Synopsis of the Ph.D. Thesis on

Assessment of combination therapy of Melatonin & GABA in Diabetic mouse models and evaluation of correlation between Leptin & T2D susceptibility

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Introduction:

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia resulting from defective insulin secretion and/or increased cellular resistance to insulin. DM is classified into two main categories: type 1 diabetes (T1D), an autoimmune disease characterized by progressive loss of functional β-cell mass resulting from insulitis, which leads to insulin deficiency and type 2 diabetes (T2D), a metabolic disorder manifested by insulin resistance in peripheral tissues due to impaired insulin signaling cascade (Kahn, 2003). The pathogenesis of T2D is more complex, comprising different degrees of β-cell failure and insulin resistance. Obesity-induced insulin resistance, and insulin secretion defects are the major risk factors for T2D (Klöppel *et al.,* 1985; Kahn, 2003). In both conditions, glucose levels in the blood increase and the heighted levels in due course of time induce chronic oxidative stress and stimulate pro-inflammatory cytokines production (leptin, resistin, TNF-α, etc.), ultimately leading to pancreatic β-cell loss and chronic hyperglycemia (Cnop *et al.,* 2005). The progressive deterioration of β-cell function and mass is a crucial parameter in the development of T1D/T2D. Therefore, it is essential to develop therapies that prevent or even reverse the deterioration of β-cell function. Despite the most recent advances in diabetes care, patients suffering from diabetes still display, a shortened life expectancy and a worsened quality of life as compared to healthy individuals. Islet transplantation has been an ultimate treatment for subjects with severe T1D, but its application is limited due to the lack of donors and the need for intense immunosuppression (Sharples *et al.,* 2016). Thus, it is pertinent to develop novel therapeutic approaches that can increase the survival rate and proliferation of endogenous pancreatic β-cells and immunosuppressive effects. However, none of the current therapies effectively achieve both the goals (Atkinson *et al.,* 2014). Monotherapies often fail in the long run due to other diabetes-related complications and the patient's lifestyle. Hence, combination therapy is recommended for long-term glycemic control (Jeon *et al.,* 2018).

The annual overall monotherapy failure rate was 17% in Korean T2D patients enrolled in the KNDP cohort study. According to this study, metformin is associated with a lower risk of monotherapy failure than sulfonylureas and meglitinides. When metformin monotherapy fails, appropriate alternatives such as thiazolidinedione, α-glucosidase inhibitors, DPP4 and SGLT2 inhibitors should be chosen considering the patient's complications. Still, monotherapy does not achieve glycemic control, combination therapy with different mechanisms of action should be initiated promptly (Jeon *et al.,* 2018; Rhee et al., 2017).

Melatonin:

Changing lifestyle trends such as a tendency to nocturnality and intake of high caloric diets cause disturbance in the sleep/wake cycle and circadian rhythms, which favors the occurrence of diabetes (Scheer *et al*., 2009). Melatonin, a pineal hormone, has a role in circadian rhythm regulation, acts as an antioxidant (Galano *et al.,* 2011), anti-inflammatory agent (Chahbouni *et al.,* 2010), and is also functionally linked to glucose metabolism (Mulder *et al.,* 2009). There might be an association between melatonin and T2D based on the findings that insulin secretion is inversely proportional to plasma melatonin concentration (Peschke *et al.,* 2013), and pinealectomy leads to loss of melatonin which further causes hyperinsulinemia as melatonin keeps a check on insulin secretion (Nishida *et al.,* 2003). Melatonin is reported to mediate its action via two receptors: MT1 (MTNR1A) and MT2 (MTNR1B), which are also expressed in pancreatic islets (Ramracheya *et al*., 2008). In rodent β-cells, the predominant effect of melatonin is in the reduction of insulin release through inhibition of the Gi–cAMP–PKA or cGMP pathways; while in human islets, studies suggest that melatonin increases intracellular calcium levels via IP3 signaling and stimulates glucagon and insulin release from α-cells and β-cells, respectively (Ramracheya *et al*., 2008). Further, melatonin is shown to induce β-cell regeneration in streptozotocin (STZ)-induced diabetic mice and β-cell regeneration in human islets transplanted in mice (Patel *et al.,* 2022). Insulin secretion and β-cell survival have been reported to be improved in response to melatonin signaling, by decreasing β-cell apoptosis and oxidative stress in human islets exposed to chronic hyperglycemia and in islets from patients with T2D (Costes *et al*., 2015). Moreover, studies on rodents have suggested that melatonin administration reduces body fat and HbA1c levels, increases GLUT4 expression and insulin sensitivity in peripheral tissues in a diet-induced obese T2D mouse model (Karamitri and Jockers, 2019; Patel, 2021). Hence, the present study was aimed to explore the potential beneficial effects of melatonin administration in T1D and T2D rodent models.

