Evaluation of AKT1 Mediated MST1 Regulation by PTP1B Inhibition via Viscosol in Streptozotocin and High Fat Diet-Induced Type 2 Diabetic Mice Model

By

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In the Name of ALLAH, the Most Gracious, the Most Mercifu

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CERTIFICATE

This thesis, submitted by **Ms. Irum Nasir** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Dated: **October 16, 2023**

Declaration

I hereby declare the authenticity and originality of the work presented in this thesis. it is an honest embodiment of my scholarly research. It is written and composed by me. No part of this thesis has ever been published or submitted for another degree or certificate.

IRUM NASIR

Dedicated to my beloved family and friends for their continuous support, love, compassion, and blessings.

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AK strain transforming	AKT
Activation Transcription Factor 4	ATF4
Adenosine Triphosphate	ATP
Adipose Tissue Hypoxia	ATH
AMP-Activated Protein Kinase	AMPK
Apoptosis Signaling Kinase 1	ASK1
Acetyl-CoA carboxylase	ACC
Bcl2 associated agonist of cell death	BAD
Bcl-2 associated X	BAX
B-Cell Lymphoma 2 Gene	Bcl-2
Bcl-2 Homology-3 (BH3)	BIM
Body mass index	BMI
C/EBP Homologous Protein	CHOP
Catalase	CAT
C-Jun N-Terminal Kinase	JNK
Damage-Associated Molecular Patterns	DAMPs
Diabetes Mellitus	DM
Endoplasmic Reticulum	ER
Eukaryotic Translation Initiation Factor-4e-Binding Protein1	4E-BP1
Fas Ligand	FASL
Forkhead Box O1	Foxo1
Free fatty acids	FFAs

Ga-Binding Protein	GABP
Gasdermin E	GSDME
Glucokinase	GCK
Glucose Transporter 2	GLUT2
Glucose Transporter 4	GLUT4
Glucose-Regulated Protein 78	Grp78
Glutamyl-Cysteine Ligase	GCL
Glutathione Reductase	GSR
Glycogen Synthase	GS
Glycogen Synthase Kinase 3b	Gsk3β
Grb-2-Associated Protein	Gab1
Growth-Factor-Bound Protein 10	Grb10
High Fat Diet	HFD
Il-1 Receptor Antagonist	IL-1Ra
Inositol-Requiring Enzyme 1α	IRE1α
Insulin dependent diabetes mellitus	IDDM
Insulin Receptor	IR
Insulin Receptor Substrate 1	IRS1
Insulin Receptor Substrate 2	IRS2
Interleukin-18	IL-18
Interleukin-1β	IL-1β
Large Tumour Suppressors 1/2	LATS1/2
Leucine-Rich Repeats	LRR
Luminal Binding Protein	BIP

Major histocompatibility complex	MHC
Malnutrition-related diabetes mellitus	MRDM
Mammalian Sterile 20-Like Kinase 1	MST1
Mitogen Activated Protein Kinase	MAPK
Messenger Ribonucleic Acid	mRNA
Mps-One Binder 1	Mob1
mTOR Complex 1	mTORC1
mTOR Complex 2	mTORC2
NAD(P)H: Quinone Oxidoreductase	Nqo1
Neurofibromatosis Type 2	NF2/Merlin
NIMA Related Kinase 7	NEK7
NLR family Pyrin domain containing 3	NLRP3
Non-esterified fatty acids	NEFAs
Non-insulin dependent diabetes mellitus	NIDDM
Nuclear Factor Kappa B	NF-kB
Pancreatic and Duodenal Homeobox 1	PDX1
Pathogen-Associated Molecular Patterns	PAMPs
Peroxiredoxin1	Prdx1
PH Domain Leucine-Rich Repeat Protein Phosphatase	PHLPP2
Phosphatidylinositide-3-Kinase	PI3K
Phosphatidylinositol-3,4,5-Triphosphate	PIP3
Phosphatidylinositol-4,5-Bisphosphate	PIP2
Phosphoinositide-Dependent Protein Kinase 1	PDK1
Pleckstrin Homology	PH

Protein Kinase C	PKC
Protein Tyrosine Phosphatase1B	PTP1B
Protein tyrosine phosphatases	PTPs
Reactive Oxygen Species	ROS
Salvador	Sav1
Signal Transducer and Activator of Transcription 1	STAT-1
Src Homology 2 Domain	SH2
Standardized mortality ratio	SMR
Sterol-regulatory element binding protein-1C	SREBP-1C
Streptozotocin	STZ
Superchiasmatic Nucleus	SCN
Tea Domain	TEAD
Thioredoxin-1	Trx1
TNF Death Receptors	TNFR
Toll-Like Receptor 4	TLR4
Tuberous Sclerosis Complex 1	TSC1
Tuberous Sclerosis Complex 2	TSC2
Tumor Necrosis Factor Alpha	TNF-α
TNF Receptor-Associated Factor 2	TRAF2
Type 1 diabetes mellitus	T1DM
Type 2 Diabetes Mellitus	T2DM
Unc-51 Like Autophagy Activating Kinase 1	ULK1
Unfolded Protein Response	UPR
Voltage-Dependent Ca2+ Channels	VDCC

ABSTRACT

Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disease which is distinguished by impaired insulin secretion, and *β*-cells death. MST1 is a widely distributed serine/threonine kinase and is the main upstream regulator of Hippo pathway. MST1 is recognized as a major factor responsible for inducing apoptosis in *β*-cells. Several studies have reported the hyperactivation of MST1, mTORC1, PHLPP2, and NLRP3 inflammasome in pancreatic cells in T2DM. In T2DM, PTP1B is over-expressed in many tissues such as liver, pancreas, and adipose tissue. Collected data shows that PTP1B overexpression is indirectly correlated with MST1 activation which is itself regulated by AKT1 in pancreatic *β*-cells. Therefore, we hypothesized that the PTP1B inhibitor, Viscosol which is isolated from *Dodonaea viscosa*, could inhibit the activation of MST1 and its mediated pathways related to apoptosis, autophagy, inflammation, and lipotoxicity. For this purpose, we designed HFD-low dose STZ-induced T2DM mice model and treated it with Viscosol. We assessed the in vivo effect of Viscosol against MST1-mediated apoptosis, autophagy, lipotoxicity, and inflammation. Furthermore, we determined the relative mRNA expression levels of our specific markers of insulin, Hippo, PHLPP2, mTORC1, and NLRP3 signalling pathways. The data revealed the reduction of PTP1B, MST1, PHLPP2, mTORC1 and NLRP3 and their down regulatory markers in our compound treated group. Moreover, in our compound treated group, we analyzed the inhibition of apoptosis, retrieval of PI3K/AKT signalling, autophagy activation and inhibition of lipid accumulation in the pancreas. Our findings suggest that Viscosol offers protective benefits against T2DM, demonstrating anti-apoptotic, hypoglycemic, anti-inflammatory, and hypolipidemic activities in the pancreas. This implies Viscosol's potential as a therapeutic agent for T2DM and related complications, primarily through the inhibition of PTP1B and MST1.

Keywords: T2DM, PTP1B, MST1, AKT1, Pancreatic *β*-cells, Apoptosis, *Dodonaea viscosa*

1. Introduction

1.1. Diabetes

Diabetes Mellitus (DM) is a long-term metabolic disorder that results in hyperglycemia because of reduced β-cells activity and insulin resistance, which eventually results in insulin insufficiency (Cole & Florez, 2020). World Health Organization (WHO) describes DM as a chronic metabolic condition characterized by eminent glucose levels in the blood that damage the kidneys, blood vessels, eyes, heart, and nerves. Type 2 Diabetes Mellitus (T2DM) is accountable for 90% of diabetes incidents due to insulin deficiency and resistance (Stumvoll *et al*., 2005; Weyer *et al*., 1999). Diabetes symptoms include polydipsia, polyuria, polyphagia visual impairment, weight loss, and genital yeast infections. Severe ketoacidosis can lead to dehydration, coma, and even death (Zimmet, Alberti, & Shaw, 2001).

Over 400 million people are affected by diabetes globally. The two main forms of diabetes (types 1 and 2) are distinguished by pancreatic β-cell malfunction and, in some circumstances, loss of β-cell mass that results in hyperglycemia triggered by absolute or relative insulin insufficiency (Miani *et al*., 2018).

1.1.1. Prevalence and Impact of T2DM on Global Health

The incidence of DM is expanding worldwide, more particularly in urbanized regions. The prevalence of DM starts at the age of 55 years, with no masculinity discrimination. The prevalence of T2DM is predicted to reach 7079 cases per 100,000 people globally by 2030, indicating a persistent rise across all geographical regions. In lower-income countries, distressing trends of rising prevalence of diabetes are found (Abdul *et al*., 2020).

International Diabetes Foundation (IDF) Diabetes Atlas 10th edition reported 537 million cases of diabetes globally, and this digit is estimated to extend to 643 million in 2030 and 783 million in 2045. Pakistan ranked 3rd, having the highest reported incidence of diabetes among people aged 20 to 79 in 2021, with 33 million cases and a 30.8% prevalence compared to India and China. Comparing IDF 9th and 10th Reports, there is an increase of 13.6 million diabetes cases from 2019-2021, because of an insalubrious lifestyle. Pakistan ranked 4th with 0.4 million annual deaths due to

diabetes. Diabetes account for 29.5% of total annual deaths, and the death rate among those under 60 is 35.5%. In Pakistan, 8.9 million undiagnosed people live with diabetes (*IDF Diabetes Atlas-10th Edition*, 2021).

Diabetes has a negative impact on an individual's functioning and quality of life, which causes severe morbidity as well as early death (Ramtahal *et al*., 2015). It has been suggested that these changes have been caused by rising levels of sedentary behavior and consumption of unhealthy food, both of which are associated with elevated fasting blood glucose concentrations and higher body mass index (BMI) (Lone *et al*., 2017). T2DM is prevalence is higher in people having elevated BMI (Mahanta *et al*., 2013).

1.2. Causes of Type 2 Diabetes Mellitus

1.2.1. Obesity and Sedentary Lifestyles

Obesity is associated with the release of free fatty acids (FFAs) into blood circulation and the distribution of FFAs to body tissues at a much faster rate (Mittendorfer *et al*., 2009). Elevated levels of FFAs in plasma play a substantial role in the insulin resistance of the liver and muscles in obese individuals (Karpe *et al*.,2011). An extreme quantity of body fat buildup causes T2DM and increases the incidence of T2DM with increased BMI. The prevalence of T2DM linked with obesity resulted in complicated biological and physiological processes responsible for changes in adipose tissue biology, multi-organ insulin resistance, and β-cell biology (Scherer, 2022). Excessive buildup of triglycerides in adipose, escalates adipocyte size and involves structural transforming to allocate the basis required for maintaining the larger adipocyte mass (Crewe *et al*., 2017; Scherer, 2016; Smith *et al*., 2019). An increase in visceral fat is an important factor that contributes to insulin resistance because an increased release of FFAs directly into the blood circulation is based on the association between visceral adipose tissue mass, insulin resistance, and the probability of developing T2DM (Kissebah *et al*., 1982; Nielsen *et al*., 2004). However, in obese individuals, only 20% of FFAs supplied to the liver derive from the lipolysis of visceral fat, while the remaining 80% come from the lipolysis of subcutaneous fat (Nielsen *et al*., 2004).

Adipose tissue hypoxia (ATH) is considered a significant factor in obesity-related disorders, which resulted in macrophage infiltration, inflammation, diminished adiponectin secretion, prominent leptin levels, adipocyte demise, ER stress, and mitochondrial malfunctioning in white adipose tissue because of obesity. ATH inhibits adipogenesis and triglyceride synthesis, leading to increased FFAs in blood circulation. Reactive oxygen species (ROS), FFAs, diacylglyceride, hypoxia, ceramide, and *TNF-α*, have been shown to trigger the activation of IKK*β* and JNK1 in adipose tissue, muscle and liver (Ye, 2009), causing insulin receptor substrate 1 (IRS-1) suppression, impeding insulin signaling and causing insulin resistance. This process contributes to impaired glucose regulation observed in conditions like T2DM (Aguirre *et al*., 2000; Z. Gao *et al*., 2006; Z. Gao *et al*., 2002). Upon activation, IKK*β* phosphorylates *IκBα* causing its ubiquitination-mediated subsequent degradation in proteasome, which allows NFκB translocation into nucleus, where it initiates transcription of various genes responsible for inflammatory and immune system responses (Rui *et al*., 2001). IKK*β* and JNK1 act as primary regulators of insulin signaling in liver, adipose tissue, and muscle, leading to insulin resistance. Hyperglycemia and obesity further enhance this effect by stimulating IKK*β* and JNK1, contributing to impaired insulin responsiveness and insulin resistance in these tissues (J. Zhang, Gao, Yin, Quon, & Ye, 2008).

The adipose tissue secretes non-esterified fatty acids (NEFAs), linking *β*-cell malfunction and increased insulin resistance (Kahn *et al*., 2006). ROS and endoplasmic reticulum (ER) stress pathways are produced by saturated fatty acids (like palmitate), which has been associated with apoptosis. Saturated fatty acids play role in apoptosis through the stimulation of ROS and ER stress pathway (X. Li *et al*., 2021).

1.2.2. ER Stress

The ER is responsible for calcium storage, lipid synthesis, protein secretion, trafficking, and processing, necessary for cell survival, differentiation, growth, and proliferation (Flamment *et al*., 2012; Villalobos-Labra *et al*., 2018). Numerous factors, including obesity, a high-fat diet, pharmaceutical drugs, oxidative stress, and hyperglycemia, can produce ER stress (Bánhegyi *et al*., 2007; Ozcan *et al*., 2004).

Numerous resident proteins, including PTP1B, which also contribute to ER stress, are activated when calcium sources are depleted as a result of ER disruption (Amodio *et al*., 2009). ER, stress triggers a protective mechanism called Unfolded Protein Response (UPR), which helps to avoid the overproduction of misfolded proteins and rehabilitate ER balance. When UPR fails to restore ER homeostasis, pro-inflammatory and apoptotic signals are activated (Marciniak & Ron, 2006). UPR activation in the context of ER stress causes inflammation and lipid accumulation, which affects insulin production, *β*-cells dysfunction, and reduce insulin sensitivity, collectively resulting in insulin insensitivity in numerous tissues such as adipose, skeletal muscle, and liver (Flamment *et al*., 2012; Han *et al*., 2013; Salvadó *et al.*, 2015). In addition to impairing insulin signaling by reducing autophagy, ER stress interferes with glucose transport by regulating the expression of GLUT-4 and the synthesis of insulin receptors (Han *et al*., 2013; Yang *et al*., 2010).

Through two luminal domains, the ER stress transducers control the suppression of protein synthesis and degradation of mRNA. The first domain detects misfolded proteins, while second domain conveys signals to cytosol and nucleus when the stabilization processes collapse (Eletto *et al*., 2014) and the same process is used for the commencement of the apoptosis (Mustapha *et al*., 2021). The Bcl-2 protein family and caspases are two protein families that are involved in the apoptotic pathway, which is the basis for differentiating two paths of apoptosis (H. C. Lee & Wei, 2000; Lindenboim *et al*., 2020). The external pathway, associated with *Fas* or *TNFR* (Bruni *et al*., 2018), and activated when death receptors (such as CD9 and TNFR) are bound on the cell surface. In pancreatic *β*-cells, TNF-α and cytokines stimulate the expression of Fas. This induction initiates the stimulation of nuclear factor κb (NF-κB) and signal transducer and activator of transcription 1 (STAT-1), triggering a cascade of caspases activation that ultimately implements the effector mechanism (Anuradha *et al.*, 2014). The internal pathway is connected to the mitochondria and is associated with Bcl-2 family proteins (Popgeorgiev *et al*., 2018; Singh *et al*., 2019). Cytochrome c is released from mitochondria into the cytoplasm which causes the activation of caspase-9, which subsequently cleaves executive caspases-3/7 (Miani *et al*., 2018). The functioning of mitochondria is regulated by an

intricate group of proteins known as the *Bcl-2* family (Morris *et al.*, 2021; Youle & Strasser, 2008).

1.2.3. Mitochondrial Dysfunction

The mitochondrial-associated ER membrane (MAM), a connection between the ER and mitochondria, is essential for retaining Ca2+ stability. (Hayashi *et al*., 2009). Mitochondria are crucial in several metabolic disorders such as T2DM (Burgos-Morón *et al*., 2019). They play a role in cellular homeostasis, Ca2+ metabolism, apoptosis, autophagy, and adenosine triphosphate (ATP) synthesis (Einarson *et al*., 2018). Mitochondrial activity is linked to ROS generation in hyperglycemic conditions (Gerber & Rutter, 2017; Yu, Robotham, & Yoon, 2006). In the presence of elevated glucose concentration, morphological changes of mitochondria are an upstream pivotal factor in ROS production, emphasizing the vital role of mitochondrial dynamics as a principal regulator of mitochondrial function (Yu *et al*., 2006). In diabetic patients and animal models, altered mitochondrial size and shape have been observed. In comparison to healthy controls, mitochondria in T2DM are smaller in size (Kelley, He, Menshikova, & Ritov, 2002). Different cell types, such as heart, liver, or pancreatic cells, experience mitochondrial fragmentation as a result of hyperglycemia (Paltauf-Doburzynska *et al.*, 2004; Yu *et al*., 2006). The insulin-resistant hepatocytes have swollen and disrupted mitochondria. (Vanhorebeek *et al*., 2005). Mitophagy is a particular type of autophagy that occurs in mitochondria and is in charge of locating and removing malfunctioning mitochondria that cause mitochondrial ROS to be produced in a cell (Burgos-Morón *et al*., 2019).

