]. In support of these findings, we have also observed an increased expression of pro-inflammatory adipokines such as TNF-α [16], IL1β [28] and resistin [29]. Furthermore, epigenetic modifications like miRNA regulation, DNA methylation, and post-translational modifications have also been suggested to regulate mRNA expression of adipokines [27,30,31]. In this context, the observed increased mRNA expression could be due to any of these reasons.

As against the transcript levels, plasma Omentin-1 levels were significantly lowered in T2D patients. Studies by other research groups substantiate our results on protein levels [4,32]. There are several explanations put forward for the reduced circulatory Omentin-1 levels in diabetic conditions. First of all, the incidence of decreased Omentin-1 in the circulation could be a consequence of either inhibited translation or decreased stability of mRNA or protein. Secondly, Yan et al. [7] have shown circulating Omentin-1 levels and adiponectin levels to have a direct correlation. Interestingly, we have observed reduced adiponectin levels in our population [33]. One of the studies has suggested that adiponectin may have a regulatory influence on Omentin-1 levels [34]. However, future studies are needed in this direction to unravel the intricate relations if any. Dysregulation of blood glucose levels with the increased propensity towards T2D and diabetic complications have been shown to be associated with sleep disturbances [35]. Moreover, it has also been reported that circadian rhythms can influence metabolic processes of adipose tissue and also expression and secretion of adipokines [36,37]. Such regulation is likely to be mediated by melatonin by way of its action on VAT either through its membrane receptors or via an action on the sympathetic nervous system [38]. The possible mechanisms of action of melatonin on Omentin-1 may be corresponding to its effect on the levels of adiponectin. From our previous study, we have observed reduced plasma melatonin levels in T2D patients [39]. The reduced Omentin-1 levels might contribute towards the progression/development of T2D. The underlying mechanism for the differential expression of mRNA and protein levels needs to be investigated in depth through *in-vivo* studies.

As discussed above, in obesity-induced diabetic individuals, there are altered levels of pro-inflammatory (TNF-α) and anti-inflammatory (adiponectin) adipokines. Omentin-1 is reported to manifest its anti-inflammatory activity by inhibiting TNF-α through JNK pathway in healthy individuals [40]. Circulatory Omentin-1 is used as a biomarker of diabetes, obesity, atherosclerosis, inflammatory disease, metabolic syndrome, and cancer [6,2] and in this context, the same could be considered in our Gujarat T2D population. However, its polymorphic sites are not associated with the disease. Further studies on *Omentin-1* expression in larger sample size are required to validate our results.

To our knowledge, this is the only study that ascribes an association between *Omentin-1* polymorphisms, its transcript and protein levels with biochemical parameters in Gujarat population. Thus, our results contribute to an understanding of the role of Omentin-1 in obesity-

induced T2D.

The current study suggests *Omentin-1* might be regulated by multiple factors at transcriptional as well as translational levels, while genetic polymorphisms are not associated with T2D. We observed an association of the AT genotype of rs2274907 with increased BMI levels. The reduced Omentin-1 protein levels might be influenced by increased pro-inflammatory adipokines and epigenetic modifications. These factors are known to be induced by a sedentary lifestyle and an unhealthy diet. The Omentin-1 levels might also be regulated by anti-inflammatory adipokine and melatonin. Thus, all these factors could be involved in the development of dyslipidemia and obesity-induced T2D (Fig. 3).

5. Conclusion

Our study suggests that although *Omentin-1* genetic variants are not associated with T2D, its reduced protein levels could play a role in T2D susceptibility.

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Competing Interests

The authors declare that no competing interests exist.

Author Contributions

RB conceived the idea and designed the experiments. MN provided adipose tissue samples. NR, RP, and SP performed the experiments. SJ performed the bioinformatics data analysis. NR did the data acquisition, performed the data analysis and wrote the original draft. RB and AVR contributed to the critical revision and approval of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.03.011.

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Intron specific polymorphic site of *vaspin* gene along with vaspin circulatory levels can influence pathophysiology of type 2 diabetes

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ABSTRACT

Vaspin, an insulin-sensitizing adipokine, has been associated with type 2 diabetes (T2D). The present study aimed to investigate the distribution of genotypes and high-risk alleles of *vaspin* genetic variants (rs77060950 G/T and rs2236242 A/T), in Gujarat subpopulation (India). Genomic DNA isolated from PBMCs was used to genotype *vaspin* polymorphisms by PCR-RFLP and ARMS-PCR from 502 controls and 478 patients. RNA isolated from visceral adipose tissue (VAT) of 22 controls and 20 patients was used to assess *vaspin* transcript levels by qPCR while the vaspin titre of the subjects was assayed using ELISA. Phenotypic characteristics of Fasting Blood Glucose (FBG), BMI and plasma lipid profile were estimated and analyzed for the genotype-phenotype correlation. We identified a significant association of rs2236242 A/T with T2D as the TT genotype conferred a 3.087-fold increased risk. The TT genotype showed association with increased FBG, BMI and Triglycerides levels. Increased GA, GT and TA haplotype frequencies, decreased VAT transcript and vaspin protein levels in T2D patients was observed, which were further negatively correlated with FBG and BMI. In conclusion, rs2274907 A/T polymorphism is strongly associated with reduced *vaspin* transcript and protein levels, and related metabolic alterations that may play a role in the advancement of T2D.

1. Introduction

Central obesity, an integral part of the metabolic syndrome, has long been viewed as a risk factor for type 2 diabetes mellitus (T2D). Adipocytes produce many biomolecules, collectively known as adipokines, playing a key role in metabolism, inflammation, and immunity. Since the discovery of leptin, many other adipokines have been discovered forming the crux of homeostasis between the anti- and pro-inflammatory macrophages [1]. Vaspin, a member of serpin A12, was initially discovered in visceral adipose tissue (VAT) of Otsuka Long-Evans Tokushima fatty rat [2,3]. It is an anti-inflammatory adipokine reported to inhibit kallikrein 7 (a protease degrading insulin). It promotes cell proliferation, inhibits apoptosis and ameliorates ER stress in-vitro [3,4]. There are also a few studies establishing the favourable effect of exogenous recombinant vaspin on insulin sensitivity and glucose tolerance [2,5].

In humans, reduced vaspin protein levels have been correlated with increased Body Mass Index (BMI) and reduced insulin sensitivity in adults [6,7], and obese women having Polycystic Ovary Syndrome (PCOS) [8,9]. Thus, the emerging line of evidence supports the concept of vaspin playing a significant role in the progression towards obesity induced T2D. *Vaspin* consists of 6 exons and 5 introns and is located on chromosome 14q32.13. Single nucleotide polymorphisms (SNPs) of *vaspin* are well explored of which, intronic polymorphic sites (intron 2 rs77060950 G/T and intron 4 rs2236242 A/T) have been investigated in relation to various diseases like T2D [10,11], PCOS [12], Metabolic Syndrome [13,14], Coronary Artery Disease (CAD) [15], Nonalcoholic Fatty Liver Disease (NAFLD) [16], obesity [17], and End Stage Renal Disease (ESRD) [18].

The predictions for India indicate that cases of T2D will rise to 74.9 million by 2030 [19] with the Gujarat population being the second highest [20]. We have reported the genetic predisposition of *TNF-α*, *resistin* and *omentin-1* in T2D [21–23]. We, thus, aimed to investigate the distribution of genotypes and high-risk alleles of *vaspin* present in

Abbreviations: T2D, type 2 diabetes; FBG, Fasting Blood Glucose; BMI, Body Mass Index; TC, Total Cholesterol; HDL, High-Density Lipoprotein; TG, Triglycerides; LDL, Low-Density Lipoprotein; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; ARMS-PCR, Amplification Refractory Mutation System-Polymerase Chain Reaction

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Table 1
Distribution of genotype and allele frequencies of *vaspin* polymorphisms in T2D patients and controls.

SNP	Genotype or allele	Controls (Frequency)	Patients (Frequency)	*p* for HWE	*p* for Association	Odds ratio	(95% CI)
		(n = 250)	(n = 250)				
Vaspin	GG	201 (80.40)	180 (72.00)	(C)	R	-	-
intron 1 G/T **(rs77060950)**	GT	45 (18.00)	61 (24.40)	0.4267	0.0606[a]	1.514	0.9764 to 1.582
	TT	4 (1.60)	9 (3.60)		0.1187 [a]	2.513	0.7543 to 3.897
	G	447 (0.89)	421 (0.84)	(P)			
	T	53 (0.11)	79 (0.16)	0.1896	0.0824[b]	1.376	0.9588 to 1.9

n: number of Patients/Controls, R:reference group, CI: Confidence Interval, Odds ratio: the allele frequency distribution, P:Patients, C: Controls, [a]Patients vs. Controls (genotype) by the chi-squared test with 2×2 contingency table.
[b]Patients vs. Controls (allele) by chi-squared test with 2×2 contingency table. Statistical significance was considered at $p < 0.025$ as per Bonferroni's correction.

SNP	Genotype or allele	Controls (Frequency)	Patients (Frequency)	*p* for HWE	*p* for Association	Odds ratio	(95% CI)
		(n = 500)	(n = 478)				
Vaspin	AA	259(51.80)	187(39.12)	(C)	R	-	-
intron 4 A/T	AT	206(41.20)	213(44.56)	0.4895	**0.0095**[a]	1.432	1.095 to 1.873
(rs2236242)	TT	35 (7.00)	78 16.32)		**0.0001**[a]	3.087	1.986 to 4.767
	A	724 (0.72)	587 (0.61)	(P)			
	T	276 (0.28)	369 (0.39)	0.1903	**0.0001**[b]	1.649	1.363 to 1.995

n: number of Patients/Controls, R:reference group, CI: Confidence Interval, Odds ratio: the allele frequency distribution, P:Patients, C: Controls, [a]Patients vs. Controls (genotype) by the chi-squared test with 2×2 contingency table.
[b]Patients vs. Controls (allele) by chi-squared test with 2×2 contingency table. Statistical significance was considered at $p < 0.025$ as per Bonferroni's correction.

the intronic region (intron 2 rs77060950 G/T and intron 4 rs2236242 A/T) and correlate them with any alterations in the transcript and protein levels. Alongside this, we also performed a genotype-phenotype correlation with various metabolic parameters to understand their association.

2. Materials and methods

2.1. Study participants

The presented work abided by the principles of the Helsinki Declaration and was sanctioned by the Institutional Ethical Committee for Human Research (IECHR: FS/IECHR/2016-9) as described elsewhere [21]. The study protocol followed was as designed by Rathwa and colleagues [21].

2.2. Anthropometric parameters, lipid profiling and DNA extraction

The height and weight of the study participants were measured to calculate BMI. Participants were subjected to overnight (12 h) fasting and their venous blood samples (3 ml) was withdrawn to estimate Fasting Blood Glucose (FBG), Total Cholesterol (TC), Triglycerides (TG), Low-Density Lipoprotein (LDL) and High-Density Lipoprotein (HDL) from plasma and genotyping was carried out from the genomic DNA extracted from PBMCs as described elsewhere [21].

2.3. Genotyping

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS) methods were used for *Vaspin* polymorphisms (rs77060950 G/T and rs2236242 A/T). The primers used are as shown in Table S1. The PCR reaction mixture composition and protocol were as described previously [21].

2.4. Vaspin transcript and protein levels

The primers used for the assessment of *vaspin* and *GAPDH* transcript levels are shown in Table S1, and protocols as described earlier were used [21]. The vaspin protein levels were determined by 3,3,5,5′-tetramethlybenzidine (TMB) based sandwich ELISA (RayBio, Norcross, GA, USA; sensitivity: 2 ng/ml) using anti-vaspin antibody and anti-vaspin antibody labelled with HRP to capture and detect vaspin protein respectively. Each step was followed by through washings. The readings were measured at 450 nm to estimate the vaspin levels against a standard curve using MultiSkan (Thermo Fischer, USA). ELISA run duplicates were maintained to ensure accuracy and precision.

2.5. Statistical analyses

Unpaired *t*-test was employed to assess the statistical significance present in FBG, lipid parameters, age, gender, BMI, vaspin transcript and protein levels between T2D patients and control subjects followed by one-way ANOVA Tukey's test for multi-group comparison. Pearson's chi square test was used to examine if intron 2 rs77060950 G/T and intron 4 rs2236242 A/T conformed to HWE and to compare the distribution of genotype and allele frequencies in patients and controls after Bonferroni correction. The strength of the associations obtained was quantitated using Odds ratios (ORs) with 95% confidence intervals (CIs). The analyses were carried out using GraphPad Prism 6 software.

3. Results

3.1. Clinical characteristics

The clinical characteristics of controls and T2D patients varied significantly as mentioned earlier [21], and the clinical characteristics of obese controls and T2D patients are shown in Table S1.

3.2. Genetic analyses of vaspin polymorphisms

The distribution of genotype and allele frequencies of rs77060950 G/T and rs2236242 A/T are shown in Table 1. *Vaspin* rs77060950 G/T was not associated with T2D ($p > 0.05$) and was hence stopped after a preliminary assessment. The distribution of genotype frequencies for rs2236242 A/T was consistent with Hardy-Weinberg expectations in

Table 2
Haplotype frequencies of *Vaspin* polymorphisms in T2D patients and controls.

Haplotype (*Vaspin*rs77060950 G/T, rs2236242 A/T)	Controls (Freq. %) (n = 500)	Patients (Freq. %) (n = 478)	*p* for Association	$p_{(global)}$	Odds ratio [95% CI]
GA	511.60 (57)	632.24 (65)	**0.0053**	7.36×10^{-6}	0.714 [0.590–0.864]
GT	312.40 (35)	243.76 (25)	$\mathbf{2.46 \times 10^{-6}}$		1.619 [1.324–1.980]
TA	46.40 (5.2)	72.76 (7.5)	**0.0441**		0.678 [0.464–0.992]
TT	25.60 (2.8)	23.24 (2.5)	–		–

CI: confidence interval. (Frequency < 0.03 in both groups has been dropped and was ignored in the analysis).

both the groups ($p > 0.025$) and it was associated with T2D (genotype and allele frequencies, $p < 0.0001$). The AT genotype was associated with increased risk for T2D with an odds ratio (OR) of 1.432 while the mutant homozygous TT genotype increased the risk by 3.087-fold. The mutant allele 'T' of rs2236242 was associated with the risk of T2D having an OR of 1.649.

3.3. Haplotype and linkage disequilibrium (LD) analysis

The estimated frequencies of the haplotypes for rs77060950 G/T and rs2236242 A/T did not vary between both the groups (global $p = 7.36 \times 10^{-6}$) (Table 2). Yet, GA ($p = 0.0053$), GT ($p = 2.46 \times 10^{-6}$) and TA ($p = 0.0441$) haplotypes were associated with T2D risk. The LD analysis showed that the polymorphisms of *Vaspin* (rs77060950 G/T and rs2236242 A/T) were in low linkage disequilibrium ($D' = 0.10$; $r^2 = 0.001$) (Fig. S2).

3.4. The analysis of association of vaspin polymorphisms with metabolic profile

rs77060950 G/T did not show any association with FBG, BMI and plasma lipids ($p > 0.05$) (Table 3). Further, rs2236242 TT genotype was associated with increased FBG ($p = 0.0001$), BMI ($p = 0.0001$) and TG ($p = 0.0065$) but was not associated with TC, LDL and HDL levels ($p > 0.05$).

3.5. Vaspin transcript levels and their association with vaspin polymorphisms, and a correlation with metabolic profile:

After normalization with *GAPDH* expression, a 2.26-fold ($p = 0.028$) decrease in the expression of *vaspin* transcript levels were observed in T2D patients by $2^{-\Delta\Delta Cp}$ analysis (Fig. 1A). However, there was no association between *vaspin* transcript levels and their polymorphisms ($p > 0.05$) (Fig. 1B). Spearman's correlation analysis showed no correlation between *vaspin* transcript levels and metabolic profile ($r^2 = 0$, $p > 0.05$) (Table 4).

3.6. Plasma vaspin protein levels and their association with vaspin polymorphisms and metabolic profile:

Reduced plasma vaspin protein levels were observed ($p = 0.0001$) in T2D patients (Fig. 2A) and obese patients ($p = 0.0001$) (Fig. 2B). Further, no association was observed between vaspin protein levels and *vaspin* polymorphisms ($p > 0.05$) (Fig. 2C). Spearman's correlation analysis showed a negative correlation between plasma vaspin protein levels and BMI ($p = 0.0307$) and FBG ($p = 0.0006$), and no correlation with the lipid profile ($p > 0.05$) (Table 5).

4. Discussion

Adipocytes and beta-cells dysfunction are the hallmarks of T2D pathogenesis and numerous factors contribute towards it, the most prominent ones being obesity and genetic predisposition [24]. A few studies have been assessed on polymorphisms of adipokine with T2D susceptibility in the Indian population.

Our results revealed no association of *Vaspin* rs77060950 G/T with either T2D risk or with any other parameters. Similar results have been documented in the German population (24). *Vaspin* intronic polymorphism rs2236242 A/T is significantly associated with T2D. This polymorphism has been studied in different populations concerning different diseases/disorders (Table 6).

The TT genotype showed a 3.087-fold increased risk for T2D. We report for the first time that the mutant T allele predisposes an individual towards the risk of T2D unlike in other populations. Kempf *et al.* [10] have reported an association of rs2236242 towards the risk of T2D; however, the functional consequences of rs2236242 polymorphism have not been explored. It is hypothesized that this polymorphic site might have an impact on the stability of mRNA or splicing efficiency of the transcribed product [10].

Correlation analysis reveals *vaspin* rs2236242 TT genotype to be significantly associated with metabolic risk factors marked by higher FBG, BMI, and TG in T2D patients. Parallel results have been observed in other populations too [14,17]. Assessment of VAT *vaspin* transcript and plasma levels reveal a significant reduction in their levels in T2D patients as also observed in other populations [26–28]. Several reports

Table 3
Vaspin polymorphisms and genotype-phenotype correlation analysis with the metabolic profile.

Genotype	FBG (mg/dl)	BMI (kg/m^2)	TG (mg/dl)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
(rs77060950) Vaspin intron 1 G/T						
GG	128.4 ± 2.2	25.7 ± 0.2	154.9 ± 3.2	166.6 ± 1.4	106.4 ± 1.2	40.9 ± 0.9
GT	116.7 ± 5.2	25.7 ± 0.2	156.7 ± 7.9	169.5 ± 2.9	106.3 ± 1.9	38.9 ± 2.1
TT	122.4 ± 1.9	25.6 ± 1.1	151.4 ± 18.9	153.1 ± 7.9	101.3 ± 5.4	41.3 ± 1.6
p value	0.0867	0.2056	0.8876	0.0739	0.1669	0.1899
(rs2236242) Vaspin intron 4 A/T						
AA	120.3 + 22.3	24.9 ± 2.3	132 ± 27.2	167.6 ± 37.15	101.1 ± 82.90	39.9 ± 12.5
AT	129.2+11.3	26.4 ± 1.0	135.8 ± 21.3	165.3 ± 33.85	97.88 ± 91.16	40.4 ± 13.1
TT	**134.4+7.2**	**27.5 ± 0.8**	**161.0 ± 11.2**	166.2 ± 37.24	96.4 ± 76.58	38.3 ± 12.6
p value	**< 0.0001**	**< 0.05**	**0.0063**	0.8984	0.8911	0.3398

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

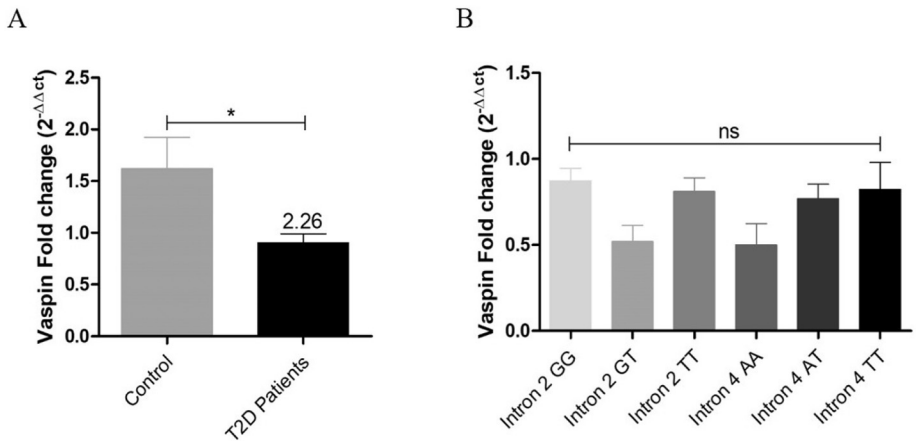

Figure 1.. A) Relative fold change of *vaspin* transcript levels in controls and patients. T2D patients showed 2.26-fold ($p = 0.028$) decrease in *vaspin* transcript levels as estimated by the $2^{-\Delta\Delta Cp}$ method (Controls n = 22; T2D patients n = 20). B) Association of *vaspin* polymorphisms with their transcript levels. *Vaspin* polymorphisms showed no association with their transcript levels ($p > 0.05$).

Table 4.
Correlation analysis of *vaspin* transcript levels with the metabolic profile.

Parameters	r^2	p
BMI (kg/m^2)	0.4135	0.6990
FBG (mg/dL)	-0.3989	1.2120
Triglycerides (mg/dL)	-0.5676	1. 6510
Total Cholesterol (mg/dL)	-0.0909	0.7904
HDL (mg/dL):	0.7900	0.9091
LDL (mg/dL)	0.6515	0.0789

$p > 0.05$, non-significant. n = 20

Table 5.
Correlation analysis of plasma vaspin protein levels with metabolic profile.

Parameters	r^2	p
BMI (kg/m^2)	-0.2514	**0.0307**
FBG (mg/dL)	-0.4695	**0.0006**
Triglycerides (mg/dL)	-0.0971	0.4765
Total Cholesterol (mg/dL)	-0.0743	0.5659
HDL (mg/dL):	-0.5174	0.6872
LDL (mg/dL)	-0.0561	0.7080

$p > 0.05$, non-significant. n = 40

have put forward the concept that the compensatory ability of vaspin secretion gradually declines with the severity of diabetes or the onset of cardiovascular diseases resulting in a slow fall in vaspin levels [2,24,28].

Obesity is a chronic low-grade inflammation that regulates the levels of pro- and anti-inflammatory adipokines by macrophage polarization [29]. Resistin, a pro-inflammatory adipokine is reported to be elevated in T2D conditions [30]. The pro-inflammatory effects of resistin are known to initiate cAMP-mediated activation of PKA and NF-κB-mediated transcription of various inflammatory adipokines i.e TNF-α and IL-1β [31]. We have observed similar results in our population indicating an imbalance of adipokines in the form of increased levels of TNF-α [21], resistin [22] and IL1-β [32] in T2D patients. Such activation of the NF-κB pathway and increased production of pro-inflammatory adipokines also seem to decrease the levels of anti-inflammatory adipokines as observed by us (adiponectin and omentin-1) in T2D patients [23,33]. A similar observation for the anti-inflammatory cytokines (apelin, IL-10 etc.) has been made by other workers as well [34]. Furthermore, we have also reported the possible involvement of angiotensin convertase enzyme (ACE) I/D polymorphisms in the same population [35]. The ACE 'D' allele is associated with increased angiotensin II [36] which may further reduce the adiponectin levels. Further, the role of circadian rhythm has been implicated earlier in regulating metabolic processes of adipose tissue, and in the expression and secretion of adipokines [37,38]. The metabolic regulation is

Table 6.
Association of *vaspin* rs2236242 A/T with various disorders.

Sr.no.	Associated with	Population	References
1.	T2D	German, Chinese	[10,25]
2.	CAD	Chinese	[15]
3.	Obesity	Egyptian	[17]
4.	Metabolic syndrome	Egyptian	[14]
5.	Metabolic syndrome	Iranian	[12]
6.	PCOS	Iranian	[13]
7.	ESRD	Iranian	[18]

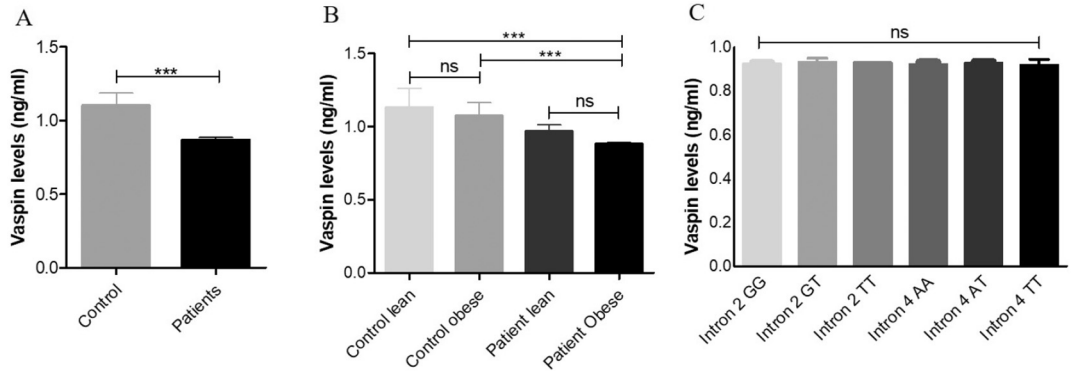

Figure 2.. Plasma Vaspin protein levels in A) controls vs. patients B) control and patients (lean vs. obese). Our results indicated a significant reduction in plasma vaspin protein levels in T2D patients ($p < 0.0001$) and obese T2D patients ($p < 0.0001$) (Controls n = 40; T2D patients n = 40). C) Association of *vaspin* polymorphisms with plasma vaspin protein levels. *Vaspin* polymorphisms showed no association with vaspin protein levels ($p > 0.05$).