GABA:

Gamma (γ)- Aminobutyric acid (GABA) initially identified as an inhibitory neurotransmitter and produced by pancreatic β-cells in large quantities has emerged as a new anti-diabetic dietary supplement, (Adeghate and Ponery, 2002). GABA exerts its biological effects by activating GABA receptors ($GABA_AR$ and $GABA_BR$) that are expressed in a variety of peripheral tissues, including pancreatic islet cells and immune cells such as T and B lymphocytes (Tian *et al.,* 2004). Within an islet, GABA acts via $GABA_AR$ on the α-cells and suppresses glucagon secretion as a result of

membrane hyperpolarization; whereas on the β-cells, it enhances insulin secretion through membrane depolarization (Rorsman *et al.,* 1989). Therefore, GABA plays an important role in the regulation of islet cell function and glucose homeostasis. GABA treated diabetic mice have been shown to display higher plasma insulin and reduced glucagon level, normalized glycemic control, and improved metabolic state (Rathwa *et al.,* 2022; Purwana *et al.,* 2014) . It stimulates β-cell replication, protects β-cells against apoptosis, attenuates insulitis, regulates islet-cell function and glucose homeostasis, and suppresses detrimental immune reactions (Rathwa *et al.,* 2022; Liu *et al.,* 2017; Tian *et al.,* 2004; Tian *et al.,* 2011; Purwana *et al.,* 2014). Hence, the present study was aimed to explore the potential beneficial effects of GABA administration in combination with melatonin in T1D and T2D rodent models.

Leptin:

Leptin (LEP), a pro-inflammatory adipokine, encoded by the ob gene located on chromosome 7q31.3, is associated with food intake and appetite, energy homeostasis, basal metabolism, and insulin secretion (Mantzoros, 1999; Yannakoulia *et al.,* 2003). Leptin levels were elevated in obesity and positively correlated with total body fat (Mantzoros, 1999; Yannakoulia *et al.,* 2003). Moreover, high leptin levels in obesity indicate a state of leptin resistance implicating impaired leptin receptor sensitivity and action (Meyers *et al.,* 2008). Apart from its central and peripheral action/effects on different tissues, leptin can also exerts multiple actions on peripheral blood mononuclear cells (PBMCs) (Sanchez-Margalet *et al.,* 2003). *In vitro* studies showed that leptin can directly induce expression of its receptors on PBMCs and thus contributes to an inflammatory response (Zarkesh-Esfahani *et al.,* 2001). Leptin exerts its important physiological effect by binding to leptin receptor (LEPR), which is a single transmembrane protein that belongs to the class I cytokine receptor family. The *LEPR* gene is located on chromosome 1p31, and its encoded product (LEPR) is distributed in a variety of tissues including the brain, adipose tissue, skeletal muscle, liver, pancreatic islets, and immune cells. Studies on LEPR have advanced an understanding of the mechanism of body weight regulation, energy homeostasis and it may also influence the onset of obesity, T2D, and metabolic syndromes (MetS) (Klok *et al.,* 2007; Meister *et al.,* 2000). Leptin circulates in a free form or is bound to its soluble receptor (sOb-R) (Sinha *et al.,* 1996). The sOb-R is the cleaved product of the extracellular domain of a membrane-bound leptin receptor, and its elevated concentrations indicate dysregulated leptin signaling (Brabant *et al.,* 2000). In obese adults, high serum leptin levels could reduce sOb-R levels and were associated with leptin resistance, although the molecular mechanism

has not yet fully understood (Chan *et al.,* 2002). The Leptin resistance is associated with insulin resistance and hence, it plays a crucial role in T2D development (Myers *et al.,* 2010). Several *LEP* and *LEPR* gene polymorphisms have been studied in different populations for their potential association with serum leptin levels, obesity, T2D, and MetS (Ghalandari *et al.,* 2015). Among these variants, the *LEP* (G-2548A *rs7799039* G/A; 5'UTR *rs2167270* G/A) and *LEPR* (exon 6 Q223R *rs1137101* A/G; exon 14 K656N *rs1805094* G/C) single nucleotide polymorphisms (SNPs) have been studied in detail in different populations (Ghalandari *et al.,* 2015). There are only a few studies on these polymorphisms in Indian population (Bains *et al.,* 2020; Dar *et al.,* 2019; Dasgupta *et al.,* 2015; Murugesan *et al.,* 2010) showing their association with obesity, BMI and T2D. However, no such studies have been perfomed in Gujarat polulation. Hence, we aimed to investigate *LEP* and *LEPR* genetic variants and their transcript levels in PBMCs, protein levels in plasma, and genotype-phenotype correlation with various metabolic parameters as well as T2D in Gujarat population.

Hypothesis of the study:

From the above mentioned studies, we have hypothesized that melatonin (M) and GABA (G) alone, and the combination (M+G) treatment may ameliorate diabetes manifestations in rodent diabetic models by promoting β-cell regeneration and increase glucose homeostasis and insulin sensitivity. In addition, *LEP* and *LEPR* polymorphisms may alter leptin and sOR-b levels which may further contribute to dyslipidemia and T2D risk in Gujarat population.

Significance of the study:

The proposed study will help in understanding the therapeutic effects of melatonin along with GABA in diabetes. The genetic association study of SNPs will help in understanding the role of *LEP* and *LEPR* in T2D risk and will help to identify the potential risk associated haplotypes for disease the susceptibility. In addition, these findings would be helpful for developing prognostic markers and alternative treatment strategies for T2D. Overall, this knowledge eventually can be of use in future translational research leading to the development of targeted drug therapy for diabetes.

Proposed Objectives:

1. **To assess the efficacy of melatonin, GABA and combination therapy in amelioration of diabetic manifestations in streptozotocin induced T1D mouse model.**

(a) Assessment of glucose tolerance.