1.2.4. Pancreatic *β*-Cells Dysfunction

Pancreatic *β*-cells have an essential role in maintaining glucose homeostasis by producing and releasing insulin. Insulin is a peptide which promotes intake of glucose and amino acids, glycogen synthase (GS) activity, metabolism of protein, cell growth and division, and inhibits lipolysis (Wilcox, 2005). Insulin is produced as proinsulin, which consists of a single polypeptide made of A and B chains, and a C-peptide linking both the chains (Weiss *et al*., 2000). The proinsulin to insulin conversion occurs in the trans-Golgi network and requires dissociation of C-peptide bonds from both chains A and B. In addition to carboxypeptidases, convertases, C-peptide and

significant Ca2+ concentrations, secretory granules also store insulin and C-peptide (Fu, Gilbert, & Liu, 2013; Howell, 1984). Glucose is the prime inducer of insulin secretion in pancreatic *β*-cells (Komatsu *et al*., 2013). When extracellular glucose level is elevated, glucose is transported by the glucose transporter 2 (GLUT2) inside *β*-cells. When glucose enters the cytoplasm, glucokinase (GCK) converts glucose into glucose-6-phosphate that enters the Krebs cycle in mitochondria, improving ATP/ADP ratio in cytosol. This increased ATP/ADP ratio facilitated the closing of potassium ATP channels. Reduced K+ conductance causes depolarization of membrane and facilitates the opening of voltage dependent Ca2+ channels (VDCCs), allowing Ca2+ entrance into the cell. This intracellular Ca^{2+} triggers the fusion of the insulin vesicles with the membrane causing the release of insulin hormone (Henquin, 2000).

Many mechanisms can cause a loss in function or even the destruction of *β*-cells as human pancreatic *β*-cells are not replaced, and seems incapable of renewing themselves, after 30 years (Perl *et al*., 2010). These mechanisms include inherited traits, genetic anomalies, insulin resistance, autoimmunity, concurrent diseases, inflammation, and environmental variables. Multiple low-dose streptozotocin injections cause diabetes through the stimulation of cell-specific immune-mediated destruction and cell-intrinsic apoptotic cascades (Ardestani *et al*., 2019). In T2DM, *β*-cells malfunction and decreased *β*-cells mass leading to the expansion of physiologically distinct ailments in insulin-resistant people. Numerous stimuli including glucotoxicity, lipotoxicity, proinflammatory cytokines, ER stress and oxidative stress, contribute to *β*-cells degeneration (Donath *et al*., 2003). During T2DM progression, *β*-cells experienced distinct changes in mass, phenotype, and function. Insulin resistance is caused by hyperglycemia and hyperlipidemia and enhanced insulin secretion to preserve normal glucose levels (Butler *et al*., 2003). Elevated blood glucose induces secretion of insulin hormone from pancreatic *β*-cells through the activation of protein kinase C (PKC), inhibition of ATP-gated potassium excretion and calcium infiltration (M. E. Cooper, 2012). In the beginning, excessive insulin production fully satisfies the body's need for the hormone, but as cells become overloaded with insulin, increasing insulin resistance (Chawla *et al*., 2011;

Kalupahana *et al*., 2012; Lee & Lee, 2014). Although this compensatory stage may extend for many years, it may eventually result in *β*-cells dysfunction and death. T2DM can cause a 60% reduction in *β*-cells mass (Butler *et al*., 2003).

1.2.5. Insulin Resistance

Obesity is marked by insulin resistance in peripheral tissues (muscle, adipose tissue, liver). This resistance escalates insulin demand, prompting pancreatic *β*-cells to adapt by increasing in number and function. Consequently, excessive insulin production occurs, leading to hyperinsulinemia and metabolic dysregulation linked to obesity and T2DM (Chiasson & Rabasa-Lhoret, 2004; Shanik *et al*., 2008). Insulin resistance and hyperinsulinemia occur before the onset of hyperglycemia, which only happens when *β*-cells fail to reimburse peripheral insulin resistance. At the onset of T2DM, cytokines, FFAs, and hyperglycemia have been suggested as contributors to *β*-cell degeneration. Additionally, diminished mitochondrial coupling of *β*-cells glucose metabolism and insulin secretion has been associated as a potential factor in *β*-cell degeneration in T2DM (MacDonald *et al*., 2009; E. Zhang *et a*l., 2019). Insulin resistance in insulin-sensitive tissues like adipose tissues, skeletal muscle, and the liver is the key pathological mechanism driving the progression of T2DM. Restricted glucose uptake in insulin-responsive tissues impaired insulin signaling pathways, notably PI3K/AKT pathway (Yudhani *et al*., 2023). Insulin action impairment seems to be in muscle cells and adipocytes, with flawed glucose transporter 4 (GLUT4) localization leading to diminished insulin-mediated glucose transport (Hunter & Garvey, 1998).

Insulin resistance leads to impaired insulin signaling including desensitization of the insulin receptor, destruction of IRS and their functioning, suppression of PI3K pathway, and inability to suppress Foxo1-triggered gene transcription, resulting from IRS1 and IRS2 inhibition. Each IRS1 and IRS2 molecule has 40 possible serine/threonine phosphorylation sites that can be regulated by MAPK, p38a, mTOR, PKC, and JNK, this can either promote IRS decomposition or prevent IRS-related PI3K activation (Copps & White, 2012; Guo, 2013; Qi *et al*., 2013; X. J. Sun & Liu, 2009). FoxO1 dephosphorylation at AKT sites stimulates FoxO1 stabilization and transcriptional expression, leading to increased hyperglycemia and gluconeogenesis.

In T2DM, elevated dephosphorylation of FoxO1 at S^{253} was observed in nucleus of the heart and liver (Altomonte *et al*., 2003; Battiprolu *et al*., 2012). Insulin resistance and T2DM can be induced in mice by activating NF-kB, a crucial regulator of pro-inflammatory reactions, by IKKb overexpression in the liver (Cai *et al*., 2005). TNFa increases insulin resistance by lowering IRS1 through JNK or S6K activation (Z. Gao et al., 2002; J. Zhang et al., 2008). FFAs from food intake or carbohydrate conversion serve as an essential energy source as well as signals which regulate protein kinases such as PKC, and JNK to inhibit insulin signaling (Holzer *et al*., 2011; Talukdar *et al*., 2010). Saturated fatty acids interact with fetuin-A, a glycoprotein released by the liver, which stimulates Toll-like receptor 4 (TLR4). The activated TLR4 activates NF-Kb, causing recruitment of c-SRC, resulting in activation of JNK and impairment of insulin function (Holzer *et al*., 2011).

1.3. Types of Diabetes

In 1965, WHO introduced the first diabetes classification system, categorized by age, recognizing various forms, including infantile, young, adult, and elderly by age, WHO also renowned other types of diabetes such as juvenile-type; brittle, insulin-resistant, gestational, pancreatic, and endocrine ("Diabetes Mellitus. Report of a WHO Expert Committee.," 1965).

In 1980, WHO published a diabetes classification that included two types of diabetes: type 1 or insulin-dependent diabetes mellitus (IDDM) and type 2 or non-insulin dependent diabetes mellitus (NIDDM) ("WHO Expert Committee on Diabetes Mellitus: Second Report.," 1980). In 1985, an updated version of 1980's Classification was issued. The words "type 1" and "type 2" were excluded in this study, but IDDM and NIDDM were kept and introduced a third category called malnutrition-related diabetes mellitus (MRDM) ("Diabetes Mellitus. Report of a WHO Study Group.," 1985). 1980 and 1985 reports introduced gestational diabetes mellitus (GDM), and the 1999 classification system was established on clinical stages which advance from normal to severe hyperglycemia. WHO omitted MRDM, and reinstated type I and type II names due to lack of evidence (Alberti & Zimmet, 1998).

1.3.1. Type 1 Diabetes Mellitus

An autoimmune process causes type 1 diabetes mellitus (T1DM) by attacking the pancreatic *β*-cells that produce insulin. *β*-cells destruction leads to impaired insulin secretion, hyperglycemia development, and ultimately leading to T1DM with very little or no insulin production. Genetic predisposition and an environmental stimulus including viral infection, initiate the autoimmune response which ultimately causes *β*-cells destruction (Atkinson *et al*., 2014; Craig et al., 2009). Symptoms of TIDM are ketoacidosis and reduced body mass index (BMI) are the common symptoms of T1DM (Thomas *et al*., 2018). Ketoacidosis may be the first disease symptom in some people, especially adolescents, and kids (Jackson *et al*., 2001).

70% to 90% of people with T1DM have antibodies besides glutamic acid decarboxylase (GAD65), ZnT8 transporter, islet antigen-2 (IA-2), and correlations with immune-related genes (Eisenbarth, 2007). More than 50 loci, responsible for TIDM, have been recognized via genome-wide association studies (N. Cooper *et al*., 2017). Genes in major histocompatibility complex (MHC) that contain HLA classes I (HLA-*A*, HLA-*B*, HLA-*C*) and II (HLA-*DRB1*, HLA-*DQA1*, HLA-*DQB1*, HLA-*DPA1*, HLA-*DPB1*) have greatest effect on genetic risk. Approximately 30% of disease susceptibility comes from variations in the HLA genes, which are among the most polymorphic in the human genome (C. C. Robertson & Rich, 2018).

1.2.2. Type 2 Diabetes Mellitus

T2DM is the most predominant metabolic diseases in the world, primarily triggered by impaired insulin excretion by *β*-cells and incompetence of insulin-responsive tissues (Roden & Shulman, 2019). The onset of T2DM involves many organs including the pancreas, adipose tissues, skeletal muscles, kidneys, brain, liver, and small intestines (DeFronzo, 2009). T2DM reduces life expectancy by up to one decade (Einarson, Acs, Ludwig, & Panton, 2018). The disease affects not only adults, but also increasing numbers of children and adolescents (*GLOBAL REPORT ON DIABETES*, 2016).

T2DM is a widespread category of diabetes, accounting for 90–95% of all cases. The primary issue in T2DM is insulin resistance, but as the condition progresses into a chronic hyperglycemic state, *β*-cell apoptosis occurs, resulting in a complete

deficiency of insulin (Association, 2018). As one of the three *PPAR* isoforms, Peroxisome proliferator-activated receptor gamma (*PPARG)* regulates adipogenesis, lipid metabolism, and insulin sensitivity, a factor that influences T2DM susceptibility in different ancestries (Ringel *et al*., 1999; Yen *et al*., 1997). Numerous reports have described that PPARG SNPs are crucial in regulating lipid and glucose metabolism. According to epidemiological studies, the missense Pro12Ala variant, commonly known as rs1801272, is the most prominent in the entire coding region of the *PPARG* gene, located in exon B (Stumvoll & H. ring, 2002). Yen et al. first reported the effect of the Pro12Ala variant on the development of T2DM (Yen *et al*., 1997).

1.2.3. Gestational Diabetes

A fluctuating condition that develops during pregnancy called gestational diabetes (GD) raises the long term possibility of developing T2DM (Bellamy *et al*., 2009). Blood glucose levels, higher than average but yet below those that indicate diabetes, indicate the presence of the disorder ("Diagnostic Criteria and Classification of Hyperglycaemia First Detected in Pregnancy: A World Health Organization Guideline.," 2014). Besides women who have hyperglycemia in the early stages of pregnancy, GD develops in women who cannot secrete enough insulin to counteract the reduced effectiveness of insulin (insulin resistance) due to increased hormone production by placenta as pregnancy develops (Organization, 2013).

1.4. Insulin Signaling Pathway

Upon insulin binding, the activation of the Insulin receptor (IR) tyrosine kinase triggers a cascade where multiple substrates such as IRS1-4, SHC, Grb-2-associated protein (GAB1), DOCK1, and CBL are recruited and phosphorylated. This process subsequently activates both Ras/MAPKs and PI3K/AKT pathways (White, 2003). IRS-1 and IRS2 are broadly dispersed, where IRS-2 is essential in hepatocytes and *β*-cells, and *IRS-1* in endothelial cells and vascular smooth muscle cells (VSMCs). *IRSs* upon Tyr-phosphorylation act as anchoring points for proteins that have SH2 domains (Siddle, 2012). Phosphorylation of PIP2 to PIP3 is catalyzed by PI3K upon activation. PIP3 functions as second messenger, enabling the anchoring and stimulation of *PH* (pleckstrin homology) domain-containing proteins, such as PDK1. AKT is phosphorylated at Thr^{308} and *PKC* at Thr^{410} as a result of PDK1 activation. AKT is

also phosphorylated at Ser^{473} by mTORC2 rather than PDK1, as mTORC2's most crucial function (Jacinto *et al*., 2004; Sarbassov *et al*., 2005). Glycogen synthase kinase 3b (*Gsk3b*), is one of AKT's downstream targets, activating the glycogen synthase (GS) (Taguchi & White, 2008). AKT also phosphorylates Bad for the prevention of apoptosis, and AS160 for the activation of the Rab10GTPase and GLUT4 translocation. CREB-regulated transcription coactivator 2 (CRTC2), which boosts hepatic gluconeogenesis, is phosphorylated and repressed by AKT (Wang *et al*., 2010). Activated AKT inhibits FoxO proteins such as FoxO1, FoxO3, and FoxO4, impeding their role in the metabolic process, apoptosis stimulation, and cell growth inhibition (Myatt & Lam, 2007). PI3K-AKT is also responsible for regulating the raptor-mTOR pathway, which controls cell growth and metabolism (Taguchi & White, 2008). AKT phosphorylation inactivates TSC2, preventing its interaction with TSC1, enabling RhebGTPase to activate mTORC1 (Inoki *et al*., 2002). By phosphorylating 4E-BP1, mTORC1 triggers the release of *eIF4E*, which interacts with translation initiation complex and promotes protein translation (Hara *et al.*, 1997). A serine/threonine kinase, p70S6K, activates the S6 ribosomal protein and stimulates translation by triggering numerous proteins of mRNA translation machinery (Ma & Blenis, 2009). Specific phosphatases, such as PTP1B, stop the insulin receptor cascade from signaling. Under a high-fat diet, mice showed impaired insulin sensitivity with enhanced *PTP1B* gene expression (Elchebly *et al*., 1999). PTEN is a crucial negative controller of insulin signaling due to its role in dephosphorylation of PIP3 to PIP2 in PI3K signaling pathway (Nakashima *et al*., 2000; Simpson *et al*., 2001).

1.5. Mammalian Sterile 20-like Kinase (MST)

MST belongs to a large family of kinases (serine/threonine) called sterile 20 proteins (Ste20p). Due to significant similarity between the catalytic domains of the human members and Ste20p of Saccharomyces cerevisiae, about 30 human members of Ste20p have been discovered (Pombo et al., 2007). Ste20 family members contain a distinct kinase domain and a structurally distinct region involved in modulation (Dan, Watanabe, & Kusumi, 2001). Mammalian Ste20 family comprises two fundamentally distinctive subfamilies, P21 activated kinase family (PAK) containing the C-terminal

kinase domain and germinal center kinase (GCK) family containing N-terminal kinase domain. MST1 belongs to GCK family (Creasy, Ambrose, & Chernoff, 1996). PAK is divided into PAKI-II; on the other hand, GCK is divided as GCK I-VIII (Boomer & Tan, 2005; Dan et al., 2001; Nogueira et al., 2008; Strange, Denton, & Nehrke, 2006). The kinase domain of PAKs is found in COOH terminus and p21 GTPase-binding domain in N-terminus promotes binding to small GTPases like Cdc42. PAK members are prominent mediators of apoptosis, cell migration, and growth (Hofmann et al., 2004). The Kinase domain of GCKs is sited in the N-terminus, but GCKs lack GTPase binding domains. GCKs play a role in physiological processes, particularly immune response, and cell volume homeostasis. (Pombo *et al*., 2007; Shui *et al*., 2007). MST1 and MST2 belong to GCK II subfamily while MST3 and MST4 belongs to GCK III subfamily (Ling, Lu, Yuan, & Lai, 2008).

1.5.1. MST1

Mammalian sterile 20-like kinase 1 (MST1), commonly referred to as STK4, KRS2, and YSK3 (Ardestani *et al*., 2014), is a substantially expressed serine/threonine kinase that is the main upstream signaling kinase of Hippo pathway. It is involved in a variety of cellular functions, including morphological development, proliferation, stress responses, and apoptosis (Avruch *et al*., 2012; Ling *et al*., 2008). In Homo Sapiens, MST1/STK4 gene is located on Chromosome 20 (44,966,494-45,080,021), consisting of 113,528 bases. While in Mus Musculus, it is located on Chromosome 2 (164,074,138-164,155,524) with 81,387 bases. It is in the cytoplasm and nucleus. The caspase-cleaved form of MST1 cycles between the cytoplasm and nucleus. MST1, a mammalian homolog of Drosophila Hippo, is a 487 amino acid protein (Creasy *et al*., 1996), having a molecular weight of 5541 Daltons in humans and 55630.16 Daltons in mice at the molecular level. MST1 protein consists of two subunits, 18kDa MST1-N subunit and a 37 kDa MST1-C subunit**.** MST1 comprises of N-terminal catalytic domain and C-terminal non-catalytic tail. The COOH-terminal half of MST1 has two different functional domains, an autoinhibitory domain spanning a 63-amino acids region and a coiled coil dimerization domain spanning 57 amino acids region (Creasy *et al*., 1996). SARAH domain, spanning 433 to 480 amino acids region in C-terminus, plays role in MST1 homodimerization and heterodimerization with the SARAH domain of adaptor proteins (Hwang *et al*., 2007). Different MST1 isoforms display distinct level of kinase activity in regard to substrates (Anand et al., 2008).