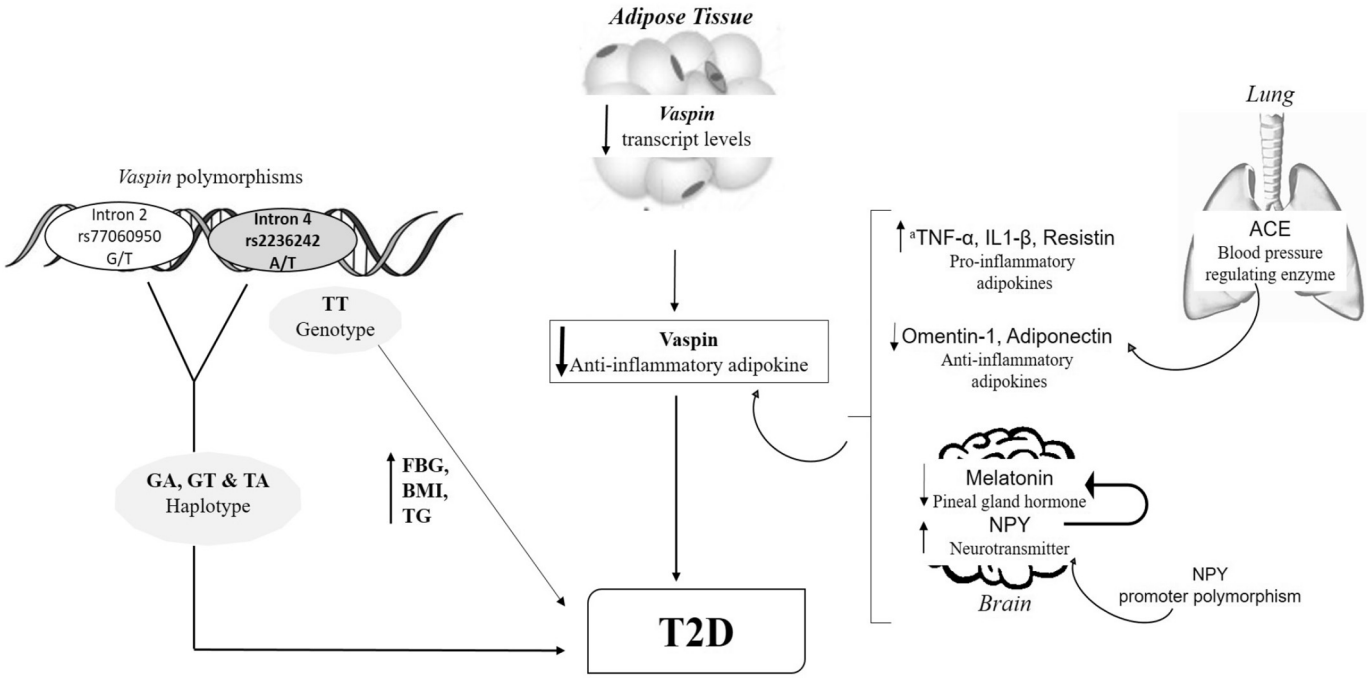

Figure 3.. Proposed mechanism of vaspin in obesity induced T2D. [a]*Vaspin* rs2236242 A/T is associated with the risk of T2D, homozygous TT genotype increases the risk of T2D by 3.087-fold. GA and TA haplotypes are associated with the risk of T2D. *Vaspin* rs2274907 TT genotype is associated with the metabolic profile (FBG, BMI and TG). The reduced[b] transcript and plasma vaspin protein levels might be influenced by the imbalance of [c]pro-inflammatory / [d]anti-inflammatory adipokines and melatonin. The anti-inflammatory adipokine- adiponectin and melatonin are down-regulated by [e]ACE and [f]NPY respectively.

predicted to be mediated by the action of melatonin, a pineal gland hormone, on VAT receptors or via the sympathetic nervous system [39,40]. We have previously reported decreased plasma melatonin levels in T2D patients [41]. We have observed the involvement of NPY promoter polymorphism regulating NPY levels which further reduces melatonin levels [33]. Apparently, in this context, the reduced levels of the anti-inflammatory adipokine-vaspin seen in T2D patients could be a direct or indirect consequence of reduced melatonin levels.

A possible mechanistic summary depicting the possible role of *vaspin* polymorphisms, and its altered transcript and protein levels in obesity and dyslipidemia associated with T2D is shown in Fig. 3.

This is the first study ascribing an association between *vaspin* rs2236242 A/T polymorphism and its plasma protein levels with metabolic parameters in the Gujarat population (India). Our findings are suggestive of ethnic differences being one of the essential contributors in the progression of T2D.

Thus, our findings open new avenues to understand the role of vaspin in obesity induced T2D.

5. Conclusion

Our results indicate that *vaspin* rs2274907 A/T polymorphism is strongly associated with its reduced transcript and protein levels, and related metabolic alterations that may play a role in the advancement of T2D.

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Declaration of competing interest

The authors declare that no competing interests exist.

Authors contribution

RB conceived the idea. NR designed and performed the experiments. NR performed the data acquisition, data analysis and wrote the original draft. NP, SP, and RP did the data acquisition, review and editing of the manuscript. RB and AVR contributed to the critical revision and approval of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.117285.

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REVIEW

β-cell replenishment: Possible curative approaches for diabetes mellitus

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Islet neogenesis;
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Regenerative medicine

Abstract *Aims:* Diabetes mellitus (DM) is a disorder of heterogeneous etiology marked by persistent hyperglycemia. Exogenous insulin is the only treatment for type 1 diabetes (T1D). Islet transplantation is a potential long cure for T1D but is disapproved due to the possibility of immune rejection in the later stage. The approaches used for treating type 2 diabetes (T2D) include diet restrictions, weight management and pharmacological interventions. These procedures have not been able to boost the quality of life for diabetic patients owing to the complexity of the disorder.
Data synthesis: Hence, research has embarked on permanent ways of managing, or even curing the disease. One of the possible approaches to restore the pancreas with new glucose-responsive β-cells is by their regeneration. Regeneration of β-cells include islet neogenesis, dedifferentiation, and trans-differentiation of the already differentiated cells.
Conclusions: This review briefly describes the islet development, functions of β-cells, mechanism and factors involved in β-cell death. It further elaborates on the potential of the existing and possible therapeutic modalities involved in the *in-vivo* replenishment of β-cells with a focus on exercise, diet, hormones, small molecules, and phytochemicals.

Introduction

Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by persistent hyperglycemia and, the number of individuals with diabetes has continued to grow over the years. It is mainly classified into type 1 diabetes (T1D) and type 2 diabetes (T2D). Other rare forms of diabetes are directly inherited [1]. T1D constitutes less than 10% of the total cases of diabetes worldwide and is triggered by

Abbreviations: T1D, type 1 diabetes; T2D, type 2 diabetes; FBG, fasting blood glucose; PDX-1, pancreatic and duodenal homeobox 1; NKX6.1, NK6 homeobox 1; NKX2.2, NK2 homeobox 2; GLUT2, glucose transporter 2; ChREBP, carbohydrate response element-binding protein; CR, calorie restriction; GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory polypeptide; PPAR-γ, peroxisome proliferator-activated receptor-γ; GABA, γ-AminoButyric Acid; TZD, thiazolidinediones; DYRK, dual-specificity tyrosine phosphorylation-regulated kinase; DPP IV, dipeptidyl peptidase-IV; Tregs, regulatory T cells.

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autoimmune-mediated destruction of pancreatic β-cells which often develops in childhood. On the contrary, T2D accounts for over 90% of the patients and is marked by insulin resistance in peripheral tissues due to impaired insulin signaling. Both forms of diabetes are associated with secondary complications that affect multiple organs [1]. There are several factors associated with T2D viz. genetic predisposition [2–4], ER stress [5], obesity [1], etc. To date, extensive research has been carried out with the perspective to understand their molecular mechanisms and possible therapies. A cure remains a far cry, even though various diabetes management approaches have been attempted. The existing therapies only help alleviate hyperglycemia and other symptomatic characteristics. Gaining insights into the possible modes of β-cell preservation is crucial at this juncture, hence an understanding of the signaling mechanisms of β-cell development and regeneration can open new treatment avenues.

Pancreas (understanding the insulin-mine)

The pancreas stems as an outpocketing of the primitive gut endoderm [6]. The adult mammalian pancreas is a diversified organ made of exocrine and endocrine cells. Exocrine cells, represented by enzyme-producing acinar, make up 95% of the pancreatic mass. The endocrine component of the pancreas is arranged as islets of Langerhans which are globular clusters of cells scattered throughout the exocrine tissue and form a minor 1–2% of the total organ mass [7]. The differentiation of islet cells, a complex process occurring during the embryonic period, is under the control of many transcription factors.

Development of islets

During embryogenesis, the pancreas develops as an endodermal anlage governed by signals from neighboring cells. Regulation of the pancreatic development is via the transcription factors PDX-1 (pancreatic and duodenal homeobox-1), PTF1-a (pancreas associated transcription factor 1-a) and HLXB9 (homeobox HB9) influencing the ventral and dorsal aspects of the anlage. The proliferation of the progenitor cells is by fibroblast growth factors (FGFs). The differentiated β-cells eventually express increased levels of PDX-1, NK6 homeobox 1 (NKX6.1), NK2 homeobox 2 (NKX2.2) and PAX-6 transcription factors. 'Postmitotic" cells are hormone-expressing cells that stop dividing [8,9]. The fetal pancreatic development shows differentiation into endocrine and exocrine cells by the 12th week. The mature pancreas formation occurs in the 20th week. Perinatal malnutrition leads to the development of T2D later in life, due to the inability of β-cells to adapt to additional demands placed by aging. The mature pancreas gains full functionality in 6-month-old infants; however, there are limited studies available due to lack of availability of the specimens [9].

Unlike the rapidly renewing gastrointestinal cells, the pancreatic cells do not show the same property. Nevertheless, the β-cells tend to retain potency throughout life. It continues to multiply at a moderately slow pace during adult life. During obesity-induced insulin resistance, increased β-cell mass occurs as a compensatory physiological feedback. However, the reduced peripheral insulin sensitivity worsens the situation leading to β-cell death. Interestingly, an increase in β-cell mass can also be observed during pregnancy to compensate for the increased demand. It accounts for 3–5% of the total cell mass. Further, it is unknown whether neogenesis from precursor cells is contributing to the β-cell mass increase during pregnancy [8–10].

Islets of Langerhans

It comprises of alpha (α), beta (β), delta (δ), epsilon (ε) and pancreatic polypeptide (PP) cells. These cells express peptide hormones such as glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide identical to their function [7]. β-cells make up the bulk of cells (60%), a central core surrounded by other cells. α-cells comprise 30% of the islet mass while δ and PP cells make up the rest [11]. Histologically, the islets appear as colonies of endocrine tissue suspended within the acinar matrix, derived from single progenitor cells. However, lineage tracing shows that each islet is polyclonal in origin [7,11]. Significant work elucidates the transcription factors involved in β-cell development. The overall molecular mechanism revealed by many research groups defines various transcription factors, signaling pathways, and molecules.

Functions of β-cells

The β-cells mainly produces bioactive insulin, in response to nutrients, hormones, and nervous stimuli, to maintain the plasma glucose levels in the physiological range. β-cells also control the functioning of neighboring cells through autocrine and paracrine signaling. The intricate signaling involves downstream activation of various pathways that modulates glucose metabolism. Glucose is the key modulator of insulin secretion sensed by glucose transporter 2 (GLUT2) on β-cells [12]. It activates the transcription factor carbohydrate response element-binding protein (ChREBP) that triggers glycolysis and β-cell proliferation [12].

A combination of factors governs β-cell mass maintenance: (a) replication of existing β-cells, (b) differentiation of new β-cells from ductal and extra-islet precursor cells (neogenesis), (c) formation of new β-cells from other endocrine cells (transdifferentiation) and (d) β-cell apoptosis [13].

Dynamic β-cell mass modifies both functions and mass to maintain the glycemic level within a very narrow physiological range. Neogenesis is accountable and widely accepted for the initial embryonic formation of the endocrine pancreas prenatally. However, its occurrence after birth is debatable because of differential observations in lineage-tracing experiments. Various studies from different models and species over a period conceived the

notion that progenitors have a role in the renewal and growth of islets after birth [13].

β-cell dysfunction/death (why mine gets exhausted?)

Mechanism

Reduced β-cell mass and function marks the clinical onset of diabetes mellitus. Autoimmunity plays a significant role in β-cell apoptosis in T1D backed by cytokine activated CD8+ cytotoxic-T cells [14]. On the contrary, β-cell loss in T2D is by various factors including obesity. Hypertrophy and hyperplasia of adipose cells and macrophage infiltration causes the pathophysiology of obesity. Macrophages and inflamed adipocytes secrete inflammatory cytokines. It leads to the desensitization of insulin-responsive tissues resulting in insulin resistance. Glucolipotoxicity and oxidative stress in β-cells are caused by persistent hyperglycemia and dyslipidemia. It jeopardizes the β-cell integrity and functioning [5].

The accumulation of free fatty acids (FFA) and diacylglycerol (DAG), and the generation of high levels of reactive oxygen species (ROS) also contribute to both β-cell and adipocyte dysfunction. Mitochondrial dysfunction is investigated for their involvement in β-cell dysfunction. Insulin secretion from β-cells and adipokine secretion from the adipose tissues are both dependent on mitochondrial integrity. The excessive availability of nutrients hampers mitochondrial biogenesis. Mitochondrial dysfunction induces β-cell apoptosis and fatty liver disease. It stalls adipocyte differentiation and alters the balance of pro versus anti-inflammatory adipokines [4,14,15]. There are many factors and mechanisms associated with apoptosis of β-cells as described in Table 1.

Proposed therapies focusing on β-cell regeneration (replenishing the mine)

The current therapies targeted for T1D are insulin pumps, multiple-injection regimens, and insulin analogs though they often do not achieve the target glycated hemoglobin levels. The islet transplantation and stem cell therapy has long been proposed [26]. The success of transplantation of islet cells with restoration of blood glucose in diabetic rats was demonstrated for the first time more than 40 years ago [27,28]. However, loss of islets during transplantation, islet death, anoxia, and engraftment are some of the causes that reduce β-cell mass and contribute to early failure of the graft. Additionally, the shortage of donor islets and cost-effectiveness are significant obstacles to the widespread application of islet transplantation as a curative procedure. The pharmacological interventions for T2D involves two approaches: i) insulin secretion from β-cells and ii) insulin mediated glucose uptake from peripheral tissues [1]. Patients develop tolerance against these drugs within a few years of treatment, which poses a challenge for the development of new medicines. Since control of glucose levels can thwart the devastating complications of diabetes, research now focuses on β-cell replacement therapy, which can be accomplished by regeneration of the deficient β-cells in the pancreas. Therefore, the focus is on the cost-effective therapeutic strategies to preserve or expand the β-cell mass and function for DM.

However, it is a challenging task because β-cells are not capable of undergoing regeneration in adult humans and are considered a more quiescent cell type as compared to hepatocytes [29]. Moreover, the challenge for the T1D cure is resetting the immune system and blocking the autoimmunity, while for T2D it is to maintain glycemic control and discover drugs targeting multiple diabetes-related complications. Another problem is that we are still not fully aware of the regulatory intricacies of human β-cell proliferation and it is complicated to understand this due to the different experimental models used for these studies [30]. Hence, more research is getting orientated towards identifying the molecules involved and their modes of action towards β-cell regulation.

Current modalities having the power of regeneration (searching the elixir)

The essential treatment for T1D is insulin administration along with optimal nutrition. The first line of therapy involves calorie restriction and physical activity for the borderline to early-stage T2D. When the desired glycemic control is unachievable, the patient is prescribed medicines. Conventional medications for T2D like metformin, sulfonylureas and insulin to lower glucose work by different mechanisms. However, due to undetermined reasons, most of them lose their efficacy with time, resulting in progressive β-cell deterioration.

The modalities encompassed in the present review have properties to enhance either β-cell mass, function, or regeneration. β-cell mass is the total weight of β-cells within the pancreas. It is regulated by the equilibrium between formation (replication of existing cells and neogenesis/transdifferentiation) and death (apoptosis/necrosis) of β-cells as well as individual cell volume (atrophy/hypertrophy) [31,32]. The β-cell function is the quantitative correlation between insulin sensitivity and insulin action to adapt and sense the metabolic environment in healthy individuals [25]. The β-cells are usually formed by two pathways: replication of already differentiated β-cells or neogenesis from putative islet stem cells. β-cells are formed even from the existing differentiated cell conversion (acinar cells, α-cells, etc.) [31,32].

In this section, we will elaborate on the regenerative properties of the existing/proposed modalities. The etiology and course of T1D and T2D are remarkably diversified. Some of these modalities have not been explored in terms of autoimmune responsiveness and insulin sensitivity.

Exercise and diet: do they regenerate β-cells?

Exercise has a final or an intermediate objective of improving or attaining physical fitness. However, it

Table 1 Factors involved in β-cells apoptosis and their underlying mechanism.

Sr. No.	DM	Factor	Mechanism	References
1.	T1D	Autoimmunity	Macrophages and dendritic cells are the first cell types to infiltrate the pancreatic islets. It presents MHC and β-cell peptides to naive CD4+ T cells that circulate in the blood and lymphoid organs. Concurrently, activated TH1 CD4+ T cells produce IL-2 that activates β-cell antigen-specific CD8+ T cells. It differentiates into cytotoxic T cells and gets incorporated into the pancreatic islets inducing the destruction of β-cells. Furthermore, CD8+ T cells and activated macrophages release granzymes, perforin, cytokines, and ROS. Thus, the above all act synergistically to destroy β-cells and leads to autoimmune diabetes.	
		Insulin Resistance (IR)	The role of insulin resistance in T2D is well known, but recent reports suggest the role of insulin resistance in T1D at the level of skeletal muscle and liver. I) Skeletal muscle IR is due to decreased glucose transport into myocytes from impaired insulin-sensitivity. Serine phosphorylation of IRS-1 in obese T1D individuals is due to the ectopic fat. The increased levels of intramyocellular lipids (IMCLs) and plasma FFAs activate serine kinases i.e., IκB kinase-β. It preferentially phosphorylates serine on IRS-1 causing decreased glucose transport. II) The liver regulates glucose homeostasis by mediating between gluconeogenesis and glycogenolysis (fasting state), and glycogen storage (fed state). Insulin suppresses gluconeogenesis via inhibition of phosphoenol pyruvate carboxykinase (PEPCK), promotes glycogen synthesis through stimulation of glycogen synthase kinase 3 (GSK-3) and inhibition of glucose 6-phosphatase (G6Pase). In T1D, all these three actions are impaired by higher doses of insulin.	[18,19]
2.	T2D	Insulin Resistance	In T2D patients, various factors such as inflammation, obesity, ER stress, or mitochondrial dysfunction triggers IR. The impaired insulin signaling pathway leads to severe complications.	[20]
		Glucotoxicity	Chronic exposure to aberrant hyperglycemia has harmful effects on cell survival, insulin secretion, and sensitivity. It is mediated through a mechanism called glucotoxicity and leads to persistent β-cell worsening. Caspase-mediated apoptosis is a key factor: I) Increasing demand for insulin placed on β-cells stresses their ER to produce more proinsulin. ER stress results in an accumulation of unfolded proteins and activates the unfolded protein response. It may cause β-cell apoptosis (mediated by stress kinases and transcription factors). II) Chronic hyperglycemia leads to a long-term increase in cytosolic Ca^{2+} and mitochondrial dysfunction that ends up as pro-apoptotic signal. It decreases the number of mitochondria and alters their morphology, manifests associated impaired glucose-stimulated insulin secretion, by way of impaired oxidative phosphorylation, decreased mitochondrial Ca^{2+}, and decline in ATP generation. These lead to the activation of apoptotic pathways. III) Hyperglycemia increases the metabolic flux into the mitochondria and impels excessive generation of ROS forming oxidative stress. Mitochondrial oxidative phosphorylation, glucose auto-oxidation, non-enzymatic glycation, PKC activation, and various metabolic pathways produce excessive ROS. It also damages the β-cells by inducing defective insulin biosynthesis and secretion, and ultimately apoptosis. Disruption of mitochondrial membrane integrity and mitochondrial DNA mutation promotes apoptosis.	[21,22]
		Lipotoxicity	Prolonged exposure of amplified FFA levels elicits toxic FA metabolites in the islet cells ("lipotoxicity"). β-cell death is mediated by the following: I) FA-induced protein kinase B inhibition leads to the downregulation of the anti-apoptotic factor Bcl-2. Also, excessive de novo ceramide is involved. II) Ceramide activates NF-κB, which upregulates the expression of inducible nitric oxide synthase (iNOS). It enhances nitric oxide and peroxynitrite formation and inhibition of the mitochondrial respiratory chain complexes I and III to promote ROS mediated apoptosis.	[22]
		Inflammation	Adipokines are hormones/cytokines secreted by adipose tissue with a role in the "adipo-insular" axis. Some (such as TNF-α, leptin, resistin) act as pro-inflammatory cytokines and contribute to β-cell failure. Others are an anti-inflammatory (vaspin, omentin-1, adiponectin) and exert protective effects on β-cell function and survival.	[4,14,15,23,24]
		Islet Amyloid	The 37-amino-acid polypeptide amylin is the chief component of the amyloid deposits that appear in the islets of Langerhans. The fibrillar form of the amylin peptide mediates toxicity that requires direct interaction of the fibrils with the cell surface. Cell death consists of RNA and protein synthesis; and is categorized by chromatin condensation, membrane blebbing, and DNA fragmentation, signifying that amylin causes islet cell apoptosis.	[25]

showed improved glucose tolerance and enhanced β-cell regeneration in rodent models. Exercise has a protective effect on β-cell mass through activation of signaling pathways and the reduction of pro-inflammatory cytokines in T1D. The physical performance affects the NF-κB pathway and reduces iNOS activity in various tissues in humans and murine models. These contribute to the downregulation of various pro-apoptotic factors like caspase-3 which favors cell survival [33]. Moderate aerobic exercise treatment involves β2-AR expression to increase intracellular cAMP levels and the subsequent suppression in Tregs (Treg cells; FOXP3+CD4+CD25+). Treg cells maintain homeostasis of the cellular immune responses that can prevent chronic inflammatory and autoimmune diseases [34]. Interestingly, in T2D patients, exercise has been shown to reduce pancreatic fat and improve β-cell function [35,36]. However, further molecular mechanisms need to be elucidated.

The dietary intervention has long been considered as a first-line therapy for diabetes management by researchers and clinicians around the globe. Interestingly, several reports focus on the benefits of customized diets on insulin signaling pathways and β-cell functionality i.e. calorie restriction (CR). CR is a reduction in calorie intake without cutting down on vital nutrients [37]. However, there are very few reports on β-cell regeneration by CR. We have observed increased CR diet-induced insulin sensitivity in the peripheral tissues [38]. Cheng et al. have shown the effect of a fast-mimicking diet on NGN-3, a progenitor cell marker that further induces PDX-1, the major β-cell-mediated transcription factor. The authors showed that in human T1D pancreatic islets, fasting conditions decrease PKA and mTOR activity while promotes SOX2, NGN-3, and insulin expression [39]. However, immunosuppressants are required to combat the autoreactive immune response. A new strategy of time-restricted diet has been proposed and studied. It showed that pro-inflammatory markers influence the circadian rhythm and enhance the β-cell responsiveness in prediabetic men [36]. However, several reports suggest a dietary intervention in T2D progression by reversing insulin resistance and restoring β-cell functionality has no effect on β-cell regeneration [40]. Thus, limitations of dietary approaches for the β-cell regeneration in DM drives the need for alternative strategies.

Hormones and β-cell regeneration

The hormones such as insulin, glucagon, glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP), gastrin, cholecystokinin (CCK), prolactin, and growth hormone (GH) have been correlated with β-cell mass. They are known to modulate β-cell growth and differentiation [41–47]. Reports suggest that GH, prolactin and placental lactogen could stimulate β-cell proliferation and insulin gene expression *in-vitro* and in T1D [41,48]. The studies showed the mechanism of GIP, gastrin, and CCK hormones in enhancing β-cell proliferation in the T2D model but not in T1D. Amongst the above-listed hormones, the most important is the "gut hormones - incretins (GLP-1 and GIP)" implicated in β-cell regeneration [42,45,49]. Buteau et al. [45] had shown for the first time that GLP-1 may increase PDX-1 expression and is involved in islet differentiation. Further, the processing of proglucagon to GLP-1 in α-cells enables it to act on β-cells in a paracrine manner. GLP-1 has demonstrated efficacious properties like β-cell apoptosis reduction, enhanced β-cell proliferation and survival in the preclinical studies in T1D model [49]. GIP plays a role in β-cell survival (*in-vivo*) and proliferation (*in-vitro*) in the T2D model. Additionally, these hormones also stimulate insulin secretion and delay gastric emptying. The gastrointestinal hormone-like gastrin enhances the dedifferentiation and reprogramming of ductal cells that work on the CCKB receptor promoting β-cell neogenesis [43,44]. CCK regulates β-cell apoptosis and mitogenesis [43]. Although CCK is not an incretin hormone, it is a potential therapeutic candidate as it enhances insulin secretion in diabetes patients on exogenous administration. It also regulates β-cell apoptosis and mitogenesis [43]. The recently identified gut peptide, Obestatin, plays a crucial role in increasing β-cell mass, pancreatic regeneration, and decreasing cell apoptosis. It also regulates insulin and glucagon. Obestatin induces these effects through increased cAMP, phosphorylation of PI3K/Akt (survival and proliferative pathways), and extracellular signal-related kinase (ERK)1/2. The *in-vitro* and *in-vivo* evidence suggests obestatin as an emerging therapeutic potential for diabetes management [50]. Furuya et al. [51] have found that the ligand-bound thyroid hormone (TH) receptor helps in the reprogramming of pancreatic acinar cells into β-cells in the T1D rodent model. THα activates Akt and induces the expression of NGN3, PDX-1, and MAFA in the acinar cells. They promote β-cell regeneration during postnatal development via PI3K signaling. Furthermore, parathyroid hormone-related protein present in pancreatic β-cells reported enhancement of the β-cell proliferation after partial pancreatectomy [52]. Ghrelin is a ligand of growth hormone secretagogue (GHS) receptor. It is known that acylated and un-acylated ghrelin helps in reducing blood glucose levels by inducing insulin secretion and increasing β-cell proliferation in T1D condition. GHS also has other metabolic effects by targeting sites downstream of peroxisome proliferator-activated receptor-γ (PPAR-γ) [53]. These properties of GHS makes it a suitable candidate for the therapeutic intervention of diabetes. This strategy may replace the need for insulin injections in the future. Thus, hormones encompassed in this section have demonstrated their effects on β-cell regeneration although their molecular mechanisms have not been studied in-depth or are limited to T1D/T2D conditions. These various molecules are depicted in Fig. 1.