(b) Assessmentof β-cell regeneration by β-cell proliferation, islet neogenesis and α to β-cell trans-differentiation.

(c) Assessment of β-cell apoptosis.

2. To assess the efficacy of melatonin, GABA and combination therapy in the amelioration of diabetic manifestations in high fat diet (HFD) induced T2D mouse model.

(a) Evaluation of glucose tolerance and insulin sensitivity.

(b) Estimation of plasma melatonin, leptin and insulin levels.

(c) Estimation of mRNA expression and enzyme levels of glucoregulatory enzymes (*GCK, G6Pase, FBPase, PEPCK, GP* and *GS*) in liver.

(d) Estimation of mRNA expression of mitochondrial biogenesis markers (*SIRT1* & *PGC1α*) in skeletal muscle.

(e) Estimation of mRNA expression of *MTNR1B, GLUT4* and lipid metabolism genes (*ACC1* & *ATGL*) in adipose tissue.

(f) Estimation of Oxygen Consumption Rate (OCR) of mitochondrial complexes in skeletal muscle.

(g) Estimation of protein expression of insulin signaling pathway in skeletal muscle.

(h) Determination of β-cell mass.

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

(a) Study the association of following *LEP* and *LEPR* polymorphisms with T2D:

- *LEP* -2548 G/A (rs7799039)
- *LEP* 5' UTR +19 G/A (rs2167270)
- *LEPR* Q223R A/G (rs1137101)
- *LEPR* K656N G/C (rs8179183)

(b) Estimation of *LEP* & *LEPR* mRNA expression in PBMCs of T2D patients and controls.

(c) Estimation of plasma LEP and sOb-R levels in T2D patients and controls.

(d) Study the possible genotype-phenotype correlation of LEP and LEPR in T2D.

Results:

<u>Objective1</u>: To assess the efficacy of melatonin, GABA, and combination therapy in amelioration of diabetic manifestations in streptozotocin induced T1D mouse model.

The study was aimed to evaluate the therapeutic potential of melatonin and GABA in STZ-induced T1D mouse model. Forty male BALB/c mice (7-8 weeks old) were used in this study. For T1D induction, 32 BALB/c mice were given five consecutive intraperitoneal (i.p.) injections of 50 mg/kg body weight (BW) STZ, freshly dissolved in 0.1 M cold sodium citrate buffer (pH 4.5). Diabetes was confirmed two weeks later in mice having fasting blood glucose (FBG) >350mg/dL. The diabetic mice were then randomly assigned to four groups: i. Diabetic Control (DC); ii. Melatonin (M) treated; iii. GABA (G) treated; and iv) M+G treated. Melatonin was administered at a dose of 0.5 mg/kg BW i.p., (Patel *et al.*, 2022) and GABA was given at a dose of 18 mg/day (Liu *et al.*, 2017) by oral gavage. The treatment was given for six weeks along with bromodeoxyuridine (BrdU) on alternative days at a dose of 100 mg/kg BW i.p. to assess the β-cell proliferation. FBG levels and BW were measured once a week. Intraperitoneal glucose tolerance test (IPGTT) was performed after 6 weeks of treatment. Thereafter, mice were sacrificed, and the pancreas was harvested and subjected to immunohistochemistry (IHC) studies for assessing the β-cell regeneration (β-cell proliferation, neogenesis, and trans-differentiation) and apoptosis. Our results suggest that monotherapies and the combination therapy significantly reduced FBG levels (M, $p<0.001$; G, $p<0.001$; M+G, $p<0.001$) by increasing plasma insulin levels (M, $p<0.01$; G, $p<0.001$; M+G, $p<0.001$) with a consequent increase in glucose tolerance (M, $p<0.001$; G, $p<0.001$; M+G, $p<0.001$) as compared to DC group, post-treatment. Furthermore, final FBG levels in all the drug-treated groups were also significantly reduced as compared to their initial levels (M, $p<0.01$; G, $p<0.001$; M+G, $p<0.001$). IHC analyses revealed that the monotherapies and the combination therapy significantly induced β-cell proliferation (M, $p<0.01$; G, $p<0.01$, M+G, $p<0.001$) and trans-differentiation (S, $p<0.05$; M, $p>0.05$; S+M, $p<0.05$), but not the neogenesis ($p>0.05$). In addition, we observed a reduced β-cell apoptosis in the drug-treated mice as compared to DC mice (TUNEL+ cells: M, $p<0.01$; G, $p<0.05$, M+G, $p<0.01$). These findings suggest that monotherapies are as effective as combination therapy in ameliorating T1D and can be used as a future therapy for β-cell regeneration in diabetes patients.

<u>Objective2</u>: To assess the efficacy of melatonin, GABA and combination therapy in the amelioration of diabetic manifestations in high fat diet (HFD) induced T2D mouse model.