1.5.1.1. MST1 Activation by Auto-phosphorylation and Caspase-3 Cleavage

In MST1, different phosphorylation sites Thr^{175}, Thr^{177}, Thr^{183}, Thr^{187}, Ser^{327}, and Thr^{387}, have been identified. For kinase activity, Thr183 and Thr^{187} in the *MST1* activation loop were found to be particularly important (Glantschnig *et al*., 2002; Graves *et al*., 2001). Autophosphorylation of Threonine 183, a major site for photoactivation, in subdomain VIII of MST1 is necessary for kinase activation. *MST1* dimer mediates intermolecular autophosphorylation to phosphorylate Thr^{183} (Glantschnig *et al*., 2002). MST1 is autophosphorylated at a Thr^{183} in an activation loop and get activated in reaction to stress or apoptotic signals (Glantschnig *et al*., 2002; Praskova *et al*., 2004). Two caspase cleavage sites, DEMD326S and TMTD349G, between auto-inhibiting domain and kinase domain, have been identified. Caspase mediated cleavage at these specific sites causes the activation of MST1 protein during apoptosis (K. K. Lee *et al*., 2001; S Ura *et al*., 2001). As a result, MST1 exists as a 54 kDa protein in its whole and as a 36 kDa caspase-cleaved form (Graves *et al*., 1998). This cleavage also promotes the nuclear translocation of kinase domain by eradicating the nuclear export signals present in C-terminal domain from N-terminal catalytic domain (S Ura *et al*., 2001). MST-N form migrates to the nucleus and induces chromosomal condensation and apoptosis (Graves *et al*., 1998; Lee *et al*., 1998; Nagata, 1997; Taylor *et al*., 1996). Removal of autoinhibitory domain and varying substrate specificity make caspase-cleaved MST1 section more active and its activity is almost nine times greater than full-length MST1 protein (Creasy *et al*., 1996). Additionally, it has been shown that it promotes development of a positive feedback cycle in sustaining the apoptotic stimulus by activating caspases and further stimulating MST1 cleavage (Ardestani & Maedler, 2016). Earlier, It has been proposed that activated MST1 induces the phosphorylation of MST1 at Thr^{183} and possibly Thr^{187} to activate other MST1 proteins (Glantschnig et al., 2002). MST1 phospho-regulation is even increased to control the cleavage of its own caspase at Ser^{327}, but only in the absence of caspase-3 (Glantschnig *et al*., 2002). MST1 upregulation is sufficient to trigger apoptosis in a variety of cells by activating SAPK (stress-activated protein kinase)/JNK (Graves *et al*., 1998), p53 (Lin *et al*., 2002), and FoxO (Lehtinen *et al*., 2006). MST1 cycles quickly and continually through the nucleus via *DAP-4* (Lehtinen *et al*., 2006; S Ura *et al*., 2001; Lin *et al*.,

2002), where catalytic fragment produced during apoptosis causes chromatin condensation (Cheung *et al*., 2003; S Ura *et al.*, 2001).

1.5.1.2. Transphosphorylation of MST1

MST1 activation is governed by transphosphorylation by numerous protein kinases like AKT mediated inhibitory phosphorylation at T^{387} site (Jang *et al*., 2007), JNK mediated phosphorylation at S^{82} residue (Bi *et al*., 2010), phosphorylation by *c-Abl* at Y^{433} residue (Xiao *et al*., 2011), mTORC2 mediated inhibitory phosphorylation at S^{438} residue (Sciarretta *et al*., 2015), and by CK2 at S^{320} residue (Servas *et al*., 2017). MST1 is activated by increased levels of phosphorylation at S^{82}, T^{183}, and Y^{433}, while MST1 activity is inhibited by increased levels of phosphorylation at T^{120}, T^{387}, and S^{438}. Additionally, TAO 1/2/3 kinases are capable of mediating the phosphorylation of Thr^{183} in the activation loop (Boggiano *et al*., 2011; Poon *et al*., 2011).

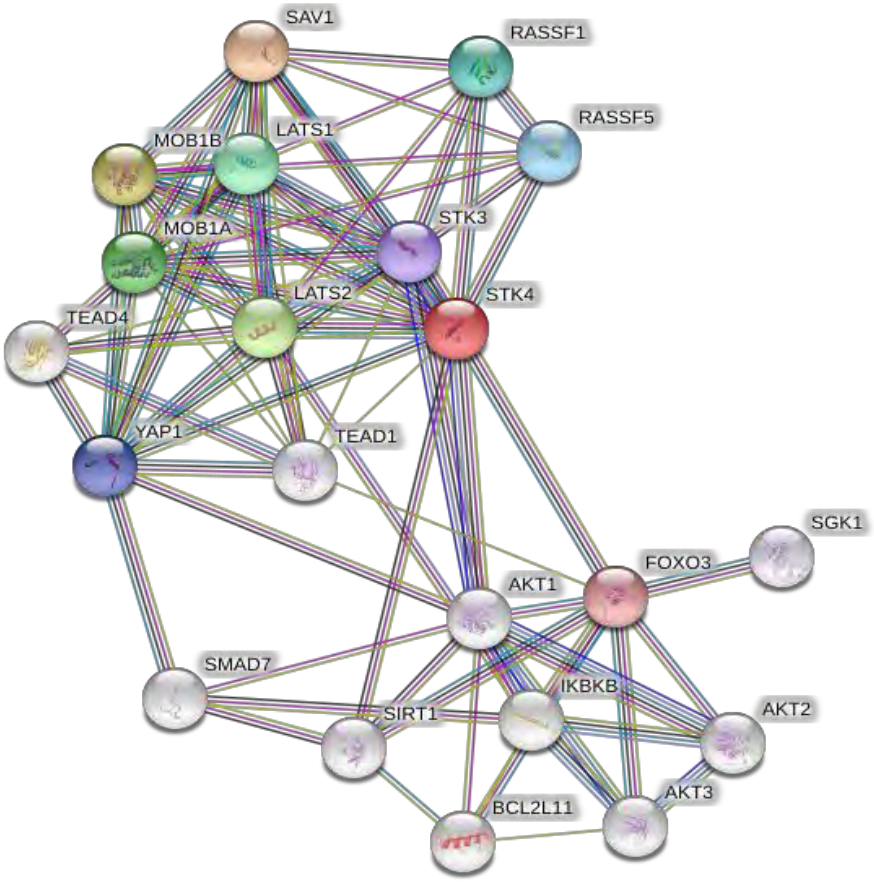

Figure 1.1: MST1 protein interaction.
(https://www.sinobiological.com/resource/mst1/proteins.)

1.5.2. MST1 and Redox State

Oxidative stress instigate the activation of *MST1* (Graves *et al*., 1998). Thioredoxin-1 (Trx1), a preserved cell reinforcement protein known for its ability to facilitate disulfide reduction, is associated with SARAH domain of Mst1 to restrict the autophosphorylation and homodimerization of MST1 inhibiting MST1. H2O2 cancels this cooperation and eventually results in MST1 initiation. TNF-α accelerated the homodimerization and MST1 activation while delaying the actual communication between Trx1 and MST1 (Chae *et al*., 2012). Under oxidative stress, peroxiredoxin1 (Prdx1) lowers H2O2 into H2O and O2 and interacts with MST1 and activates MST1. Morinaka *et al*., demonstrated that peroxiredoxin1 (Prdx1) interacts with MST1 under oxidative stress conditions and is required for activation of MST1 by H2O2 (Morinaka *et al*., 2011). In liver cells, MST1/2 removal resulted in enhanced expressions of a group of antioxidant enzymes crucial for the eradication of ROS. Elevated expressions of these antioxidant enzymes, such as γ-glutamyl-cysteine ligase (GCL), glutathione reductase (GSR), catalase (CAT), cytosolic thioredoxin (Txn1), copper/zinc superoxide dismutase (SOD) and mitochondrial thioredoxin (Txn2), increases glutathione (GSH) accretion which causes activation of crucial transcription factor for production of YAP, GA-binding protein (GABP) in MST1 deficient liver (Chinenov *et al*., 1998; H. Wu *et al*., 2013).

1.5.3. Regulation of MST1 Activation by JNK

MST1 is phosphorylated at serine 82 in N-terminal region by JNK, which increases activity of MST1. JNK inhibition resulted in diminished MST1 activity, nuclear translocation, and MST1-induced apoptosis (Bi *et al*., 2010). MST1 appears to activate the JNK and p38 MAPK kinase pathways in mammals through MKK3/MKK6 and MKK4/MKK7 respectively (Graves et al., 1998). The process that causes chromatin condensation in apoptosis is MST1 cleavage by caspases, which is followed by MST1-facilitated activation of JNK (Seiji Ura *et al*., 2007). MST1 was recognized as a peculiar substrate of JNK in apoptosis and JNK is upstream controller of MST1-FoxO signaling in stress-prompted apoptosis, supporting the idea that JNK induces apoptosis independent of c-Jun transactivation (Seiji Ura *et al*., 2007). Once activated, *MST1* activates *JNK* independent of caspase cleavage. *MST1* phospho-

regulation is even further extended to modulate its own caspase cleavage at Ser^{327}, however, only where and when *Caspase-3* is absent (Glantschnig et al., 2002). Therefore, a number of kinases, such as Cdk1, MST1, and JNK1, causes the separation of 14-3-3 proteins from apoptosis inducing factors such as BH3-only proteins BAD and BAX along with the transcription factor *FoxO* and *c-Abl* (Lehtinen & Bonni, 2008; Lehtinen *et al*., 2006; Sunayama *et al*., 2005; Tsuruta *et al*., 2004; Z. Yuan *et al*., 2008, 2009).

1.5.4. Histone H2B and JNK1 Activation by MST1 to Induce Apoptosis

JNK is part of the MAPK family which phosphorylate c-Jun at specific Ser residues (63 and 73) on its N-terminal end. JNK acts as a proapoptotic kinase, mediating cellular apoptosis triggered by different stimuli, including IL-1β (Verma & Datta, 2010). JNK phosphorylation has a significant role in β-cell destruction, and its inhibition has been considered a promising strategy to protect β-cells and enhance insulin sensitivity in proinflammatory conditions (Bonny *et al*., 2001; Kaneto *et al*., 2004). The PI3K-AKT pathway acts as a potent repressor of JNK in pancreatic islets, serving as anti-apoptotic mechanism (Aikin *et al*., 2004). In human islets or INS-1E cells, persistently activated JNK which overexpress MST1, induced apoptosis in β-cells (Ardestani *et al*., 2014). MST1 triggers apoptosis by histone H2B phosphorylation at a preserved Ser^{14} site in mammalian cells and Ser^{10} site in Saccharomyces cerevisiae. Histone variations are essential for condensation of chromosomes that is an essential event in apoptosis. One of the significant modifications is histone H2B phosphorylation at Ser^{14}. Cheung *et a*., reported a 34 kDa nuclear H2B protein kinase phosphorylated at Ser^{14} in cells suffering from apoptosis. The cleavage of MST1 during apoptosis and the phosphorylation of H2B had a comparable time course, and caspase 3 inhibition prevented both processes (Ahn *et al*., 2005; Cheung *et al*., 2003).

1.5.5. AKT1-mediated MST1 regulation and PTP1B inhibition in T2DM management

The protein tyrosine phosphatase (PTP1B) is a tail-anchored protein with a single transmembrane domain in the c-terminus, to get anchored to intracellular membranes like the ER or mitochondrial membrane (Anderie *et al*., 2007). PTP1B is anchored to

the ER with 35 hydrophobic amino acid residues, which helps in directing it towards the cytoplasmic face of ER (Frangioni *et al.*, 1992). PTP1B's ability to interact with substrates is constrained by its anchoring to a particular subcellular site, which also plays a part in controlling its activity (Tonks, 2003). PTP1B dephosphorylates the phospho-tyrosine sites of insulin receptor (IR) to negatively control the insulin signaling pathway (Salmeen *et al*., 2000). In mice with PTP1B overexpression, insulin-mediated *INSR* tyrosine phosphorylation is reduced by 35%, PI3K activity is reduced by 40–60%, and glucose transport is reduced as well. TIIDM and obesity are associated with overexpression of PTP1B in livers (Zinker et al., 2002), pancreas (S. Liu *et al*., 2014), adipose tissues (Venable *et al*., 2000), and muscles of rodents (Ali *et al*., 2009). Main role of PTP1B is to control the signaling of two essential hormones, insulin and leptin, which play crucial roles in controlling cellular metabolism and glucose homeostasis. Insulin signaling is downregulated due to the dephosphorylation of active IR and IRS by PTP1B (S. Zhang & Zhang, 2007). PTP1B over activity has been linked to insulin signaling down-regulation and is taken as a promising therapeutic target in treatment of DM (Uddin et al., 2018) and alleviates insulin resistance (Montalibet & Kennedy, 2005).

MST1 acts as direct inhibitor of AKT1. *β*-cell apoptosis induced by MST1 is facilitated by inhibition of PI3K/AKT signaling transduction (Ardestani *et al*., 2014). MST1 blocks AKT1 in both its fully mature form and in caspase-cleaved form, both species located in cytoplasm and nucleus, respectively, and both forms have ability of diminishing AKT1 activity on their own (Cinar *et al*., 2007). PTP1B is considered as a novel substrate for AKT as PTP1B phosphorylation at Ser^{50} by AKT suppresses PTP1B activity and reduces its capacity to dephosphorylate the IR, potentially acting as a positive feedback mechanism to amplify insulin signaling (Ravichandran *et al*., 2001). PTP1B deprivation reversed the IRS-1 regulated PI3K/AKT/FoxO1 signaling and the inhibition of the gluconeogenic enzyme in hepatic cells (González-Rodríguez *et al*., 2010). AKT also acts as an inhibitor of MST1. AKT negatively regulates MST1 activity by phosphorylating it at T^{120} and T^{387} sites, resulting in inhibition of MST1 autophosphorylation, its kinase activity, cleavage, and nuclear translocation (Cinar *et al*., 2007). MST1 inhibition via AKT enhances cell survival and reduces MST1's

kinase activity on *FoxO3* (Jang *et al*., 2007). PI3K/AKT signaling pathway play crucial role in insulin gene expression and secretion of insulin and *β*-cell survival (Assmann *et al*., 2009; Bernal-Mizrachi *et al*., 2001; Tuttle *et al*., 2001). AKT blocks activation of MST1 and *β*-cell apoptosis prompted by high glucose stimulus (Ardestani *et al*., 2014).

1.5.6. Regulation of FOXO3-FOXO1 by MST1

MST1 actively phosphorylates and activates forkhead box proteins (*FOXO*), that induces the production of pro-apoptotic genes like *FasL* and *TRAIL* in extreme stressful situations (Lehtinen *et al*., 2006). MST1 phosphorylation by JNK at Ser^{82} exerts a consequential effect on the MST1-FoxO3 signaling pathway. Ser^{207} is recognized as the MST1 phosphorylation site in FoxO3 and is responsible for FoxO-dependent transcription and demise of cells (Lehtinen *et al*., 2006; Yuan *et al*., 2009). MST1 plays critical role in activating pro-apoptotic transcription factor *FoxO1* by phosphorylating it at Ser^{212}, facilitating the detachment of FoxO1 from 14-3-3 protein (Lehtinen *et al*., 2006; Z. Yuan *et al*., 2008). JNK1 also interferes with the interaction between 14-3-3 protein and c-Abl, and FoxO (Sunayama *et al*., 2005; Yoshida *et al*., 2005). AKT, which is activated by insulin/IGF signaling, phosphorylates FoxO1/3 at multiple sites. This phosphorylation leads to the binding of 14-3-3, facilitating the exit of *FoxO1/3* from the nucleus. On the other hand, MST1 stimulates the phosphorylation at different sites within the forkhead domain (specifically FoxO1 at Ser^{212} and FoxO3 at Ser^{207}), preventing the binding of 14-3-3 protein and stimulating the entry of FoxO1/3 into the nucleus. The impact of MST1-catalyzed phosphorylation of FoxO1/3 on FoxO DNA binding varies depending on the involvement of particular DNA site (Brent *et al*., 2008). Consequently, the transcriptional outcomes are probably dependent on the specific chromatin state and the surrounding cell environment. The cellular localization of FoxO1 plays an essential role in determining the localization of PDX1, and their expression levels are inversely correlated. The regulation of FoxO1 involves both JNK and AKT activity. JNK activity encourages the nuclear importation of FoxO1, leading to the transfer of PDX1 from the nucleus. Conversely, AKT-mediated phosphorylation of FoxO1 causes its localization in the cytoplasm, while PDX1 is translocated into the nucleus.

The JNK-PDX1 pathway appears to be a vital signaling system that transmits short- and long-term glucose stimulation signals, potentially mediating the dual influence of glucose on *β*-cell survival and function (Ardestani *et al.*, 2014).

