Synopsis of the Ph.D. thesis on

Adiponectin gene polymorphisms in type 2 diabetics and evaluating the therapeutic potential of pitavastatin and L-glutamine in combination on type 2 diabetes mouse model

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For the degree of

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Introduction

Many modern day "Lifestyle Disorders" are emerging, especially obesity and type 2 diabetes mellitus (T2D). T2D is a multifactorial and polygenic disorder (Qatanani & Lazar, 2007) characterized by hyperglycemia resulting from insulin resistance, eventual pancreatic β-cell loss, and a decline in incretin function (Knop *et al.,* 2007). By the time T2D is diagnosed, islet function is often reduced by 50% as compared to healthy controls (Garber, 2011). As progressive deterioration of β-cell mass and insulin resistance due to obesity are the hallmark features in the development of T2D (Leitner *et al*., 2017), it is important to develop therapies that can arrest or even reverse β-cell mass reduction and insulin resistance. While most of the current therapies target to improve insulin sensitivity by modulating hepatic function (Biguanides and Thiazolidinediones), enhancing anti-inflammatory hormones having insulin sensitizing properties such as adiponectin and control of incretins seem to be more promising. Overaccumulation of visceral adipose tissue (VAT) has been identified as one of the major driving factors towards T2D. VAT is an important regulator of metabolic homeostasis by virtue of the adipokines (pro-inflammatory and anti-inflammatory) that it secretes.

Adiponectin in T2D pathogenesis:

Adiponectin, an anti-inflammatory adipokine, has been identified to have anti-diabetic properties (Iwaki *et al.,* 2003). *ADIPOQ* gene located on chromosome 3q27 codes for the 30 kDa adiponectin protein (Vasseur *et al*., 2003). This gene spans 17 kb and consists of 3 exons and 2 introns (Nakatani *et al*., 2005), and is exclusively expressed in white adipose tissue. Adiponectin is found in various polymorphic forms in plasma (Pajvani *et al*., 2003; Waki *et al*., 2003). Apart from its insulin sensitizing action, adiponectin is also responsible for free fatty-acid combustion via PPARα activation, which decreases the triglyceride content in liver and skeletal muscle thereby enhancing insulin sensitivity (Yamauchi *et al*., 2003). Various single nucleotide polymorphisms (SNPs) of *ADIPOQ* gene (-11391G/A, -11377C/G, +10211T/G, +45T/G and +276G/T) have been reported to be associated with T2D (Jaziri *et al*., 2010; Thirunavukkarasu *et al.,* 2014).

Pitavastatin:

Pitavastatin is a member of statin family which acts on dyslipidemia by lowering cholesterol levels. It is an inhibitor of HMG-CoA reductase, the enzyme that catalyses the first step of cholesterol synthesis. It has been reported that pitavastatin amongst its other counterparts could maximally increase adiponectin levels (Inami *et al.,* 2007; Nomura *et al.,* 2012; Mita *et al.,* 2013). In T2D human patients, pitavastatin improves glycated haemoglobin levels and

ameliorates insulin resistance (Huang *et al.*, 2016; Yang *et al.*, 2016). Further, pitavastatin has been found to preserve GLUT4 expression in diet induced obesity mouse model (Ishihara *et al.*, 2010). As adiponectin enhances insulin sensitivity by decreasing the hepatic and skeletal muscle triglyceride content, its decreased levels contribute to high level of circulating free fatty acids (Medina-Urrutia *et al.*, 2015). The presence of surplus energy reserves in hepatocytes decrease insulin-induced glucose uptake by inactivating AMPK. Dyslipidemia being a T2D associated comorbidity can be rectified by statins. Additionally, there are mixed reports on increased serum adiponectin and decreased C-reactive protein levels when treated with statins (Matsubara *et al*., 2012) and in amelioration of insulin resistance in T2D patients (Cui *et al.*, 2018). Atorvastatin has been reported to preserve β-cells in streptozotocin-induced diabetic mouse model by reducing ER stress (Chen *et al*., 2014) however there are no reports till date on the action of pitavastatin on β-cell preservation or restoration.