As the melatonin and GABA treatment helped in combating β-cell loss in the T1D mouse model, it

was interesting to study whether this combination could also improve HFD-induced T2D manifestations. To investigate the effects of M+G in T2D, a diet-induced T2D model was established. To develop T2D model, 32 male C57BL/6J (6-7 weeks old) mice were fed with HFD and 8 mice were fed with a normal chow diet (NCD) for 30 weeks. T2D was confirmed 30 weeks later, having two consecutive readings of FBG >200mg/dL. HFD fed mice were randomly assigned into four groups: i. HFD; ii. Melatonin (M) treated; iii. GABA (G) treated; and iv.) M+G treated. Melatonin was administered at a dose of 10 mg/kg BW i.p. (Patel, 2021) and GABA was given at the dose of 12 mg/day (Liu *et al.,* 2017) by oral gavage. The treatment was given for six weeks. FBG levels and BW were measured once a week along with food and water intake. IPGTT and intra-peritoneal insulin tolerance test (IPITT) were carried out at the end of the treatment. 1 ml of blood was collected, and tissues (Pancreas, adipose tissue, skeletal muscle, and liver) were harvested to assess the various parameters. Our results suggest that the monotherapies (M,$p<0.01$; G, $p<0.01$) are as efficacious as that of the combination therapy ($p<0.001$) in reducing FBG levels, increasing glucose tolerance (S, $p<0.01$; M, $p<0.05$; M+G, $p<0.001$) and insulin sensitivity (S, $p>0.05$; M, $p>0.01$; S+M, $p<0.001$). The BW of the HFD group was significantly increased compared to the normal chow diet (NCD) group ($p<0.001$). However, the treated groups did not significantly affect the final BW compared to their initial BW ($p>0.05$). Assessment of food and water intake revealed that no significant difference was observed in the food intake after 6 weeks of treatment ($p>0.05$). However, a significant reduction in water intake was observed in combination treated groups after 6 weeks of treatment ($p<0.05$). In addition, plasma lipid levels (TG, TC, and LDL) were restored in all the treated groups as compared to HFD group (TG: M, G, M+G, $p<0.001$; TC: M, $p<0.01$; G, M+G, $p<0.01$; LDL; M, G, M+G, $p<0.01$). Furthermore, assessment of plasma insulin, leptin, and melatonin levels revealed that there was hyperinsulinemia and hyperleptinemia along with decreased melatonin levels in the HFD group as compared to NCD ($p<0.001$), and these levels were restored in the drug-treated groups (insulin: M, M+G, $p<0.01$; G, $p<0.05$; leptin: M, M+G, $p<0.05$; melatonin: M, M+G, $p<0.001$) as compared to HFD group. Further, mRNA expression and specific activity of glucoregulatory enzymes from liver were monitored. *Glucokinase* (*GCK)* enzyme activity was significantly increased in the M+G group ($p<0.05$) as compared to HFD group, suggestive of increased glucose uptake. *Glucose 6-phosphatase* (*G6Pase*) expression was significantly reduced in all the treated groups (M, G, $p<0.05$; M+G, $p<0.01$) compared to HFD group, indicating the increased glucose uptake. Similarly, *glucose transporter 2* (*GLUT2*) expression was significantly reduced in GABA and M+G groups (G, M+G, $p<0.05$) as compared to HFD group. Furthermore,

the gene expression and activity of *fructose 1,6-bisphosphatase* (*FBPase*) and *phosphoenolpyruvate carboxykinase* (*PEPCK*) were significantly increased in the HFD group ($p<0.05$ and $p<0.01$, respectively) as compared to NCD groups, and significantly reduced in M & M+G (M, $p<0.05$; M+G, $p<0.01$) and G & M+G groups (G, $p<0.01$; M+G, $p<0.001$), respectively, suggesting the reduced gluconeogenesis. Additionally, there was a significant reduction in the gene expression (M, $p<0.05$; G, $p<0.05$; M+G, $p<0.05$) and activity of *glycogen phosphorylase* (*GP*) (M, $p<0.001$; G, $p<0.01$; M+G, $p<0.001$). All the drug-treated groups as compared to HFD showed reduced glycogenolysis. However, no significant difference in *glycogen synthase* (*GS*) expression was observed in any of the drug-treated groups ($p>0.05$) compared to HFD. Nevertheless, increased liver glycogen content in all the drug-treated groups (S, M, S+M, $p<0.001$) suggested for an increase in glycogenesis. Moreover, the expression of crucial markers for mitochondrial biogenesis i.e., *sirtuin 1*(*SIRT1*) and *peroxisome proliferator-activated receptor-gamma coactivator 1 alpha* (*PGC1α*) showed significant increase in all the drug-treated groups as compared to HFD (SIRT1: G, $p<0.01$; M, M+G, $p<0.001$; PGC1α: M, G, $p<0.05$; M+G, $p<0.01$). Furthermore, lipid metabolism markers i.e. *acetyl-CoA carboxylase1* (*ACC1*) expression was reduced in both HFD and the drug-treated groups (M, G, M+G, $p<0.001$), and *adipose triglyceride lipase* (*ATGL*) expression was significantly reduced in all the drug-treated groups (G, $p<0.05$; M, M+G, $p<0.001$) as compared to HFD, suggesting for the improved lipid metabolism. Additionally, we monitored absolute gene expression of *MTNR1B* and *Glucose transporter 4* (*GLUT4*) in adipose tissue by ddPCR. The results showed an increased copy number/µl of the mentioned genes in the HFD group as compared to NCD group, suggesting for insulin resistance. Moreover, the copy number/µl was reduced in all the treated groups, indicating for the reduced insulin resistance. Further, the respiratory control ratio **(**RCR) of state 3/state 4 for mitochondrial complexes I, II, III, and IV ($p<0.001$, $p<0.001$, $p<0.01$, $p<0.05$, respectively) was significantly reduced in HFD group as compared to NCD. A significant increase in the RCR was observed in all the treated groups for CIII (M, $p<0.05$; G, $p<0.05$; M+G, $p<0.01$) and CIV (M, $p<0.01$; G, $p<0.05$; M+G, $p<0.01$). However, the RCR of state 3/state 4 for CI was significantly increased only in the M+G ($p<0.05$) and for CII in M ($p<0.05$) and M+G ($p<0.001$) treated group. These results are suggestive of the increased mitochondrial respiration in the treated groups as compared to HFD group. Further, western blot analysis of the proteins involved in insulin signaling pathway revealed that IR1β ($p<0.05$), pAkt Ser473/Akt ($p<0.05$), and GLUT4 ($p<0.05$) levels were significantly down regulated and pIRS Ser307/IRS ($p<0.05$) levels were significantly up regulated in HFD ($p<0.05$), suggesting the insulin resistance condition. Upon treatment, the