1.6. PHLPP2: Linking MST1 and AKT in Cellular Signaling

PHLPP was found in superchiasmatic nucleus (SCN) of the hypothalamus in a rat (Shimizu *et al*., 1999). PHLPP family contains PHLPP1α having1205 amino acids, PHLPP1*β* having 1717 amino acids, and PHLPP2 having 1323 amino acids (Brognard *et al*., 2007; T. Gao *et al*., 2005). The PHLPP1*α* and PHLPP1*β* are the splice variants of the same gene, traced on chromosome 18q21.33. PHLPP1*β* has a 56 kDa N-terminal region that distinguishes it from PHLPP1*α*. The PHLPP2 gene is located on chromosome 16q22.3 (Brognard *et al*., 2007). PHLPP2 gene is located on chromosome 8 and consists of 1355 amino acids. Both PHLPP1 and PHLPP2 share the same domain, including PH domain, leucine-rich repeats (LRR), a C-terminal PDZ ligand, and a PP2C phosphatase domain. Additionally, PHLPP1*β* and PHLPP2 have Ras association domain (RA domain) preceding PH domain (Brognard *et al*., 2007; T. Gao *et al*., 2005). PHLPP phosphatases are powerful promoters of cell death as they inhibit proliferative pathways (O'Neill *et al*., 2013). Increased expression of PHLPP1/2 results in *β*-cells disaster in diabetes. PHLPP levels are significantly raised in metabolically stressed *β*-cells in diabetic humans and rodents. Genetic inhibition of PHLPPs significantly enhances *β*-cell progression and function in diabetic experimental models both in vitro and in vivo, as well as in primary human diabetic islets (Lupse *et al*., 2021).

AKT kinase, the primary target of PHLPPs, plays a vital role in stimulating cell survival in *β*-cells. The phosphorylation of various substrates by AKT exerts a positive influences on transcription and secretion of insulin as well as growth and survival of *β*-cells (Assmann *et al*., 2009; Bernal-Mizrachi *et al*., 2001; Tuttle *et al*., 2001). For complete catalytic activity, AKT1 necessitates phosphorylation at Ser^{473} in hydrophobic motif and Thr^{308} in the activation loop (Jacinto *et al*., 2006). PHLPP2 specifically dephosphorylates the Ser^{473} site of AKT in cells, leading to reduced AKT activity, increased apoptosis, and diminished cell proliferation (T. Gao *et al*., 2005). PHLPPs exhibit different substrate preferences for definite AKT isoforms. PHLPP1

primarily targets the dephosphorylation of AKT2, while PHLPP2 dephosphorylate AKT1. However, the specificity may vary in different tissues and cellular states (Brognard *et al*., 2007; T. Gao *et al*., 2005). In *β*-cells, *PHLPP* targets *MST1* as its second downstream molecule, crucial for cell survival. MST1 serves as an apoptotic factor under diabetic conditions, affecting *β*-cell function and survival (Ardestani *et al*., 2019, 2014). PHLPP causes the dephosphorylation of MST1 at Thr^{387}site, activating MST1 and its subsequent downstream targets such as p38 and JNK, causing apoptosis. The PHLPP-AKT-MST1 axis controls cell proliferation and apoptosis, possibly depending on cell nature (Qiao *et al*., 2010). PHLPP1/2 expression increases due to *mTORC1* overactivation in diabetic models, making them significant phosphatases in this context (Lupse *et al*., 2021).

1.7. MST1 and Hippo Pathway in Apoptosis of Pancreatic *β*-Cell

MST1, a potent pro-apoptotic kinase, is found in *β*-cells and have a crucial role in managing *β*-cells death and dysfunction, leading to reduced insulin secretion (Faizah *et al*., 2020; Ardestani *et al*., 2014). MST1 serves as the core kinase within the Hippo pathway, expressed fully in pancreatic *β*-cells (Ardestani & Maedler, 2018). Because of limited presence of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase, pancreatic *β*-cells are extremely susceptible to ROS (Al-Nahdi *et al*., 2018). Numerous external triggers activate apoptosis in cells through MST1/MST2, resulting in the generation of active catalytic fragment by caspase cleavage, that is involved in programmed cell death process (Praskova *et al*., 2008). In T2DM, the distinct apoptosis progression eventually induces the loss of *β*-cells mass, accompanied by morphological alterations such as cell rounding, formation of vesicle, and chromosome condensation (Galluzzi *et al*., 2015; Marchetti *et al*., 2020).

Hippo pathway regulates apoptosis in response to metabolic changes related to T2DM, involving key elements such as MST1, NF2, LATS2, and YAP (Ardestani *et al*., 2014; J. Liu *et al*., 2020; Shu et al., 2019; Ting Yuan *et al*., 2021; Ting Yuan *et al*., 2017). This pathway is also responsible for maintaining organ size through a balance of apoptosis, proliferation, and tissue regeneration (Chang *et al*., 2019; Rausch & Hansen, 2020). The final cell count in an organ depends on the interplay between cell proliferation and apoptosis, both of which are influenced by the Hippo

pathway's activity (Tao et al., 2021; S. Wu, Huang, Dong, & Pan, 2003). NF2 serves as the initial activator of the Hippo pathway, influencing cell growth and proliferation (Y. Li *et al*., 2015). Activation of Hippo pathway by NF2 involves two mechanisms, one of which activates MST-Sav along with MST1/2 dependent phosphorylation of LATS1/2, while the other recruits LATS1/2 to the cell membrane (Yin *et al*., 2013). The interaction of Ras association proteins with RASSF and MST kinases at plasma membrane triggers phosphorylation and transactivation, activating Hippo pathway and apoptosis (Hwang *et al*., 2014; Iwasa *et al*., 2018; Oceandy *et al*., 2019). MST1 and LATS2 are core kinases that induce *β*-cell apoptosis (T Yuan *et al.*, 2014). MST1/2 and LATS1/2 kinase cascade are essential for Hippo pathway regulation. MST1/2 and the regulatory protein Salvador (Sav1) activate LATS1/2, along with the regulatory protein MOB1. The Hippo pathway regulates YAP, a major downstream effector, by inhibiting it through LATS1/2-mediated phosphorylation at Ser^{127}, leading to its sequestration, degradation and prevention of transcriptional activity (Harvey *et al*., 2013; Zhao, Li *et al*., 2010). YAP exerts its effect through TEAD transcription factors to modulate cellular functions like cell-survival and apoptosis. Any component disturbance within kinase core results in YAP-driven escalation in proliferation and increased resistance to apoptosis in various tissues (Zhao *et al*., 2010).

In diabetic conditions, MST1 was found to be highly activated in *β*-cells of human and mouse, leading to apoptosis through mitochondrial-dependent pathway by the upregulation of BIM. The increased MST1 activity is triggered by various cellular stress factors, such as oxidative stress, glyco-lipotoxicity, and proinflammatory cytokines, resulting in apoptosis by stimulating mitochondrial pro-apoptotic protein BIM. This process involves changes in Bax/Bcl-2, cytochrome c release, and the activation of caspase-9 and caspase-3, ultimately leading to cell death (Ardestani & Maedler, 2016). In human pancreatic islets, there is a strong relationship between BIM and MST1, regulated by JNK and AKT. Caspase-3 and JNK play dual roles as both upstream activators and downstream targets of pro-apoptotic signaling cascade in *β*-cells, further exacerbating apoptosis. MST1 is cleaved in response to many apoptotic signals, comprising those from the Fas receptor and TNFα. MST1

stimulates cell death by regulating various downstream targets, such as LATS1/2, histone H2B, FoxO members, the intrinsic mitochondrial pro-apoptotic process, JNK, and caspase-3 activation (Cheung *et al*., 2003; Bi *et al*., 2010; Avruch *et al*., 2012). MST1 activation in diabetic *β*-cells leads to impaired insulin secretion by promoting the proteasomal degradation of crucial *β* cell transcription factor, PDX1 which is essential for insulin production (Ardestani & Maedler, 2016). Specific phosphorylation of certain residues within PDX1, such as Ser^{61} and Ser^{66} by GSK3, negatively regulates its function, targeting PDX1 proteasomal degradation under oxidative stress (Boucher *et al*., 2006).

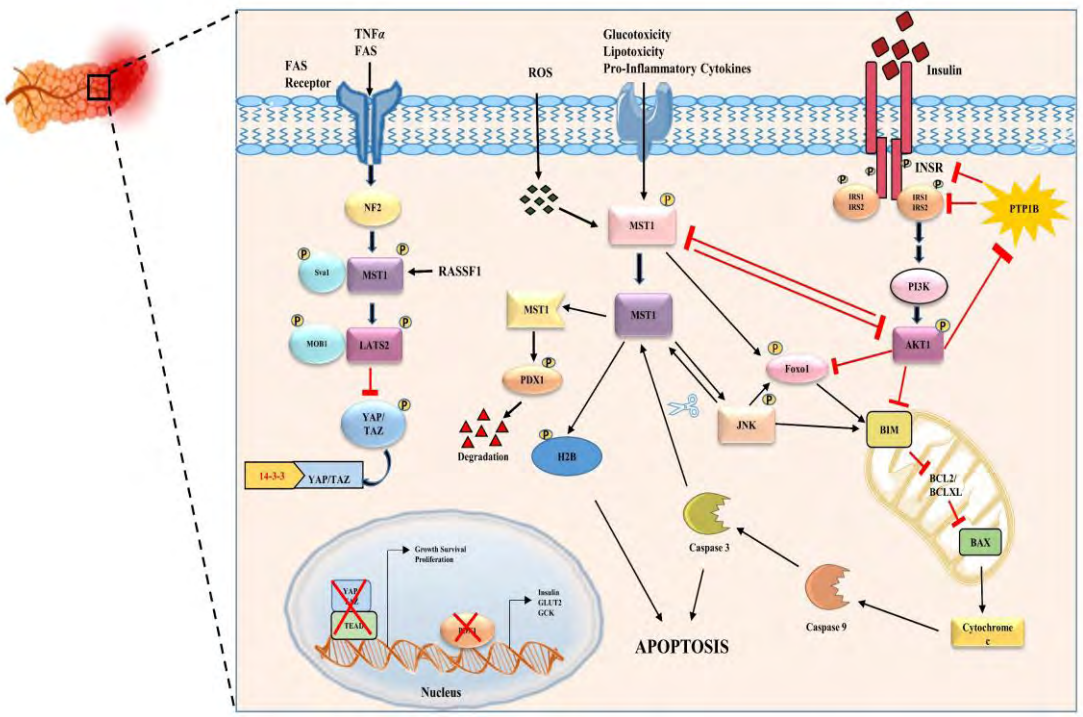

Figure 1.2. Apoptosis induced in Pancreatic *β*-cells through Mitochondrial and Hippo Pathway.

1.8. mTORC1 mediated Autophagy inhibition and Lipogenesis in Pancreas

mTOR is a nutrient-responsive serine-threonine kinase, generates the mTORC1 and mTORC2, which are structurally and functionally unique. mTORC1 consists of catalytic subunit mTOR and Raptor, Deptor, mLST8/GbL, and PRAS40. Growth hormones, such as insulin and IGF-1, trigger the activation of mTORC1. Nutrients such as glucose, amino acids, and high ATP/AMP ratio, all activate mTORC1.

mTORC1 promotes protein, lipid, and nucleotide synthesis, thus regulating the intracellular metabolic state (Laplante & Sabatini, 2012; Saxton & Sabatini, 2017). In human pancreatic islets with T2DM, an increased expression of mTORC1 activation was observed which is associated with *β*-cell death and dysfunction. Experiments involving the suppression of mTORC1-S6K1 signaling, either through genetic or pharmacological means, resulted in the restoration of insulin excretion in human *β*-cells. This suggests that increased mTORC1 activity compromises function of *β*-cell function (Ting Yuan, Rafizadeh, *et al*., 2017). In patients with T2DM and in islets and *β*-cells exposed to high glucose concentrations, a significant increase in mTORC1 activity was noticed, while mTORC2 signaling was reduced (Ting Yuan, Rafizadeh, *et al*., 2017). Hyper-activation of mTOR signaling in *β*-cells have detrimental effects, including direct phosphorylation of IRS1/2 by mTORC1, leading to their degradation and impairment of insulin signaling, resulting in insulin resistance. Additionally, mTORC1 phosphorylate and activate Grb10, disrupting the interaction between IR and IRS1/2, causing IRS2 degradation, and causing impairment of insulin signaling pathway (Warren *et al*., 2016; Wick *et al*., 2003).

mTORC1 is a well-known inhibitor of autophagy and its persistent activation negatively impacts autophagic circulation and triggers *β*-cells death (Ardestani *et al*., 2018; Bartolomé *et al*., 2014). The AMPK-mTORC1-autophagy signaling pathway controls insulin secretion and *β*-cell survival, but continuous nutritional stress, such as that associated with T2DM, suppresses AMPK in pancreatic *β*-cells, activating mTORC1 and, as a result, affecting autophagy and mitophagy, which results in dysfunctional *β*-cells and diabetes (Pepin *et al*., 2016; Ruderman & Prentki, 2004; Y. Sun *et al*., 2008). AMPK plays a crucial role in autophagy by directly phosphorylating ULK1. AMPK can activate ULK1 by phosphorylating it at multiple sites, including Ser^{317}, Ser^{777}, Ser^{467}, Ser^{555}, Ser^{637}, and Thr^{574}. Conversely, mTORC1 inhibits ULK1 by phosphorylating it at Ser^{317} and Ser^{777}. Activation of AMPK can be suppressed by mTORC1 through enhanced phosphorylation of ULK1 at Ser^{757}, leading to decreased autophagy (Egan *et al*., 2011; Kim *et al*., 2011; Kubli & Gustafsson, 2014). LATS2-facilitated *β*-cell apoptosis by triggering activation of Rag-mTORC1. Under stress- and diabetic conditions, mTORC1 hyperactivation and LATS2 activation interact in

collaboration to inhibit compensatory machinery of *β*-cell, resulting in *β*-cells death, malfunction, and ultimately metabolic insufficiency and diabetes (Ting Yuan *et al*., 2021).

Lipotoxicity has developed as a potential factor in the deterioration of *β*-cell functions (R. P. Robertson *et al*., 2004; R H Unger & Zhou, 2001). In the *β*-cells of diabetic animals, lipid accumulation, compromised glucose stimulated insulin secretion (GSIS), and disrupted gene expressions in *β*-cell, have been reported (R H Unger & Zhou, 2001; Roger H Unger, 2002). Elevated SREBP-1c expression in islets and liver of various diabetic animal models has been proposed (Kakuma et al., 2000). In islets and INS-1E cells experiencing glucolipotoxicity, AMPKα regulates lipogenic enzymes (ACC and FAS) (D. Zhang, 2018).

mTORC1 hyperactivation was indicated by elevated expression of its downstream targets such as S6K1 and S6 (Ting Yuan *et al*., 2021). mTORC1 plays a role in promoting de novo lipogenesis through the SREBP1 dependent pathway. This can be achieved either by phosphorylation of S6K1 (Düvel *et al*., 2010) or by modulating the localization of Lipin1 and expression of Srebp1 (Peterson *et al*., 2011). The regulation of *Srebp1* by mTORC1 entails the nuclear entry of Lipin1. When Lipin1 is dephosphorylated and catalytically active, it facilitates nuclear alteration and regulate the effects of mTORC1 on expression of Srebp target genes, Srebp promoter action, and nuclear Srebp protein richness (Peterson *et al*., 2011). Both Srebp1 and Srebp2 promote proliferation downstream of mTORC1, and their activation is mediated by S6K1 (Düvel *et al*., 2010).

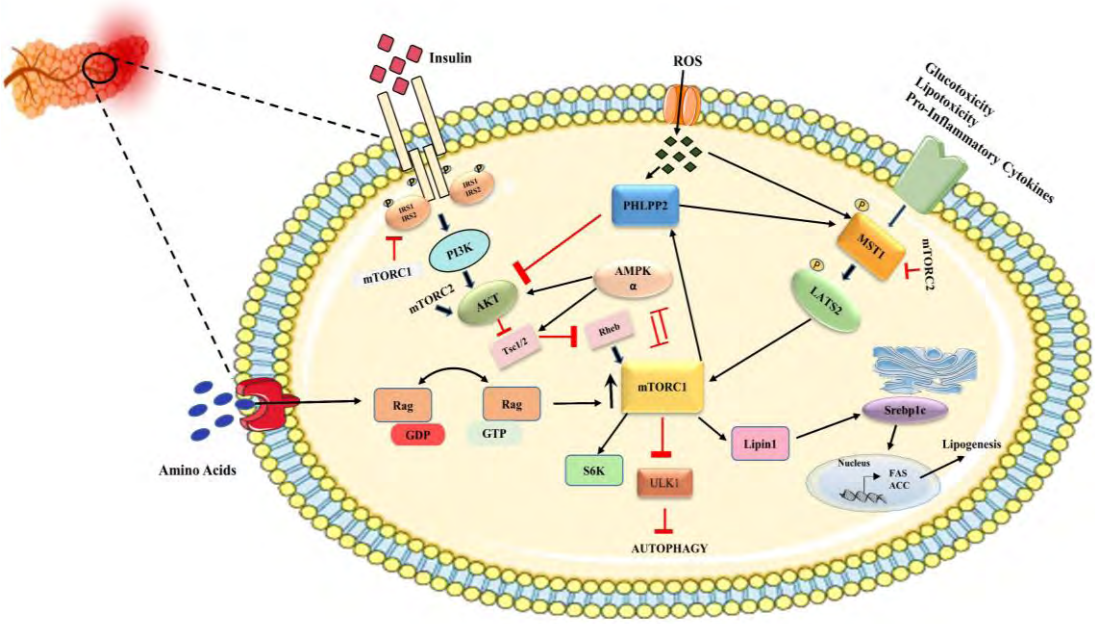

Figure 1.3: Autophagy Inhibition and Lipogenesis in Pancreatic *β*-cells.