L-glutamine:

L-glutamine plays an important role in the intermediary metabolic pathways (Wu, 2009). As the precursor of peptides, proteins, neurotransmitters and nitrogenous bases, it is used to produce energy in various organs (Darosa *et al.*, 2015). This amino acid also maintains cell proliferation, immune function, acid-base balance and regulation of gene expression (Newsholme *et al.*, 2011). Interestingly, L-glutamine levels are reduced in T2D (Mansor *et al*., 2015; Lee *et al*., 2017). It has been extensively studied as a Glucagon-like peptide-1 (GLP-1) secretagogue (Reimann *et al.*, 2004). GLP-1 binds to its receptor on β-cell bringing about alternate insulin secretion pathway and β-cell preservation (Preitner *et al.*, 2004). L-glutamine has also been reported to be as effective as DPP-4 inhibitor (Bonet et al., 2014). L-glutamine supplementation has been found to increase the expression of key PI3K signalling molecules (PI3K, PDK1, and GLUT4) and promote AKT phosphorylation, GLUT4 translocation and glucose uptake in the presence of insulin during hyperglycemia (Świderska *et al*., 2018). However, its regenerative properties have not been studied.

Hypothesis:

We hypothesize that genetic polymorphisms in the adiponectin gene may contribute to predisposition towards T2D risk in Gujarat population. We also hypothesize that pitavastatin would increase the adiponectin levels and normalize the dyslipidemia while L-glutamine would increase the GLP-1 levels leading to insulin secretion and β-cell survival in High Fat Diet (HFD)+ Streptozotocin (STZ) induced T2D mouse model. Combination of pitavastatin and L-glutamine may also ameliorate T2D in a synergistic / additive manner.

Significance of the study:

The present study aimed to explore the association of adiponectin gene polymorphisms with T2D in Gujarat population and its genotype-phenotype correlation with various metabolic parameters and adiponectin levels. We also aimed to evaluate the therapeutic effect of pitavastatin and L-glutamine in combination on the High Fat Diet (HFD)+ Streptozotocin (STZ) induced T2D mouse model. The first part of the study may help us identify potential genetic markers for making an effective prognosis for the development of T2D while the second part may help pave avenues for drug discovery having a more holistic approach.

Proposed Objectives:

I. To assess the association of *ADIPOQ* polymorphisms with T2D in Gujarat population and to study the possible genotype-phenotype correlation with plasma adiponectin levels.

1. To study the association of following single nucleotide polymorphisms with T2D
    a) *ADIPOQ* promoter -11377 C/G (*rs266729*)
    b) *ADIPOQ* intron 1 +10211 T/G (*rs17846866*)
    c) *ADIPOQ* exon 2 +45 T/G (*rs2241766*)
    d) *ADIPOQ* intron 2 +276 G/T (*rs1501299*)
2. To estimate the plasma High Molecular Weight (HMW) and total adiponectin levels.
3. To study the possible genotype-phenotype correlation of plasma adiponectin levels and risk towards T2D and various metabolic parameters.

II. To investigate the therapeutic potential of small molecule enhancers for adiponectin & GLP-1 secretion in mouse model of glucose intolerance/insulin resistance.

1. To establish HFD+STZ induced type 2 diabetes mouse model.
2. To evaluate glucose tolerance and insulin sensitivity.
3. To estimate plasma insulin and adiponectin levels, and lipid profile.
4. To study transcript levels of glucoregulatory enzymes and enzyme activities in liver.
5. To study transcript levels of genes involved in mitochondrial biogenesis and ETC complex activities in skeletal muscle.
6. To study expression of proteins involved in insulin signaling pathway in skeletal muscle.

7. To study the effect on pancreatic β-cell regeneration and β-cell death.

Results:

<u>Objective I</u>: To assess the association of *ADIPOQ* polymorphisms with T2D in Gujarat population and to study the possible genotype-phenotype correlation with plasma adiponectin levels.