levels of IR1β were up regulated considerably in all the treated groups (M, $p<0.05$; G, $p<0.05$; M+G, $p<0.01$) along with pAkt/Akt in M and M+G (M, $p<0.05$; M+G, $p<0.01$) as compared to HFD group. Moreover, the levels of pIRS/IRS were significantly down regulated in M and M+G groups ($p<0.05$), suggesting for the improved insulin sensitivity. Intriguingly, GLUT4 levels were significantly up regulated only in M+G treated groups ($p<0.05$) as compared to HFD group. Finally, all the drug-treated groups showed a significant decrease in β-cell mass and islet number per pancreatic section (G, $p<0.01$; M, M+G, $p<0.001$; M, G, M+G, $p<0.001$, respectively) as compared to HFD group, suggesting for an improvement in beta-cell hyperplasia and hypertrophic condition. The above results conclude that the monotherapies are as effective as combination therapy in ameliorating HFD-induced T2D manifestations by improving the metabolic parameters and modulating the glyco-lipid metabolism in liver and adipose tissue, respectively, and enhancing the mitochondrial function in the skeletal muscle. Additionally, it also increases peripheral insulin sensitivity and restores the β-cell mass. The possible mode of action of melatonin and GABA and their combinatorial effects on the amelioration of diabetes manifestations in peripheral tissues (pancreas, liver, skeletal muscle, and adipose tissue) are shown in Fig. 1A.(Cecon and Jockers, 2018; Karamitri and Jockers, 2018; Ye *et al.*, 2017; Wang *et al.*, 2017; Hatazawa *et al.*,2017; Befroy *et al.*,2007)

<u>Objective3</u>: To assess genotype- phenotype correlation of leptin (LEP) and its receptor (LEPR) in Gujarat T2D patients and controls.

According to the Helsinki Declaration, the study was performed and approved by the Institutional Ethical Committee for Human Research (IECHR: FS/IECHR/2016-9). In this study, 439 T2D patients (males/females) and 451 (males/females) healthy controls from Gujarat population participated. The patients showing FBG >125mg/dl, and controls exhibiting FBG <110mg/dl with no prior history of T2D were included in the study. Body mass index (BMI) was calculated by measuring height and body weight. 3 ml of venous blood was drawn from the participants after 12h of overnight fasting in K_3EDTA coated tubes to estimate FBG, total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL) using commercially available kits. LDL was calculated using Friedewald's (1972) formula. Genomic DNA and RNA were isolated from PBMCs of controls and patients. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype *LEP* (*rs7799039* G/A; *rs2167270* G/A) and *LEPR* (*rs1137101* A/G; *rs1805094* G/C) polymorphisms and qPCR to

estimate *LEP* and *LEPR* transcript levels. For the genotype-phenotype correlation analysis, metabolic and plasma lipid profiles were used. Plasma protein levels of leptin and its soluble receptor (sOb-R) were estimated using commercially available ELISA kits. The distribution of genotype frequencies for all the investigated polymorphisms followed the Hardy-Weinberg equilibrium (HWE) in both patient and control groups ($p>0.05$). The genotype and allelic frequencies of *LEP rs7799039* G/A and *rs2167270* G/A polymorphisms and *LEPR rs1805094* G/C polymorphism did not differ significantly between patients and control groups ($p>0.025$); hence, were discontinued after an initial assessment. However, the GG genotype ($p=0.009$) and mutant allele 'G' ($p=0.02$) of *LEPR rs1137101* A/G were associated with increased risk for T2D with an odds ratio (OR) of 1.66 and 1.24, respectively. A haplotype evaluation of two polymorphic sites of *LEPR rs1137101* A/G and *rs1805094* G/C revealed that the haplotypes differed significantly between patients and controls (global $p=0.026$), and the susceptible disease haplotype 'GG' was prevalent in patients ($p=0.018$). Moreover, the genotype-phenotype correlation analysis showed that GG genotype of *LEPR rs1137101* A/G polymorphism was associated with elevated FBG ($p=0.027$) and TC ($p=0.025$) levels. Additionally, LEP mRNA levels were significantly increased ($p<0.0001$) whereas *LEPR* mRNA levels were decreased in patients ($p<0.05$) as compared to controls. Further, the plasma leptin was significantly increased in T2D patients ($p=0.0047$) and sOb-R levels were reduced considerably in T2D patients ($p=0.0294$) as compared to controls. The correlation analysis also showed a positive correlation of leptin with BMI ($p=0.0001$) and TG ($p=0.0163$), and sOb-R protein levels with BMI ($p=0.0001$), FBG ($p=0.0434$), and TG ($p=0.0084$). Thus, our findings revealed that the genetic variant of *LEPR rs1137101 A/G*, along with elevated leptin and decreased sOb-R protein levels, could pose a risk towards T2D susceptibility in Gujarat population.