1.9. MST1-Mediated Inflammasome Activation in the Pancreas

T2DM involves chronic inflammation, with inflammasomes being essential indicators of metabolic failure and *β*-cell instability (Sepehri *et al*., 2017; Tong *et al*., 2017). Inflammasomes are cytosolic complexes that respond to various signals and trigger an inflammatory response (K. Yang *et al*., 2021). The inflammasome composed of NLRP3, pro-caspase 1, and ASC, is extensively investigated. This complex of multiple proteins triggers production of Interleukin-1*β* (IL-1*β*) and Interleukin-18 (IL-18) (G. Li *et al*., 2016; K. Yang *et al*., 2021). Inflammasome activation is a main regulator in inducing pancreatic islets impairment. In pancreatic islets, prolonged hyperglycemia leads to ROS accumulation, resulting in an increase in TXNIP levels which activates the inflammasome NLRP3, leading to IL-1*β* maturation through caspase-1 dependent processes (Sokolova *et al*., 2018). Additionally, in high fat diet-induced diabetic rodents, there is macrophage infiltration in the pancreas, which further promotes IL-1*β* production and apoptosis of pancreatic cells, ultimately compromising islet function and leading to insulin resistance (Jourdan *et al*., 2013; Schroder, Zhou, & Tschopp, 2010).

IL-1β plays diverse roles in the regulation of metabolism and inflammatory responses, influencing insulin excretion, and promoting apoptosis of *β*-cell, potentially

contributing to T2DM (Sepehri *et al*., 2017; Tong *et al*., 2017). Enhanced glucose concentrations cause apoptosis in human pancreatic *β*-cells through the interaction between constantly expressed Fas ligand and elevated Fas. In response to high glucose levels, *β*-cells produced IL-1*β* independent of an immune-interceded process. However, IL-1*β* production can be counteracted by the presence of IL-1 receptor antagonist (IL-1Ra), a naturally existing anti-inflammatory cytokine present in *β*-cells. The interaction between IL-1*β* and IL-1Ra play a critical role in progression of diabetes (Maedler & Donath, 2004).

Due to lipotoxicity, glucotoxicity, and pro-inflammatory cytokines, pancreatic beta-cells in T2DM experience elevated stress, which activates MST1 and LATS1/2 signaling proteins in the Hippo pathway and inhibits YAP and TAZ (J. Liu *et al*., 2020; Shu *et al*., 2019; Ting Yuan, Maedler, *et al*., 2017). In human derived pancreatic MIA PaCa-2 cells, JNK and p38 phosphorylation induced by IL-1*β*, was observed together with elevated levels of ER stress indicators, namely *BiP*, *GADD34*, *CHOP* and *ATF4* (Verma & Datta, 2010). IL-1*β* contributes to FasL-induced caspase-3/-7 activation which was relying on JNK1/2, BIM and Bid with increased cytochrome c release, ultimately leading to caspase-3/7 activation (Lutz *et al*., 2014).

The *NLRP3* inflammasome stimulation occurs by binding of Nek7 and NLRP3, resulting in the maturation and release of IL-1*β* upon caspase-1 activation. LPS stimulation can directly induce the interactivity between Nek7 and NLRP3, resulting in the oligomerization and initiation of inflammasome. Additionally, JNK has been involved in activation of *NLRP3*. In the context of L. monocytogenes infection, JNK-Nek7-NLRP3 signaling pathway is activated in macrophages. Mst1/2 has a vital role in this mechanism, facilitating the formation of Nek7-NLRP3 complex through *JNK*, thereby mediating the activation of NLRP3 inflammasome (A. Gao *et al*., 2021). It has been described that caspase-3 cleaves GSDME to produce a cytotoxic GSDME-N fragment upon activation, causing pore formation and pyroptosis. Caspase-3 activation is traditionally related with apoptosis, but it also promotes pyroptosis (Rogers *et al*., 2019, 2017). Gasdermin-mediated pores trigger mitochondrial apoptotic pathways by releasing cytochrome c and initiating apoptosome formation, demonstrating GSDMD-N also has a similar activity (Rogers *et al*., 2019). Pyroptosis

can also result from the cleavage of GSDME by caspases 1, 3, and 7 (Jiang, Gu, Zhao, & Sun, 2019). These findings reveal diverse roles of caspases and gasdermin proteins in different cell death mechanisms.

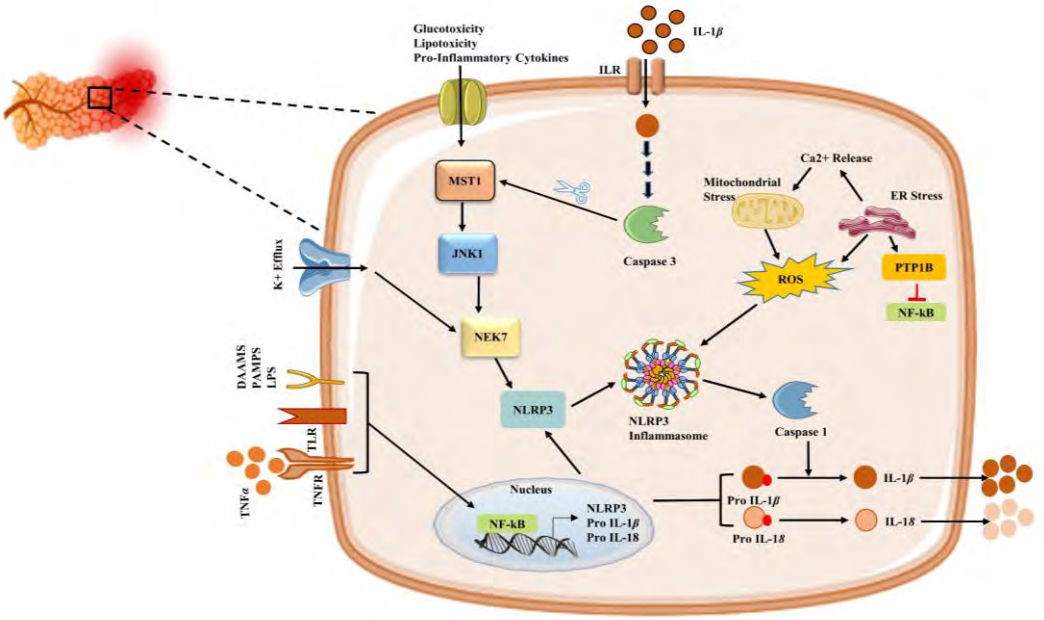

Figure 1.4: MST1 mediated Inflammasome Activation in Pancreatic *β*-cells.

1.10. Viscosol

In a study by Ziauddin *et al*., the anti-diabetic effect of each extracted polyphenolic component from aerial portions in the methanolic extract was evaluated. The main source of PTP inhibitors is Dodonaea viscosa. The most effective substance against PTP1B was found to be Compound 4 (viscosol), which is 5,7-dihydroxy-3,6-dimethoxy-2-(4-methoxy-3-(3-methyl but-2-enyl) phenyl)-4H-chromen-4-one (Uddin *et al*., 2018). The powerful bioactive substance was discovered as a pale-yellow substance having molecular-weight of 412.1522 and chemical formula C23H24O7. With 13.5µM IC50 value and greater inhibitory action as compared to other isolated chemicals, viscosol is a powerful inhibitor of PTP1B. Moreover, it was discovered through kinetic analysis that compound (4) more efficiently inhibited free enzymes than the enzyme-substrate complex, as shown by KI = 4.1 0.2 M and KIS = 26.4 0.4 M, respectively. Additionally, it was deduced from the investigation that this compound functions via mixed inhibition type 1 mechanism (Uddin *et al*., 2018).

Viscosol is an inhibitor of protein tyrosine phosphatases (PTPs), such as PTP1B. By inhibiting PTP1B, viscosol prevents the dephosphorylation of insulin receptors (IR) and insulin receptor substrates (IRS), thus allowing the PI3K/AKT signaling pathway to remain active and AKT to inhibit MST1. Consequently, this leads to MST1 inhibition, enhancing the activation of downstream targets and promoting cell growth and survival. The in vivo antidiabetic effect of Viscosol has been determined (Sohail *et al*., 2022).

1.11. Aims and Objectives

This research focuses on exploring the inhibitory effects of a potential flavonolic compound, viscosol, on MST1 in a low-dose STZ-induced HFD T2DM mice model. We have previously identified, the compound's antidiabetic activity through *in vivo* and *in vitro* studies. However, the current study aims to explore the role of MST1 in NLRP3 activation in the pancreas and hypothesizes that IL-1β activates MST1 via caspase-3 activation. The primary objective is to assess viscosol's potential in mitigating MST1-mediated apoptosis and inflammation in the T2DM model. By conducting this research, valuable insights will be gained into viscosol's mechanism of action in inhibiting MST1. The findings could pave the way for the improvement of new therapeutic strategies for managing diabetes-related inflammation.

The specific aims of the present research are.

- Explore MST1's impact on insulin signaling, β-cell apoptosis, the Hippo pathway's role in β-cell death, and its activation of NLRP3-driven inflammation.
- Investigate the influence of mTORC1 on autophagy regulation within pancreatic β-cells.
- Assess how Viscosol affects MST1 inhibition, subsequently influencing insulin signaling and pancreatic β-cell mass restoration.

2. MATERIALS AND METHODS

2.1. Animal Selection

The mice breed (Mus Musculus, C57BL/6) was bought from the primate laboratory of the NIH, Islamabad. Only the adult male mice with an average weight of 25–30g and average age of 8–12 weeks were chosen for the experiment. For acclimation, we confined each of those mice for a week at primate facility of Quaid-I-Azam University. Water and common pellet food were provided to all the mice. Throughout the experiment, mice were subjected to 12-hour cycles of light and darkness while being held at a standard temperature of 27°C.

2.2. Ethical Approval

The experiment was conducted in accordance with established protocols, and all prerequisites for its completion were accomplished. The research was approved by the Bioethics Committee of Quaid-i-Azam University, Islamabad.

2.3. Study Design

Each group contains triplicate mice, and all mice were assigned intermittently to one of three groups: Group-1 was designated as normal; Group-2 was designated as the streptozotocin-induced diabetic group (STZ); and Group-3 was designated as the streptozotocin-induced diabetic compound treated group (STZ+C or compound treated). Normal standard pellet food and water were supplied to Group-1 mice. A single intraperitoneal injection of saline (500µl) was injected to the Control group. Low dose streptozotocin (STZ) injections were administered intraperitoneally for a period of five days (40 mg/kg) to STZ group. High-fat diet (HFD) was also given to these mice. The first dose was administered following a 24-hour fasting, and the other ones were administered after fasting of 4–6 hours. Following injection, they had a 10% glucose solution and continued to take HFD. HFD and intraperitoneal streptozotocin injection (40 mg/kg) were administered to the compound-treated group for five consecutive days. They were also provided with a 10% glucose bottle. Following the onset of diabetes, the glucose bottle and HFD were removed and the intraperitoneal injection of Viscosol was injected. The normal pellet diet contains dietary components as 12.1% carbohydrate ,4.1% fat and 22.2% protein as total kcal

percentage, while HFD contained dietary components as 17% of carbohydrates, 58% fat and 25% protein as total kcal percentage.

Table 2.1. Groups of experimental mice used in the current study.

Serial No.	**Groups**	**Names**
1	Group-1	Normal
2	Group-2	STZ-HFD induced Diabetic Group
3	Group-3	STZ-HFD induced Compound treated

2.4. Diabetes Induction and Drug Treatment

As stated above, the mice were segregated into three groups and on the first day, weight and blood glucose levels were measured, and readings were taken from all groups. A digital glucometer (Roche Diagnostic, ACCU-CHEK Instant S, Mannheim, Germany) was used to measure blood glucose level (BGL) of mice. The repeated low dose of streptozotocin (Bioworld, CAT # 41910012-3) was closed to enable the mouse model to resemble the T2DM human model pathophysiology. In STZ-HFD induced diabetic group, all mice were kept at overnight fasting for the first injection, and 4-6 hours for the rest of the injection, only tap water was available to that group. The streptozotocin drug (Bio plus Fine Research Chemical, CAT # 41910012-3, Bioworld), was dissolved in saline, vortexed and was administered according to the body weight of mice (40mg/Kg), for 5 consecutive days. After every STZ injection, the mice group was provided with 10% glucose, along with HFD. The very next day after the last injection, a 10% glucose bottle was removed, and mice were kept on the HFD and normal water. Mice developed diabetes over a period of 9 to 10 days. Mice exhibiting fasting blood glucose levels exceeding 250 mg/dl (>11.1 mM) were categorized as diabetic. Blood glucose levels of all the mice were constantly monitored by a digital glucometer before euthanization. In Group-3, STZ was given consecutively for 5 days through intraperitoneal injection, provided with HFD and

10% glucose. The induction of diabetes in this group is similar to Group-2 (STZ-HFD induced) as described above. When diabetes was induced in mice, HFD was not provided. On day 11, the compound, which was extracted from the Dodonaea viscosa, was solubilized in 1% dimethyl sulfoxide (DMSO) solution and given to mice of Group-3 (33mg/kg) by a single intraperitoneal injection. After compound treatment, the blood glucose levels of mice were regularly monitored over a span of 7-days. On the 17th day, after blood glucose measurement, this group was euthanized for further analysis. The body weight of all mice was regularly monitored throughout the whole study.

2.5. Dissection of Mice and Organ Collection

All the mice were fasted overnight, given access to tap water, and their body weight and blood glucose levels were measured before being dissected at the final stage of the research. Mice were dissected in accordance with IACUC standards. All the equipment and glassware from the dissection box were autoclaved before use. A cooling chain was maintained throughout the dissection. After euthanization, mice were put on a dissection board and pinned in the claws and paws to put the abdomen in the front. The organs were thoroughly cleaned to remove all the blood and debris by first washing them in chilled PBS buffer with pH range of 7.2–7.4 and then again washing them in cold distilled water. The extracted organs were subsequently stored at -80°C for further experimental procedures.

2.6. RNA Extraction and Purification

For molecular analysis, RNA was extracted from pancreatic tissue samples (stored at -80 °C), from all the experimental animals. Invitrogen RNA kit (RNA Minikit, Invitrogen, Cat No # 1218301 8A) was used to extract RNA from the tissues. For RNA extraction, 50-100mg of tissue was taken. Liquid nitrogen was used to rapidly freeze the tissue sample in the mortar, and the frozen tissue was then crushed with a pestle. Pestle and mortar were properly sterilized before use. During the grinding process, 1ml of lysis buffer along with 10µl beta-mercaptoethanol was added to lyse the tissues. The homogenized mixture was placed into an RNase-free tube, followed by centrifugation at 2000g for 5 minutes at 4°C by using a centrifuge machine (Eppendorf Centrifuge 5415R, Hansburg). At the bottom, pellet was formed and the

supernatant was discarded. After that, the lysate was poured into a sterile homogenized eppendorf tube and centrifuged at 12,000g for 2 minutes. Homogenate was also run through an 18 to 21 gauge-syringe needle marked 21 G×1/4 (0.8 mm×32mm) 5-10 times to homogenize the tissues properly. The homogenate was mixed with 100-200µl of chloroform and vortexed for 15 seconds. Homogenate was incubated for 3 minutes at room temperature. After incubation, we centrifuged the sample at 12,000g for 15 minutes at 4°C. Several layers were dispersed throughout the entire solution. The supernatant, containing the RNA, was separated carefully and the remaining solution was discarded.

The supernatant was moved to a new RNase-free Eppendorf and 70% chilled ethanol was then added in a ratio of 1:1. Samples were vortexed after giving it a vigorous shake and transferred to the 700µl of sample, centrifuged at 12,000g for 15 seconds, the flow through liquid was removed. To purify the solution, we transferred it into the spin cartridge tube provided with the kit which had a collection tube below and repeated the above step until all the supernatant and ethanol mixture is passed through the spin cartridge. As an aqueous solution passed through the membrane, RNA remained bound to the membrane. 700µl Wash buffer 1 was then added to spin cartridge followed by centrifugation of the cartridge at 12,000g for a duration of 15 seconds. A new collection tube was placed with in the spin cartridge after discarding the solution passed down the filter membrane and added 500µl wash buffer II. The centrifugation was done at 12,000g for 15 seconds. To improve purification, wash buffer II was reintroduced, followed by centrifugation of the cartridge at 12,000g for a duration of 15 seconds. After washing steps, an empty spin at 12000g for 2 minutes was given to remove the remaining wash buffer from the membrane filter. A recovery tube was placed under the spin cartridge and 50-70 µl of RNase free water was poured into it and centrifuged at 12,000 g for 1 minute after incubation of 1 minute at room temperature. The quality of extracted and purified RNA was assessed using a Nanodrop machine (Colibri Spectrophotometer, Berthold Detection System GmbH 75173 Pforzheim, Germany). The concentration and the absorbance ratio of A260/A280 were measured.