Adiponectin, an anti-inflammatory adipokine, is an important determinant of the status of insulin sensitivity. Various association studies of adiponectin (*ADIPOQ*) gene single nucleotide polymorphisms (SNPs) and metabolic diseases like T2D, and cardiovascular diseases have been reported earlier. However, results are varied and ambiguous due to the apparent contradictions. Hence, we aimed to investigate (1) the association between *ADIPOQ* SNPs: −11377C/G, +10211T/G, +45T/G and +276G/T for the risk towards T2D and, (2) genotype-phenotype correlation of these SNPs with various biochemical parameters in Gujarat population. Age and sex matched genomic DNA of 500 diabetic patients and 500 controls from Gujarat ethnicity were genotyped using PCR-RFLP and TaqMan assay. Plasma HMW and total adiponectin levels were estimated by ELISA. Results suggested: (i) association of +10211T/G (p<0.001) and +276G/T (p=0.008) with risk towards T2D, (ii) reduced HMW adiponectin/ total adiponectin ratio in Gujarat patients (p<0.001) and its association with +10211T/G (p<0.001) and +276G/T (p<0.001), (iii) association of the above SNPs with increased Fasting Blood Glucose (FBG), Body Mass Index (BMI), Triglyceride (TG), Total Cholesterol (TC) in Gujarat patients (p<0.001), and (iv) increased GGTG haplotype in obese patients (p=3.87x10^{-5}) of Gujarat population. Reduced HMW adiponectin, in the backdrop of obesity and *ADIPOQ* genetic variants might alter metabolic profile posing risk towards T2D.

<u>Objective II:</u> To investigate the therapeutic potential of small molecule enhancers for adiponectin & GLP-1 secretion in mouse model of glucose intolerance/insulin resistance.

New therapeutic approaches such as targeting the declining levels of insulin sensitizing hormones along with decreased insulin secretion or action would result in the development of a holistic therapy. As β-cell dysfunction and eventual death in the pre-context of dyslipidemia is a central parameter of T2D, we developed a High Fat Diet (HFD)+ streptozotocin (STZ) induced mouse model to mimic these conditions. We thereafter explored the action of pitavastatin and L-glutamine alone and in combination in HFD+STZ induced T2D mouse model. 9-10 weeks old male C57BL/6J mice (n=32) were fed with HFD for 18-20 weeks to induce obesity and insulin resistance while non-diabetic control group was fed with Normal

Chow Diet (NCD; n=8). Body Weight (BW) and FBG levels were monitored weekly. The HFD fed mice (n=32) were then administered with STZ for 3 consecutive days (40 mg/Kg b.w i.p) to induce beta cell destruction. The HFD+STZ treated animals were randomly assigned into four groups: 1) Diabetic Control (DC), 2) Pitavastatin (P), 3) L-glutamine (LG), and 4) P+LG treated (n=8 respectively). Mice were treated with pitavastatin (0.5mg/Kg b.w in diet) and/ L-glutamine (500 mg/Kg b.w p.o.) daily for 6 weeks along with BrdU (100 mg/Kg b.w i.p.) to monitor β-cell proliferation. Significant amelioration of insulin sensitivity (p<0.001) and glucose tolerance (p<0.001) along with reduced FBG (p<0.001), triglyceride (p<0.001), total cholesterol (p<0.001), low-density lipoprotein (p<0.01), and insulin (p<0.001) levels were observed in pitavastatin+L-glutamine (P+LG) group subjected to six-week treatment. The specific activity of the gluco-regulatory enzymes, fructose 1,6-bisphosphatase (p<0.001), and phosphoenolpyruvate carboxykinase (p<0.001), and *GLUT2* (p<0.001) transcript levels in liver were reduced. In addition, an increased gene expression of *glucokinase* (p<0.001) and *glycogen synthase* (p<0.01) along with increased glycogen content were also observed (p<0.001) in the combination treated group. Further, the oxygen consumption rate of mitochondrial complexes I (p<0.001), II (p<0.001) and III (p<0.001) and mitochondrial biogenesis as indicated by *SIRT1* (p<0.001), *PCG1α* (p<0.01) and *TFAM* (p<0.001) transcript levels were restored in the P+LG treated group. Western blot analysis of the proteins involved in insulin signalling pathway showed an increase in the $pIRS_{Ser307}$/IRS (p<0.05) and $pAKT_{S473}$/tAKT (p<0.001) ratios, IR1β (p<0.01) in the monotherapies and combination treated group suggesting an increase in the insulin sensitivity in the skeletal muscle. However, results on protein expression of GLUT4 and PPARα in skeletal muscle will be discussed in the thesis. In addition, a significant increase in islet number as a result of β-cell regeneration (proliferation) (p<0.001) and reduced β-cell death (TUNEL) (p<0.001) was also observed in the P+LG treated group. However, no translocation of apoptosis inducing factor (AIF) was observed in any of the groups suggesting absence of caspase-independent cell death. The monotherapies showed only a marginal enhancement in the above parameters, thus put together their action was synergistically enhanced as shown in Figure1.