Small molecules/agents known to cause regeneration

GLP-1 mimetics/receptor agonists

GLP-1 is secreted by the intestinal L-cells induce insulin secretion. It also acts on β-cells via its receptor GLP-1 receptor (GLP-1R) and activates adenylate cyclase to form cAMP. The transient rise in cAMP levels activates PKA and

Epac2 altering the ion channel activity and closing the β-cell ATP-sensitive potassium channels. It leads to an increased intracellular Ca^{2+} that causes insulin release. The activation of this alternate insulin secretion pathway devoid of GSIS made it a promising intervention for T2D as it could even alleviate the exhausted β-cells. GLP-1 also enhances PDX-1 that promotes differentiation, proliferation, and survival of β-cells in T2D [42]. Recent research has shown that the activation of GLP-1R leads to β-cell proliferation and neogenesis. Simultaneously, it inhibits β-cell apoptosis in pancreatic exocrine cells and diabetic rodents making it a possible intervention for T1D [42]. The specific mechanisms involved are not well defined but it involves activation of PKC and MAPK, the synergistic interaction of transforming growth factor-β (TGF-β), and SMAD transcription factor activity. In this context, GLP-1R agonists like liraglutide and exenatide emerge as the therapeutic potential for DM. It is reported that exendin-4, a GLP-1 mimetic and equivalent to exenatide, differentiates human fetal islet and pancreatic ductal cells into insulin-producing cells in T2D [42]. The proliferative action of GLP-1R agonists in *in-vitro* mediates the transactivation of the epidermal growth factor receptor, which leads to an increase in PI3K and activation of Akt-protein kinase B (PKB). They also encourage β-cell replication via IRS-2 signaling. It also activates cAMP/PKA, PI3K, and MAPK signaling pathways, and up-regulates cell-cycle regulator cyclin D1 expression. GLP-1R dependent inhibition of β-cell apoptosis is associated with diminished levels of pro-apoptotic proteins i.e. active caspase 3, thioredoxin interacting protein, PARP cleavage, up-regulation of pro-survival factors (Bcl-2, Bcl-xL), and inhibition of apoptosis protein-2 [54]. Exendin-4 also showed a significant decrease in islet inflammation, improved β-cell mass, and glucose tolerance in NOD mice [42,49]. Besides studies of exendin-4 in combination with anti-CD3 therapy demonstrated a positive effect on β-cell mass in NOD mice [49]. Numerous GLP1 synthetic secretagogues are manufactured to date with only L-glutamine being a naturally available one. However, it's regenerative or neogenic properties have not been studied directly. We have seen a significant increase in the number of insulin-positive cells upon L-glutamine treatment on the HFD-STZ T2D mice model primarily by replication [55]. Therefore, the beneficial effects of GLP-1R agonists seen in T1D and T2D preclinical models need further validation and investigation on its molecular mechanisms.

GIP/GIPR analogs

GIP is a 42-residue long incretin hormone secreted by intestinal K-cells, in response to nutrient ingestion like GLP-1 and enhances glucose-stimulated insulin secretion. GIP also enhances insulin biosynthesis and promotes β-cell proliferation and survival [42]. Though the pathway remains elusive, it is suggested that it mediates secretagogue and proliferative properties via cAMP/PKA, PKA/CREB, and MAPK. The antiapoptotic effect is by PI3K/Akt activation with subsequent phosphorylation of FOXO1. Kim et al. [56] has reported a significant reduction in islet cell apoptosis in diabetic rats when treated with GIP. It phosphorylates

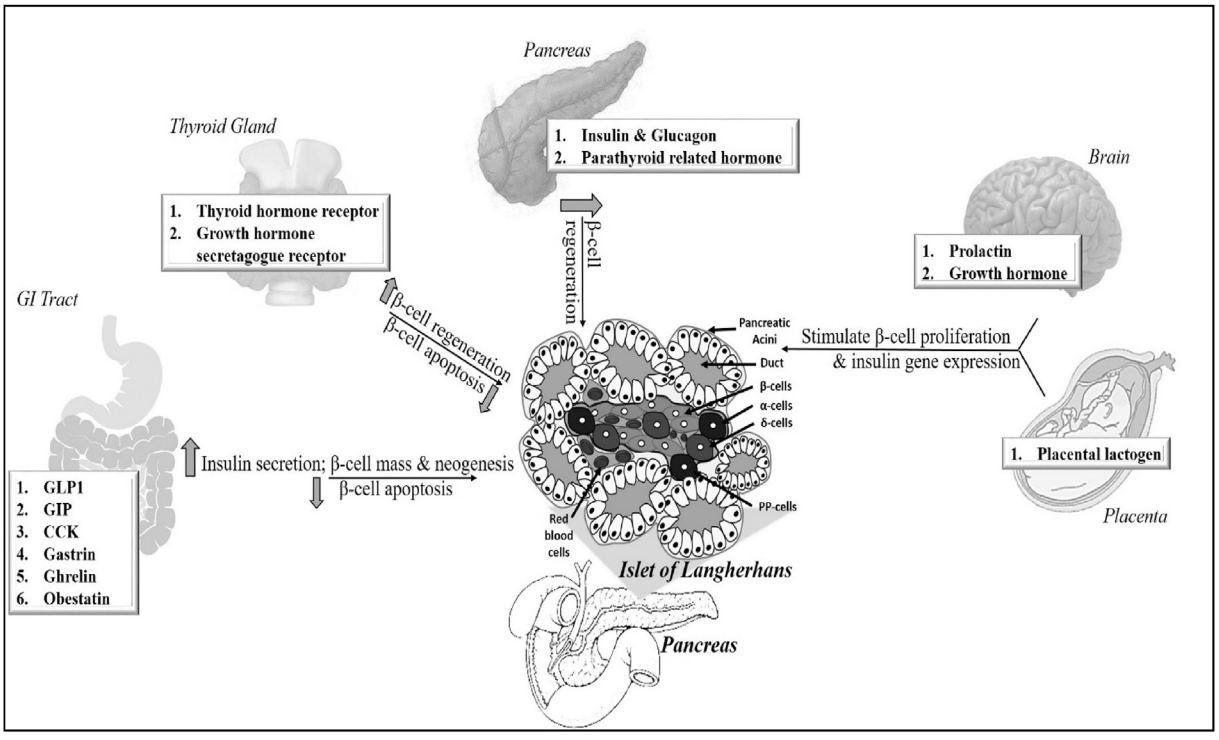

Figure 1 The effect of hormones on pancreatic islets and β-cells: 1. Prolactin, GH and placental lactogen stimulate β-cell proliferation 2. Insulin, Glucagon, and parathyroid related hormone stimulate β-cell regeneration 3. Thyroid hormone receptor and GH secretagogue receptor reduce β-cell apoptosis. 4. GLP-1, GIP, CCK, Gastrin, Ghrelin, and Obestatin increase β-cell mass, neogenesis and insulin secretion.

FOXO1 via PI3K/Akt, resulting in reduced expression of pro-apoptotic Bax gene and up-regulation of the anti-apoptotic Bcl-2 gene [56]. GIP-R analogs (GIP1–30 and GIP1–42) induce alternative conformational changes in different tissues, may be due to differences in the membrane environment caused by the different responses [57]. This possibility could influence the progress of clinically relevant GIP analogs. Thus, GIP/GIPR analogs are considered as a therapeutic potential in T1D. The evidence of reducing protein levels of Bax is encouraging, yet advanced research is needed.

Dipeptidyl peptidase-IV (DPP IV) inhibitor

Numerous DPP-IV inhibitors are studied in the area of β-cell proliferative and regenerative properties. These inhibitors inactivate the DPP-IV serine proteases by binding competitively to its active site and thus increases the half-life of the incretins. The first DPP-IV inhibitor, sitagliptin, was approved in 2006. It opened doors for more drugs such as saxagliptin, vildagliptin, linagliptin, and alogliptin [1]. They are prescribed as an additional therapy for T2D patients with severe diabetic conditions [58]. There are reports which have demonstrated the effects of DPP-IV inhibitors on β-cell proliferation in the T2D mouse model and may improve T1D pathogenesis. However, further clinical studies are needed to substantiate the reported studies [59]. Effective therapy of T1D/late-stage T2D requires efficient suppression of the autoimmune processes and restoration of islet β-cells.

Sodium-glucose co-transporter 2 (SGLT2) inhibitors

SGLT2 inhibitors, a novel class of oral anti-diabetes treatment, selectively target the SGLT2 protein. The Na^+ concentration-dependent proteins are found in the small intestine (SGLT1) and kidneys (SGLT2). SGLT2 inhibitors block the symporters from reabsorption of the excess glucose thereby reducing hyperglycemia [60]. In the last decade, many researchers have explored the β-cell regenerative properties of these inhibitors. Cheng et al. reported the protective effect of empagliflozin on pancreatic β-cell from glucotoxicity-induced oxidative stress. They demonstrated an increase in cell proliferation marker Ki-67 expression and enhanced β-cell area/total pancreatic area in the T1D mice model [61]. Subsequently, Takahashi et al. reported that luseogliflozin administration improved glucose intolerance and increased the expression levels of MAFA, PDX-1, NKX6.1, and GCK. It also decreased β-cell apoptosis and preserved β-cell mass in db/db mice [62]. Dapagliflozin when administered in the T2D model could provide a long-lasting effect in preserving pancreatic β-cell mass [63].

Thiazolidinediones (TZD)

TZDs act as nuclear hormone receptor PPARγ ligands. PPARγ, a transcription factor highly expressed in adipose

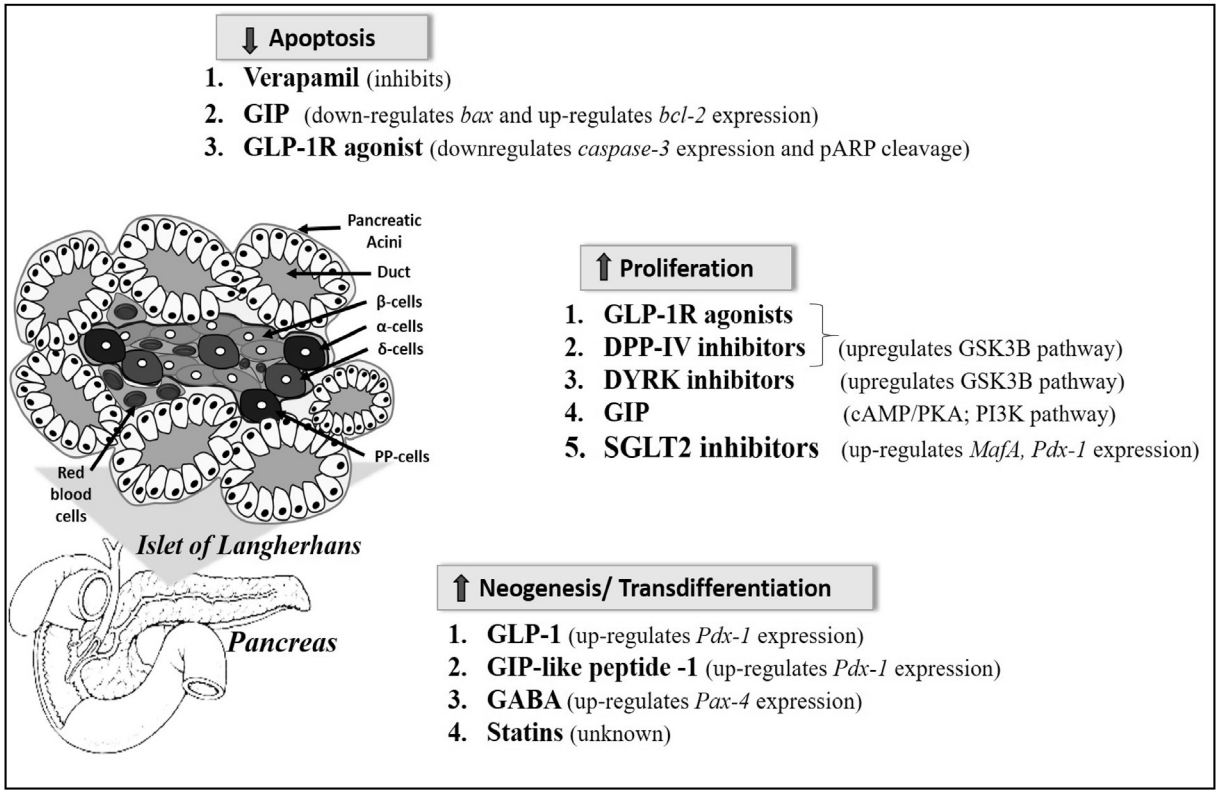

Figure 2 The effects of small molecules on pancreatic β-cells: 1. GIP GLP-1R agonists and TZDs reduce β-cell apoptosis. 2. GLP-1R agonists, DPP-IV inhibitors, DYRK inhibitors, GIP and SGLT2 inhibitors promote β-cell proliferation. 3. GLP-1, GIP-like peptide-1 and GABA induce neogenesis or transdifferentiation of various cells to β-cells.

tissue, regulates gene expression and oversees nutrient homeostasis [1]. PPAR-γ activation has several insulin-sensitizing consequences like decrease in circulating FFAs and triglyceride concentrations, cycle favoring glucose utilization, and increased production of GLUT-4. It suppresses the production of pro-inflammatory cytokines and increases the production of adiponectin from adipose tissue [1]. TZDs protect human islets from the detrimental effects of hyperglycemia, lipotoxicity, and inflammation. The reduced β-cell apoptosis and amyloid formation results in improved glucose-stimulated insulin secretion and β-cell survival through reduced β-cell apoptosis and amyloid formation [1,20]. In a recent study by Hirukawa et al. [64], PPARγ agonists showed protective effects on pancreatic β-cells by the augmentation of IRS-2 expression in db/db mice [64]. However, due to the adverse side-effects of pioglitazone including increased risk of fluid retention, heart failure, and bone fracture, the use of this agent has become limited in clinical practice.

Dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) inhibitor

Many novel compounds such as amniopyrazine compounds [GNF4877], 5-iodo-tubericidin [5-IT] an adenosine analog [65,66], and harmine based inhibitor [65] have been screened recently. They are proliferative agents derived from human cadaveric islets with labeling indices in the 1.5%–3% range [65–67]. They are immunolabelled in Ki67, EdU, BrdU, and PCNA. Interestingly, all these molecules are kinase (DYRK1A) inhibitors. Furthermore, these compounds also inhibit the nuclear factor of activated T-cell (NFAT) kinases, and glycogen synthase kinase-3 beta (GSK3β). It leads to NFAT nuclear localization, needed for β-cell proliferation. NFAT transactivates cycle activating genes (encoding cyclins E and A) and represses cell cycle inhibitor genes (CDKN1C, CDKN2A, and CDKN2B) [65,66]. The mechanism involves dephosphorylation of NFAT by calcineurin, translocation into the nucleus, activation of cell cycle promoting genes, and entry into the cell cycle. The kinase, DYRK1A, serves as

Table 2 Phytochemicals and its role in β-cell regeneration.

Plant	Animal model		Treatment	Effect on β-cell	References
	Strain	Mode of Diabetes Induction			
O. integrifolia	Adult female mice of BALB/c strain (7–8 weeks old)	Partial pancreatectomy	Flavonoid rich fraction (FRF) of *O. integrifolia* for a period of 21 days at a dose 250 mg/kg of body weight.	Newly formed islets budding off from ducts. Up regulation of INS1/2, REG-3α/γ, NGN-3, and PDX-1 involved in β-cell neogenesis	[76,77]
U. dioica	Adult Wistar Kyoto male rats (8–10 weeks old)	Single dose STZ (50 mg/kg, i.p.)	Aqueous ethanolic extract of *U. dioica* leaves (0.625 and 1.25 mg/kg/day) for 28 days	Recovery of the structural integrity of islets and increase in β-cell number	[78]
A. linearis	Male Balb/c mice (9–11 weeks old)	Single dose STZ (200 mg/kg, i.p.)	(Z)-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid 1, a water-soluble extract (a dose of 10 mg/kg body weight) for 11 days	Protects β-cell by preventing loss of expression of anti-apoptotic protein B-cell Lymphoma –2	[79]
C. longa	Young male mice	Single dose STZ (60 mg/kg, i.p.)	Curcumin 200 mg/kg body weight every day in a single dose for 12 weeks	Inhibits lymphocytes infiltration in the islets of Langerhans and keeps the number of islets and β-cells	[80]
M. charatia	Neonate Wistar pups (2-days-old)	Single dose STZ (100 mg/kg, i.p.)	Ethanolic extract of *M. charatia* fruit pulp (400 mg/kg/day) for 28 days	Improvement of HOMA % β-cell function, by well-formed islets. Increase in β-cell number and area	[81]
G. sylvestre	Young male mice	Single dose STZ (100 mg/kg, i.p.)	*G. sylvestre* extract (400 mg/kg) for 28 days	Induces anti-diabetic effect	[82]
A. Sativum	Male Sprague –Dawley rats	Single dose STZ (50 mg/kg, i.p.)	Raw garlic homogenate (250 mg/kg) orally for a period of 4 weeks	Increase in insulin positive islets cells	[83]
A. indica	Young male mice	Single dose STZ (100 mg/kg, i.p.)	*A. indica* 200 mg/kg for 28 days	Recovery of islet architecture	[84]

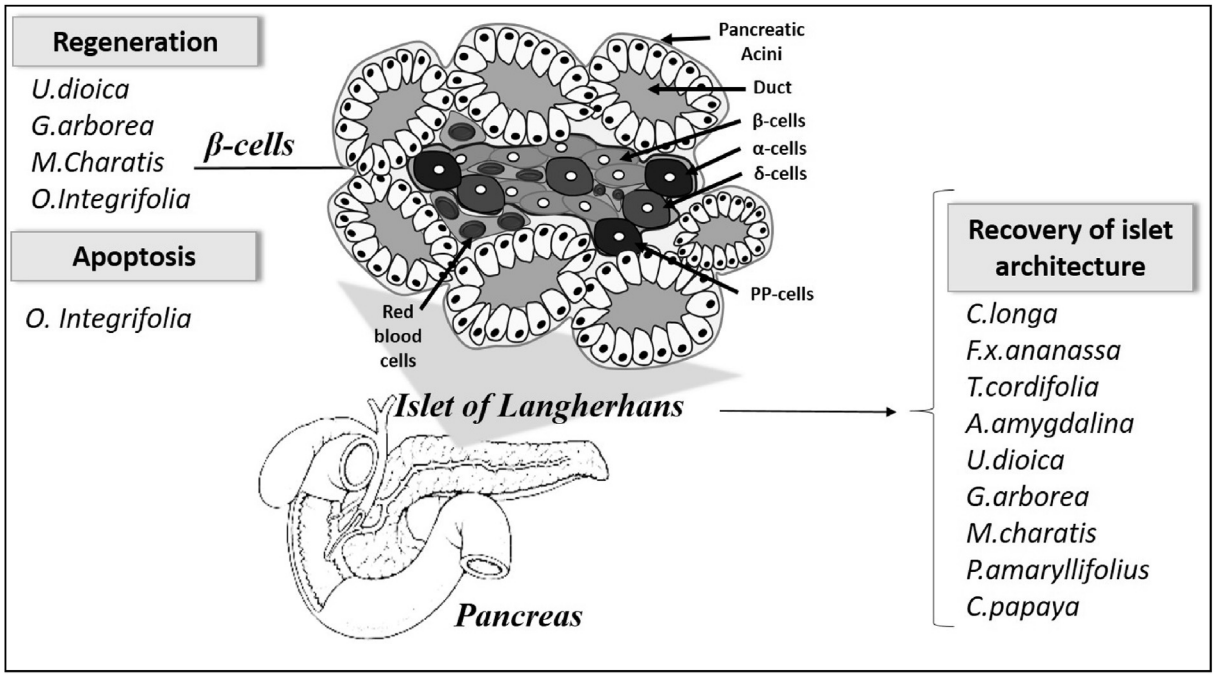

Figure 3 The effect of phytochemicals on pancreatic β-cells: Several reports are indicating the role of phytochemicals. 1. U.dioica, M.charatia, and A.sativum increase the islet and β-cell number. 2. A.linearis and O.integrifolia reduce β-cell death and O.integrifolia also upregulates gene expression of INS-1/2, NGN-3, PDX-1 and REG-3α/γ. 3. C.longa, U.dioica, M.charatia, G.sylvestre, and A.indica influence and recover the architecture of pancreatic islets.

the terminator in this process by re-phosphorylating NFAT and ultimately applies the brake on the cell cycle. Thus, DYRK1A inhibitors appear to be good candidates [65,66]. These DYRK1A inhibitors candidates have been studied individually or in combination with other drugs. The combined effect of DYRK1A and TGF-β inhibition preserves the function of the differentiated β-cell. Extensive research of DYRK1A inhibitors was carried out in T2D. It is encouraging, as the residual β-cell mass is substantially higher in T2D than T1D. These inhibitors must overcome the issues of autoimmunity in T1D.

γ-AminoButyric acid (GABA)

GABA, a major inhibitory neurotransmitter, has proven a role in islet-cell hormone homeostasis, preservation of the β-cell mass, suppressing detrimental immune reactions and consequent apoptosis [38,68]. The positive effect of GABA treatment is documented in T1D and T2D murine models. GABA therapy protects NOD animals from diabetes and a similar effect is detected in *in-vivo* models [69,70]. Also, GABA generally regulates cytokine secretion from human PBMCs and suppresses β-cell-reactive CD8+ CTLs in T1D models [68,70]. These propose the role of GABA as an immunosuppressant. GABA may act as an inducer of α-to-β-like cell conversion *in-vivo* upon prolonged exposure in the STZ-induced mouse model [68]. However, the mechanism requires further elucidation. The encouraging reports of GABA on T1D lead to studies on T2D models as well. The activation of GABAA-Rs and GABAB-Rs receptors (GABA receptors on β-cell) can induce β-cell replication and activation of α-cell GABAA-Rs can promote their conversion into β-cells [68]. GABA has been reported to promote human β-cell replication and islet cell survival in *in-vivo* and humanized mice [68]. Combined GABA and sitagliptin (DPP-IV inhibitor) therapy have demonstrated β-cell regenerative effects in various diabetic mouse models [68]. The up-regulation of PDX-1 expression contributes towards β-cell replication. We have observed reduced FBG levels, improved whole-body insulin responsiveness indicated by increased insulin levels, insulin sensitivity, glucose tolerance, and promoted β-cell proliferation in combination therapy of GABA and calorie restriction [38]. All these data indicate the potential of GABA to stimulate the growth and function of insulin-producing β-cells and act as an immunosuppressive agent for diabetes therapy.

Statins

Statins are commercially available in managing dyslipidemia comorbidities associated with T2D. Marchand *et al.* have shown the role of statins on β-cell regeneration wherein atorvastatin induces neogenesis in T1D model [71]. Adiponectin is a hormone secreted by adipose tissue which enhances insulin sensitivity by decreasing the hepatic and muscle triglyceride content. The decreased levels contribute to the high level of circulating FFAs [1,15]. Additionally, there are mixed reports on increased serum adiponectin and decreased C-reactive protein levels when treated with statins [72]. We have demonstrated the β-cell regenerative effect of statin i.e. pitavastatin administration in HDF-STZ induced T2D mice models [54]. The effects of small molecules on β-cell are summarised (see Fig. 2).

Phytochemicals known to cause regeneration

From ancient times, India has been a pioneer country for the knowledge of ayurveda and has propagated various plants and plant-derived products for medicinal purposes. Recent studies have shown that plants contain many bioactive compounds that are responsible for their medicinal properties. Such non-nutritive plant molecules providing various health benefits or disease-preventing effects are termed as "Phytochemicals". Phytochemicals are found in fruits, vegetables, cereals, grains, and plant-based beverages such as tea, coffee, wine and beer. Some of the phytochemicals are lycopene in tomato, isoflavones in soy, flavonoids in fruits, carotenoids in carrots, etc. Phytochemicals from cruciferous vegetables (broccoli, cauliflower, cabbage, kale, brussels sprouts etc.) are used to treat certain tumors [1,73]. Several new studies are being conducted on phytochemicals concerning T1D [71] and T2D [1,73–75] cure and management and are represented in Table 2 and Fig. 3.

Conclusions

There have been many advances made to understand and enhance the regenerative potential of β-cells. The major pancreatic cell types (islet, acinar, and ductal) have a certain degree of plasticity to expand existing β-cells and induce transdifferentiation into β-cells. Encouraging findings from recent studies suggest the possibility of endogenous replenishment in humans. Yet, it is unclear how enhanced regeneration could be achieved in humans to restore normoglycemia. Mechanistic and developmental studies in rodents have laid the foundation for identifying small molecules and drugs already approved for their medical use that could enhance both regenerative pathways. It has created a new epoch for the cell-based therapies that may be improved, safe and versatile over exogenous insulin. The crucial β-cell transcription factors, PDX-1, NGN3, and MAFA, have been targeted in the mentioned modalities for creating effective therapies. Even so, the challenge remains as to how the interventions could be focused on β-cells alone. There are other major obstacles to overcome the issue. Of foremost importance is tackling the autoimmune attack of T1D so that regenerative strategies can have a long-lasting beneficial effect. There have only been a few studies that have attempted to replenish β-cells in NOD mice. Fortunately, a new approach to protect β-cells from an autoimmune attack is the encapsulation of transplanted islets and to increase immune tolerance by increasing the Treg cell population. A clinical trial also revealed that immunosuppression in T1D patients has a positive effect on β-cell regeneration [85–88]. For T2D, it is still undetermined whether the pancreatic cells are receptive to experimental stimulation that promotes growth, expansion, and differentiation. If the cells have become senescent, it is unlikely that they could be regenerated unless the senescence pathway is targeted. The advanced technologies of using humanized diabetic rodent models, artificial intelligence and machine learning could drive and elucidate the development of new therapies for diabetes research.