Conclusions:

Our *in vivo* studies suggest that the monotherapies and combination therapy can ameliorate T1D and T2D in mouse models by inducing β-cell regeneration, by improving glucose and lipid metabolism and increasing insulin sensitivity in peripheral tissues. In addition, our population study suggests significant association of *LEPR rs1137101* A/G polymorphism with T2D in Gujarat population. Overall, these findings suggest that increased circulating leptin and low sOb-R protein levels along with *LEPR* polymorphism might increase the risk of T2D in Gujarat population. The results are summarized in Fig.1A & B.

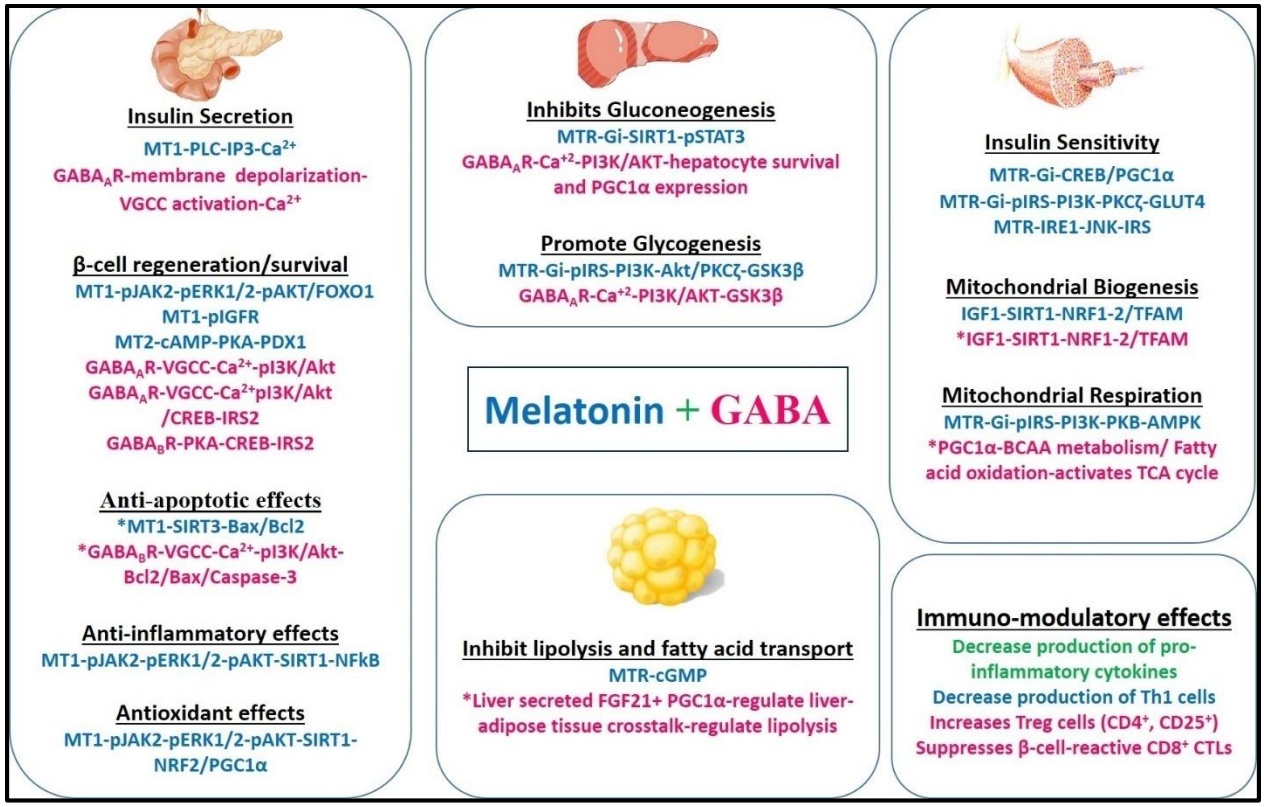

Figure 1A. Possible mode of action of Melatonin and GABA and their combination in the amelioration of diabetes manifestations in peripheral tissues. Our *in vivo* studies suggest that monotherapies and M+G therapy reduce fasting blood glucose levels, increase plasma insulin levels and glucose tolerance, promote β-cell regeneration and reduce β-cell apoptosis in T1D mouse model. It also showed its potential in ameliorating T2D manifestations by improving glucose and lipid metabolism, increasing insulin and leptin sensitivity in peripheral tissues, improving mitochondria biogenesis, and restoring β-cell mass.

*Hypothesized pathway.