Conclusion:

Our population-based study showed that *ADIPOQ* +10211T/G and +276G/T polymorphisms were significantly associated with T2D in Gujarat population. Further, the GGTG haplotype showed the risk towards developing obesity-induced T2D. Also, +10211T/G and +276G/T

polymorphisms were significantly associated with increased FBG, dyslipidemia and reduced HMW: total adiponectin levels. Pitavastatin in combination with L-glutamine ameliorates T2D synergistically by improving glucose homeostasis, lipid profile, mitochondrial biogenesis and β-cell survival.

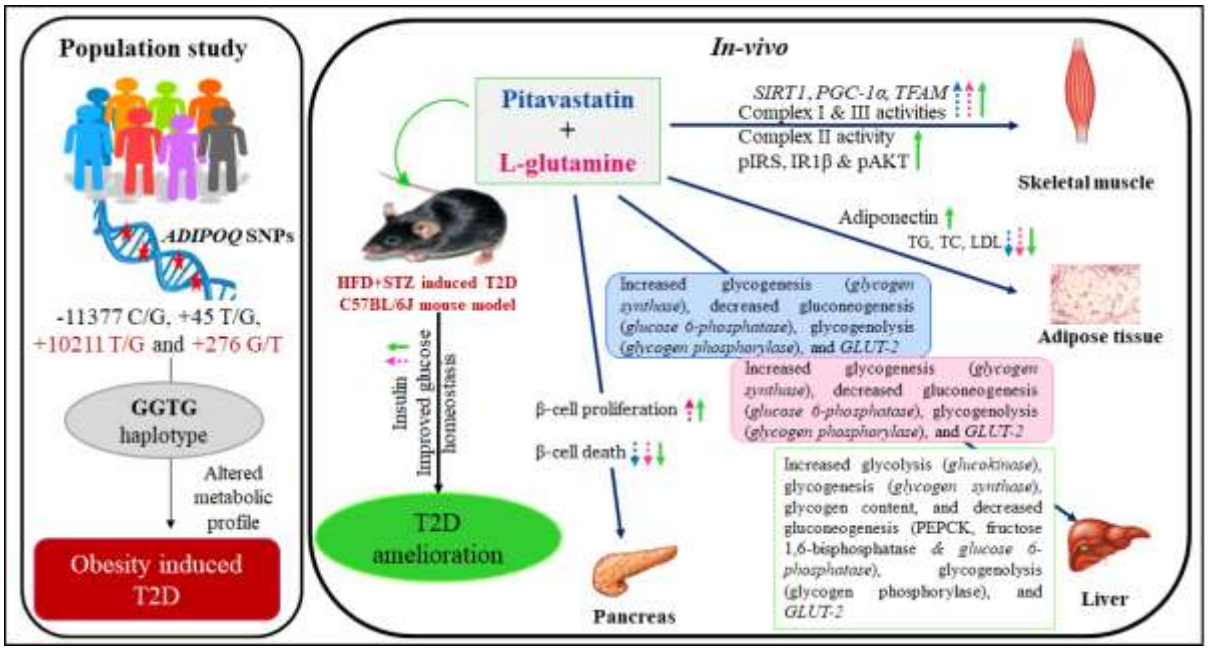

Figure 1: Our population study suggested an association of *ADIPOQ* +10211 T/G and +276 G/T with altered metabolic profile and risk towards T2D, and the haplotype GGTG (-11377 C/G, +45 T/G, +10211 T/G and +276 G/T) conferred risk towards the development of obesity induced T2D. Further, pitavastatin and L-glutamine could induce normolipidemia and reduce the transcript levels of *glucose 6-phosphatase, glycogen phosphorylase* and *GLUT2* in liver whilst increasing *SIRT1, PGC1α* and *TFAM*, mitochondrial complex I and III activities in skeletal muscle. Both pitavastatin and L-glutamine could also reduce β-cell death individually and L-glutamine also could induce β-cell proliferation. In combination they could additionally increase mitochondrial complex III activity in skeletal muscle, and *glucokinase* transcript levels with a concomitant decrease in PEPCK and fructose 1,6-bisphophatase activities in liver. The combination also induced normoadiponectinemia, increased phosphorylation of IRS and AKT, increase IR1β protein levels in skeletal muscle, and insulin levels. As a whole the drugs could work together synergistically to bring normolipidemia, reverse mitochondrial dysfunction, decrease gluconeogenesis, glycogenolysis, glycogen synthesis and *GLUT-2*. The combination therapy could also reduce β- cell death, increase insulin levels, enhance glucose tolerance and insulin sensitivity thus being able to ameliorate T2D in HFD+STZ induced mouse model.