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Author contributions

RB, AVR, and NR conceived the idea and designed the subtopics. NR wrote the first four aspects (1, 2, 3 and 4) and the 5th point was co-written by NR (5.1, 5.3.5, 5.3.7, 5.3.8 and 5.4), RP (5.2), SP (5.3.1–4), MI (5.3.6) and SR (5.4). NR and NP created supporting integrative images. AVR and RB were involved in active discussion throughout the review writing, critically evaluated the review and offered suggestions for the integrative figures.

Declaration of competing interest

The authors declare that no competing interests exist.

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Association of melatonin & *MTNR1B* variants with type 2 diabetes in Gujarat population

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ABSTRACT

Aim/hypothesis: Melatonin is a circadian rhythm regulator and any imbalance in its levels can be related to various metabolic disorders. Melatonin and the genetic variants of Melatonin Receptor 1B (*MTNR1B*) are reported to be associated with Type 2 Diabetes (T2D) susceptibility. The aim of the present study was to investigate i) plasma melatonin levels ii) Single Nucleotide Polymorphisms (SNPs) of *MTNR1B* and iii) Genotype-phenotype correlation analysis in T2D patients.

Methods: Plasma and PBMCs were separated from venous blood of 478 diabetes patients and 502 controls. Genomic DNA was isolated from PBMCs. PCR-RFLP was used for genotyping. Melatonin was estimated from plasma samples by ELISA.

Results: Our study suggests: i) decreased plasma melatonin levels in T2D patients and, ii) association of *MTNR1B* rs10830963 GG genotype with increased Fasting Blood Glucose (FBG).

Conclusion: It can be concluded that reduced titer of melatonin along with altered FBG due to *MTNR1B* genetic variant could act as a potent risk factor towards T2D in Gujarat population.

1. Introduction

A disturbed circadian rhythm is strongly related to Type 2 Diabetes (T2D) and insulin resistance in recent years [1]. Melatonin, a pineal hormone, is known to regulate circadian rhythm and sleep [2]. Melatonin mediates its action through two receptors; MT1 (*MTNR1A*) and MT2 (*MTNR1B*) present in various tissues including pancreatic islets [3,4]. The finding that insulin secretion and plasma melatonin levels are inversely correlated suggests a possible association between melatonin and T2D [5].

It is well-known that T2D is a multifactorial and polygenic metabolic disorder [6]. Substantial variation between different ethnic populations has been reported with regard to the genetic architecture underlying T2D [7,8]. *MTNR1B* (13.16 kb) comprises of two exons, one intron, and 5′- and 3′-flanking regions [9]. Recent studies have identified genetic polymorphisms within *MTNR1B* i.e. rs4753426, rs10830962, and rs10830963 (−1193 C/T, 5′ UTR G/C and intron C/G respectively) to be associated with higher fasting glucose levels, impaired insulin secretion, increased risk of T2D and gestational diabetes in different ethnicities [10,11]. Reduced melatonin levels and an increased melatonin signaling are known to be the risk factors for T2D [12,13].

The aim of this study was to examine whether i) plasma melatonin and polymorphisms in *MTNR1B* (rs4753426 C/T, rs10830962 G/C, and rs10830963 C/G) are associated with T2D in Gujarat population and, ii) the genotype-phenotype correlation of the above-mentioned Single Nucleotide Polymorphisms (SNPs) and plasma melatonin with the metabolic profile are associated with T2D. This is the first genetic association study of *MTNR1B* variants with T2D and metabolic profile 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

Abbreviations: MTNR1A, Melatonin Receptor 1A; MTNR1B, Melatonin Receptor 1B; TC, Total Cholesterol; TG, Triglycerides; LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; BMI, Body Mass Index; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2013/1). The importance of the study was explained to all the participants and a written consent was obtained from all diabetes patients and control subjects. The study group included 478 T2D patients (213 males and 265 females) and 502 control subjects (251 males and 251 females). Further, the T2D subjects recruited for the study displayed fasting blood glucose (FBG) levels > 125 mg/dL. BMI (weight kg/height m^2) was calculated by recording height and weight.

2.2. Blood collection, DNA extraction, and lipid profiling

Three ml venous blood was drawn from diabetes patients and ethnically matched control subjects between 8:00 AM to 10:00 AM and collected in K_3EDTA coated tubes (Greiner Bio-One, North America Inc., North Carolina, USA). Plasma was separated and stored at −20 °C for evaluation of lipid profile and assay of melatonin. 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 MTNR1B SNPs by PCR-RFLP

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used to genotype the three *MTNR1B* polymorphisms. The primers used for genotyping are mentioned in Table S1. 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 25 mM dNTPs (Puregene, Genetix Biotech), 1.0 µL of 10 mM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.2 µL (5 U/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 S1) 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 S1. 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 digestion 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. Estimation of plasma melatonin

Plasma levels of melatonin in age and sex-matched patients and controls were measured using human melatonin ELISA Kit (Glory Science Co., Ltd, TX, USA) as per the manufacturer's protocol. The melatonin levels were estimated in the study subjects between the ages of 35–50 years only as the melatonin levels are known to decrease significantly after the age of 50 years.

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-squared analysis. The distribution of the genotypes and allele frequencies of *MTNR1B* polymorphisms for patients and control subjects were compared using the chi-squared test with 2 × 2 contingency tables respectively using Prism 5 software (GraphPad software Inc; San Diego CA, USA). P values less than 0.017 for genotype and allele distribution 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^2values for the pair of the most common alleles at each site were obtained using http://analysis.bio-x.cn/myAnalysis.php [14]. Plasma melatonin levels in patient and control groups were plotted and analyzed by unpaired t-test using Prism 5 software. 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 Prism 5 software after adjusting confounding variables like age and sex. P values less than 0.05 were considered significant for all the association analysis. The statistical power of detection of the association with the disease at the 0.017 level of significance was determined by using the G* Power software.

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 controls. However, LDL did not differ in the study groups ($p = 0.9322$).

3.1. Association of MTNR1B polymorphisms with T2D

The genotype and allele frequencies of the investigated *MTNR1B* polymorphisms (rs4753426 C/T, rs10830962 G/C, and rs10830963 C/G) are summarized in Table 2 while the representative gel images for PCR-RFLP analysis of *MTNR1B* polymorphisms 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 ($p > 0.05$).

Furthermore, genotype and allelic frequencies of *MTNR1B* polymorphisms were found to be statistically indifferent ($p > 0.017$) with

Table 1
Baseline characteristics of diabetes patients and controls from Gujarat population.

	Controls (Mean ± SD)	Patients (Mean ± SD)	P value
	(n = 502)	(n = 478)	
Age	39.64 ± 16.35 yr	55.99 ± 10.42 yr	–
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	.06 ± 7.3	–
Family history	NA	64 (14%)	–

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

Table 2
Genotype and allele frequency distribution of *MTNR1B* polymorphisms in T2D patients.

Gene/ SNP	Genotype or allele	Controls (Frequency)	Patients (Frequency)	*p* for Association	Odds ratio	(95% CI)
		(n = 481)	(n = 426)			
MTNR1B rs4753426 (−1193 C/T)	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)			
		(n = 470)	(n = 417)			
MTNR1B rs10830962 (5' UTR G/C)	GG	122 (0.26)	114 (0.27)	R	–	–
	GC	226 (0.48)	205 (0.49)	0.8546[a]	0.9707	0.7065 to 1.334
	CC	122 (0.26)	98 (0.24)	0.4212[b]	0.8596	0.5946 to 1.243
	G	470 (0.50)	433 (0.52)	0.4198[c]	0.9261	0.7685 to 1.116
	C	470 (0.50)	401(0.48)			
		(n = 489)	(n = 434)			
MTNR1B rs10830963 (intron C/G)	CC	169 (0.35)	133 (0.31)	R	–	–
	CG	259 (0.53)	266 (0.61)	0.0663[a]	1.305	0.9819 to 1.734
	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 2 × 2 contingency table, [c] Patients vs controls (allele) using chi-square test with 2 × 2 contingency table, Values are significant at $p < 0.017$ due to Bonferroni's correction.

Bonferroni's correction for multiple testing as shown in Table 2. This study has 95% statistical power for the effect size 0.11, 0.05 and 0.17 to detect association of *MTNR1B* promoter polymorphisms at $p < 0.017$ in T2D patients and controls.

3.2. Haplotype and linkage disequilibrium analyses of MTNR1B polymorphisms

A haplotype evaluation of the three polymorphisms of *MTNR1B* (rs4753426, rs10830962, and rs10830963) 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 3.

The LD analysis revealed that the three polymorphisms investigated in the *MTNR1B* were in low to high LD association (Fig. S2). In particular, rs4753426: rs10830962 (D' = 0.44, $r^2 = 0.18$) and - rs4753426: rs10830963 (D' = 0.68, $r^2 = 0.24$) showed moderate LD association. rs10830962: rs10830963 showed high LD association (D' = 0.87, $r^2 = 0.47$).

3.3. Correlation of MTNR1B polymorphisms with FBG, BMI and plasma lipids

Correlation of *MTNR1B* polymorphisms showed that rs10830963 GG genotype was found to be associated with increased FBG ($p = 0.02$) (Table 4). However, it was not associated ($p > 0.05$) with BMI and plasma lipids (TC, TG, HDL, LDL). Further, rs4753426 and rs10830962 also did not show any association with FBG, BMI and plasma lipids ($p > 0.05$).

3.4. Plasma melatonin levels and their correlation with FBG, BMI and plasma lipids

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. 1).

Spearman's Correlation analysis revealed that there is no correlation between melatonin levels and BMI, FBG and plasma lipids ($R^2 = 0$, $p > 0.05$) (Table 5).

4. Discussion

Disturbances in sleep have been implicated in the dysregulation of blood glucose levels and have also been reported to increase the risk of T2D and diabetes complications. Melatonin, a pineal hormone regulates energy metabolism by maintaining circadian rhythms [15]. Genome-wide association studies have shown a close association between *MTNR1B* polymorphisms and fasting hyperglycemia and T2D [16].

Our study on genetic variants of *MTNR1B* (rs4753426, rs10830962, and rs10830963), 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 rs10830963 GG genotype 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 population [17,18]. However, this SNP does not show any correlation with obesity-linked parameters in T2D [19]. Moreover, several studies in different populations have found no association between *MTNR1B* polymorphisms and T2D [19,20,21]. Interestingly, a large-scale meta-analysis when

Table 3
Haplotype frequencies of *MTNR1B* polymorphisms in T2D patients and controls.

Haplotype (*MTNR1B* rs4753426 C/T, rs10830962 G/C, and rs10830963 C/G)	Patients (Freq. %) (n = 459)	Controls (Freq. %) (n = 501)	*p* for Association	$p_{(global)}$	Odd 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 case and control has been dropped and was ignored in the analysis).

Table 4
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 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 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 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 $p < 0.05$.

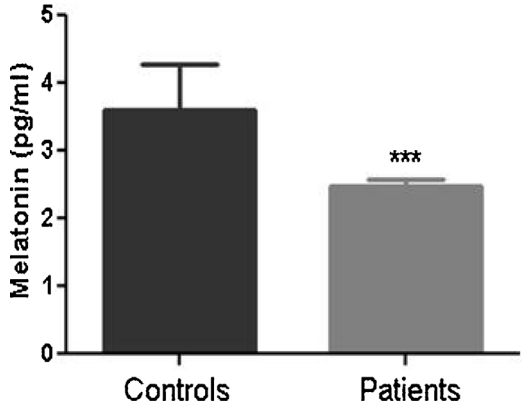

Fig. 1. Plasma melatonin levels in controls and patients. Comparison of findings showed a significant decrease in the melatonin levels in patients compared to controls ($p < 0.001$).

Table 5
Correlation analysis of plasma melatonin with BMI, FBG, and plasma lipids.

	Melatonin	
	R^2	*p*
BMI (Kg/m^2)	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].

stratified according to the ethnicity revealed that *MTNR1B* rs10830963 is associated with T2D in Caucasians but not in Asians [22]. Our observation of association of G allele with FBG stands substantiated by a meta-analysis [23] and a GWAS study [16,24]. However, they also found an association with increased T2D risk which is not seen in our study. Further, Takeuchi et al. have shown rs10830963 polymorphism to be associated with elevated FBG in two Asian populations [25]; 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 [26,27]. Our study also shows a clear correlation between lowered melatonin levels and T2D risk, a finding supported by the observations of Peschke et al. [28] and McMullan et al. [29]. 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.

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 lipids. Yildiz et al. have also reported that melatonin does not show any correlation with any of the anthropometric and biochemical parameters [30].

The *MTNR1B* risk allele genotype (GG) has been associated with insulin resistance [31,13] and an increase in *MTNR1B* expression by 2–4 fold in human pancreatic islets [13]. Gaulton et al. revealed that the G allele of rs10830963 favorably binds to NEUROD1 in islet cells further increasing FOXA2-bound enhancer activity and MTNR1B expression leading to increased risk of T2D [32]. 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 [13,33]. Moreover, our previous study has shown an association between *NPY* polymorphism, and related elevation in NPY levels, and T2D [34]. In addition, NPY has been reported to mimic the potential of light to suppress melatonin secretion [35]. Apparently, the elevated NPY levels in our T2D patients and decreased melatonin levels and *MTNR1B* variant could together account for elevated FBG and T2D risk as shown in Fig. 2.

5. Conclusion

T2D patients showed reduced melatonin levels and association of *MTNR1B* rs10830963 GG genotype with increased FBG levels, suggesting an important link between melatonin, *MTNR1B*, and FBG in T2D. For the first time, we report a possible involvement of melatonin and its receptor gene variant conferring a risk towards T2D in Gujarat population.

Author contributions

RB conceived the idea and designed the experiments. RP performed the experiments. RP, SP, and NR contributed to data acquisition. Data analysis and manuscript writing were performed by RP. RB and AVR did critical revision and approved the manuscript.

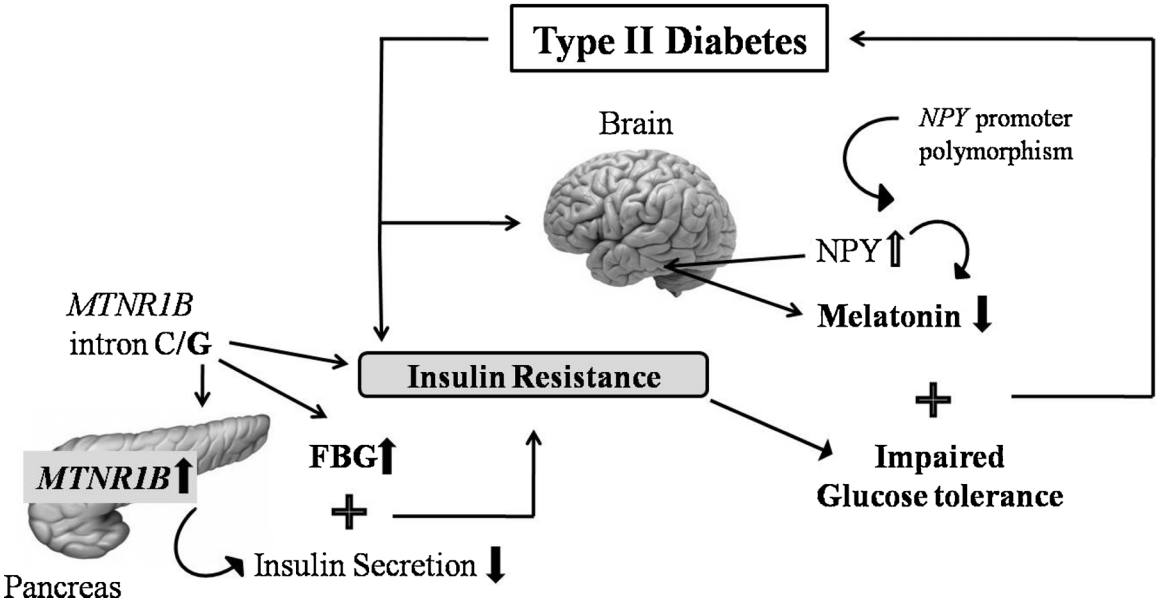

Fig. 2. Role of melatonin and its receptor genetic variant in T2D susceptibility. *NPY* and *MTNR1B* polymorphisms might modulate their respective gene expression. A rise in NPY levels might lead to a reduction in melatonin levels and *MTNR1B* rs10830963 GG genotype might increase FBG levels. The increased *MTNR1B* expression could also contribute to impaired glucose tolerance by reducing insulin secretion. Thus, altogether decreased melatonin along with its receptor genetic variant confers risk towards T2D.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.04.058.

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REVIEW ARTICLE

Treatment Avenues for Type 2 Diabetes and Current Perspectives on Adipokines

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Abstract: ***Background*:** Diabetes has turned into a pandemic disorder that is affecting millions of people worldwide. Industries are aggressively racing and pursuing research towards the discovery of antidiabetic drug and the current global sale of such drugs are ever on the increase. However, in spite of such massive level of expenditure thereof, WHO projects that by 2030, diabetes will rank as the 7th leading cause of mortality.

***Objective*:** It is in this context that we have reviewed here the various approaches available and possible towards diabetes management. This review also includes the WHO guidelines for controlling the glycemic levels, which must be known and followed by clinicians for a better diabetes management.

***Conclusion*:** Despite having a wealth of FDA-approved therapeutic options for type 2 diabetes majorities of the patients are not able to achieve the appropriate glycemic control due to various factors. The development of new options with actions at multiple foci of diabetic manifestation and better efficacy may potentially help in improving the current scenario of T2D management.

Keywords: Type 2 diabetes, therapeutics, gut and brain derived molecules, adipokines, small molecule drugs, phytochemicals.

1. INTRODUCTION

While the past history of mankind has, and is still witnessing the loss of human life due to malnutrition, the modern world is trying to combat diseases caused by unhealthy and excessive eating patterns in developed and developing countries. Although progress in science and technology has enhanced the quality of life, on the flip side, affluence has decreased physical activity especially amongst the economically privileged section of the society. This has led to the increased incidence of lifestyle related disorders such as obesity, impaired lipid profile, hypertension, and diabetes proving to be of great concern to public health. Although sedentary life style and modern dietary patterns have been related with type 2 diabetes (T2D), the interaction of genetic factors has also been suggested to have a role in diabetes manifestation [1].

Prediction based on current trend indicates that by 2030 there would be about 552 million obese and diabetic individuals. Current trends suggest that obesity and T2D have assumed pandemic proportions [2]. India alone is home to more than 65.1 million diabetics [3].

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1.1. Clinical Journey of T2D

T2D is characterized by hyperglycemia resulting from insulin resistance, eventual pancreatic β-cell failure and decreased incretin function [4]. By the time T2D is diagnosed, islet function is often reduced by 50% as compared to healthy controls [5]. The involvement of impaired α-cell function has also been implicated in the pathophysiology of T2D. A hampered α-cell functioning leads to a constitutive rise in the glucagon levels which keeps the blood glucose high even post meal [6].

Research has shown that 74% predisposition towards T2D is due to lifestyle and only 26% due to genetic factors (https://cpmc.coriell.org/about-the-cpmc-study/health-conditions-and-drug-response/type-ii-diabetes/risk-factors-type2-diabetes).

Till the present, several factors have been associated with T2D like obesity, inflammation, mitochondrial dysfunction, hyperinsulinemia, lipotoxicity/hyperlipidemia, genetic background, endoplasmic reticulum (ER) stress, aging, oxidative stress and steatosis [7]. An extremely high correlation between the T2D and obesity has been established in which obesity (adiposity) has been shown to be the major cause of insulin resistance and consequent diabetic manifestations [8]. T2D is a classical metabolic disease, but it is also associated

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Table 1. Glycemic Index (WHO).

Sr no.	Blood Sugar Classification		Fasting (mmol/L)	2-h post-glucose load (mmol/L)
1	Normal		5.5	4.4- 7.7
2	Pre-diabetic	Impaired glucose tolerance	<7.0	7.8-11.0
		Impaired fasting glucose	6.1-6.9	<7.8
3	Diabetic		≥7.0	≥11.1

with a 2-4 fold increased risk of cardiovascular disease [9-12].

A recent report in 2013 established that the rising levels of obesity in South Asians are largely due to nutrition, lifestyle and demographic transitions, ever more due to faulty diets and physical inactivity, in the background of genetic predisposition [13]. In the backdrop of rich nutritious diet, T2D results from dysfunctional carbohydrate metabolism.

1.2. The Multi-Tasking Hormone: Insulin

Insulin is a metabolic hormone produced by pancreatic β-cells present in the islets of Langerhans. It is released into the blood stream in response to a rise in blood glucose level post meal and exhibits diversified effect on various tissues. Primarily, insulin mediates glucose uptake by muscle, fat, and liver cells; and it also stops glucose production in the liver by inhibiting gluconeogenesis. Alongside, insulin also stimulates the liver and muscle tissue to store excess glucose in the form of glycogen. In a healthy person, these functions together maintain the blood glucose and insulin levels in a harmonic balance [14].

Physiologically, insulin is secreted at basal levels between meals to keep a check on the hepatic glucose output. However, post meal it is secreted at higher levels to aid glucose uptake and this occurs in two phases. In the first phase, it reduces basal glucagon secretion, and in the second, *i.e.* 10 min after glucose exposure, the secretion is sustained until normoglycemia is achieved [15]. In T2D, the first phase of insulin response is almost eliminated or is severely blunted [16]. The loss of β-cell function appears to be accompanied by a reduction in β-cell mass [5] which regulates overall insulin secretion. Due to impaired insulin release, the blood glucose levels tend to remain high post meal that is eventually compensated by the second phase of insulin release achieving normoglycemia [16].

Thus, the decreased insulin function leads to chronic hyperglycemia (during both fasting and postprandial stages) and acute glycemic fluctuations. Table **1** represents the classification of diabetics and pre-diabetics based on the glycemic index as prescribed by the World Health Organization (WHO) [17].

1.3. Insulin Resistance

Insulin resistance is a pathophysiological condition wherein insulin-induced glucose uptake is impaired in the insulin-responsive tissues *i.e.* liver, muscle and adipose tissues evoking pre-diabetes/impaired glucose tolerance. Pre-diabetes is associated with high blood HbA1C levels and is the first pathophysiological condition to set in [18], eventually leading to hyperinsulinemia as the β-cells produce a large amount of insulin in an effort to control blood glucose levels [7]. Unchecked/ undetected pre-diabetic stage in the due course of time develops into T2D as the β-cells get exhausted in the process of compensating for the insulin resistance [19].

Most individuals with insulin resistance remain unaware that they are in their pre-diabetic stage for many years until they develop T2D, which is a serious lifelong disorder.

Obesity and the malfunctioning of β-cells have been firmly associated with each other but the molecular pathway is still undefined [20]. Adiposity or obesity has been directly coupled with adipose tissue inflammation and is marked by amplified pro-inflammatory cytokines such as TNF-α. However, it is a mystery whether the low-grade chronic inflammation is adequate to cause islet dysfunction [21-24]. Other factors such as glucolipotoxicity, amyloidosis, failure of β-cell expansion and dedifferentiation and β-cell apoptosis, have also been associated with obesity [18, 25-28]. Thus, though T2D is a multifactorial, polygenic disorder, obesity seems to play a major role in the onset of this disorder [29].

1.4. Obesity: A Cause of Insulin Resistance

Since the discovery of insulin in 1920 and its role in T2D, it has been used as a mono-hormonal therapy for treating diabetic patients [14]. However, the unraveling of additional hormones having glucoregulatory effect has expanded our horizon for search towards innovative therapies for T2D management.

Obesity, caused due to an over accumulation of adipose tissue, is not just a cosmetic concern but a medical condition as well. Excessive body (adipose) accumulates over a period of time to an extent that it starts having a negative effect on one's well-being [30]. In 1962, J. Neel theorized the "thrifty gene hypothesis" to partially explain the rise in obesity-related diseases in the world [31]. The hypothesis tries to explain that, various genes that promote the efficient utilization and storage of fuel might have been selected by nature to favor the survival of the human race during famines. Whereas today, in time of food abundance, the "same genes" make human predisposed to obesity and T2D [32]. Hormonal or other disturbances/imbalances in the early developmental periods may also lead to a thrifty gene phenotype predisposing individuals to diabetes in the adult stage on exposure to potential diabetogenic agents/conditions [33]. Adipose tissue, apart from serving as a store house of en-

ergy, also secretes bioactive peptides, termed 'adipokines/ adipocytokines', which act locally and distally by autocrine, paracrine and endocrine modes [34]. They interact with central and peripheral organs such as brain, liver, and skeletal muscles thus playing an important role in many physical processes [35]. Till date, over 100 adipokines have been identified and studied like leptin, resistin, adiponectin, visfatin, omentin-1, TNF-α, IL-6, etc. Increased production of most adipokines in obese individuals influences multiple functions such as appetite and energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism and homeostasis [36].

Researchers have also found a gripping statistics for a substantial number of T2D patients being lean with BMI <25 [37-39]. Such cases of T2D have been found to be associated with malnutrition [40, 41], smoking [42], alcoholism [43], predisposition to genetic modulators [44], and also impaired adipose expandability [45]. Genome-wide association studies (GWAS) have identified approximately 50 genetic loci to be associated with T2D in lean and obese individuals [46-48]. The road towards the development of T2D remains many and since the cure is still obscure, the approach is restricted to T2D management by achieving glycemic targets.

2. GLYCEMIC TARGETS FOR T2D PATIENTS

The most important goal to prevent and delay diabetes-related complications is to maintain the glycemic target. It has been noticed that achieving glycosylated hemoglobin (HbA1c) level below 6.5% reduces microvascular complications in T2D [49]. Intensive control of blood-glucose levels using sulphonylureas or insulin drastically reduces the risk of complications in patients with T2D when compared with conventional treatment [49, 50].

The WHO [17] recommends four options for diagnosing diabetes as shown in Table **2**.