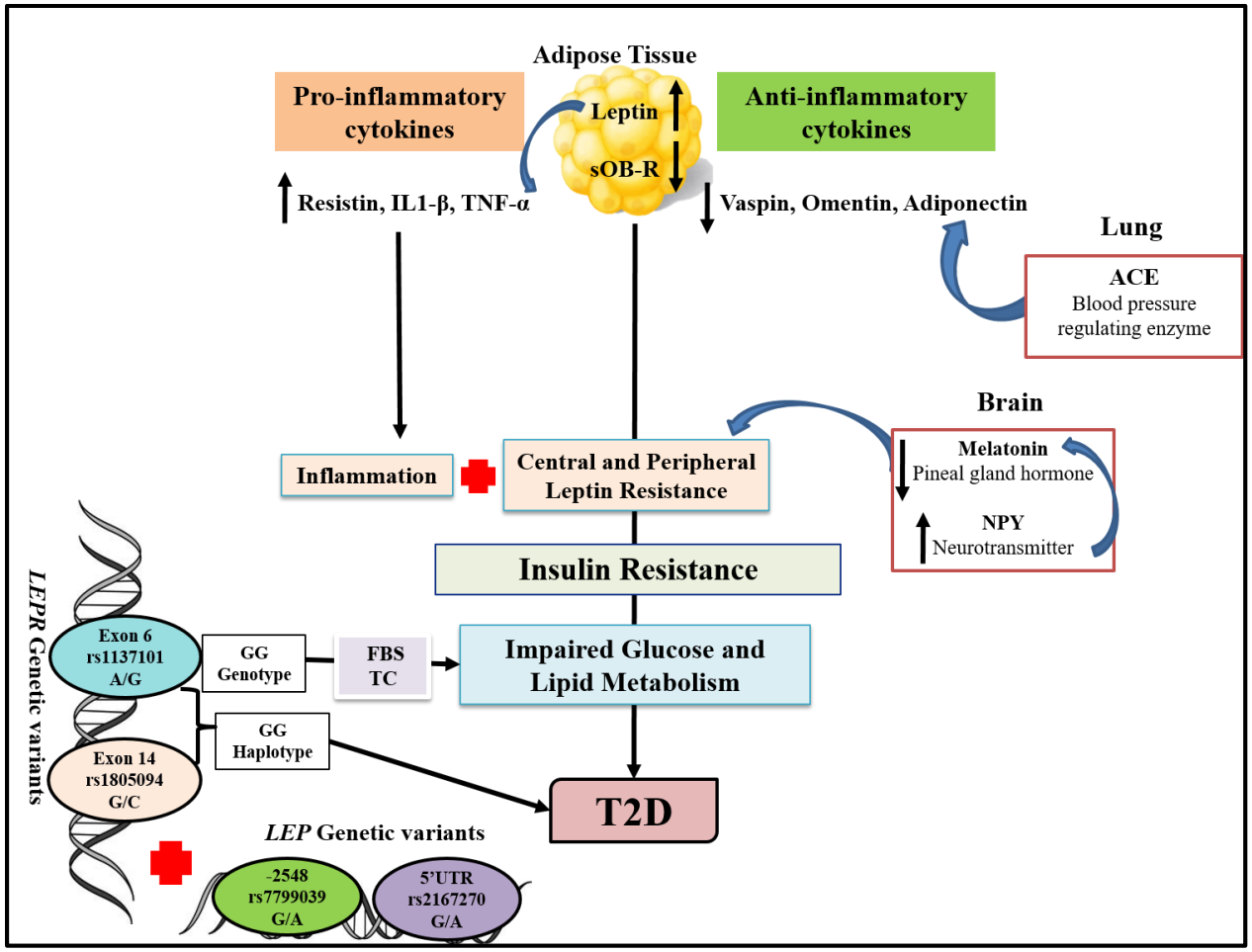

Figure 1B. Role of leptin (LEP) and leptin receptor (LEPR, sOb-R), and altered adipokine levels in T2D. *LEPRrs1137101* A/G polymorphism is significantly associated with T2D, and the homozygous GG genotype and haplotype increase the risk of disease by 1.66 and 1.35 fold, respectively. Moreover, the GG genotype shows a strong association with elevated FBG and TC levels. The increased leptin and decreased sOb-R levels might be responsible for leptin resistance. Hyperleptinemia further elevates the TNF-α levels, which play an important role in the imbalance of pro-inflammatory/anti-inflammatory.

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Achievement:

1. Awarded Gujarat State Government - Scheme of Developing High-quality research (SHODH) fellowship 2020.

Publications:

1. Patel, R., **Parmar, N.,** Rathwa, N., Palit, S. P., Li, Y., Garcia-Ocaña, A., & Begum, R. (2022). A novel therapeutic combination of sitagliptin and melatonin regenerates pancreatic β-cells in mouse and human islets. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 119263. **(IF:4.74)**
2. Rathwa, N., **Parmar, N.,** Palit, S. P., Patel, R., Bhaskaran, R. S., Ramachandran, A. V., & Begum, R. (2022). Calorie restriction potentiates the therapeutic potential of GABA in managing type 2 diabetes in a mouse model. *Life Sciences*, *295*, 120382. **(IF:5.03)**
3. Patel, R., **Parmar, N.,** Palit, S. P., Rathwa, N., Ramachandran, A. V., & Begum, R. (2022). Diabetes mellitus and melatonin: Where are we? *Biochimie*.**(IF:4.08)**
4. Rathwa N, Patel R, Palit SP, **Parmar N**, Rana S, Ansari MI, Ramachandran AV, Begum R. (2020). β-cell Replenishment: Possible Curative Approaches for Diabetes Mellitus. *Nutrition, Metabolism and Cardiovascular Diseases*. 30(11):1870-1881. **(IF:3.7)**
5. Rathwa N, **Parmar N,** Palit SP, Patel R, Ramachandran AV, Begum R. (2020) Intron specific polymorphic site of vaspin gene along with vaspin circulatory levels can influence pathophysiology of type 2 diabetes. *Life sciences* 243:117285. **(IF:3.44)**