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Publications:

1. **Palit SP**, Patel R, Jadeja SD, Rathwa N, Mahajan A, Ramachandran AV, Dhar MK, Sharma S, Begum R. (2020) A genetic analysis identifies a haplotype at adiponectin locus: Association with obesity and type 2 diabetes. *Sci Rep.*, 10:2904. (IF:3.99)
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Manuscript under preparation:

Repurposing statin and L-glutamine to replenish β-cells in hyperlipidemia

Oral/poster presentations:

1. **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. *(*Received YUVA scholarship award for participation in the conference*).

2. **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. Poster presentation delivered at ICRED- 2019, 37th Annual Conference of the International Conference on Reproductive Biology and Comparative Endocrinology (19-21 January 2019) at School of Liberal Studies and Education, Navrachana University, Vadodara-391410, Gujarat, India (*Received Best Poster Award*).
3. **Pramanik S**, Patel R, Rathwa N, Ramachandran AV, Begum R. Haplotype at adiponectin locus and its remarkable association with type 2 diabetes. Oral presentation delivered 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. (*Received 1st prize for best poster*)
4. **Pramanik S**, 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 Dec 2017 (*Received Best Poster Award*).
5. **Pramanik S**, Patel N, Rana S, Ramachandran AV, Begum R. Association of Adiponectin Genetic Variants with Type 2 Diabetes. Poster presentation delivered at International Conference on Reproductive Biology and Comparative Endocrinology & The 35th Annual Meeting of The Society for Reproductive Biology and Comparative Endocrinology, 9-11 February 2017 held at Department of Animal Biology, University of Hyderabad, Hyderabad, India.
6. **Palit SP**, Rathwa N, Patel R, Rana S, Patel N, Ramachandran AV, Begum R Association of Adiponectin and Resistin genetic variants with Type 2 Diabetes. Poster presentation delivered at Two-day National Symposium on Omics…to Structural Basis of Diseases, 30 Sept. and 1 Oct. 2016 held at The M. S. University of Baroda, Vadodara, Gujarat, India
7. 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.
8. Patel R, **Pramanik S**, 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. Poster presentation delivered at American Diabetes Association 79th Scientific Sessions (7-11 June 2019) at Moscone Center, San Francisco-94103, California, USA.
9. 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. Poster presentation delivered at International Conference on 'Proteins, miRNA and Exosomes in Health and Diseases' held at The M. S. University of Baroda, Vadodara, Gujarat, India on 11-13 December 2018.
10. Rathwa N, **Palit SP**, Patel R, Dhimmar H, Bhati H, Parmar N, Ramachandran AV, Begum R. Genetic Variants of Omentin-1 And Vaspin: Association with Obesity And

Dyslipidemia Related To Type 2 Diabetes Susceptibility. Poster presentation delivered at International Conference on Reproductive Physiology and Comparative Endocrinology & The 36th meeting of SRBCE, 20 – 22 January 2018 held at BITS Pilani, KK Birla Goa Campus, Goa, India.

11. 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.
12. 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.
13. Rathwa NN, Patel R, **Pramanik S**, Parmar NR, Ramachandran AV, Begum R. Calorie restriction in combination with GABA ameliorates type 2 diabetes. Poster presentation delivered at American Diabetes Association 79th Scientific Sessions (7-11 June 2019) at Moscone Center, San Francisco-94103, California, USA.
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18. 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. Poster presentation delivered at International Conference on 'Proteins, miRNA and

19. Patel R, Rathwa N, **Palit SP**, 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. Oral presentation delivered at International Conference on Reproductive Physiology and Comparative Endocrinology & The 36th meeting of SRBCE, 20 – 22 January 2018 held at BITS Pilani, KK Birla Goa Campus, Goa, India.

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