2.7. Complementary DNA (cDNA) Synthesis

After RNA isolation and quality assessment, cDNA was synthesized from total purified RNA (1 µg) by the RivertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific™, Catalog number: K1621). For cDNA synthesis, oligo dT primers were used. A total of 20 µl of the reaction mixture was made for each reaction to generate 500ng of cDNA from total purified RNA. First, the master mix was prepared which includes RT buffer, dNTP's, oligo dT primer, reverse transcriptase enzyme and RNase inhibitor in appropriate amount as mentioned in the Table 2.2. In each labeled PCR tube, the calculated volume of RNA and nuclease-free water/deionized water were added, along with the master mix. The reaction mixture was placed in a PCR machine and allowed to incubate at 42°C for a duration of 1 hour (T3 Thermoblock, Biometra, Germany). Finally, the reverse transcriptase enzyme was inactivated by heating the tubes for 5 minutes at 70°C. The newly synthesized cDNA was then stored at -20°C.

Table 2.2. cDNA synthesis protocol

Serial No.	Chemical composition	Volume (µl)
1	RT Buffer	2
2	dNTP's	2
3	Oligo dT primer	0.8
4	Reverse Transcriptase	2
5	RNase Inhibitor	2
6	RNA	1µg
7	Nuclease free water	Remaining volume

2.8.RT-qPCR

We performed the real-time PCR by using the My Go Pro PCR (My Go PCR system, IT-IS life sciences) and Eva Green® qPCR Mix Plus (HOT FIREPol®, LOT No. 08242520.1) along with a particular set of primers (Table 2.5). According to optimized protocol, primers (100µM) and the cDNA stock were diluted by 1:10. We prepared a final reaction mixture of 10µl (Table 2.3) in 8-well RT-PCR tubes (0.1ml

8-tube strips). We made fresh dilutions for each reaction to obtain better results. VAPA (VAMP-Associated Protein A) was used as a housekeeping gene in pancreas. The program settings that were used to proceed our RT-qPCR, is listed in Table 2.4. The relative mRNA expression levels of specific genes of interest were calculated by using $2^{-\Delta\Delta CT}$ method.

Table 2.3. Composition and Calculations of RT-qPCR reaction mixture for one reaction (volume 10µl).

Serial No.	Composition	Volume (µl)
1	Eva Green	2
2	Forward Primer (1:10)	1
3	Reverse Primer (1:10)	1
4	cDNA (1:10)	6

Table 2.4. MyGo Pro PCR Profile for RT-qPCR.

Serial No.	Program Profile	Internal settings	Temperature (°C)	Ramp (°C/s)	Time duration (s)
1	Hold	Hold	95	5	5
2	3-step Amplification	Denaturation	95	5	5
		Annealing	60	5	45
		Elongation	72	5	17
3	Pre-melt Hold	Hold	95	5	10
4	High resolution melting	Initial stage	60	4	60
		Final stage	97	4	15

2.9. Statistical Analysis

Descriptive statistics were calculated by one-way ANOVA for our data using GraphPad Prism software (version 8.02). Results were displayed as mean ± SD. The differences were found to be significant at 0.05 p-value. All the experiments were performed in triplicate.

Table 2.5. List of Primers for RT-qPCR.

Sr. No.	Primer Name	Sequence (5' to 3')	Length	Tm (°C)	Amplicon Size (bp)
1	mMst1 F	CACATTCCGTAAGCCAGAGC	20	60.8	97
	mMst1 R	CTGAGTGGCTGTGGCTCTCT	20	60.8	
2	mPhlpp2 F	CTAAAGAACCCGTCTCCAAGC	21	60.2	87
	mPhlpp2 R	CACCCAAAGGGAAGTGAAGA	20	60.1	
3	mJnk1 F	GGGTGCTGATGCTTTCAGAT	20	60.2	98
	mJnk1 R	CAGAGGGTACACGGCTTCC	19	60.7	
4	mLipin1 F	GATTCCAATGATGCGAGACC	20	60.4	83
	mLipin1 R	GCCCCACGTAATTCATTGTC	20	60.2	
5	mBim F	CACCTGCTGTGTGCTTCCTA	20	60	89
	mBim R	GCTGGCCTAAAGCAGTGAAC	20	60	
6	mBcl2 F	GGCTCCCTTCATGAAATCCT	20	60.4	101
	mBcl2 R	AGAACCCCTGTCTCCAAAGG	20	60.5	
7	mH2bl1 F	AGGAGATATCCAGCAGGCAGT	21	60.2	114
	mH2bl1 R	ATGGATGGAGCTTAGGAATGAA	22	59.9	
8	mNf2 F	AGACAACACCAGTGGGCTTC	20	60.2	105
	mNf2 R	TCACTGTTGCTGGGAGTCAG	20	60	
9	mLats2 F	GAGAGCTGGTGGGTGTGACT	20	60.3	93
	mLats2 R	CTGTGAATGGCAAGATACGGT	21	60	
10	mCasp3 F	AATAGCCCTGCAGCCCAT	18	60.6	64
	mCasp3 R	GAGCACAGTCTCCCTGAGGAT	21	60.8	
11	mAkt1 F	CAGCAAGAAGGCACCAGAA	19	60.1	83
	mAkt1 R	GAAGCAGCTGTGACCTCTCC	20	60.1	
12	mPdx1 F	ACTTGAGCGTTCCAATACGG	20	60.1	85
	mPdx1 R	CTTAGCTTGCTCAGCCGTTC	20	60.3	
13	mIRS1 F	AAGCACTGTGACACCGGAA	19	60.3	72
	mIRS1 R	CTTCGTGACCAGCTGTCCTT	20	60.4	
14	mPI3K F	GAGACAGGATGGGTCAAGGA	20	60.0	132
	mPI3K R	CAAAGCAACACAGGAGAGCA	20	60.2	
15	mPtpn1-F	GCATAGGACAGTGGTAATGCG	21	60.5	123

	mPtpn1-R	AACTCACAGGGAAAGCAGAGG	21	60.8	
16	mFoxo1a-F	CTGAGAAGTGCCTGATGAGGAT	22	60.8	117
	mFoxo1a-R	GAAAGACACACACCAAGCCAC	21	60.6	
17	mmTorc1-F	CCAGGAGGACATTTGTTCAGA	21	60.1	96
	mmTorc1-R	CACTGAACACAGTAGAGCCAGTG	23	60.0	
18	mSrebp1c-F	GGACCTTTGTCATTGGCTGT	20	60.0	62
	mSrebp1c-R	GCATGGTCCTGATTGCTTG	19	60.2	
19	mIl1b F	AGGGGACATTAGGCAGCAC	19	60.1	78
	mIl1b R	AGTGCGGGCTATGACCAA	18	60.2	
20	mNlrp3 F	AGCCCTCCTTCACCATCAG	19	60.2	68
	mNlrp3 R	CACAAGCCTTTGCTCCAGA	19	60.1	
21	mVapa F	TGTAGCCTTGGCAGTCCTG	19	60.0	88
	mVapa R	CTGGTGCTCAGAAGGCAGA	19	60.3	

3. Results

3.1. Quantitative Analysis of Targeted Gene Expression through RT-qPCR

After synthesizing the cDNA, we conducted RT-qPCR to examine the relative mRNA expression levels of our specific genes of interest in pancreas by using primers. The RT-qPCR data underwent manual analysis using the ΔΔCT method in Microsoft Excel, and the statistical analysis was accomplished using GraphPad Prism software (Version 8.0.2.263).

In Pancreas, we were interested in evaluating the role of MST1 on different signaling pathways which are affected in T2DM. In our study, we investigated pancreatic *β*-Cells from control mice, HFD-STZ-induced diabetic mice (labelled "STZ"), and HFD-STZ- induced compound treated group. We aimed to assess the mRNA levels of genes pivotal to insulin-mediated regulatory pathways, including the insulin signaling, mTORC1 signaling, hippo pathway, and associated inflammation. In subsequent mRNA expression graphs, "STZ" represents the HFD-STZ-induced diabetic model, while "STZ+C" indicates the compound-treated group.

3.1.1. MST1 mediated Insulin Signaling Inhibition

In pancreatic *β*-cells of mice, we analyzed the relative mRNA expression of insulin signaling markers using RT-qPCR. To normalize the data, we used *VAPA* (VAMP-Associated Protein A) as the housekeeping gene.

Through RT-qPCR, we examined the relative gene expression of key initiators of insulin signaling pathways, including *IRS1*, *PI3K* and *AKT1*. Notably, all these genes exhibited significant downregulation in STZ-HFD diabetic group, indicating a pronounced alleviation in their expression levels. But in compound treated group, relative mRNA expression level of *IRS1*, *PI3K* (approximately 3-fold) and *AKT1* (1.5 fold) were significantly increased. *PDX1* expression was downregulated in STZ-HFD diabetic group while its expression was increased by 2-fold in case of compound treated group. The relative *MST1* (3-fold) and *PTPN1*(8-fold) gene expressions were upregulated in STZ-HFD-induced T2DM and found to be downregulated in STZ-HFD compound treated group (Figure 3.1).

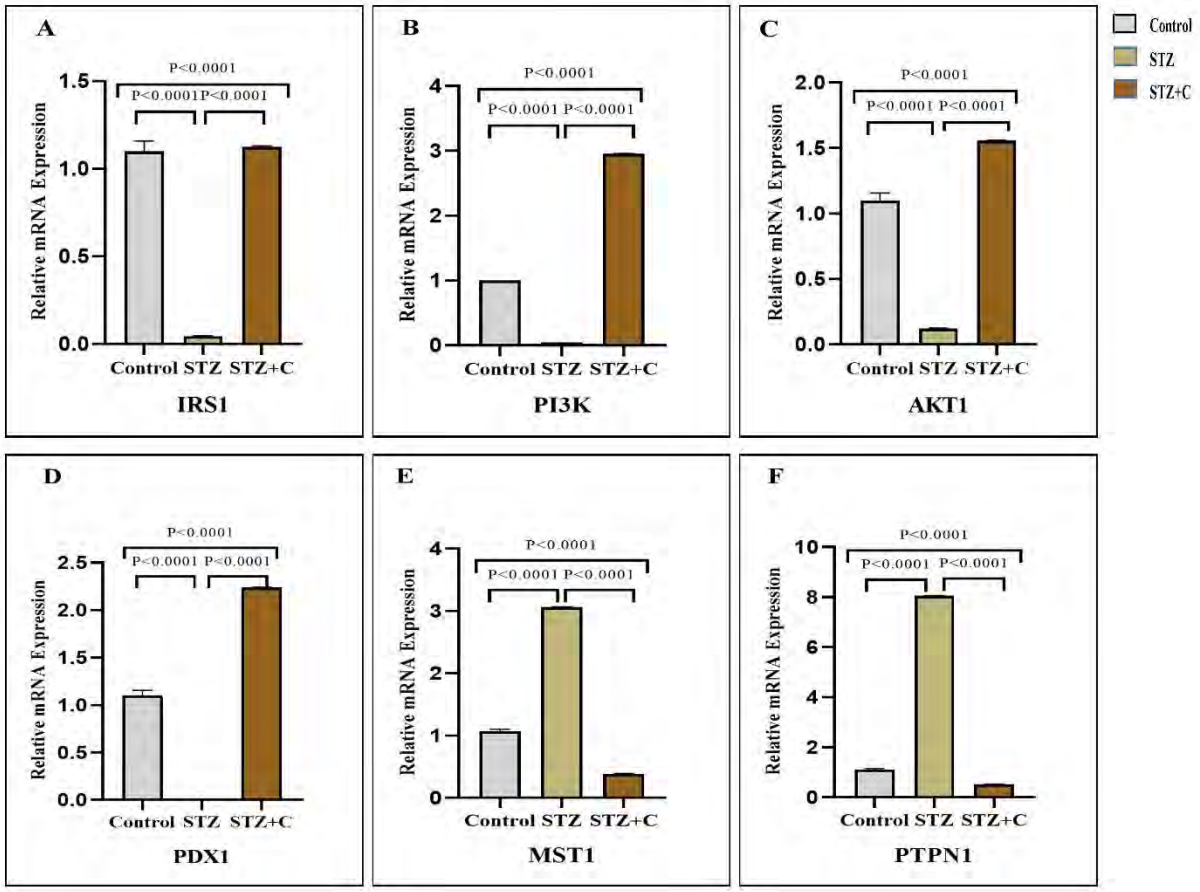

Figure 3.1. Relative Expression Analysis of Insulin Signaling Markers. **A.** Relative mRNA expression of *IRS1* in *β*-cells. The *IRS1* expression was relatively higher in STZ+C group as compared to the control group, and it was significantly reduced in STZ group. **B.** Relative mRNA expression of *PI3K* in *β*-cells. The *PI3K* expression was increased significantly in STZ+C group. In STZ group, it was found to be significantly reduced. **C.** mRNA expression of *AKT1* was examined in *β*-cells. *AKT1* expression was enhanced in the STZ+C group and it was decreased in STZ diabetic group. **D.** Relative mRNA expression of *PDX1* in *β*-cells. *PDX1* relative mRNA expression was found relatively higher in STZ+C compound group while it was reduced in STZ diabetic group. **E.** Relative mRNA expression of *PTPN1* in *β*-cells. *PTPN1* relative mRNA expression was significantly high in STZ group while it was significantly reduced in STZ+C and Control group. **F.** Relative mRNA expression of *MST1* in *β*-cells. The relative expression of *MST1* was significantly increased in STZ

group and found to be decreased in STZ+C and Control group. All results were found significant ($P < 0.05$).

3.1.2. PHLPP2 Regulating AKT1 and MST1 Activity

We investigated the expression of *PHLPP2* in STZ-HFD diabetic group, compound treated and control groups. *PHLPP2* expression was increased by 6-fold in STZ-HFD diabetic group as compared to compound and control groups. *MST1* expression was also increased by 3-fold in STZ-HFD diabetic group and *mTORC1* showed increased expression by approximately 8-fold in STZ-HFD diabetic group. They showed decreased expressions in compound treated and control groups. *AKT1* was downregulated in STZ-HFD diabetic group and showed increased expression by 1.5-fold in compound treated group (Figure 3.2)

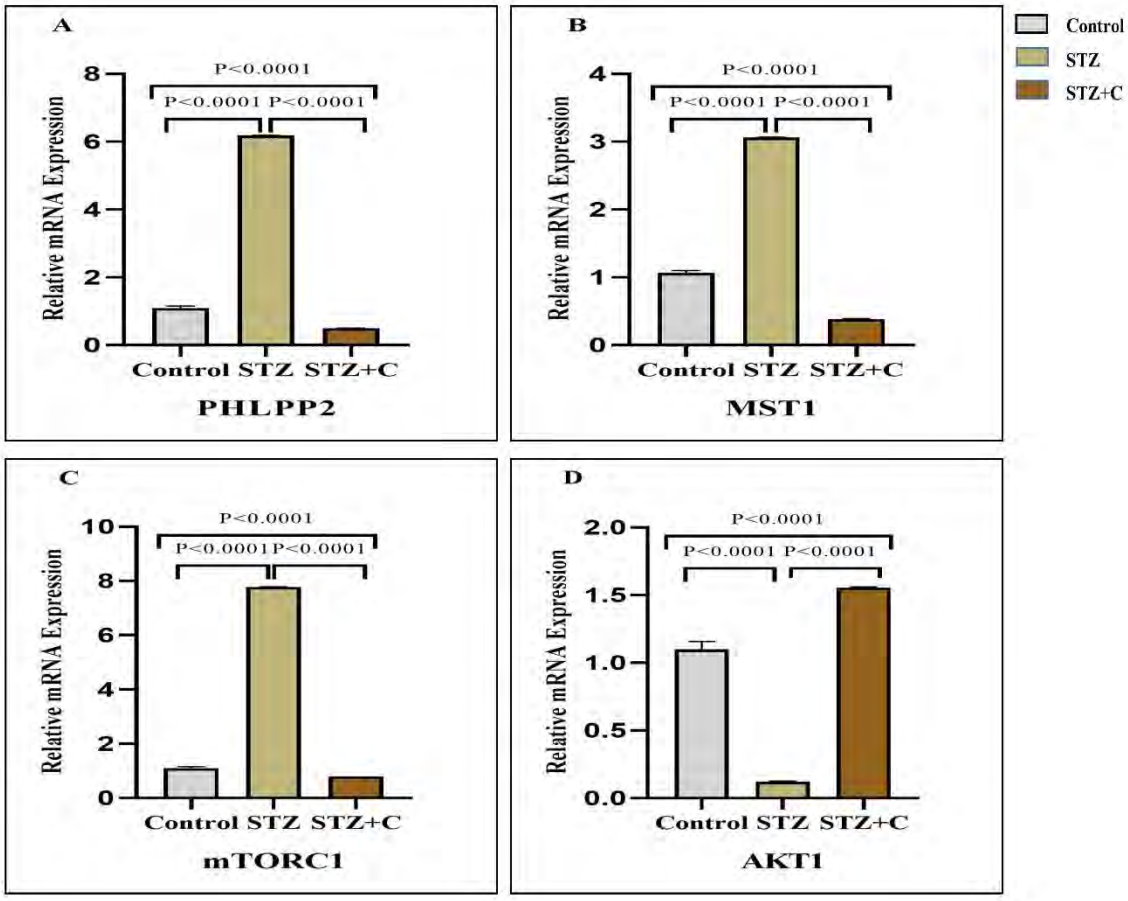

Figure 3.2. *PHLPP2* Expression in Pancreatic *β*-Cells Death. **A.** Relative mRNA expression of *PHLPP2* was examined. *PHLPP2* expression was elevated in STZ-HFD diabetic group, and it was reduced in STZ+C and Control groups. **B.** *MST1* expression

was increased in STZ-HFD diabetic group, and it was decreased in STZ+C and Control groups. **C.** The relative mRNA expression of *mTORC1* was significantly enhanced in STZ-HFD diabetic group while it showed diminished expression in compound treated and control group. **D.** *AKT1* showed increased expression in STZ-HFD diabetic group, and it was decreased in STZ+C and Control groups. All the results were significant ($P < 0.05$).