Table 2. Glycemic targets for T2D patients.

Glycemic Targets	
Fasting Plasma glucose	<7 mmol/L
2-hour plasma glucose	< 11.1 mmol/L
HbA1c	< 6.5%
Random plasma glucose	<11.1 mmol/L

Though the disease can be taken care of by using various anti-diabetic drugs or subcutaneous injections, they do not offer the extent of glycemic control provided by functional pancreatic β-cells.

3. LIFESTYLE INTERVENTIONS FOR THE TREATMENT OF DIABETES

Interventions involving dietary and physical activity changes are widely used and appear to be the most successful approaches for improving long-term weight maintenance and health status [51]. Exercise as a physical activity is suggested to serve as first line therapy for obesity and diabetes [52]. Exercise is known to contribute to glucose homeostasis and improve diabetic manifestations thus decreasing the incidence of diabetes significantly [53, 54]. It brings about significant changes in molecules of insulin signaling pathway and glycogenesis (GLUT4, protein kinase B (PKB), glycogen synthase (GS)) along with lipid profile markers *i.e.* reduction in plasma LDL, total cholesterol, triglyceride levels and TC/HDL ratio [55-57]. It also decreases the levels of pro-inflammatory cytokines like IL-6, C-reactive protein (CRP), TNF-α and IL-1β [58-60] and modulates adipokines such as leptin, resistin, apelin and ghrelin [61, 60]. Interestingly, exercise appears to be a new *modus operandi* for adipose tissue remodeling and modulation of uncoupling protein 1 (UCP1) in brown adipose tissue for improving diabetic manifestation [62]. A profound change in white adipose tissue (WAT) in response to exercise training is the mechanism by which the whole-body metabolic health is improved. Exercise also increases the number of beige cells in WAT that express UCP1, Tbx1, Tmem26, and Cd137 as well as markers of vascularization (*e.g.*, Vegfa, Pdgf, Angptl2) [63]. Moreover, it also promotes mitochondrial biogenesis in skeletal muscle helping ameliorate diabetic manifestations [64, 65]. Hence, exercise induced protein molecules apart from exerting a favorable influence on overall heath can also improve glucose and lipid metabolism and so could serve as a novel therapeutic target. Besides physical activity, people nowadays are also inclined towards calorie restricted diet.

Calorie restriction (CR) is fast developing as a new dietary intervention even though it has its own limitation such as its result reproducibility. CR is described as a reduction in caloric intake, typically by 20-40% of ad libitum consumption while maintaining sufficient intakes of protein and micronutrients to avoid malnutrition [66, 67]. Ideally, dietary treatment should aim to ensure adequate growth and development by reducing excessive fat accumulation and avoiding the loss of lean body mass taking care of overall well-being and preventing cyclical weight regain [68]. There are various dietary approaches for weight loss *i.e.*, low-fat, high-protein, low glycemic index and calorie restricted diets. In this context, *Dietary Guidelines* have recommended certain foods to be consumed less - "foods to reduce" (*i.e.*, saturated and trans fats, cholesterol, sodium, added sugar, refined grains, alcohol) and foods to be consumed more - "foods to increase" (*i.e.*, fruits, vegetables, whole grains, low-fat dairy and protein foods, oils). There is no standard definition of a "high-protein diet;" however, intake of protein greater than 25% of the total energy or 1.6 g/kg per day of body weight can be considered high [69]. As carbohydrates vary in the degree to which they raise blood glucose and insulin levels, a term "glycemic index" (GI) has been coined indicative of the property of carbohydrate-containing food [70]. A low GI diet is a precise blend of low-fat and low-carbohydrate dietary regime. Recommendations for this dietary approach are based not only on GI but also takes into account the nutritional content of the diet as a whole [71].

CR attenuates the degree of oxidative stress [72] and increases expression of genes involved in mitochondrial function and biogenesis such as *PPARGC1A, TFAM,* and *SIRT1* [73]. Elevated rate of whole body fat oxidation in response to calorie restriction was observed along with decreased lev-

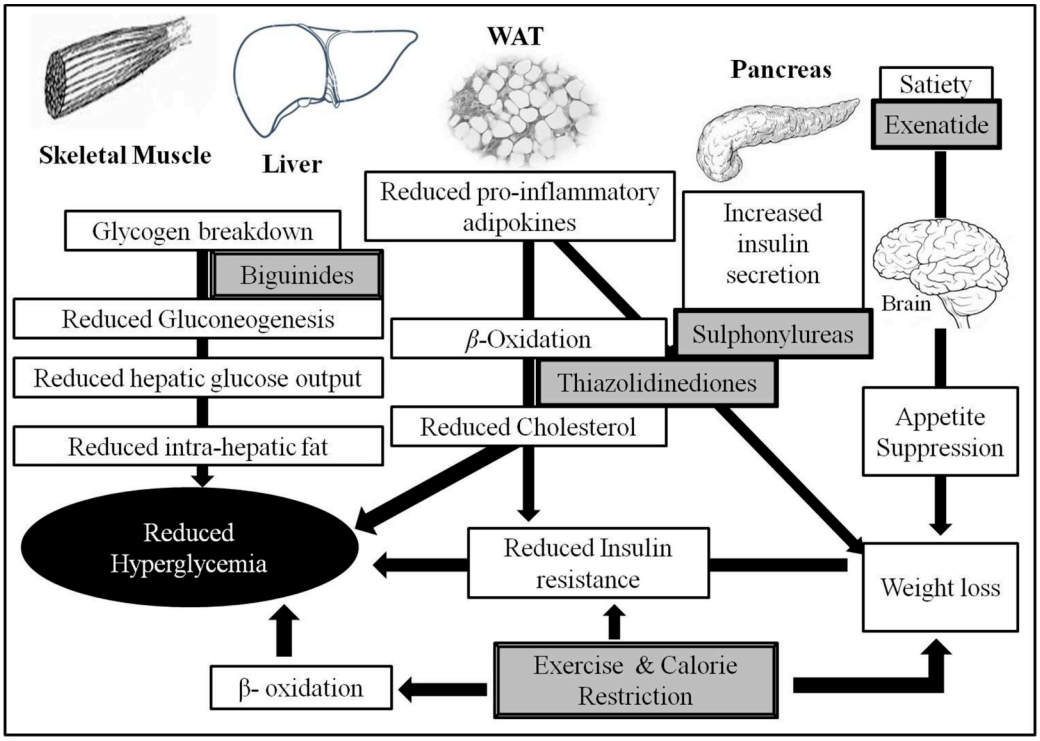

Fig. (1). An illustration showing plethora of effects of exercise, calorie restriction and drugs contributing to reduced hyperglycemia.

els of fatty acid synthesis in liver [74], improved fasting glucose levels besides offering protection from other cardio metabolic risk factors [75], and also reduced pro-inflammatory adipokines [76]. The tight direct relationship between the phosphorylation (and therefore activation) of ERK and $p70^{S6K}$ along with the phosphorylation of $IRS1^{S612}$ and $IRS1^{S632/635}$ implicates ERK and MTOR/ $p70^{S6K}$ as the kinases responsible for the phosphorylation of these sites in the liver as observed in obesity-induced insulin resistance. However, CR diminished activities of these kinases ameliorating insulin resistance [77] as shown in Fig. (**1**).

Thus CR was and still is the traditional first-hand method to control T2D. However, the CR regime would vary from person to person and thus needs to be designed in a person-specific manner. Conversely, strict caloric restriction may work negatively by increasing the risk of hypoglycemia [78], leading to a decreased bone density, ketosis, *etc* [79].

4. THERAPEUTIC MODALITIES AND TARGETS

4.1. Current Strategies (Synthetic Small Molecule Drugs/ Oral Hypoglycemic Agents)

The characteristics of most widely used monotherapeutic modalities are tabulated in Table **3**. The choice of initiating a glucose lowering strategy is based on the level of glycemic control required. When the level of glycemia is high (*e.g.*, A1C >8.5%), therapeutants with a rapid glucose-lowering capacity, or potentially earlier initiation of combination therapy, are recommended. Similarly, when glycemic levels are closer to target goals (*e.g.*, A1C <7.5%), CR or medications with lower hypoglycemic potential may be considered [80]. Below is an overview of traditional and newer/emerging agents used in T2D.

4.1.1. Biguanides

Metformin is the most widely prescribed blood-sugar-lowering drug in the world and is the first line of medication for T2D. It belongs to a class of drugs called biguanides. American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) have jointly recommended metformin as the initial drug to be prescribed if nutritional therapy and exercise prove to be inadequate [81]. Metformin limits glucose production from liver by inhibiting gluconeogenesis and glycogenolysis while increasing insulin sensitivity so that glucose is taken up by muscle, fat, liver, and other types of cells. Metformin monotherapy on an average lowers A1C by approximately 1.5% and it is generally well-tolerated, with the most frequent undesirable effects being gastrointestinal in nature [80]. The major advantage of metformin is that it does not cause hypoglycemia while having positive effect on serum lipids and lipoproteins as compared to other classes of small molecule [82-84]. Metformin non-competitively inhibits the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase and mitochondrial complex I, resulting in an altered hepatocellular redox state, reduced conversion of lactate and glycerol to glucose, and decreased hepatic gluconeogenesis. Alternatively, it has been shown that in mouse hepatocytes, metformin leads to the accumulation of AMP, which inhibits adenylate cyclase, reducing the levels of cAMP and protein kinase A (PKA) activity, and further inhibiting phosphorylation of crucial protein targets of PKA, while blocking glucagon-dependent glucose release from hepatocytes [85-88]. Further, AMPK which is activated by metformin might play a key role in long-term effects of metformin by improving lipid metabolism and mitochondrial function in the liver [89].

Table 3. Characteristics of most widely used monotherapeutic modalities.

Group	Class	Generic name	Side effects
Biguanides	Sensitizer	Metformin	Weight loss, GI upset
Thiazolidinediones		Rosiglitazone Pioglitazone	Weight gain Peripheral edema
Alpha glucosidase inhibitors	----	Acarbose Miglitol	GI upset
Sulfonylureas	Secretagogue	Chlorpropamide Glibenclamide Glimepiride Glipizide Tolazamide Tolbutamide	Hypoglycemia Weight gain
Glinides		Nateglinide Repaglinide	Weight gain
Exenatide	GLP-1 analog	Byetta	Weight loss
Dipeptidyl peptidase-4 inhibitors	DPP-4 inhibitors	Sitagliptin Saxagliptin Linagliptin	----

4.1.2. Sulfonylureas

Sulfonylurea is an insulin secretagogue *i.e.* it lowers glucose levels by triggering insulin secretion from *β*-cells. It closes the potassium channels by binding to adenosine triphosphate (ATP)-sensitive potassium channels and thereby leads to subsequent opening of calcium channels resulting in the exocytosis of insulin. Though the first generation sulfonylureas were efficacy wise similar to metformin, they were however known to cause severe episodes of hypoglycemia. The second-generation sulfonylurea agents (*e.g.*, glipizide, glimepiride) have comparatively lesser side effects [90]. Sulfonylureas bind to and close ATP-sensitive K^+ channels (KATP) on pancreatic beta cell membrane which depolarizes the cell by preventing potassium ions from exiting. This depolarization opens voltage-gated Ca^{2+} channels leading to Ca^{2+} influx. This rise in intracellular calcium leads to increased fusion of insulin granulae with the cell membrane, and therefore increased secretion of (pro) insulin [91].

4.1.3. Glinides

Glinides (*i.e.*, repaglinide, nateglinide) are a similar class of insulin secretagogues like sulfonylurea but bind differently and have a shorter circulating half-life. It depolarizes *β*-cell membrane leading to insulin granule exocytosis and also acts as peroxisome proliferator-activated receptor-gamma (PPARγ) agonist leading to glucose uptake [92, 93]. This necessitates frequent administration. Like metformin and sulfonylurea, glinides too have a similar efficacy of reducing A1C by 0.5 – 0.8% and they also pose a risk of weight gain [80]. Moreover, they have not been associated with episodes of hypoglycemia [94, 95].

4.1.4. α-Glucosidase Inhibitors

The mode of action of α-Glucosidase inhibitors (*e.g.*, Acarbose & Miglitol) is very different from the above classes discussed. They work by reducing the rate of digestion of polysaccharides in the proximal small intestine and thus indirectly lower the postprandial glucose levels. However, compared with metformin and sulfonylureas, they are less effective in lowering glucose reducing A1C by only 0.5% to 0.8%. These drugs function as high affinity reversible inhibitors of alpha-glucosidase, particularly pancreatic alpha-amylase and membrane-bound intestinal alpha-glucosidase. Pancreatic alpha-amylase hydrolyzes complex carbohydrates to oligosaccharides in the lumen of the small intestine while, intestinal glucosidase hydrolyses oligosaccharides, trisaccharides and disaccharides to glucose and other absorbable monosaccharides in the brush border of intestinal villi. The inhibition of these enzymes thus reduces the rate of formation of "absorbable sugars" and thus delays the rise in blood glucose concentration following meals (postprandial). This action therefore results in attenuation of postprandial plasma glucose (30-35% reduction), as well as insulin, gastric inhibitory polypeptide and triglyceride peaks [96]. *α*-Glucosidase inhibitors are commonly associated with increased gastric complications [80].

4.1.5. Thiazolidinediones (TZDs or Glitazones)

TZDs mediate their effect *via* the activation of peroxisome proliferator–activated receptor (PPARγ) largely present in adipose tissue which, modulates the expression of several genes involved in glucose and lipid metabolism, inclusive of those that code for adipocyte fatty acid binding protein, lipoprotein lipase, fatty acid transporter protein, fatty acyl-CoA synthase, malic enzyme, glucokinase and the GLUT4 [97]. Activation of PPARγ is reported to induce adi-

pogenesis and adipocyte differentiation after the activation of C/EBP-α and synergizing with it [98]. TZDs (*i.e.* pioglitazone, rosiglitazone) also increase insulin sensitivity of glucose disposing tissues. Pioglitazone treatment was reported to increase (PPAR)-γ coactivator-1α and mitochondrial transcription factor A leading to mitochondrial biogenesis. Further, it also increases the expression of genes in the fatty acid oxidation pathway such as carnitine palmitoyltransferase-1, malonyl-CoA decarboxylase, and medium-chain acyl-CoA dehydrogenase [99]. TZDs are mostly used as part of a combination therapy. The most common adverse effects associated with TZDs include weight gain, fluid retention, increased subcutaneous adiposity, macular edema, heart failure, and bone fractures [100].

4.2. Current Strategies (Synthetic Large Molecule Drugs)

4.2.1. Insulin

Amongst the various strategies, insulin is the most effective in lowering glycemia and reduces elevated A1C to, or close to, the therapeutic goal. However, because T2D patients are insulin resistant, generally a large dose is required. Insulin therapy has beneficial effects on the triglyceride and HDL-cholesterol levels but is also known to cause weight gain [101] and hypoglycemic episodes. Compared with NPH (Neutral Procaine Haledon) and regular insulin, insulin analogues with longer pharmacokinetic profiles (*e.g*. insulin glargine), as well as, analogues with very short durations of action (*e.g*. insulin lispro), decreases the risk of hypoglycemic episodes [80].

4.2.2. Incretins

Incretins are a class of enteric hormones which regulate blood glucose by stimulating insulin secretion indirectly from the *β*-cells and, the decline of *β*-cells in T2D has been linked to their impaired action [5]. The major ones in this class are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) secreted from endocrine K and L cells respectively in the small intestine in response to a rise in glucose levels. They then activate G protein–coupled receptors on pancreatic *β*-cells thereby stimulating insulin secretion [102]. GLP-1 also inhibits glycogenesis by decreasing the secretion of glucagon. Furthermore, GLP-1 is known to have an effect on the central nervous system like delayed gastric emptying and a feeling of satiety. In contrast, GIP has an effect only on glucagon secretion [103]. What makes GLP-1 a favorable agent is its property to induce insulin secretion in response to the raised blood glucose level post meal. This reduces the chances of adverse side effects such as sudden hypoglycemia [104]. Studies on T2D individuals have shown increased insulin secretion and concomitant decreased glucagon secretion on treatment with GLP-1 receptor agonists [5].

4.2.3. DPP 4 Inhibitors

Dipeptidyl-peptidase IV (DPP-4) is a ubiquitous serine protease acting on a variety of substrates ranging from hormones to chemokines to neuropeptides [105]. In the enteric system it cleaves GLP-1 and GIP secreted from gastric mucosa, trimming down their half-life to few minutes in plasma. DPP-4 inhibitors are being used to sustain the rise in GLP-1 level post meal in fasting conditions as well, thus keeping a check on hyperglycemia [106]. DPP-4 has also been identified as a novel adipokine with a significantly high expression in visceral fat of obese subjects impairing insulin signaling at Akt level in the glucose disposal organs [107]. The same group has also shown the augmented release of DPP-4 (by 50%) in response to obesity-related TNF-*α* elevation and an inhibition of the anti-lipolytic action of Neuropeptide Y (NPY) [108].

Strategies such as the development of DPP-4 resistant GLP-1 analogues (*e.g*., exenatide, liraglutide) as well as molecules that inhibit the enzymatic activity of DPP-4 (*e.g.*, sitagliptin, vildagliptin, saxagliptin) have already been extensively attempted [109, 110].

4.3. New and Emerging Therapies

Over the counter, oral drugs discussed till now mostly stimulate insulin release, suppress hepatic glucose output and assist glucose disposal but they only work towards diabetes management by controlling hyperglycemia [110]. With reference to both type 1 and type 2 diabetes, wherein there is *β*-cell loss sooner or later, the therapeutic focus has now shifted from merely controlling glycemic targets to regeneration or preservation of *β*-cell mass. A lot of work has been carried out in this context in the past few decades and as a result, a large number of agonists (*e.g*. Betatrophin) have been identified through high throughput screening that induces *β*-cell replication in animal models. In this context, work from our own group has shown flavonoid mixture from *Oreocnide integrifolia* (Urticaceae), a folklore plant, to have significant insulin secretagogue, insulinomimetic and cytoprotective effects [111]. But unfortunately, very few such molecules have been found to induce *β*-cell replication at a substantial rate from a therapeutic point of view in humans [112].

Of late, hormones and other protein molecules have also gained a great deal of focus as therapeutic agents by virtue of their biological significance encompassing an array of various functions as illustrated in Fig. (**2**) [113].

4.3.1. Brain: The Seat of Hunger and Satiety

The long posited theory, that brain was critical in the negative feedback regulation of appetite and body weight was found to be true as it was found that brain requires an incessant supply of glucose for meeting its energy demands [114] making it the highest consumer of glucose [115]. Due to this strict dependence on glucose, brain exerts regulation on blood glucose levels through an array of inter-coordinating hormones (leptin, ghrelin, NPY, glucagon-like peptide-1, insulin, *etc*.) to achieve a precise physiological balance [116-118]. While NPY and ghrelin are secreted in response to activation of the nutritional prompt "feeding center" by a fall in the blood glucose level, the rest are secreted in response to activation of "satiety center" by a rise in the glucose level, making the brain a "dual-core" system [115]. Also, reduced plasma levels of brain-derived neurotrophic factor (BDNF) have been associated with impaired glucose metabolism and type II diabetes in human subjects [119]. Simultaneously, caloric restriction and exercise have been shown to elevate BDNF levels by various mechanisms [120, 121]. Increased insulin-stimulated tyrosine phosphorylation

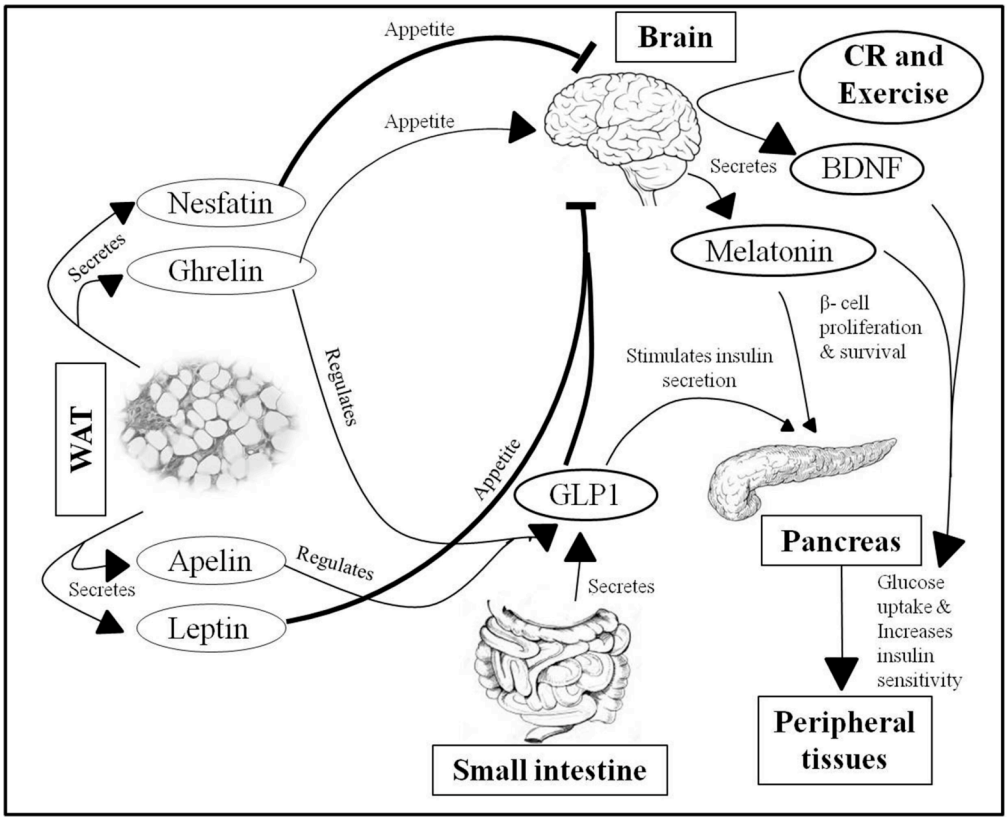

Fig. (2). The Gut –Brain axis in diabetic regulation.

of insulin receptors in liver and PI3-kinase activity in liver, skeletal muscle and brown adipose were demonstrated in *db/db* mice when administered with BDNF for 14 days [122]. Any disturbance in the energy homeostatic balance leads to conditions like hyperglycemia or T2D.

4.3.1.1. Melatonin

Melatonin, referred as the hormone of darkness, is mainly secreted by the pineal gland with a night time high and day time low [123]. Its primary role has been identified in maintaining body homeostasis and biological clock *i.e*. the sleep-wake cycle [124]. It is thus, referred as "Sleep hormone" too. Apart from regulating the "Clock genes" it has also been strongly linked to T2D [125]. Many past studies from our group have shown hypoglycemic and promotion of peripheral glucose uptake effects of melatonin in the vertebrate series based on melatonin administration and pinealectomy [126-128]. Recently, it was further shown that melatonin supplementation exhibited greater potency than estrogen replacement therapy in overcoming diabetogenic metabolic dysregulation in ovariectomized/estrogen deficient rats [129]. Studies describing the effect of melatonin on various glucose responsive tissues expressing MT2 (Melatonin receptor Type 2) are available. Accordingly, in human adipocytes, it downregulates GLUT4 expression [130], in murine skeletal muscle it enhances insulin receptor substrate-1(IRS-1) phosphorylation [131] and in mouse liver it induces glucose release [132]. Interestingly, MT1 (Melatonin receptor Type 1) and MT2 receptors are also expressed on the pancreatic islet cells [133, 134]. In the *β*-cells, it decreases insulin secretion by inhibiting cAMP and cGMP pathways while, it enhances the secretion of insulin by increasing cytoplasmic Ca^{2+} concentration *via* phospholipase C/IP3 pathway activation [135, 136]. The modulatory effect of melatonin also extends to glucagon secretion from *α*-cells apart from insulin secretion from *β*-cells [137]. Thus, a reduction in melatonin could potentially contribute to the genesis of diabetes as, a functional inter-relationship between melatonin and insulin is revealed in diabetic patients [138, 139]. Additional evidences from experimental studies are available for melatonin promoted insulin receptor tyrosine phosphorylation and production of insulin growth factor [140]. Shi *et al*. [141] further demonstrated insulin resistance and glucose intolerance in individuals with a disturbed internal circadian system which could however, be re-established by melatonin supplementation [142, 143]. Our group had earlier demonstrated increased GLUT4 expression in the muscle tissue of diabetic rats upon melatonin supplementation. In other studies, the anti-diabetogenic effects of melatonin as a de-programmer of early neonatal corticosterone induced thrifty phenotype for adult diabetogenic manifestations had also been recorded [33, 144].

Therefore, the existence of melatonin receptors on islet cells may be targeted to design pharmacotherapy for T2D. Melatonin is currently under intensive scrutiny in experimental animal models of diabetes, obesity, and metabolic syndrome [145].

4.3.2. GUT: The Second Brain

4.3.2.1. Ghrelin

Ghrelin (a gut – brain peptide) is synthesized mainly by the P/D cells of human gastric mucosa [146]. However, ghrelin is also found in many other tissues like the hypo-

thalamus, brain cortex, pituitary, adrenal, hippocampus and pancreas [147, 148]. In obese individuals ghrelin is found to be attenuated [149].

Ghrelin possesses growth hormone-releasing activity, adipogenic activity, and orexigenic activity by acting as a ligand to the growth hormone secretagogue receptor 1a (GHSR) in CNS [148, 150-152] and, its level is controlled by a number of factors like food intake, insulin and glucagon levels. Apart from playing a role in regulating energy homeostasis, the presence of ghrelin and its receptors on the pancreatic α and β-cells indicates its additional glucoregulatory role [153]. In a study by Tong and colleagues, it was reported that both supraphysiological and physiological doses of ghrelin in healthy individuals suppressed insulin secretion leading to an impaired glucose tolerance but interestingly, the insulin sensitivity was unaltered in the latter dose [154, 155]. In another independent study by Vestergaard *et al*. [156], intramuscular administration of ghrelin enhanced glucose uptake and lowered blood glucose level. They had earlier shown this effect of ghrelin to be without any effect on the insulin signaling pathway [157]. Thus, the above studies are suggestive of the pharmacological potential of ghrelin by its effect on glucose-stimulated insulin secretion (GSIS) and insulin sensitivity. Ghrelin has also been demonstrated to suppress glucose-induced insulin release *via* GTP-binding proteins and delay K+ efflux thereby regulating insulin release and glycemia [158]. There are also reports of ghrelin priming intestinal L cells for the production of GLP1 incretin hormone which improves glucose tolerance by stimulating insulin production by β-cells and decreasing glucagon production by α-cells [159]. Maintaining a critical level of serum ghrelin by its neutralization using antagonists or anti-ghrelin antibodies might be worthwhile to study. Since insulin is known to inhibit ghrelin, usage of insulin mimetics to regulate ghrelin level could also be a novel approach towards T2D management.