Manuscript under communication:

1. **Nishant Parmar,** Nirali Rathwa, Roma Patel, Sayantani Pramanik Palit, Naisargi Patel, Satyashree Shetty, AV Ramachandran, Rasheedunnisa Begum*. Exon specific polymorphic site of *LEPR* gene along with Leptin and its soluble receptor circulatory levels: The potential risk factors for Type 2 diabetes.

Manuscript under preparation:

1. A novel combination of melatonin and GABA ameliorates T1D and T2D manifestations.

Oral/ Poster presentations:

1. **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 'Nextgen genomics, biology, bioinformatics and technologies (NGBT) held at Mumbai, India on 30th September -2nd October, 2019 ****(Received best poster/oral presentation award)***.
2. Patel R, Palit SP, Rathwa N, **Parmar N**, Dhimmar H, Pancholi DA, Ramachandran AV, Begum R. "Melatonin and DPP-IV inhibitor: A novel combinatorial approach for β-cells regeneration" at 9th International Conference on 'Nextgen genomics, biology, bioinformatics and technologies (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 'Nextgen genomics, biology, bioinformatics and technologies (NGBT) held at Mumbai, India on 30th September -2nd October, 2019.
4. Rathwa N, Patel R**,** Palit SP, **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. Poster presentation delivered at NextGen Genomics, Biology, Biochemistry and Technologies (NGBT) Conference (Sep 30th to 2nd Oct 2019) at Taj Lands End, Mumbai India.
5. Rathwa NN, Palit SP, Patel R, **Parmar NR,** Ramachandran AV, Begum R. "143-LB: Calorie Restriction in Combination with GABA Ameliorates Type 2 Diabetes" at American Diabetes Association at Moscone Centre, San Francisco, California, USA from 7th-11th June, 2019.
6. Patel R, Pramanik S, Rathwa NN, **Parmar NR,** Dhimmar H, Pancholi DA, Ramachandran AV, Begum R. "112-LB: Melatonin And DPP-IV Inhibitor: A Novel Combinatorial Approach For ß-Cell Regeneration" at American Diabetes Association at Moscone Centre, San Francisco, California, USA from 7th-11th June, 2019.
7. Rathwa N, **Parmar N**, Pramanik S, Patel R, Dhimmar H, Ramachandran AV, Begum R. "Genetic Variants of Omentin-1 and Vaspin: Association with Type 2 Diabetes Susceptibility" at International Conference on Reproduction, Endocrinology and Development, School of Liberal Studies and Education, Navrachana University, Vadodara-391410, Gujarat, India from 19th-21st January, 2019.
8. Rathwa N, Patel R, Pramanik S, **Parmar N**, Ansarullah, Bhaskaran RS, 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, School of Liberal Studies and Education, Navrachana University, Vadodara-391410, Gujarat, India from 19th-21st January, 2019.
9. Patel R, Rathwa N, Pramanik S, **Parmar N**, Dhimmar H, Ansarullah, Vasu V, Ramachandran AV, Begum R. "β-cell regenerative potential of melatonin and DPP-IV inhibitor in amelioration of T1D" at International Conference on Reproduction, Endocrinology and Development, School of Liberal Studies and Education, Navrachana University, Vadodara-391410, Gujarat, India from 19th-21st January, 2019.
10. Patel R, Rathwa N, Pramanik S, **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 Diseases' held at The M. S. University of Baroda, Vadodara, Gujarat, India from 11th - 13th December, 2018.
11. Rathwa N, **Parmar N**, Pramanik S, 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 Diseases' held at The M. S. University of Baroda, Vadodara, Gujarat, India on 11th - 13th December, 2018. ***(Selected for oral presentation)***

12. Patel R, Rathwa N, Pramanik S, **Parmar N,** Dhimmar H, Ansarullah, 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 Physiology and Comparative Endocrinology (ICRPCE) and the 36th annual meeting of the Society for Reproductive Biology and Comparative Endocrinology (SRBCE), Birla Institute of Technology and Science Pilani, KK Birla Goa Campus 403726, India from 20th-22th January, 2018.
13. Rathwa N, Pramanik S, 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 Physiology and Comparative Endocrinology (ICRPCE) and the 36th annual meeting of the Society for Reproductive Biology and Comparative Endocrinology (SRBCE), Birla Institute of Technology and Science Pilani, KK Birla Goa Campus 403726, India from 20th-22th January, 2018.
14. Patel R, Rathwa N, Pramanik S, **Parmar N,** Dhimmar H, Ansarullah, 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 Physiology and Comparative Endocrinology (ICRPCE) and the 36th annual meeting of the Society for Reproductive Biology and Comparative Endocrinology (SRBCE), Birla Institute of Technology and Science Pilani, KK Birla Goa Campus 403726, India from 20th-22th January, 2018.

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