3.1.3. Pancreatic *β*-Cells Apoptosis

In pancreas, we evaluated the markers responsible for pancreatic *β*-cells death. *MST1*, *NF2*, *LATS2*, *JNK1*, *Casp-3*, *H2bl*, *FoxO1* and *BIM* were found to be elevated in STZ-HFD diabetic group, responsible for apoptosis of *β*-cells. *MST1* was upregulated by 3-fold, *NF2* by 4-fold, *LATS2* by 8-fold, *JNK1* by 5-fold, *Casp-3* by 5-fold, *H2bl* by 3-fold and *FoxO1* by approximately 5-fold. But their expression was significantly reduced in compound treated group. Expression of *BCL2* was decreased significantly in STZ-HFD diabetic group and found to be increased in STZ-HFD compound treated group as same as in control group (Figure 3.3).

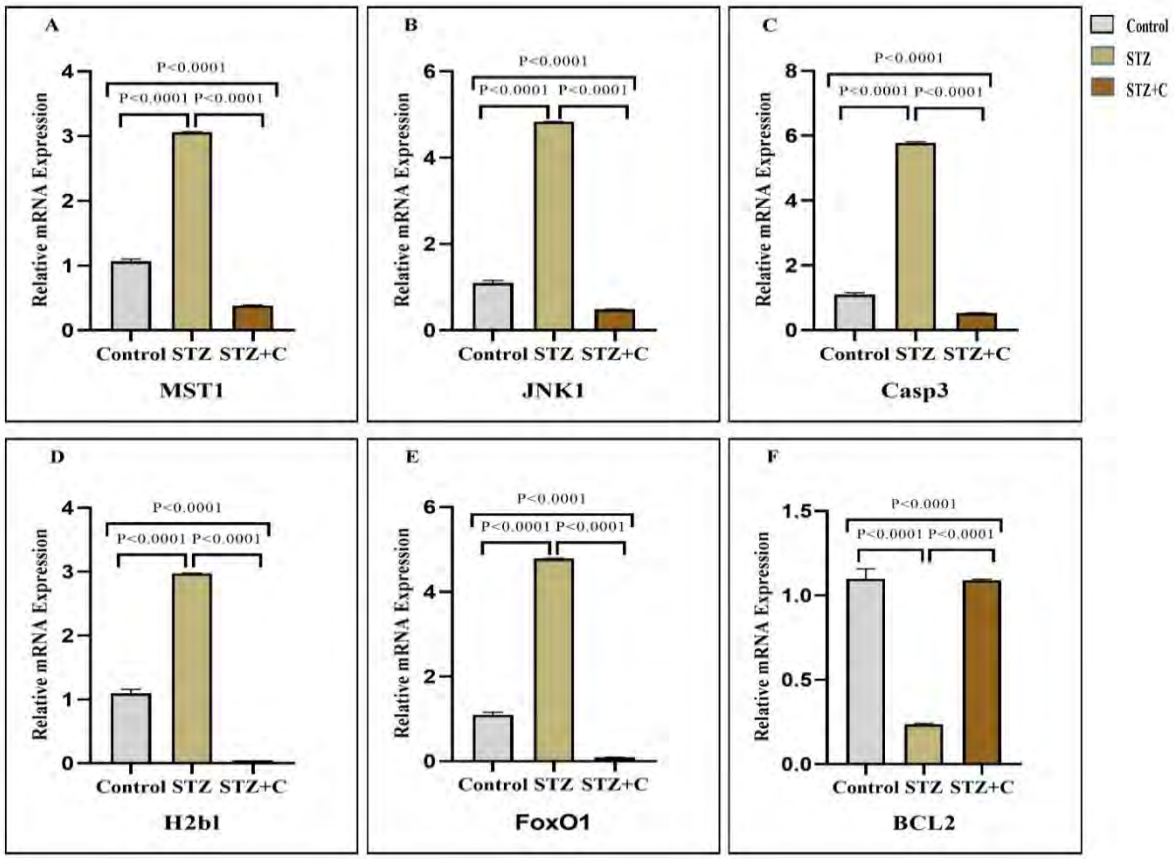

Figure 3.3. mRNA Expression Analysis of Apoptotic and Anti-Apoptotic Markers in Pancreatic *β*-cells. **A.** Relative mRNA expression of *MST1* was analyzed. *MST1* expression was significantly enhanced in STZ group while it was considerably reduced in STZ+C and Control groups. **B.** Relative analysis of *JNK1* mRNA expression was conducted in *β*-cells. *JNK1* relative mRNA expression was notably higher in STZ group but it was not detected in STZ+C and Control groups. **C.** Relative mRNA expression analysis of *Casp-3*was examined in Pancreatic *β*-cells. Relative mRNA expression of *H2bl* showed significant enhancement in STZ group and it was not expressed in STZ+C and Control groups. **D.** Relative mRNA expression analysis of *H2bl* was conducted. Additionally, the relative mRNA expression of *H2bl* demonstrated a significant enhancement in the STZ group, whereas it was not expressed in the STZ+C and Control groups. **E.** The relative mRNA expression of *FoxO1* was examined. Notably, *FoxO1* expression was significantly amplified in the STZ group, whereas it experienced a substantial decrease in both the STZ+C and Control groups. **F.** Relative mRNA expression of *BCL2* was analyzed. The relative mRNA expression of *BCL2* was significantly reduced in STZ group and it was notably expressed in STZ+C and Control groups. All the results were significant ($P < 0.05$).

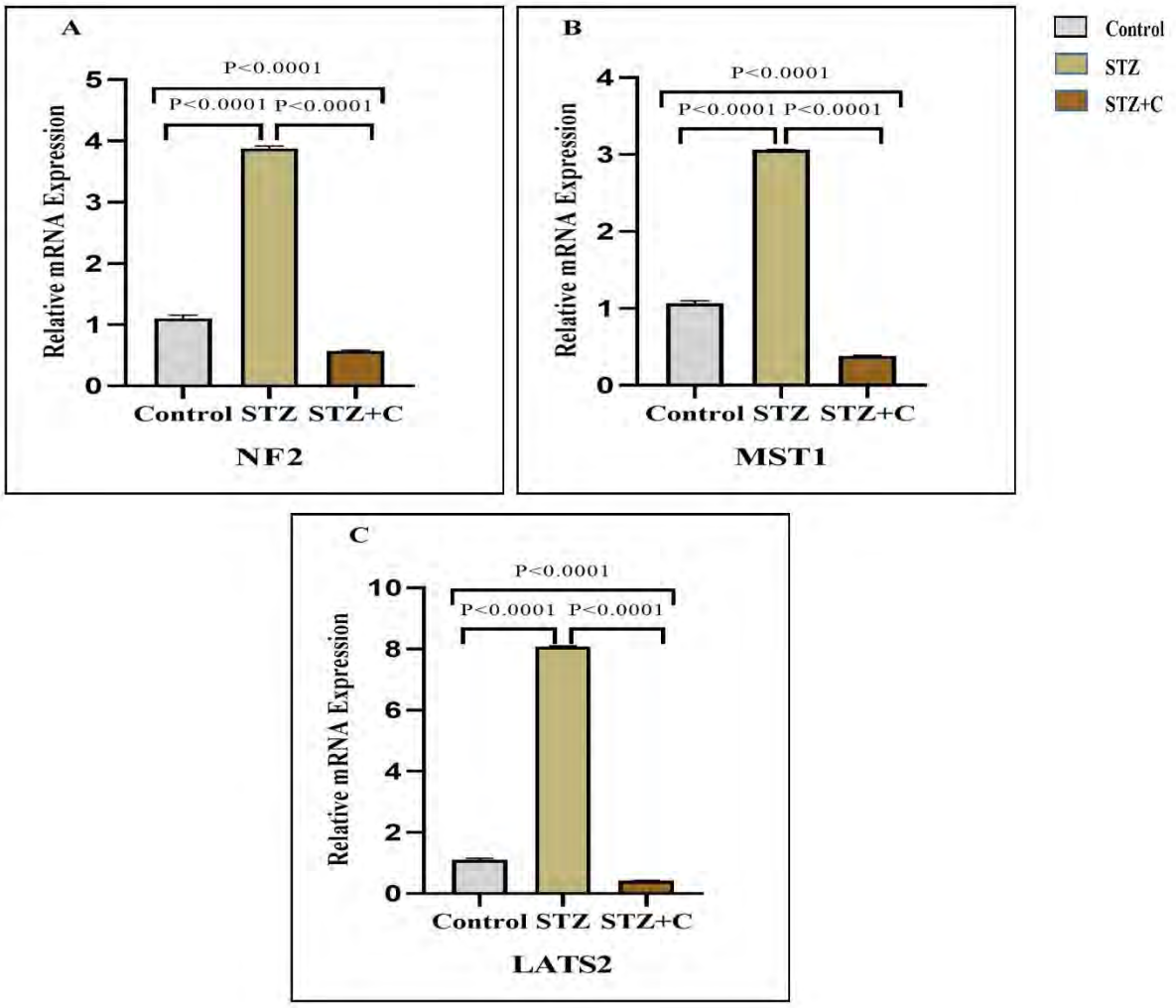

Figure 3.4. Expression of Hippo Signaling Markers in Pancreatic *β*-cells. **A.** Relative mRNA expression analysis of *NF2* was examined in *β*-cells. *NF2* relative expression was increased in STZ group while it was decreased in STZ+C and Control groups. **B.** Analysis of relative mRNA expression of *MST1* in *β*-cells. *MST1* expression was significantly enhanced in STZ group, and it was significantly reduced in STZ+C and Control groups. **C.** Relative mRNA expression of *LATS2* was analyzed in Pancreatic *β*-cells. *LATS2* expression exhibited significant enhancement in the STZ group, while it showed a considerable reduction in both the STZ+C and Control groups. All the results were found significant ($P < 0.05$).

3.1.4. mTORC1 mediated AMPK*α*/ Autophagy Inhibition

We evaluated the expressions of mTORC1 signaling markers which were involved in autophagy inhibition. The expressions of *mTORC1*, *LATS2*, *Srebp1c*, *Lipin1*, *AMPKα*

and *ULK1* were analyzed at mRNA level. Significantly increased expressions of *mTORC1* (approximately 8-fold), *LATS2* (8-fold), *Srebp1c* (8-fold), *Lipin1*(3-fold) were observed in STZ-HFD diabetic group as compared to compound treated group. The relative mRNA expressions of *AMPKα* and *ULK1* were decreased in STZ-HFD diabetic group while showed increased expression in compound treated group (Figure 3.5).

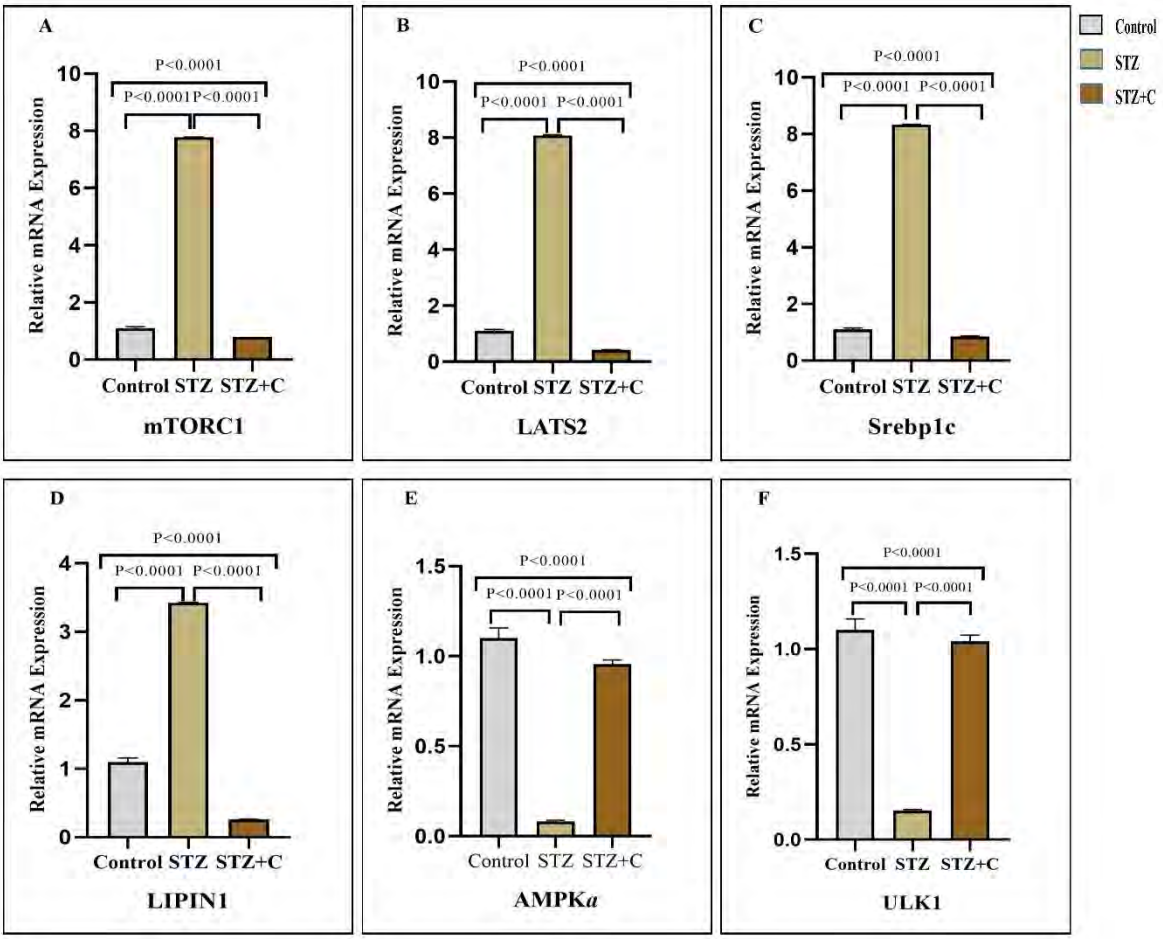

Figure 3.5. mRNA Expression of *mTORC1* Signaling Markers in Pancreatic *β*-cells. **A.** Analysis of relative mRNA expression of *mTORC1* in *β*-cells. *mTORC1* expression showed significantly increased expression in STZ group and it was deceased in STZ+C and Control group. **B.** *LATS2* mRNA expression showed substantial enhancement in STZ group while it was reduced in STZ+C and Control groups. **C.** *Srebp1c* mRNA expression showed a significant increase in STZ group, while it was

reduced in both STZ+C and Control groups. **D.** The mRNA expression of *Lipin1* demonstrated a notable increase in the STZ group, while it showed a decrease in both the STZ+C and Control groups. **E.** Relative mRNA expression of *AMPKα* was analyzed. *AMPKα* relative mRNA expression was markedly downregulated in STZ group as compared to STZ+C and Control groups. **F.** Relative mRNA expression of *ULK1* was evaluated. *ULK1* relative mRNA expression was downregulated in the STZ group compared to STZ+C and Control groups. All the results were found significant ($P < 0.05$).

3.1.5. MST1 mediated Inflammasome Activation

We evaluated the expression of inflammatory markers involved in pancreatic *β*-cells inflammation. The expression of *MST1*, *JNK1*, *NEK7*, *NLRP3* and *IL-1β* were analyzed at mRNA level. *MST1* expression was increased by 3-fold in STZ-HFD diabetic group as compared to compound treated group and control group. The expression of *JNK1* was elevated by approximately 5-fold in STZ-HFD-induced T2DM group. The expressions *NLRP3* (9-fold) and *IL-1β* (>2-fold) were elevated in STZ-HFD diabetic group. But their expressions were significantly reduced in compound treated and control group (Figure 3.6).