4.3.3. Adipokines

4.3.3.1. Adiponectin

ADIPOQ gene located on chromosome 3q27 codes for the 30 kDa adiponectin protein [160]. The protein is exclusively expressed in white adipose tissue. Adiponectin is found in various polymorphic forms in plasma. It is found in 3 major oligomeric forms; a low–molecular weight (LMW) trimer, a mid–molecular weight (MMW) hexamer, and a high–molecular weight (HMW) 12- to 18-mer [161, 162].

Normal level of adiponectin in the circulation is 2-20 μg/mL and forms 0.05% of total serum protein. Apart from its insulin sensitizing action, adiponectin is also responsible for free fatty-acid combustion *via* PPARα activation and increasing AMP: ATP ratio by AMPK activation and thus plays a pivotal role in energy metabolism [163]

The primary mechanism by which adiponectin enhances insulin sensitivity appears to be through increased fatty acid oxidation and suppression of gluconeogenesis thus decreasing the triglyceride content in liver and skeletal muscle, and enhancing insulin sensitivity [163, 164]. The mechanism of action of adiponectin on muscle appears to be through activation of AMP kinase (AMPK) and PPARα [165, 166]. In the case of liver, adiponectin promotes glucose uptake and stalls gluconeogenesis and, activates fatty acid oxidation and decreases inflammation *via* PPARα [167]. Interestingly, it has been found that adiponectin levels are compromised in obese, insulin-resistant rodent models [168] and also in obese rhesus monkeys that develop T2D [169]. More interestingly, reduced insulin sensitivity in conjunction with decreased plasma adiponectin level was also noted in these animal models [169]. Similar observations were reported in obese humans as well, particularly those with visceral obesity [170-172]. In humans, caloric restriction and physical exercise have been shown to increase circulating adiponectin levels significantly and also to attenuate the TNF-α to adiponectin ratio [173, 174].

Restoring the adipokine level or increasing AMPK and PPARα levels may in this context prove beneficial. Many such molecules have already been studied, each having its own limitations. Recently, a small-molecule adiponectin receptor agonist- Adiporon was reported to improve insulin sensitivity without altering insulin secretion [175]. However, activating adiponectin alone or increasing AMPK level might not be an ultimate answer to β-cell loss. Among the several adipokines, adiponectin has of late attracted a good deal of attention by virtue of its antidiabetic and antiatherogenic effects [176].

4.3.3.2. Resistin

Resistin gene was originally identified present on chromosome no. 19 of mouse in 2001. Resistin (12.5 kDa) is an unusual hormone in the sense that it has 11 cysteine residues out of a total of 114 amino acids [177]. In serum, resistin circulates predominantly as trimers and hexamers, with the trimer being the most bioactive form [178]. Resistin is expressed at very low levels in human adipose cells whereas; high levels are expressed in spleen, bone marrow, mononuclear leukocytes and macrophages [178-180]. Some studies have suggested that mature human adipocytes lack resistin expression, while preadipocytes do [181, 182]. Infusion of resistin in Sprague-Dawley rats resulted in weakened hepatic insulin sensitivity and glucose metabolism [183] and, chronic elevated circulating resistin levels led to increased fasting glucose, weakened glucose tolerance and decreased hepatic insulin sensitivity [184]. Resistin was also reported to induce SOCS3, resulting in the suppression of insulin-mediated signaling in adipocytes [185].

However, the function of resistin in humans has been inconclusive [186-188]. Many studies have shown a positive correlation between elevated serum resistin level and insulin resistance and obesity in humans. Sheng *et al.* [189] observed resistin to be expressed in human hepatocytes while Tsiotra *et al.* [190] and Gharibeh *et al.* [191] observed that resistin caused insulin resistance in female subjects with T2D and obese T2D patients. However, contradictory results from human studies indicate resistin gene expression and its circulating levels to be both increased and unchanged in obesity and insulin resistance [192-195]. Resistin was identified as a pro-inflammatory adipokine mediating its action *via* TNF-α by activation of NF-κB pathway [196] and recruitment of immune cells [197]. And recent study unraveled that resistin binds to adenyl cyclase associated protein 1receptor which increases cAMP and PKA activity [198]. In a study by Steppan *et al*. [199], blocking of resistin action with neutral-

izing antibody was found to improve whole-body insulin sensitivity in diet-induced obese (DIO) mice while, antisense oligodeoxynucleotide against resistin mRNA completely reversed hepatic insulin resistance in animals [200]. It is likely that resistin is a biomarker for and/or contributes to insulin resistance in specific populations.

4.3.3.3. Omentin-1

Omentin-1 is a novel 40 kDa fat depot-specific adipokine (gene bank accession number- AY549722) which has been identified from a cDNA library of visceral omental adipose tissue [201], located on 1q21.3 chromosome locus [202]. It is also known variously as intelectin-1, intestinal lactoferrin factor, endothelial lectin HL-1 or galactofuranose binding lectin. In humans, omentin is expressed as two homologous proteins, omentin-1 and -2, encoded by two separate genes located adjacent to one another on 1q22-q23. Omentin-1 is the major circulating isoform in human plasma. Omentin-2 shows 83% amino acid identity with omentin-1 [203]. Omentin mediated glucose uptake occurs *via* the phosphorylation of Akt at physiological concentrations [204, 205].

Omentin gene expression in visceral adipose tissue and circulating omentin level were reported to be decreased in obese subjects [203] associated with impaired glucose tolerance (IGT) and in T2D subjects [206-208]. However, circulating omentin levels were found to be elevated in patients with nonalcoholic fatty liver disease [209]. Omentin receptor, target tissues, and signaling mechanism remain obscure as yet, but the above studies are indicative of its potential as a therapeutant.

4.3.3.4. Vaspin

Vaspin, a visceral adipose tissue (VAT) derived serine protease inhibitor has an insulin-sensitizing effect and belongs to the serpin superfamily (Serpina12). It was found in the VAT of Otsuka Long-Evans Tokushima Fatty rat (OLETF) typified with central obesity and T2D [210]. Vaspin acts as a circulating serpin, which serves as a ligand for a cell-surface receptor complex, GRP78/MTJ-1, and exerts anti-inflammatory action in ER induced stress [211]. In another study, Nakatsuka *et al*. [212] showed vaspin to serve as a ligand for a cell-surface voltage-gated anion channel complex in endothelial cells thereby exerting anti-apoptotic, proliferative, and protective effects on the endothelium of rat models with T2D. Furthermore, vaspin also protects endothelial cells by its inhibitory action on NF-κB [213].

Increased serum vaspin was found to be associated with obesity in young Korean men [214] and also with BMI, triglycerides, fasting insulin and insulin resistance in pubertal obese children [215]. However, administration of recombinant vaspin in obese mice showed to improve glucose tolerance and insulin sensitivity suggesting the rise in vaspin levels to be a compensatory rise in response to obesity and insulin resistance. Interestingly, it was also higher in healthy females as compared to healthy males demonstrating sexual dimorphism [216]. On the other hand, several studies have failed to show even a simple correlation between serum vaspin levels and BMI [217] and insulin sensitivity [218] or with T2D [219].

Interestingly, vaspin influences insulin-induced glucose uptake *in vivo*, but not *in vitro*. Vaspin probably modulates insulin action only in the presence of its target proteases, which most probably trigger altered insulin sensitivity. Therefore, identification of vaspin's target protease is the major challenge for future studies related to vaspin. Unraveling the proteases might lead to the development of novel anti-diabetic therapy, which may improve insulin sensitivity in patients with T2D.

4.3.3.5. Leptin

Leptin is synthesized and secreted by the adipose tissue in proportion to the amount of fat deposition [220-222]. However, it mediates its action *via* brain as an anorexigenic hormone [223]. It was found that injection of recombinant leptin on daily basis into normal mice reduced their appetite while increasing their energy expenditure. This resulted in the elimination of fat deposits in a short span of time without causing hypoglycemia [224, 225]. These results made leptin a potent anti-obesity drug. However, it was soon, also, observed that leptin levels were already elevated in obese individuals [149]. Detailed leptin-based clinical trials by NIH show significant hyperleptinemia under obese conditions (www.clinicaltrials.gov and http:// www.ncbi.nlm.nih.Gov/ pubmed/). This suggests of a possible leptin resistance [226-228] which can be due to three possible reasons: i) inefficient/weakened transport of leptin across the blood-brain-barrier (BBB), ii) diminished neuronal leptin signaling in the target neurons, and iii) impaired downstream signaling cascade of target cells [229].

Leptin regulates body weight and neuro-endocrine functions apart from appetite through its receptors in CNS [220, 221]. Leptin's effect on body weight has been shown to be *via* GABAergic neurons in mice [230]. Though the mechanism through which leptin functions remains still obscure, a substantial amount of data strengthens its glucoregulatory effect [231, 232]. Thus, leptin may serve as a therapeutic solution for lean as well as obese type 2 diabetics by means of a "Brute force" effect (exogenous leptin given despite leptin resistance).

4.3.3.6. Nesfatin

Nesfatin-1was discovered for the first time in 2006 as a satiety hormone [233] and further studies [234] provided evidence for it to be another hormone involved in the regulation of energy metabolism. Specifically, it is secreted by the peripheral adipose tissue, gastric mucosa, pancreatic endocrine β-cells, and testis [235]. Intraperitoneal injection of nesfatin-1 in rats reportedly suppressed food intake in a dose-dependent manner [236]. It is also shown to work independent of the leptin pathway [233], thus making nesfatin-1 a possible mode of treatment in obese individuals with leptin gene mutation or leptin resistance [237]. There are a number of theories that explain its action; the first being by activation of the melatonin pathway and, the second being by inducing NPY secretion [235, 237-239].

Additionally, it was found that there was a significant decrease in food intake and body weight on a continuous infusion of nesfatin-1 into the third ventricle of brain in rats [233]. Downregulation of gluconeogenesis and promotion of peripheral glucose uptake were the attendant effects noticed

by such infusions [240]. However, in multiple clinical studies, it was noted that plasma nesfatin-1 levels were higher in T2D patients and was associated with the homeostasis model assessment of insulin resistance (HOMA), BMI and plasma insulin. This paradoxical elevation of plasma nesfatin-1 in T2D patients is hypothesized to be a compensatory upregulation to recompense for the metabolic stress imposed by obesity or a possible nesfatin-1 resistance [241]. Improper understanding of nesfatin-1 action precludes detailed elucidation of its role in T2D and glucoregulation. However, nesfatin-1induced activation of β-cell Ca^{+2} channels and the resultant increased insulin secretion [242] opens up avenues to explore the feasibility of nesfatin-1 in the amelioration of T2D.

4.3.3.7. Visfatin

Visfatin has recently been identified as a novel adipokine and also as a pre β-cell colony-enhancing factor [243]. Much greater expression in visceral fat tissue has formed the basis for its name [244]. However, some other studies showed visfatin to be expressed by the macrophages infiltrating adipose tissue in response to the inflammatory signals emanating from various other tissues [245, 246]. Visfatin was found to be insulin mimetic in action as it increased glucose uptake in adipocytes and myocytes. It also exerted other actions like suppressing hepatic glucose release and stimulating hepatic triglyceride accumulation and, increasing its own synthesis in pre-adipocytes in mice models [247]. Visfatin was found to exert its effect by activating insulin receptors *via* a different binding site, causing receptor phosphorylation and the activation of the downstream signaling molecules [248]. In a meta-analysis study, volunteers categorized as overweight/obese or type 2 diabetic, Chang and colleagues observed the plasma visfatin concentrations to be increased [249]. This provides hope for its exploitation as a possible diagnostic marker for diabetes.

4.3.3.8. Retinol Binding Protein 4 (RBP4)

RBP4 is a carrier protein of retinol (vitamin A alcohol) in circulation. It is bound to transthyretin in circulation and its physiological function is to prevent the kidney excretion of retinol [250].

The first key link between RBP4 and diabetes was the observation of an eminently higher plasma level of RBP4 in obese and T2D mice and humans [201] and, alleviation of insulin resistance in diet-induced obesity by an induced experimental decrease in RBP4 [251]. Yang's group also showed the expression of gluconeogenic enzyme (phosphoenolpyruvate carboxykinase) in liver and attenuation of insulin signaling in muscle by preventing insulin receptor substrate-1phosphorylation and activation of phosphatidylinositol-3-kinase in mice by RBP4.

RBP4 has also been reported to cause adipose tissue inflammation by the activation of JNK inflammatory pathway leading to the priming of antigen presenting cell (APC) activation and consequent overshooting of the balance towards differentiation of adipose tissue resident APCs into M1 macrophages exhibiting increased pro-inflammatory gene expression [252-255]. Thus, reducing the RBP4 levels can be a potential therapeutic strategy by means such as Transthyretin Antisense Oligonucleotides [256] or Anti-TNF-α therapy [257].

4.3.3.9. Apelin

Apelin was detected in adipose tissue [258] and later shown to be produced and secreted by adipocytes [259]. Apelin has been identified as an adipokine which increased during adipogenesis [259, 260]. One of the main regulators of apelin is insulin and, a close relation between insulin and apelin has been shown both *in vivo* and *in vitro* [259]. The expression of apelin in adipocytes has been shown to be increased in various mouse models of obesity associated with hyperinsulinemia. The pattern of apelin expression in adipocytes paralleled the plasma levels of insulin in mice under conditions of fasting and re-feeding. Even in *in vitro* studies involving cultured adipocytes (3T3F442A) and isolated human adipocytes, expression and release of apelin is shown to be increased on insulin treatment. Apelin receptors (APJ) find wide expression in various tissues such as stomach, heart, lung, skeletal muscle, etc. along with being expressed in hypothalamus [259, 261, 262]. One of the first actions of apelin noted was its role in energy metabolism and, the same group also demonstrated its action on intestinal glucose absorption in a murine model [263]. It was also reported to promote glucose uptake by the enterocytes by overexpression of GLUT2 channels and upregulation of GLP1 secretion [264, 265]. In peripheral tissues, apelin exhibits a glucoregulatory action by stimulating Akt and AMPK phosphorylation [264, 266]. Consequently, it was also shown that apelin treatment improved mitochondrial biogenesis [267] and insulin sensitivity in insulin-resistant obese mice [263]. Adding to the apelin quest, Xu *et al.* [268] demonstrated apelin facilitated GLUT4 translocation in C57BL/6J mice.

As apelin receptors also exist on β-cells, it is assumed to have a paracrine or autocrine regulatory action on insulin secretion thereby preventing hyperinsulinemia and contributing to improved insulin sensitivity [269]. Apelin was also shown to inhibit lipolysis in 3T3 L1 cells [270] and *in vivo* studies indicated it to be through activation of AMPK [271]. Consistent with these findings, many studies have shown increased plasma apelin concentrations in obese and/or diabetic subjects with higher insulin levels [261].

All the reported data put together suggests that apelin could play a major role in glucose homeostasis by increasing insulin sensitivity and insulin secretion along with a concomitant suppression of lipolysis.

4.3.3.10. Adipsin

Adipsin was the first adipokine to be studied [272] and, it functions in the complement pathway as factor D [273, 274]. Adipsin cleaves factor B only when in complex with C3b, catalyzing the formation of the C3 convertase (C3bBb), which can act on C3 to liberate C3a. C3a stimulates insulin secretion by localizing Ca^{2+} in β-cells only in the presence of elevated glucose levels. In addition, the half-life of C3a is very short as it is rapidly inactivated by serum carboxypeptidases to its inactive form- C3a-desArg [275]. Its levels are known to drastically decline in obese and diabetic animal models [276]. Interestingly, restoration of adipsin expression increased post prandial insulin levels in T2D patients. It was also noted that the circulating levels of adipsin were

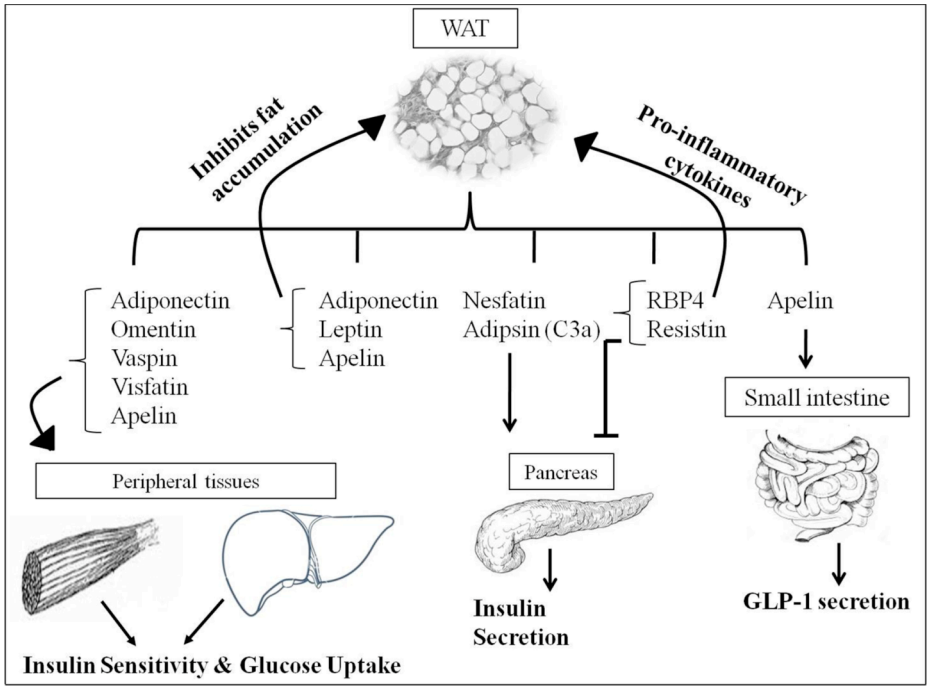

Fig. (3). An illustration of the interplay of various adipokines in maintaining glucose homeostasis.

significantly decreased in T2D patients with β-cell failure when compared with T2D patients without the evidence of β-cell failure. This observation suggested that adipsin dysregulation in diabetic patients might be one of the factors leading to β-cell insufficiency. Thus, adipsin seems to fill in the gap between adipose tissue metabolism and pancreatic β-cell function. Adipsin levels may prove to be a predicting biomarker to help a physician identify individuals at highest risk of impending β-cell failure [275].

The characteristic glucose-dependent insulin secretagogue property makes C3a an ideal drug having an in-built negative feedback coordination reducing the chances of hypoglycemia. However, strategies to overcome its short half-life need to be evaluated and its usage at the cost of generating mitochondrial oxidative stress and its long-term effects needs to be assessed in detail [276].

By and large, every adipokine discussed in this section plays a role in the maintenance of glucose homeostasis as representated in Fig. (**3**).

4.3.4. Phytochemicals

Antidiabetic plants are known to be ubiquitously present worldwide. The extracts from these antidiabetic plants exert wide ranging effects such as stimulate B cell regeneration/proliferation, exert hypolipidemic and antioxidative effects, modulate glucose metabolism, alleviate diabetic complications and also act as insulin mimetics [277]. The active ingredient in the plant extracts are, for example, bassic acid (*B. sartorum)* and, natural flavonoids like quercetin and kaempferol (*E. alatus*) and many others. They possess the capacity to reduce hyperglycemia by promoting glucose uptake and glycogen synthesis [278-280]. Dieckol, a compound isolated from *E. cava*, too exhibits antidiabetic properties by inhibiting α-glucosidase and α-amylase as with acarbose, a pharmaceutical antidiabetic [281]. Though the exact mechanism is still obscure, a study carried out by Kang *et al*. [282], suggested Dieckol to mediate its action *via* AMPK and Akt signaling pathways. In addition, the root extract of *B. aristata* and Comatin, an active ingredient from *C. comatus* were found to reduce insulin resistance and enhance glucose homeostasis [283, 284]. Studies in this direction have also demonstrated the favourable influence of not only a poly-herbal extract in rats but even single plant extract and its isolated flavonoid rich fraction on various facets of carbohydrate metabolism and β-cell neogenesis in a T2D mouse model [111, 285-287]. The active compounds obtained from medicinal herbs and their properties are shown in Table **4**.

The medicinal plants with antidiabetic properties also have bioactive compounds like (−) epicatechin (a flavonoid), marsupin (benzofuranone), and pterosupin (a dihydrochalcone) which have been shown to decrease blood glucose level in diabetics as effectively as metformin [253, 354]. A sulfated flavonoid from *P. discolor* extract was reported to inhibit aldose reductase in experimental animals and, clinical trials of the same showed anti-hyperglycemic effect [355]. There are also other active compounds like amorfrutins isolated from licorice (*G. foetida)* which mediate their action *via* activation of PPAR γ, a central player in glucose and lipid metabolism [288]. Vanillin and 4-hydroxybenzaldehyde (Table **4**) are shown to reduce insulin resistance by decreasing adipogenesis and increasing fatty acid oxidation and leptin signaling in obese rats [289]. Further, cytopiloyne has been reported to bring about insulin release from β-cells by increasing the levels of DAG and PKCα and promoting Ca^{2+} influx [290]. Additionally, capsaicin, an active constituent of chili pepper has been shown to activate AMPK in 3T3-L1 preadipocytes [322]. EGCG acts in multiple ways as mentioned in Table **4**. It affords protection against β-cell death mediated *via* islet amyloid polypeptide (IAP) *in vitro* [356] and also activates AMPK in adipocytes [322]. Resveratrol,

Table 4. **Active antidiabetic compounds from plants and their properties.**

Plant Name	Active Compound	Property	Reference
G. uralensis	Amorfrutin 1-4	Regulates Insulin Resistance	[288]
G. elata	Vanillin, 4 hydroxy-benzaldehyde	Reduces Insulin Resistance	[289]
C. verum, C. Zeylanium C. aromaticum	Cinnamaldehyde	Reduces Insulin Resistance	[290]
T. foenum-graceum	Diosgenin Galactomannan Trigoneoside Xa, Xb, X1b, XIIa, XIIb, XIIIa, Ia, Ib, Va G hydroxylisoleucine	Reduces Insulin Resistance	[291, 292]
T. divaricate E. microphylla	Conophylline	Regulates β-cell function	[293-296]
roxburghii	Kinsenoside	Regulates β-cell function	[297]
N. stellata	Nymphayol	Regulates β-cell function	[298]
S. marianum	Silybin Silydianin, Silychristin	Regulates β-cell function	[299-307]
B. pilosa	3-β-D-glucopyranosyl-1-hydroxy-6(E)-tetradecene-8,10,12-triyne 2-β-D-glucopyranosyloxy-1-hydroxy-5(E)-tridecene-7,9,11-triyne 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (cytopiloyne)	Regulates β-cell function	[308, 309, 290]
Dietary fibers from roots of A. tequilana	Inulin/Raftilose	Regulates GLP-1 function	[310-312]
japonica	Butyl-isobutyl pthalate	Glucose absorption in gut	[313]
B. vulgaris	Berberine	Decrease hyperglycemia, Increase insulin resistance, Increase pancreatic β-cell regulation, decrease lipid peroxidation	[314-319]
M. charantia	Momordicin	Decrease blood glucose	[320]
P. Clausseni-anum	2',6'-dihydroxy-4'-methoxychalcone	Decrease blood glucose	[321]
Capsicum plants	Capsaicin	Regulates insulin resistance and β-cells	[322, 323]
P. ginseng	Ginsenoside Rb1, Rb2, Rc, Rd, Re, Rf, Rg1	Regulates insulin resistance and β-cells	[324, 325]
longa	Curcumin Turmerin	Regulates insulin resistance and β-cells	[326, 327]
I. paraguariensis	3,5,-o matesaponin2	Increase GLP1 production	[328]
Z. officinale	Gingerol Shogaol	Regulates insulin receptor signaling Increase islet cell proliferation and insulin sensitivity	[329, 330]
C. sinensis	Epigallocatechin 3 gallate (EGCG)	Islet protection, Increase insulin secretion and insulin tolerance, Decrease gluconeogenesis Insulin mimetic action	[331-333]

(Table 4) contd…

Plant Name	Active Compound	Property	Reference
I. okamurae	Diphlorethohydroxy carmalol	α-glycosidase and α-amylase inhibitor	[334]
G. max	Genistein	Increase islet mass and insulin sensitivity, Activates PKA, ERK1/2, AMPK	[322, 335-338]
	Glyceollin I, II, III	GLP-1 and insulin secretion, β-cell function	[339]
A. linearis	Aspalathin Rutin	Regulates insulin tolerance and β-cell function, α-glucosidase inhibitor	[340-344]
A. vera	Aloresin A	Decrease α-glucosidase and insulin resistance	[345]
E. jambolana	FIIc	Antidiabetic and antioxidant	[346]
Rupestris C. aerea	Phenol, 2,4-bis (1,1-dimethylethyl) and z, z-6,28-heptatriactontadien-2-one	α-amylase inhibitor and antioxidant	[347]
S. sonchifolius (ECU44)	4,5-di-O-caffeoylquinic acid (CQA) and 3,5-di-O-CQA	α-amylase and α-glucosidase inhibitor	[348]
P. integerrima	Pistagremic acid	α-glucosidase enzyme inhibition	[349]
H. thebaica	Luteolin 7-O-[6"-O-α-Lrhamnopyranosyl]-β-D-galactopyranoside and chrysoeriol 7-O-β-D-galactopyranosyl(1→2)-α-L-arabinofuranoside	Ameliorate glucose and insulin tolerance, Reduces AST and ALT levels of liver	[350]
E. jambolana	LH II	Antidiabetic	[351]
E. addisoniae	2'"-dimethyldihydropyrano [5'",6'"] and isoflavanone and 2,3-dihydroauriculatin	Tyrosine phosphatase 1B (PTP1B) inhibitor	[352]

commonly found in plants has potential to activate AMPK and other downstream molecules which are shown to decrease insulin resistance in diabetic mice [357, 358].

Since diabetic manifestations involve free radical associated damage in beta cells and their apoptosis accompanied with insulin resistance and hyperglycemia a combination of these well-studied phytochemicals can effectively target the pathophysiological conditions and prove to be a better treatment paradigm than either/alone.

CONCLUSION

Type 2 diabetes is a metabolic disorder that can be prevented/ controlled through lifestyle modification, diet control, and weight management. Despite the presence of several treatment options to aid the control and management of this disorder, majority of patients with T2D do not achieve appropriate glycemic control and also suffer from major or minor side effects. Since a one stop solution seems more lucrative, most pharma and biopharma companies seem to be in a competitive race for developing novel drugs with minimal side effects. Though a total cure is still elusive, newer insight into the pathophysiology of the disease is coming to light. While synthetic small molecule drugs pose long-term side effects, modulating adipokine levels seem to be the promising approach to evade side effects. As adipokines have intricate involvement and functions in the regulation of appetite, satiety, energy expenditure and physical activity, they are the most promising contenders which, can serve as tools for weight loss interventions in the future. T2D being characterized by hyperglycemia, hyperlipidemia, and hyperinsulinemia as mentioned earlier, interventions at any one or more of these triad manifestations along with β-cell regeneration may not only help manage T2D but also alleviate the disorder to a greater extent. Though researches as of now project adipokines as potent therapeutic agents, a lack of in-depth knowledge about the mechanism(s) at the molecular level poses a major limitation. Filling up this lacuna followed by clinical studies seems the urgent need for generating highly specific therapeutic modalities.

Till then, as cure is still not in the visible realm, management of the disease tailored to improve the quality of life of individuals with T2D seems the current need.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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A genetic analysis identifies a haplotype at adiponectin locus: Association with obesity and type 2 diabetes

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Adiponectin is a prime determinant of the status of insulin resistance. Association studies between adiponectin (*ADIPOQ*) gene single nucleotide polymorphisms (SNPs) and metabolic diseases have been reported earlier. However, results are ambiguous due to apparent contradictions. Hence, we investigated (1) the association between *ADIPOQ* SNPs: −11377C/G, +10211T/G, +45T/G and +276G/T for the risk towards type 2 diabetes (T2D) and, (2) genotype-phenotype association of these SNPs with various biochemical parameters in two cohorts. Genomic DNA of diabetic patients and controls from Gujarat and, Jammu and Kashmir (J&K) were genotyped using PCR-RFLP, TaqMan assay and MassArray. Transcript levels of *ADIPOQ* were assessed in visceral adipose tissue samples, and plasma adiponectin levels were estimated by qPCR and ELISA respectively. Results suggest: (i) reduced HMW adiponectin/total adiponectin ratio in Gujarat patients and its association with +10211T/G and +276G/T, and reduced *ADIPOQ* transcript levels in T2D, (ii) association of the above SNPs with increased FBG, BMI, TG, TC in Gujarat patients and (iii) increased GGTG haplotype in obese patients of Gujarat population and, (iv) association of −11377C/G with T2D in J&K population. Reduced HMW adiponectin, in the backdrop of obesity and *ADIPOQ* genetic variants might alter metabolic profile posing risk towards T2D.

Metabolic Syndrome (MS) is the new wave of diseases that has hit the human population in the last few decades-the Metabolic Syndrome Era. It has become pandemic and with obesity and type 2 diabetes (T2D) clubbed under the MS umbrella, millions of people around the globe have come under its grip. Though obesity and T2D are ubiquitous, there exists a pattern of prevalence based on ethnicity. A recent report has identified demographic transitions, nutrition and lifestyle in the backdrop of genetic predisposition as the chief factors responsible for the rising trend of obesity associated amongst South Asians[1]. Over accumulation of visceral adipose tissue (AT) has been identified as one of the major driving factors towards T2D. Adipose tissue is an important regulator of metabolic homeostasis by virtue of the adipokines (pro-inflammatory and anti-inflammatory) that it secretes. In obese conditions, the fine-tuned balance between the pro- and anti-inflammatory adipokines gets altered leading to various metabolic disorders[2]. These bioactive peptides act locally and distally to calibrate and fine tune various metabolic pathways. Adiponectin is one such calibrator which is abundantly expressed in white adipose tissue[3]. It circulates in three polymorphic forms, low molecular weight (LMW), moderate molecular weight (MMW) and high molecular weight (HMW). Interestingly, the ratio of plasma HMW adiponectin to total adiponectin is more strongly correlated with plasma glucose levels than any of the forms alone[4]. Adiponectin gene (*ADIPOQ/APM1/GBP28*) locus, 3q27, has been strongly associated with a variety of metabolic disorders like- impaired glucose tolerance, obesity, dyslipidemia and T2D[5–7]. Studies undertaken on different ethnic groups have shown positive association of certain SNPs of the adiponectin gene with T2D[3,8–11]. However, T2D being a multi-factorial and polygenic metabolic disorder[12], significant variations have been reported concerning the genetic architecture

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underlying T2D amongst different ethnic populations[13,14]. The SNPs to be studied were selected based on the following criteria: (1) validated SNPs for frequency in Genome Wide Association Studies (GWAS), (2) SNPs with scientific evidence for their role in augmented protein synthesis. *ADIPOQ* comprises of 2 introns and 3 exons encoding for the 30 kDa adiponectin protein[15]. Four SNPs were studied, −11377C/G (*rs266729*) in promoter, +10211T/G (*rs17846866*) in intron 1, +45T/G (*rs2241766*) in exon 2 and +276G/T (*rs1501299*) in intron 2, to examine their association with T2D. Since Indian population is relatively non-homogenous, we conducted our study in native Gujarat, and Jammu and Kashmir (J&K) population independently. We also aimed to study the genotype-phenotype association of the above-mentioned SNPs with Fasting Blood Glucose (FBG), Body Mass Index (BMI), plasma lipid profile and T2D.

Materials and Methods

Study subjects. Two ethnically different populations of India, one from the western Indian state of Gujarat and another from the northern Indian state of J&K were included in the present study. This study was carried out in agreement with the Declaration of Helsinki as approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2016-9) and Institutional Ethics Review Board (IERB), Shri Mata Vaishno Devi University, Katra, J&K, India (Smvdu/IERB/13/23). It was ensured that at least five previous generations of the study subjects were of the respective ethnicities. Blood collection camps were conducted to guarantee the involvement of all the socio-economic strata in the study. The importance of the study was explained to all the participants and written consent was obtained from all patients, and age and sex-matched control subjects. The study group of Gujarat population included 475 diabetes patients (211 males and 264 females) and 493 control subjects (250 males and 243 females) while, the study group of J&K included 507 diabetes patients (282 males and 225 females) and 300 controls (140 males and 160 females) between the age group of 30 to 67 years. The T2D patients recruited for the study displayed FBG > 125 mg/dL[16]. Patients suffering from autoimmune diseases or cancer were excluded from the study. Samples of visceral (omental) adipose tissue were taken from individuals of Gujarat population undergoing bariatric surgery and fasting clinical parameters of all the study subjects are as described previously[17]. A detailed family history of the patients was recorded based on a questionnaire to collect information on first- and second-degree relatives and their history of T2D. The controls selected showed FBG < 110 mg/dL with no prior history of T2D. They were healthy and disease or infection free. The study subjects included both obese and lean individuals and their BMI (weight in kg/height in m^2) was calculated by recording height and weight.

Blood collection and DNA extraction. FBG levels were measured by prick method using glucometer (TRUEresult® - Nipro). Blood was obtained from diabetic and ethnically matched controls as per our previous study[17]. Plasma was used for lipid profiling and assaying plasma HMW adiponectin and total adiponectin levels. PBMCs were separated for DNA extraction by phenol-chloroform method. DNA was stored at −20 °C for further analysis.

Screening of *ADIPOQ* SNPs. Samples from Gujarat population were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for −11377C/G, +10211T/G and +276G/T. The PCR reaction mixture had a total volume of 20 µL as per our previous study[17] with primer dependent annealing temperatures (Table S1). The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide. Details of the restriction enzymes (Fermentas, Thermo Fisher Scientific Inc., USA) and digested products are mentioned in Table S1. 15 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 instruction. The digestion products with 50 base pair DNA ladder (HiMedia, India) were resolved on 3.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator i.e. E-Gel Imager Life Technologies (Fig. S1A–C) and uncropped images of the gels are as in Fig. S3. 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. *ADIPOQ* +45T/ G (*rs2241766*) SNP was genotyped by TaqMan real time PCR using the pre-designed assay ID c__26426077_10 for allelic discrimination, containing specific probes for each allele marked with VIC and FAM fluorescent dyes (ThermoFisher Scientific, USA). Real-time PCR was performed in 10 µl volume using LightCycler®480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. A no-template control (NTC) was used with the SNP genotyping assay. Samples with each genotype were analyzed together as an internal control. J&K samples were genotyped for −11377C/G (*rs266729)*, +45T/G *(rs2241766)* and +276G/T*(rs1501299)* in a panel using High-throughput genotyping MassArray platform (SEQUENOM)[18]. The success rate of SNP genotyping was > 95%. As a quality control measure of SNP genotyping, three duplicate samples and a negative control was included in each 96 well plate. The concordance rate for genotyping was 99.5%. Further values for SNP +10211T/G *(rs17846866)* were imputed using CEU data from 1000 genome (Phase 3) as reference dataset and analyzed using PLINK ver 1.07 as the samples were exhausted.

Plasma parameters. In Gujarat population plasma total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-c) levels were measured using commercial kits (Reckon Diagnostics P. Ltd, Vadodara, India). Low-density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's (1972) formula[19]. Human total adiponectin and HMW adiponectin ELISA Kits (Elabioscience Biotechnology Inc., USA) with a sensitivity of 0.47 ng/mL and 3.75 ng/mL respectively were used to estimate the levels of total adiponectin and HMW adiponectin in patients and controls. The plasma samples used were freeze-thawed only once. All the

plasma estimations were carried out in duplicates with % coefficient of variation within 10%. The plasma samples from J&K population were assayed for various biochemical parameters at a commercial clinical laboratory.

Determination of adiponectin transcript levels. RNA isolation and cDNA synthesis: Total RNA was isolated from visceral adipose tissue (VAT) using Trizol method. RNA integrity and purity were verified by 1.5% agarose gel electrophoresis/ethidium bromide staining and O.D. 260/280 absorbance ratio of 1.9 respectively. To avoid DNA contamination, RNA was treated with DNase I (Puregene, Genetix Biotech) before cDNA synthesis. Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostic GmbH, Mannheim, Germany) was used to prepare cDNA using one microgram of total RNA isolated, according to the manufacturer's instructions in the Eppendorf Mastercycler gradient (USA Scientific, Inc., Florida, USA). The expression of *ADIPOQ* and *GAPDH, IPO8* and *ACTB* (reference) transcripts were measured by Light-Cycler® 480 Real-time PCR (Roche Diagnostics GmbH, Manneheim, Germany) using gene- specific primers (Eurofins, Bangalore, India) as shown in Table S1. Real-time PCR was performed using Light-CyclerH 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and carried out in the Light-CyclerH 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany) as per our previous study[17].

Statistical analyses. The normally distributed data for baseline parameters were analyzed by unpaired t-test while Mann-Whitney test was used for data not following normal distribution. Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all the SNPs in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *ADIPOQ* SNPs for patients and control subjects were compared using the chi-square test with 2×2 contingency tables respectively using GraphPad Prism 5 software. The genotypes have been analyzed in an additive, dominant and recessive model as there was low genotype frequency of the homozygous minor alleles (<10% frequency). *P* values less than 0.0125 for genotype and allele distribution were considered as statistically significant as per Bonferroni's correction for multiple testing. The strength of association of the *ADIPOQ* SNPs with the risk for T2D was assessed by odds ratio (OR) with 95% confidence intervals (CI). Haplotypes and linkage disequilibrium (LD) coefficients ($D' = D/D_{max)}$ and r^2 values for the pair of the most common alleles at each site were obtained using http://analysis.bio-x.cn/myAnalysis.php[20]. Association studies of SNPs with other parameters were performed using analysis of variance (ANOVA) and Kruskal Wallis test. Adjustments for the possible confounding effects of age, sex, and BMI were also done for the samples. Relative gene expression of *ADIPOQ*, and *GAPDH, IPO8* and *ACTB* levels and fold change ($2^{-\Delta\Delta Cp}$ values) in T2D patients and control groups were plotted and analyzed by unpaired t-test. All the analyses were carried out in GraphPad Prism 5 software. *P* values less than 0.05 were considered significant for all the association studies. To predict the functional impact of non-coding polymorphisms, ENCODE prediction tool (https://www.encodeproject.org/) was employed[21].

Results

Clinical parameters differed significantly between controls and patients in both the populations of Gujarat and J&K (Tables S2 and S3). Patients had significantly higher FBG ($p < 0.0001$). Moreover, obesity related factors like BMI, TC, TG and LDL-c were significantly elevated ($p < 0.0001$, $p = 0.0360$ and $p = 0.001$, respectively) while HDL-c was significantly decreased ($p < 0.0001$) in patients as compared to controls in Gujarat population while in the J&K population BMI ($p = 0.015$), FBG ($p < 0.0001$) and TG ($p = 0.001$) levels were significantly higher in T2D patients.

Association of *ADIPOQ* SNPs with T2D. The genotype and allele frequencies of the *ADIPOQ* SNPs are summarized in Table 1. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg Expectations (HWE) ($p > 0.05$) in both the populations. Analysis of the genotype frequencies of +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) SNPs using an additive model revealed them to be significantly associated ($p < 0.0001$) while the promoter 11377C/G (*rs266729*) and exonic +45T/G (*rs2241766*) SNPs were not associated with T2D (Table 1). Further, in Gujarat population a significant association was detected for the intron 1 +10211T/G (*rs17846866*) when analyzed in the recessive model (OR = 1.797, 95% CI = 1.369–2.359, $p < 0.0001$) with T2D. Likewise, the intron 2 + 276G/T (*rs1501299*) SNP was also found to be significantly associated in the recessive model (OR = 2.05, 95% CI, 1.57–2.65, $p < 0.0001$) as shown in Table 1. However, in J&K population, only promoter −11377C/G (*rs266729*) polymorphism was found to be associated ($p = 0.0101$; OR = 1.47, 95% CI = 1.09–1.96) with T2D in the recessive model (Table 1). The frequency of mutant alleles for +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) was noted to be significantly higher in diabetic patients as compared to that of control subjects (OR = 2.33 and OR = 1.726, respectively) in Gujarat population.

Haplotype and linkage disequilibrium analysis of *ADIPOQ* SNPs. A haplotype evaluation of four polymorphic sites of *ADIPOQ* was performed in Gujarat population. The estimated frequencies of the haplotypes differed significantly between patients and controls (global $p = 7.76 \times 10^{-12}$) as shown in Table S4. The disease susceptible haplotypes were CGTG ($p = 0.0003$), CGTT ($p = 6.32 \times 10^{-5}$), GGTT ($p = 0.0207$) and GGTG ($p = 0.0030$) (Table S4). Furthermore, the GGTG ($p = 3.87 \times 10^{-5}$) haplotype in particular was found to be significantly higher in obese patients as shown in Table 2. The LD analysis revealed that the four SNPs investigated were in low to moderate LD association (Fig. S2). Haplotype and LD analyses were not performed in the J&K population as only −11377C/G (*rs266729*) was found to be associated with T2D and the genotypes of +10211T/G (*rs17846866*) were imputed.

***ADIPOQ* expression and plasma HMW adiponectin/total adiponectin ratio in patients and controls.** A significant reduction in *ADIPOQ* transcript levels was observed in Gujarat T2D patients as compared to controls after normalization with *GAPDH* expression ($p = 0.0187$) as suggested by mean ΔCp values (Fig. 1A).

SNP	N	Genotype		Allele		Odds Ratio [95% CI] (*p*-value)			
						Allelic	Additive	Dominant	Recessive
Gujarat Population									
rs266729		CC	CG + GG	C	G	1.23 [0.95–1.59] (0.118)	0.2644	1.46 [0.72–2.95] (0.1443)	1.28 [0.92–1.77] (0.1432)
Controls	286	155	131	427	145				
T2D Patients	285	137	148	402	168				
rs17846866		TT	TG + GG	T	G	2.33 [1.85–2.93] (<0.0001)	<0.0001	1.46 [0.15–2.02] (<0.0001)	1.79 [1.36–2.35] (<0.0001)
Controls	493	363	130	847	139				
T2D Patients	475	289	186	687	236				
rs2241766		TT	TG + GG	T	G	0.86 [0.64–1.18] (0.3722)	0.6704	0.74 [0.22- 2.55] (0.6325)	0.86 [0.61- 1.21] (0.3954)
Controls	467	362	105	822	112				
T2D Patients	359	287	72	642	76				
rs1501299		GG	GT + TT	G	T	1.72 [1.42–2.09] (<0.0001)	<0.0001	1.99 [1.28–3.08] (0.0018)	2.05 [1.57–2.65] (<0.0001)
Controls	489	255	216	692	250				
T2D Patients	464	172	298	579	361				
Jammu and Kashmir Population									
rs266729		CC	CG + GG	C	G	1.34 [1.05–1.69] (0.0168)	0.0365	1.26 [0.67–2.36] (0.2294)	1.47 [1.09–1.96] (0.0101)
Controls	290	151	139	423	157				
T2D Patients	503	309	194	787	219				
rs17846866[#]		TT	TG + GG	T	G	0.95 [0.70–1.29] (0.3827)	—	—	0.95 [0.71–1.27] (0.3663)
Controls	300	141	159	206	94				
T2D Patients	507	232	275	343	164				
rs2241766		TT	TG + GG	T	G	0.72 [0.52–1.02] (0.0613)	0.2041	0.646 [0.23–1.83] (0.2039)	0.71 [0.49–1.04] (0.0788)
Controls	299	251	48	545	53				
T2D Patients	507	400	107	894	120				
rs1501299		GG	GT + TT	G	T	1.09 [0.86–1.40] (0.2248)	0.7452	1.12 [0.59–2.13] (0.3670)	1.12 [0.83–1.51] (0.2247)
Controls	289	170	119	443	135				
T2D Patients	502	309	193	786	218				

Table 1. Genotype and allele frequencies distribution of *ADIPOQ* SNPs in T2D patients in Gujarat and J&K population. [#]Values were Imputed using CEU data from 1000 genome (Phase 3) as reference dataset and analyses was carried out in PLINK ver 1.07.

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

Table 2. Haplotype frequencies in lean and obese patients in Gujarat population. *Indicates haplotypes significantly associated with obesity induced T2D. Frequency <0.03 were ignored in the analysis. The haplotypes in J&K population could not be assessed as the data for +10211T/G (*rs17846866*) was imputed.

The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.84 fold decrease in the expression of *ADIPOQ* transcript levels in patients as compared to controls (Fig. 1B). Similar results were obtained for *ADIPOQ* transcript levels when normalized with *IPO8* ($p = 0.0184$) and *ACTB* ($p = 0.0344$) (Fig. S4A,C). The $2^{-\Delta\Delta Cp}$ analysis of the same showed approximately 0.87 and 0.82 fold reduction in the expression of *ADIPOQ* transcript levels in patients as shown in (Fig. S4B,D). Further, there was no significant difference observed between *ADIPOQ* transcript levels and its SNPs ($p > 0.05$) as shown in Fig. 1C. Plasma HMW adiponectin and total adiponectin levels, and their ratio monitored in 37 controls and 45 patients showed significant decrease ($p < 0.001$) in Gujarat patients as compared to controls (Fig. 1D). Healthy females showed higher HMW adiponectin/total adiponectin ratio than healthy males ($p < 0.001$) (Fig. 1E). A significant drop in the ratio was observed in diabetic males and females when compared with their healthy counterparts ($p < 0.05$ & $p < 0.01$ respectively) (Fig. 1E). There was no significant reduction in the HMW adiponectin/total adiponectin ratio between healthy lean and obese individuals. However, the

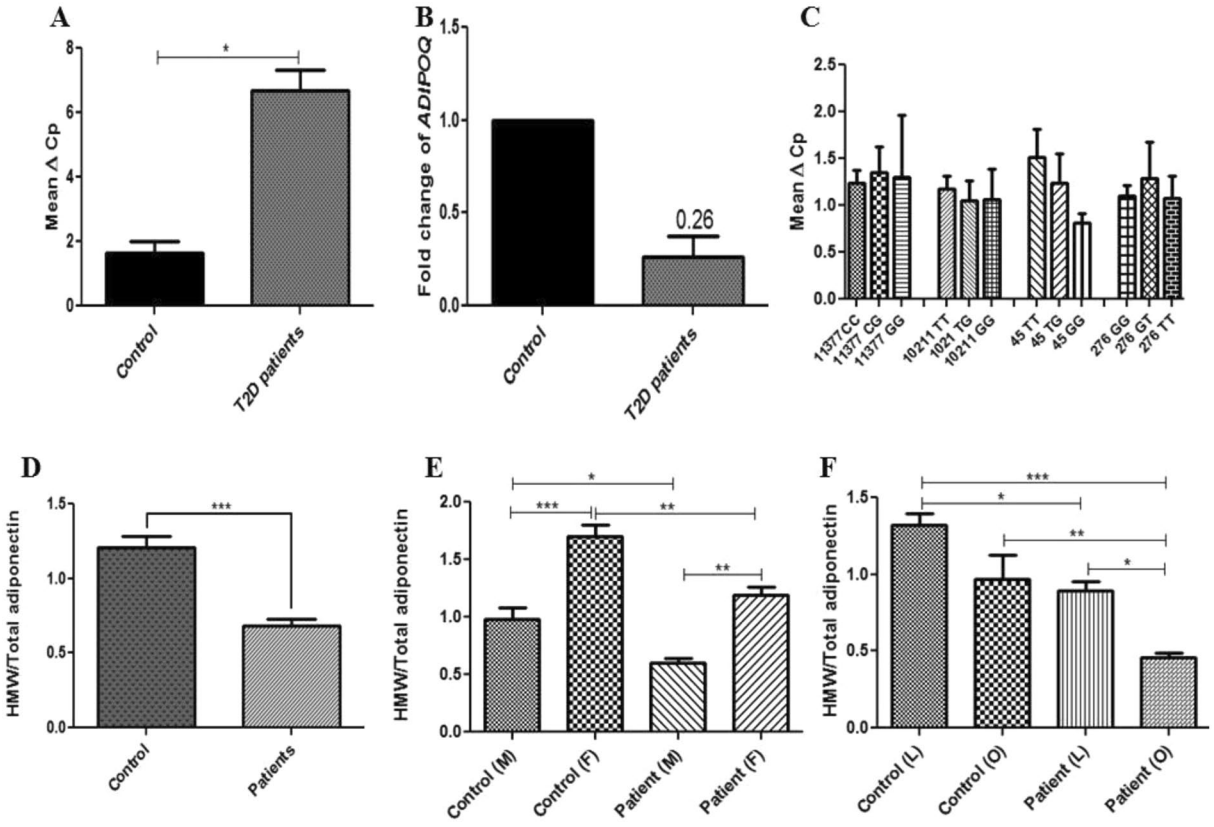

Figure 1. *ADIPOQ* transcript levels and plasma adiponectin levels in Gujarat population. (**A**) Relative gene expression of VAT *ADIPOQ* in controls and patients: Significant decrease in *ADIPOQ* transcript levels was observed in patients (Mean $\Delta Cp \pm SEM$: 1.639 ± 0.3829 v/s 6.681 ± 0.6558; $p = 0.0187$), (**B**) Relative fold change of *ADIPOQ* expression in controls and patients. Expression of *ADIPOQ* transcripts in T2D patients as compared to controls was decreased by 0.84 fold as determined by the $2^{-\Delta\Delta Cp}$ method. (Controls n = 14; T2D patients n = 10). (**C**) Association of *ADIPOQ* polymorphisms with *ADIPOQ* transcript levels. No association between *ADIPOQ* polymorphisms and *ADIPOQ* transcript levels ($p > 0.05$). HMW adiponectin/total adiponectin ratio in (**D**) controls versus patients. Plasma HMW adiponectin/total adiponectin ratio in patients were significantly lower than in controls, (**E**) control and diabetic males and females. HMW adiponectin/total adiponectin ratio in control and patient females were significantly higher than in control and patient males and (**F**) lean (L) and obese (O) control and diabetic subjects. Obese patients showed significantly reduced HMW adiponectin/total adiponectin ratio (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (Controls n = 37; T2D patients n = 45).

obese patients showed a significant drop compared to lean patients ($p < 0.05$) (Fig. 1F). Lean and obese diabetic individuals showed reduced HMW adiponectin/total adiponectin ratio as compared to their respective controls ($p < 0.05$, $p < 0.01$). The drop in the plasma adiponectin ratio was further accentuated in obese diabetic patients ($p < 0.001$) (Fig. 1F).

Association of *ADIPOQ* SNPs and their genotypes with metabolic parameters and HMW adiponectin/total adiponectin ratio. As shown in Table 3, in Gujarat population, the GG genotype of −11377C/G was associated with increased levels of TG, LDL-c and HDL-c (females). The GG genotype of +10211T/G was significantly associated with FBG, BMI, TG, TC, HDL-c and HMW adiponectin/total adiponectin ratio while the TT genotype of +276G/T was significantly associated with increased FBG, BMI, TG, TC and LDL-c and, decreased HDL-c ($p > 0.05$). Further, +45T/G was not associated with any of the parameters in Gujarat population. However, no significant association of the metabolic parameters was observed with the polymorphisms in J&K population (Table S5).