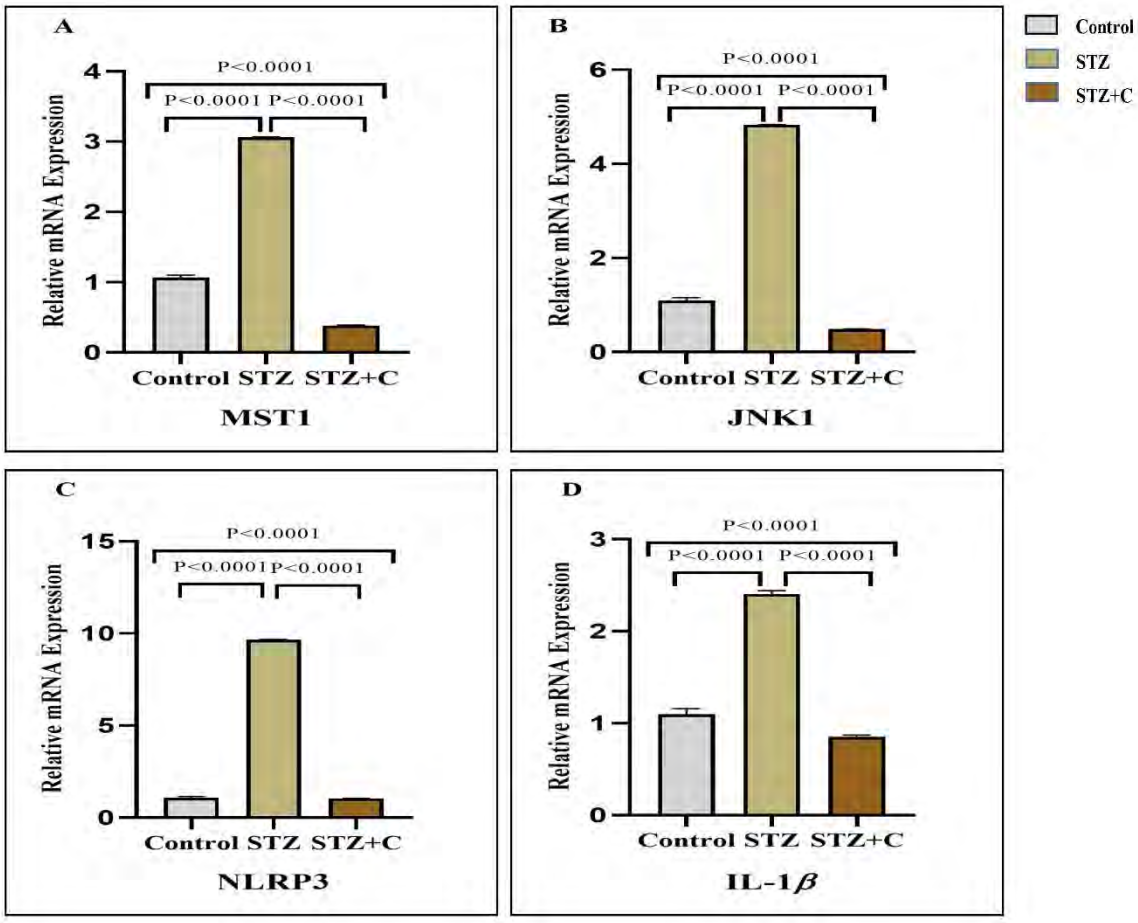

Figure 3.6. Relative mRNA Expression of Inflammatory Markers in Pancreatic *β*-cells. **A.** Relative mRNA expression of *MST1* was analyzed. *MST1* expression was higher in STZ group, and it was considerably reduced in STZ+C and Control groups. **B.** Analysis of relative mRNA expression of *JNK1* revealed a significant increase in *JNK1* expression in the STZ group, whereas it remained undetected in both the STZ+C and Control groups. **C.** The relative mRNA expression of *NLRP3* was assessed. *NLRP3* expression exhibited a notable increase in the STZ group, while showing a substantial decrease in both the STZ+C and Control groups. **E.** *IL-1β* expression was analyzed in *β*-cells. A significant rise in *IL-1β* expression was detected in STZ group, contrasting with a significant reduction seen in both the STZ+C and Control groups. All the results were significant ($P < 0.05$).

4. Discussion

T2DM is caused primarily by decreased pancreatic *β*-cell insulin excretion and inability of insulin-responsive tissues to respond to insulin (Roden & Shulman, 2019). Pancreatic *β*-cells have an essential role in maintaining glucose homeostasis by producing and releasing insulin (Wilcox, 2005). When pancreatic cells cease to function, they are unable to produce enough insulin to combat insulin resistance which results in T2DM (Rorsman & Braun, 2013; Weir & Bonner-Weir, 2004). Insulin resistance is the main problem in T2DM, but when the situation progresses into a chronic hyperglycemic state, *β*-cell apoptosis occurs, leading to an absolute insulin deficiency (Association, 2018). T2DM can cause a 60% reduction in *β*-cells mass (Butler *et al*., 2003) and is linked with obesity which resulted in complex biological and physiological developments which changed the biology of adipose tissue and pancreatic *β*-cells (Scherer, 2022). Multiple low-dose streptozotocin injections cause diabetes through the stimulation of cell-specific immune-mediated destruction and cell-intrinsic apoptotic cascades (Ardestani *et al*., 2019). ER stress is triggered by various stimuli, such as obesity, a high-fat diet, oxidative damage, hyperglycemia, and many pharmaceutical drugs (Bánhegyi *et al*., 2007; Ozcan *et al*., 2004). When calcium sources are depleted as a result of ER stress, PTP1B is activated (Amodio *et al*., 2009). PTP1B is highly expressed in tissues that are sensitive to inulin and performs a crucial function as a negative regulator of insulin signalling (Goldstein, 1993). MST1 acts as a direct inhibitor of AKT1. *β*-cell apoptosis induced by MST1 is facilitated by the inhibition of PI3K/AKT signaling transduction (Ardestani *et al*., 2014). PTP1B is considered a novel substrate for AKT as *PTP1B* phosphorylation at Ser^{50} by AKT suppresses PTP1B activity and reduces its capacity to dephosphorylate the IR, potentially acting as a positive feedback mechanism to amplify insulin signaling (Ravichandran *et al*., 2001). In 2014, a study conducted on mice with a pancreas-specific *PTP1B* deletion exhibited that the absence of *PTP1B* stimulated the proliferation of *β*-cells and led to a decrease in pancreatic *β*-cell apoptosis (Fernandez-Ruiz *et al*., 2014). PTP1B over activity has been linked to the down-regulation of insulin signaling, and is considered to be a promising therapeutic

target in the treatment of DM (Uddin *et al*., 2018) and alleviates insulin resistance (Montalibet & Kennedy, 2005).

In the current study, we examined the in vivo effects of the potent PTP1B inhibitor Viscosol, also known as 5,7-dihydroxy-3,6-dimethoxy-2-(4-methoxy-3-(3-methyl but-2-enyl) phenyl)-4H-chromen4-one, isolated from *Dodonaea viscosa*, on MST1 mediated pancreatic *β*-cell death, mTORC1-AMPK*α* autophagy inhibition and glucose metabolism. In vitro and In vivo antidiabetic efficacy of viscosol has also been reported (Sohail *et al*., 2022; Uddin *et al*., 2018). A T2DM model induced by HFD and low dose streptozotocin was used. Previous studies using HFD-Low dose STZ-induced models have also been described (Lenzen, 2008; Skovsø, 2014).

In our current study, we determined the relative mRNA expression of our targeted genes involved in different signaling cascades in the pancreas. Pancreas is a source of insulin production and T2DM occurs due to the malfunctioning of pancreatic β-cell (Cuenco & Dalmas, 2022). In pancreatic β-cells, we first evaluated the genes of insulin signaling such as *IRS1*, *PI3K*, and *AKT1*. Their alleviated gene expression has been seen in HFD-STZ induced diabetic mice group but significantly elevated in our compound-treated group. Then, we investigated the gene expressions of *PTPN1*, *MST1*, and *PDX1* and their elevated gene expressions have been seen in STZ-HFD induced diabetic group but remarkably decreased in our compound-treated group. *IRS1* and *IRS2* inhibition inactivates *PI3K* and elongates the activation of MAPKs as ERK1/2, p38, and JNK. The decreased expression of *IRS1* and *PI3K* is according to the concept that mice lacking *PI3K* catalytic subunit showed insulin resistance and type 2 diabetes (Brachmann *et al*., 2005; Cho *et al*., 2001). *MST1* act as a direct inhibitor of *AKT1*. *β*-cell apoptosis induced by MST1 is facilitated by the inhibition of PI3K/AKT signaling transduction (Ardestani *et al*., 2014). MST1 activation in diabetic *β*-cells leads to impaired insulin secretion by promoting the proteasomal degradation of the crucial *β* cell transcription factor, *PDX1* which is essential for insulin production (Ardestani & Maedler, 2016). Insulin signaling down-regulation has been related to over activity of PTP1B, which has been a promising target for the treatment of DM (Uddin *et al*., 2018). PTP1B deficient models also showed enhanced IR phosphorylation and insulin sensitivity (Koren & Fantus, 2007).

In our experiment, we witnessed the elevated expressions of *MST1* and *PTP1B*, and diminished expressions of *AKT1* in STZ-HFD induced diabetic group but decreased expressions of *MST1* and *PTP1B*, and increased expressions of *AKT1* in our compound-treated group. All these expressions of *MST1*, *PTP1B*, and *AKT1* in diabetic and compound-treated groups are supporting our hypothesis that when PTP1B inhibition via Viscosol activates IRS1 and AKT1, which upon activation phosphorylates MST1 and inhibits MST1 mediated apoptosis in pancreatic β-cells.

We investigated the expressions of *PHLPP2*-regulated genes such as *AKT1*, *MST1*, and *mTORC1*. We have seen the elevated expressions of *mTORC1*, *PHLPP2*, and *MST1* in the STZ-HFD induced diabetic group as compared to a compound-treated group where they have shown diminished expressions. While the expressions of the *AKT1* gene have been seen to be decreased in STZ-HFD induced diabetic group and increased in our compound-treated group. The diminished expressions of *AKT1* and *PHLPP2* enhanced expressions in the diabetic group confirms that PHLPP2 specifically dephosphorylates AKT1 in β-cells, leading to reduced AKT activity and increased apoptosis of β-cells (T. Gao et al., 2005). *PHLPP2* increased expressions have also confirmed the elevated expressions of *MST1* in the diabetic group, which causes apoptosis of pancreatic *β*-cells (Qiao *et al*., 2010). *PHLPP2* expression increases due to *mTORC1* overactivation in diabetic models, making them significant phosphatases in this context (Lupse *et al*., 2021). In in-vitro, and in-vivo experimental models of diabetes, and in primary human T2D islets, the genetic inhibition of PHLPPs significantly improves pancreatic β-cells survival and function (Lupse *et al*., 2021).

The metabolic stress in T2DM can induce pro-inflammatory cytokines, tumor necrosis factor-*α* (TNF-*α*), and interleukin-6 (IL-6), activating a series of processes leading to apoptosis in pancreatic *β*-cells (Ding et al., 2019; Randeria *et al*., 2019). MST1, which is a substantially expressed serine/threonine kinase, is main upstream regulator of Hippo pathway and is involved in a variety of cellular functions, including morphological development, proliferation, stress responses, and apoptosis (Avruch et al., 2012; Ling et al., 2008). We found the higher expression of *MST1*, *JNK1*, *Casp-3*, *H2bl*, and FoxO1*α* in STZ-HFD induced diabetic mice group but their

expressions were downregulated in our compound treated group. All these genes are involved in β-cells apoptosis whose elevated expressions. MST1 is phosphorylated by JNK, which increases MST1 activity. It has been reported that JNK inhibition resulted in diminished MST1 activity, nuclear translocation, and MST1-induced apoptosis (Bi *et al*., 2010). JNK phosphorylation has a significant role in *β*-cell destruction, and its inhibition has been considered a promising strategy to protect *β*-cells and enhance insulin sensitivity in pro-inflammatory conditions (Bonny *et al*., 2001; Kaneto *et al*., 2004). Caspase-3 and JNK play dual roles as both upstream activators and downstream targets of the pro-apoptotic signaling cascade in *β*-cells, further exacerbating apoptosis. MST1 stimulates cell death by regulating various downstream targets, such as LATS1/2, histone H2B, FoxO members, the intrinsic mitochondrial pro-apoptotic process, JNK, and caspase-3 activation (Cheung *et al*., 2003; Bi *et al*., 2010; Avruch *et al*., 2012). MST1 plays a critical role in activating pro-apoptotic transcription factor FoxO1*α* by phosphorylating it at Ser^{212}, facilitating the detachment of FoxO1*α* from 14-3-3 protein (Lehtinen *et al*., 2006; Z. Yuan et al., 2008). The cellular localization of FoxO1 plays an essential role in determining the localization of PDX1, and their expression levels are inversely correlated. The regulation of FoxO1 involves both JNK and AKT activity. JNK activity encourages the nuclear importation of FoxO1, leading to the transfer of PDX1 from the nucleus (Ardestani *et al.*, 2014). The expressions of *Bcl2*, an anti-apoptotic gene, have been decreased in STZ-HFD induced diabetic group but its expression was significantly enhanced in our compound-treated group. *Bcl-2* inhibits apoptosis in pancreatic *β*-cells (Iwahashi et al., 1996). In human pancreatic islets under antagonistic circumstances, 80% silencing of *MST1* slowed down the process of apoptosis, and a lower BIM value was also noted in diabetic situations (Ardestani *et al*., 2014).

Hippo pathway regulates apoptosis in response to metabolic changes related to T2DM, involving key elements such as MST1, NF2, LATS2, and YAP (Ardestani *et al*., 2014; J. Liu *et al*., 2020; Shu et al., 2019; Ting Yuan *et al*., 2021; Ting Yuan *et al*., 2017). We determined the expressions of genes involved in the Hippo Signaling pathway such as *NF2*, *MST1*, and *LATS2*. The elevated expression of these markers has been seen in STZ-HFD induced diabetic group as compared to the compound-

treated group where their expressions were significantly decreased. NF2 is the upstream regulator of MST1 in Hippo signaling (Yin *et al*., 2013). NF2 knockout in pancreatic *β*-cells could prevent apoptosis without affecting *β*-cell function (T Yuan, Gorrepati, Maedler, & Ardestani, 2016). Upon activation, MST1 activates LATS2 whose enhanced expression has been seen in our STZ-HFD induced diabetic group. LATS2 activation resulted in the inhibition of YAP which promotes the transcription of survival genes (Harvey *et al*., 2013; Zhao, Li *et al*., 2010). XMU-MP-1 has been used as an MST1 inhibitor to improve glucose tolerance in STZ-induced diabetic mice (Faizah *et al*., 2020). In in-vitro human islets and in-vivo rodent models of diabetes, neratinib as an MST1 inhibitor proved as a potential β-cell-protective drug (Ardestani *et al*., 2019).

We determined the expressions of genes involved in *mTORC1*-mediated autophagy inhibition. We have seen the elevated expressions of *mTORC1*, *LATS2*, *Srebp1c*, and *Lipin1* in the STZ-HFD-induced diabetic group while their expressions have been decreased in the compound-treated group. While the reduced expressions of *AMPKα*, and *ULK1* have been seen in STZ-HFD induced diabetic group but their increased expressions have been seen in our compound-treated group. The elevated expressions of *mTORC1* and *LATS2* in the diabetic group have proved that mTORC1 and LATS2 interact in collaboration to promote *β*-cells death (Ting Yuan *et al*., 2021). mTORC1 hyperactivation due to continuous nutritional stress associated with T2DM, downregulated the expressions of *AMPKα* and *ULK1* in pancreatic *β*-cells, resulting in protective autophagy inhibition (Pepin et al., 2016; Ruderman & Prentki, 2004; Y. Sun et al., 2008). The main cause of *β*-cells lipotoxicity is the elevated expressions of *Srebp1c* in STZ-HFD diabetic group. Srebp1c knockdown in INS-1E cells protected them against lipotoxicity and inhibited PDX1 reduction, suggesting a therapeutic possibility for treating diabetes caused by lipotoxicity (J. Li et al., 2010). Srebp1c inhibition is common to antidiabetic drugs such as leptin, metformin, adiponectin, and PPARγ agonists (Kakuma et al., 2000). In INS-1E cells and islets experiencing glucolipotoxicity, Irisin treatment leads to phosphorylation of AMPKα and inhibits the expressions of lipogenic enzymes such as ACC and FAS (D. Zhang, 2018).

We investigated the expressions of genes involved in *MST1*-mediated inflammasome activation. The elevated expressions of *MST1*, *JNK1*, *NLRP3,* and *IL-1β* have been seen in STZ-HFD diabetic group while their expressions have shown marked reduction in our compound-treated group. Elevated expressions of *MST1* have proved that it plays a role in *JNK-mediated NEK7-NLRP3* activation which is confirmed by their elevated expressions in STZ-HFD induced diabetic group but decreased in our compound-treated group (A. Gao *et al*., 2021). NLRP3 inflammasome upon activation catalyzed the release of IL-1*β* through caspase-1. IL-1*β* plays diverse roles in the regulation of metabolism and inflammatory responses, influencing insulin excretion, and promoting apoptosis of *β*-cell, potentially contributing to the progress of T2DM (Sepehri *et al*., 2017; Tong *et al*., 2017). IL-1*β* has been recognized as an initiation factor activating Casp-3 in pancreatic *β*-cells (Lutz *et al*., 2014). Diarylsulfonylurea-containing compound termed as MCC950, 3,4-Methylenedioxy-β-nitrostyrene (MNS), and CY-09 are potent inhibitors of NLRP3 (Zahid, Li, Kombe, Jin, & Tao, 2019). We witnessed the elevated expressions of *MST1*, *JNK*, *NLRP3*, and *IL-1β* in the STZ-HFD diabetic group which supported the hypothesis that IL-1*β* can activate MST1 through Casp-3 activation, and when MST1 is activated, it catalyzed the activation of NLRP3 inflammasome causing inflammation in pancreatic *β*-cells.

Concluding, in current study, we have tried to explore the effect of viscosol on the indirect inhibition of MST1 pancreatic *β*-cells of T2DM mice model. The inhibition of PTP1B via viscosol indirectly inhibited MST1 activation due to the activation of IRS1 and AKT1. MST1 inhibition in this way resulted in a proliferation of *β*-cells, activation of autophagy via AMPK*α* activation, reduction of glucolipotoxicity, and protection from inflammation.

In future, there is a need to do a lot of work on MST1's role in organs affected by T2DM. Apoptosis induced by MST1 can be observed in the brain and kidney. We can use inhibitors that can target other markers of the hippo pathway such as NF2. There is a need to identify the genetic predispositions and epigenetic changes associated with MST1 that could shed light on individual susceptibility to T2DM and potential targets.

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