Bioinformatics analyses. ENCODE data base showed that −11377C/G (*rs266729*), +10211T/G (*rs17846866*), +45T/G (*rs2241766*) and +276G/T (*rs1501299*) do not overlap with any cis-Response Elements (cREs) or display any cREs within 2 kb. Further, eQTL database GTex shows TG and GG genotypes of *rs17846866* to have significantly reduced levels of plasma adiponectin similar to our findings. However, the eQTL data for the rest of the SNPs are not available. Analysis of *rs2241766*, a synonymous exonic SNP, revealed that the glycine residue at the 15th position remains unchanged (SIFT). Further, the change in codon usage was calculated by applying a relative synonymous codon usage (RSCU) approach to understand the relevance of ribosomal pause in reduced amount of protein being expressed. The delta Relative Synonymous Codon Usage (RSCU) value for

Genotype/ Allele	FBG (mg/dL)	BMI (Kg/m^2)	TG (mg/dL)	TC (mg/dL)	HDL-c (mg/dL)		LDL-c (mg/dL)	HMW adiponectin: total adiponectin (µg/mL)
					Male	Female		
***ADIPOQ* −11377 C/G (*rs266729*)**								
CC	124.50 (50.02)	25.37 (5.28)	123.00 (79.00)	161.70 (39.47)	36.81 (10.73)	45.17 (14.02)	93.83 (37.5)	0.97 (0.48)
CG	124.70 (51.02)	25.57 (5.95)	150.00 (102.00)	162.70 (39.52)	37.59 (9.30)	34.63 (9.96)	101.90 (39.36)	1.00 (0.54)
GG	124.10 (30.64)	26.36 (5.51)	166.00 (84.00)	156.40 (37.13)	39.75 (13.25)	26.56 (1.51)	101.40 (32.03)	0.64 (0.24)
***P* value**	0.6241	0.4906	**<0.0001**	0.8671	0.7369	**<0.0001**	**0.0087**	0.2055
***ADIPOQ* +10211T/G (*rs17846866*)**								
TT	130.00 (56.13)	25.60 (5.90)	135.80 (92.00)	151.60 (27.89)	42.79 (14.38)	43.18 (14.57)	96.86 (37.5)	1.50 (0.61)
TG	132.20 (55.11)	25.33 (5.20)	138.90 (78.00)	162.20 (38.97)	41.62 (21.49)	44.16 (13.51)	96.64 (46.54)	0.86 (0.39)
GG	148.10 (56.86)	27.82 (5.60)	166.40 (85.60)	175.60 (39.02)	37.76 (12.92)	34.22 (8.07)	99.20 (37.57)	0.82 (0.36)
***P* value**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0141**	**<0.0001**	0.6024	**0.0001**
***ADIPOQ* +45T/G (*rs2241766*)**								
TT	155.40 (4.26)	26.82 (5.20)	164.00 (14.8)	163.80 (37.00)	36.62 (11.85)	40.53 (12.36)	95.79 (39.5)	0.98 (1.20)
TG	171.50 (12.96)	27.16 (5.29)	172.80 (20.3)	164.50 (44.91)	36.51 (11.00)	40.42 (14.46)	96.75 (39.26)	0.83 (0.38)
GG	122.50 (8.50)	30.05 (3.748)	103.90 (15.28)	185.70 (27.61)	34.57 (6.734)	41.27 (11.80)	94.87 (37.83)	0.82 (0.30)
***P* value**	0.3293	0.2619	0.6088	0.4735	0.9708	0.9936	0.9396	0.9284
***ADIPOQ* +276G/T (*rs1501299*)**								
GG	151.00 (53.88)	24.98 (4.53)	143.30 (78.00)	153.20 (29.34)	37.87 (12.34)	40.64 (12.52)	70.36 (27.13)	1.36 (0.63)
GT	166.90 (69.67)	27.69 (5.53)	165.20 (89.00)	154.70 (32.12)	35.78 (10.48)	39.25 (12.56)	92.99 (36.33)	0.93 (0.44)
TT	303.80 (94.54)	29.75 (4.23)	266.60 (90.00)	189.00 (25.96)	33.28 (11.93)	37.34 (6.34)	90.62 (34.1)	0.75 (0.33)
***P* value**	**<0.0001**	**0.0001**	**<0.0001**	**0.0001**	**<0.0001**	0.0831	**0.005**	**0.0006**

Table 3. Genotype-phenotype association analyses of *ADIPOQ* SNPs with metabolic parameters in Gujarat population. Data represented as Mean (SD).

the GGT to GGG codon change was calculated to be −0.31. However, no significant association of the +45T/G polymorphism was found with adiponectin levels.

Discussion

Our findings, for the first time, collectively suggest that *ADIPOQ* CGTG, CGTT, GGTT and GGTG haplotypes were associated with T2D, further GGTG was significantly associated with obesity induced T2D. Also, +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) were strongly associated with obesity induced T2D susceptibility in Gujarat population; whereas in J&K population only −11377C/G (*rs266729*) was found to be associated with T2D. The difference in the association of variants can be attributed to the ethnic differences between the two populations. The findings in Gujarat population are further linked with reduced levels of HMW adiponectin and disease-associated risk factors like FBG, BMI and lipid parameters thereby suggesting their crucial role in metabolic disease susceptibility.

Obese phenotype has been associated with a reduction in the anti-inflammatory and a boost in the pro-inflammatory adipokines. Our previous reports suggest interleukin 1β (IL1β)[22], resistin[23] and TNFα[24] to play an important role in the development of obesity, islet dysfunction and decreased insulin secretion. On the contrary, adiponectin[2], omentin-1[25], melatonin[26] and vaspin[27] are known to enhance insulin sensitivity. The normal range of total adiponectin in healthy individuals is reported to be 2–20 µg/mL[28]. The characteristic short stature of South Asians combined with visceral adiposity leads to an increased weight per area distribution defined by body mass index predisposing those to metabolic diseases[1,29–31]. Genome-wide association studies have shown a close association between adiponectin, *ADIPOQ* SNPs, fasting hyperglycemia and various metabolic diseases though varying from population to population[32–34]. Earlier studies have shown promoter −11377C/G (*rs266729*) polymorphism to have a positive association with hypoadiponectinemia and risk of developing T2D[35] and is supported by the findings in J&K population. As opposed to this, we found this SNP not to be associated with T2D or BMI in Gujarat population supporting the work by Schaffler *et al.* who also reported the absence of transcription factor binding sites at or around this SNP site[36]. However, the GG genotype of −11377 C/G (*rs266729*) did show an association with increased serum triglycerides and LDL-c, and reduced HDL-c in females. In spite of not being associated with T2D, possibly an indirect effect of other SNPs could be the reason for the observed altered association of the −11377 C/G (*rs266729*) with the serum lipid levels.

Adiponectin gene expression in an adipose tissue is regulated by a 34 bp enhancer located in the first intron[37]. Therefore, the finding of +10211T/G (*rs17846866*) located close to the enhancer in the region of the first intron affecting lipid metabolism and adiponectin levels in the present study is of significance. Though the ENCODE data base doesn't show an overlap of this polymorphism with any cREs or display any cREs within 2 kb; eQTL database GTex shows TG and GG genotypes of +10211T/G (*rs17846866*) to have significantly reduced levels of plasma adiponectin similar to our findings. Additionally, this SNP is also seen to be associated with increased BMI, FBG, TG, TC and reduced HDL-c. To date, three independent studies, including ours, have established the association of +10211T/G (*rs17846866*) with three different Indian populations belonging to different

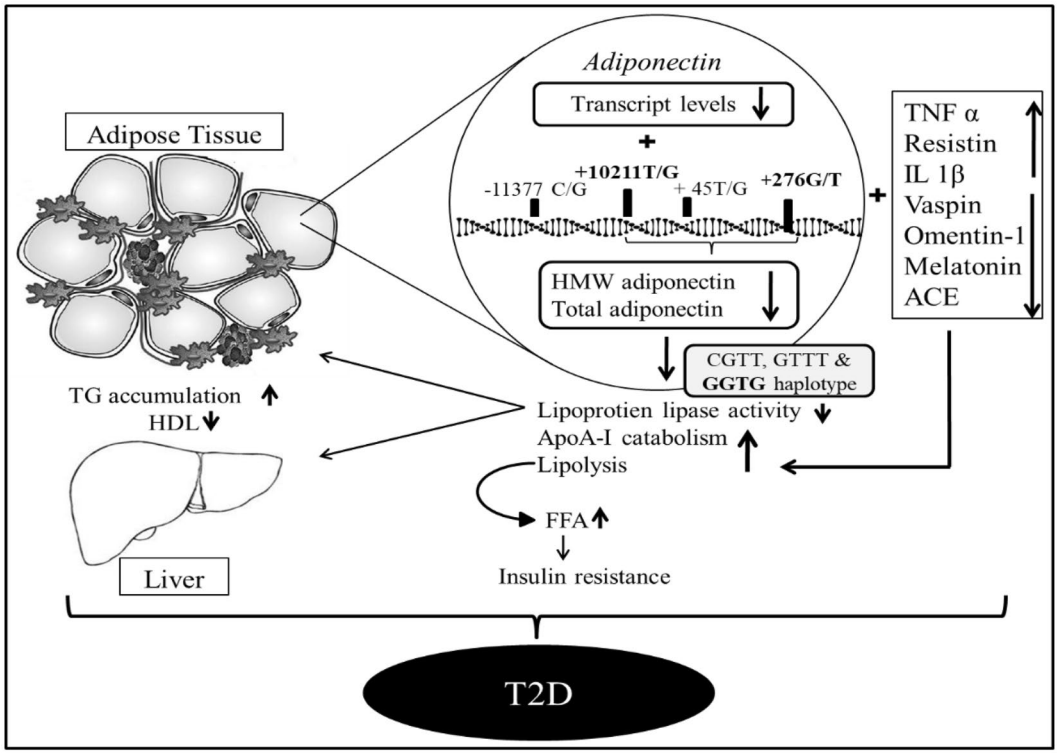

Figure 2. Role of *ADIPOQ* SNPs in T2D: The *ADIPOQ* CGTT, GTTT and GGTG haplotypes in presence of *ADIPOQ* +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) along with decreased transcript, plasma HMW adiponectin and total adiponectin, and increased TNFα, FFA, resistin leads to altered metabolic profile thereby contributing to insulin resistance and T2D in Gujarat population.

demographical and geographical regions, thus further validating the significance of this SNP[10,11]. However, the results from J&K population did not reveal any such association. +45T/G (*rs2241766*) is a synonymous SNP with a codon change from GGT to GGG. Though studies on Chinese Han population found an association between +45T/G (*rs2241766*) and insulin resistance[38]; our results show no association between +45T/G (*rs2241766*) and T2D as supported by studies on Italian, French and Swedish populations[3,8,9]. We report a significant association of +276G/T (*rs1501299*) with T2D, and serum lipid profile in Gujarat population while no association was found in J&K population. Supporting our data from Gujarat population, similar results were obtained in earlier studies in German[39], Swedish[40], Italian Caucasian[41], French Caucasian[3] and South Indian populations[35]. However, the results of the study by Hara *et al.*[42] in Japanese subjects were in accordance with the results obtained in J&K population. In Gujarat population, the TT genotype conferred approximately double risk for developing T2D against the GG genotype in +276G/T (*rs1501299*). Furthermore, +276G/T (*rs1501299*) is also found to be linked with increased BMI, FBG, TG, and TC, and reduced HDL-c in males. These findings also suggest the association of +276G/T (*rs1501299*) with Non-Alcoholic Fatty Liver Disease (NAFLD), co-morbidity associated with T2D as supported by Wang *et al.*[43]. Additionally, we have also found increased levels of TNFα, Free Fatty Acids (FFA) and resistin in obese patients[17,44]. Since TNFα is shown to be an important regulator of adiponectin multimerization[45], our observations of increased TNFα, reduced adiponectin transcript and HMW adiponectin levels in obese patients are self-explanatory. We had also reported a rise in IL1β levels in obese diabetic patients[46], asserting the rise in pro-inflammatory adipokine and drop in anti-inflammatory adipokine in obesity-associated low-grade inflammatory condition. Further, adiponectin levels show sexual dimorphism[47] and our results further confirm this as females in general demonstrated a higher tendency of HMW adiponectin/total adiponectin ratio than males. Also, a significant drop in adiponectin ratio of lean diabetic individuals was observed which was further pronounced in obese diabetic patients. Moreover, the overall plasma HMW adiponectin/total adiponectin ratio tends to be lower in subjects with the homozygous mutant allele for +10211T/G (*rs17846866*) and +276G/T (*rs1501299*). In concordance with our findings, adiponectin levels were strongly and inversely associated with diabetes risk[48,49]. Alongside, we had also reported the prevalence of a significantly high number of angiotensin convertase enzyme (ACE) I/D polymorphism in the same population[50]. The ACE D allele has in particular been shown to be associated with increased angiotensin II[51] which may be further adding to the down regulation of adiponectin. We suggest that the reduced HMW adiponectin in particular is responsible for insulin resistance as, among the adiponectin isoforms, the HMW isoform binds to its receptor with maximum affinity leading to a potent activation of 5′ AMP-activated protein kinase (AMPK). Thus, the lowered HMW adiponectin may be partly responsible for developing T2D[52]. The increased level of TG may be due to a decrease in the lipoprotein lipase activity and Very Low-Density Lipoprotein receptor (VLDLr) expression levels, which have been proposed to be modulated by adiponectin[53]. While HDL-c levels and their particle size are inversely correlated with the

catabolic rate of apolipoprotein (ApoA-I), a direct role of reduced adiponectin with increased catabolism of the major ApoA-I present in HDL-c has been proposed[54], explaining how hypoadiponectinemia leads to decreased HDL-c levels. The correlation between hypoadiponectinemia and reduced HDL-c levels, as observed by us further strengthens the hypothesis. To summarize, +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) are significantly associated with increased FBG, BMI, TG, TC and reduced HMW adiponectin/total adiponectin ratio. More importantly, the haplotype analysis reveals that individuals with GGTG haplotype in particular show an increased tendency towards obesity induced T2D[55] (Fig. 2). Thus, we may conclude that adiponectin gene is associated with T2D, nonetheless variation in the susceptibility loci within the gene depends on ethnic variation among different populations. However, further investigations to understand the mechanistic aspects of genetic variants regulating adiponectin levels are warranted in other cohorts.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

R.B. developed the concept. S.P.P. designed and performed the experiments. S.P.P., R.P. and N.R. contributed to data acquisition and data analysis was performed by S.P.P. S.D.J. contributed towards bioinformatics analyses and interpretation. A.M., M.K.D. and S.S. contributed to the data generation and analysis in J&K population. R.B. and A.V.R. contributed to the critical revision and approval of the article.

Competing interests

The authors declare no competing interests.

Additional information

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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,

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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

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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).

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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.

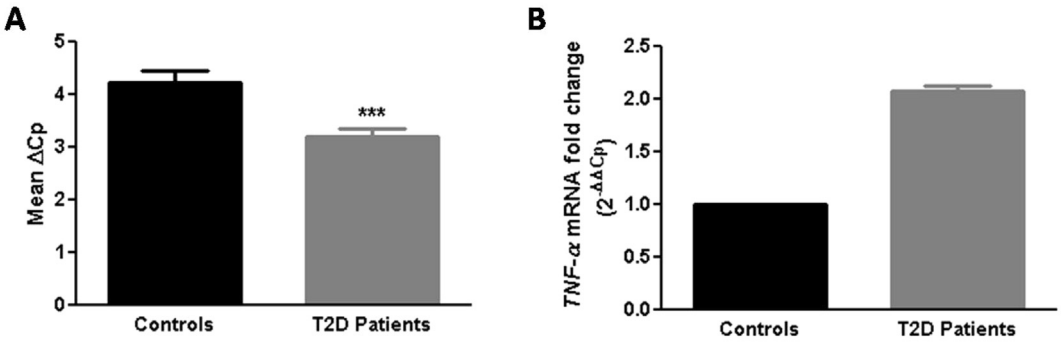

Fig. 1. a) Relative gene expression of *TNF-α* in PBMCs of controls and patients: Significant increase in *TNF-α* mRNA transcript was observed patients (Mean ΔCp ± SEM: 4.24 ± 0.21 vs 3.63 ± 0.13; $p < 0.0001$). **b) Relative fold change of *TNF-α* expression in controls and patients.** Diabetic patients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

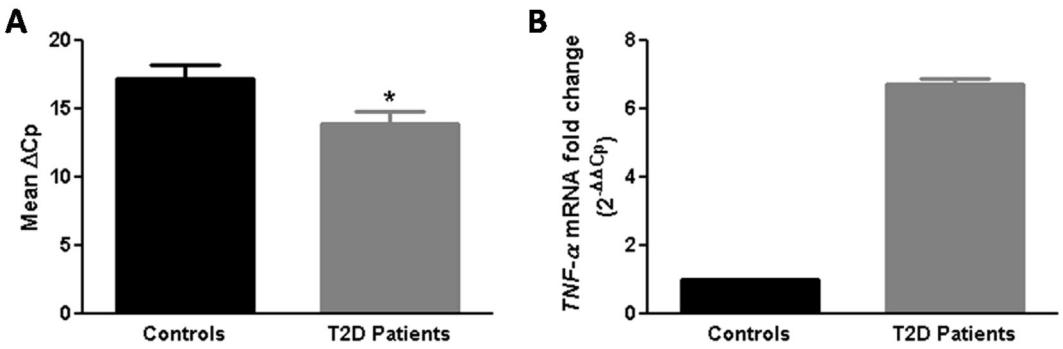

Fig. 2. a) Relative gene expression of *TNF-α* in adipose tissues of controls and patients: Significant increase in *TNF-α* mRNA transcript was observed patients (Mean ΔCp ± SEM: 17.23 ± 0.99 vs 13.87 ± 0.98; $p = 0.0381$). **b) Relative fold change of *TNF-α* expression in controls and patients.** Diabetic 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).

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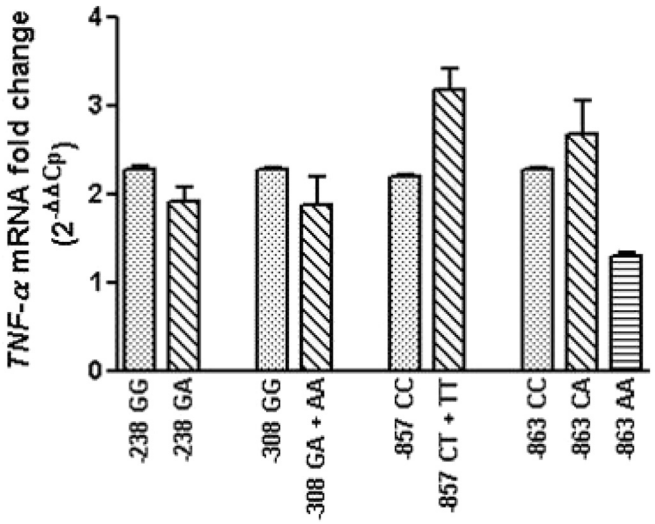

Fig. 3. Genotype-Phenotype correlation of *TNF-α* polymorphisms with its mRNA fold change. Individuals with −857 CT + TT showed 3.6 fold increase in its expression as compared to CC genotype. No difference was observed with respect to other polymorphisms and respective genotypes.

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

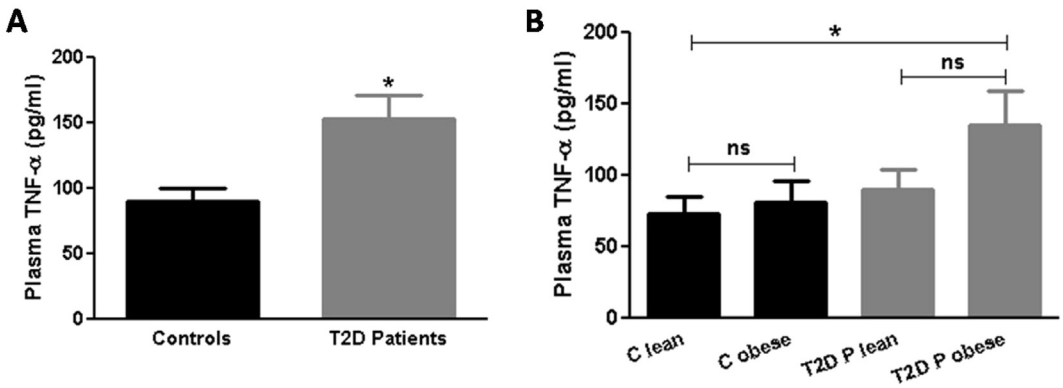

Fig. 4. Plasma TNF-α concentrations in a) controls vs patients Plasma TNF-α concentrations were increased in patients ($p = 0.0122$) and **b) control lean vs obese and patients lean vs obese.** Control lean vs patient obese showed a significant difference ($p = 0.0405$) while no difference was observed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

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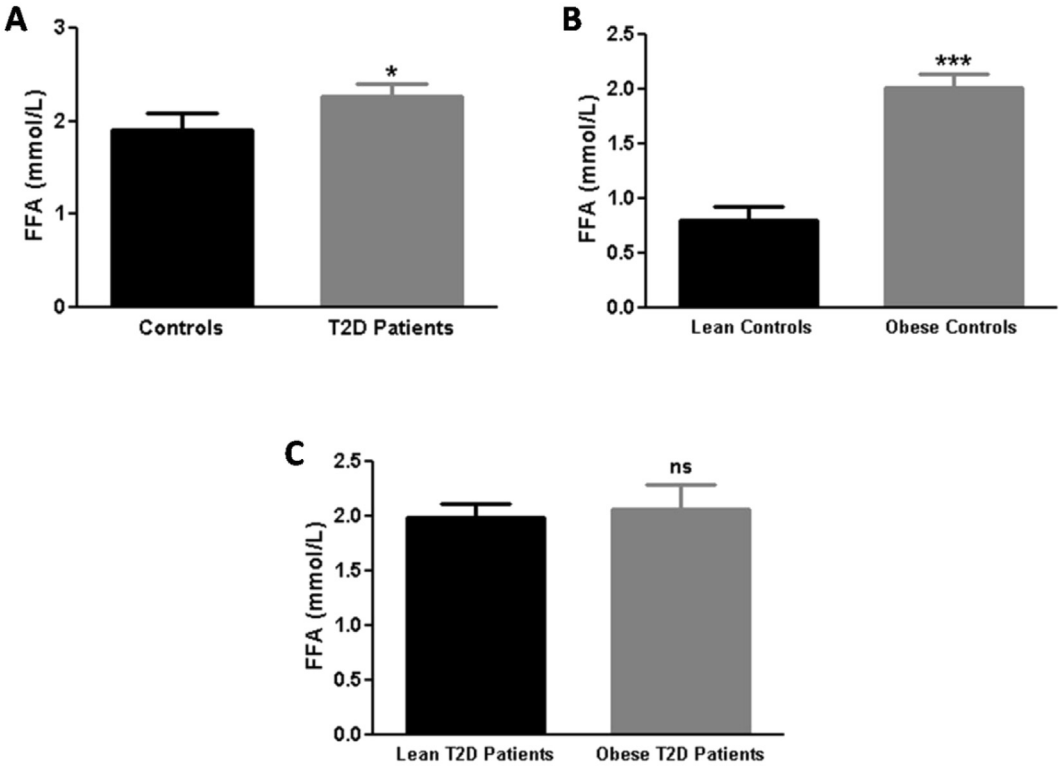

Fig. 5. Plasma free fatty acid concentrations in a) controls vs patients, Plasma FFA in patients showed significant elevation ($p = 0.0215$). **b) lean vs obese controls** Plasma FFA exhibited a significant increase in control obese as compared to control lean ($p < 0.0001$), and **c) lean vs obese patients.**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

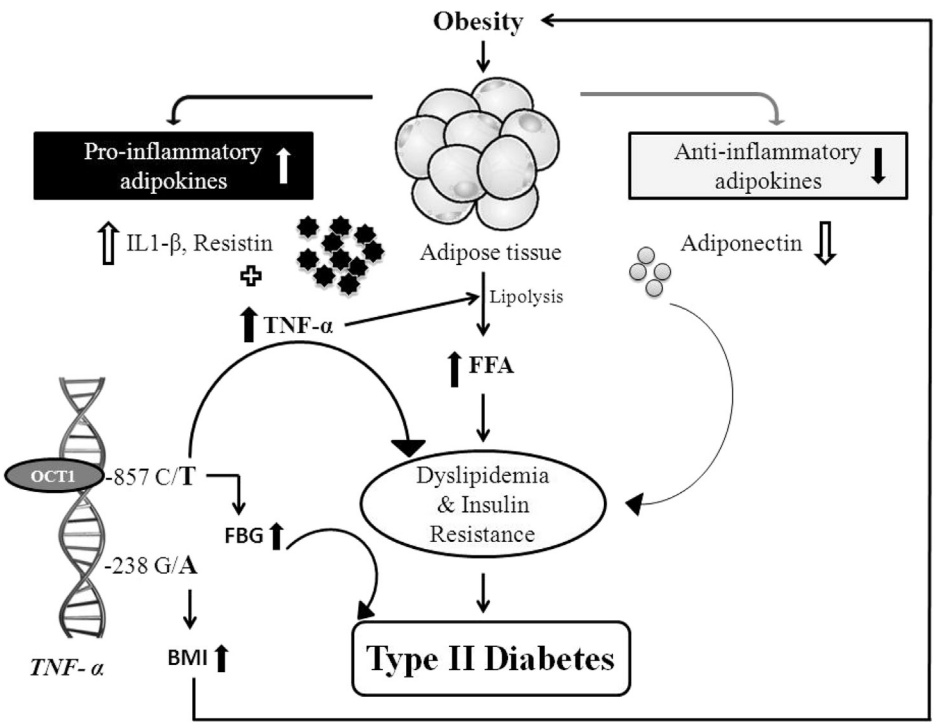

Fig. 6. Mechanism of imbalance in adipokines leading to dyslipidemia and obesity-induced T2D. In obesity, dysregulation of pro and anti-inflammatory adipokines is manifested. Genetic variant of *TNF-α* −857 T allele modulates its expression by strongly binding to the OCT1 transcription factor. Elevated 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.

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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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