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LIPOCALIN-2 AMELIORATES THE SIGNS AND OUTCOMES OF DIABETES MELLITUS IN AN ANIMAL MODEL

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LIPOCALIN-2 AMELIORATES THE SIGNS AND OUTCOMES OF DIABETES MELLITUS IN AN ANIMAL MODEL

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Biochemistry and Molecular Biology)

Under the Supervision of Professor Ernest Adeghate

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Declaration of Original Work

I, Saeeda Mohammed Al Jaberi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Lipocalin-2 Ameliorates the Signs and Outcomes of Diabetes Mellitus in an Animal Model*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Ernest Adeghate, in the College of Medicine and Health Sciences at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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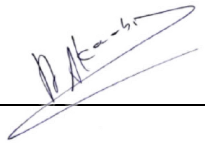
Signature  Date 26/08/2021

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Abstract

Lipocalin-2 (LCN2) is a new adipocytokine consisting of 198 amino acids. It is also referred to as neutrophil gelatinase-associated lipocalin, siderocalin, uterocalin, α1-microglobulin related protein, or 24p3. LCN2 belongs to a large group of transport proteins that are capable of carrying small and lipid soluble molecules in blood circulation. It has two membrane receptors, megalin/glycoprotein GP330, which binds human LCN2 and SLC22A17 or 24p3R, which forms complexes with mouse Lcn2 protein. LCN2 is encoded by a gene located at chromosome locus 9q34.11. LCN2 was initially isolated from neutrophil granules released at site of infection and inflammation in human and from mouse kidney cells. LCN2 kills bacteria by iron depletion during antibacterial innate immune response via sequestering bacterial ferric siderophores enterobactin (Ent). It has a protective role in infection, inflammation, injury and other forms of cellular stress. In addition, it is able to interact with and stabilize matrix metalloproteinase-9 in human neutrophils. Tissue localization and the effect of LCN2 were studied in streptozotocin-induced diabetic rats using morphological, physiological, biochemical and molecular biology techniques. This study shows that LCN2 is co-localized with insulin in normal and diabetic pancreatic β-cells. In addition, LCN2 significantly reduced the plasma levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin, cholestrol, triglycerides, low density lipoprotein and improved the levels of high density lipoprotein and total protein after the onset of diabetes. LCN2 treatment decreased the plasma levels of BUN, Urea and LIPC in diabetic animals. Moreover, the levels of insulin, C-peptide, amylin and GIP were significantly increased in diabetic rats treated with LCN2. Furthermore, LCN2 showed a significant antioxidant activity by increasing glutathione reductase, super oxide dismutase and catalase levels in pancreatic tissue of normal and diabetic animals, as well as their levels in the serum. In vitro observations show that LCN2 at a concentration of 10^{-8} M and 10^{-12} M caused large and significant increases in insulin release from the INS-1 rat insulinoma cell line. In conclusion, LCN2 ameliorates the acute and chronic complications of diabetes mellitus and appears to be a promising adjuvant therapy in the management of DM.

Keywords: Diabetes mellitus, lipocalin-2, metabolic parameters, rat, hormones, electron microscopy.

Title and Abstract (in Arabic)

يخفف ليبوكالين-2 علامات ونتائج مرض السكري في نموذج حيواني لمرض السكري

الملخص

ليبوكالين-2 مكون من 198 حمض أميني ويشار اليه أيضا بالجيلاتينيز المرتبط بالليبوكالين، سايدروكالين و يوتيروكالين. ليبوكالين-2 ينتمي لعائلة الليبوكالين وهي مجموعة من البروتينات التي تنقل الجزيئات الصغيرة مثل االحماض الدهنية، البروستاجلاندين، الريتينويدات، المنشطات والهرمونات. لدى الليبوكالين-2 اثنين من المستقبلات الغشائية المستقبل 24 بي 3 و المستقبل ميجالين. تم عزل ليبوكالين-2 في البداية من حبيبات الخلايا التي تم اطلاقها في موقع العدوى والالتهاب فالانسان ومن خلايا الكلى فالفئران. ليبوكالين-2 له دور وقائي فالعدوى والالتهابات ويقتل البكتيريا عن طريق استنفاذ الحديد، بالإضافة الى ذلك فهو قادر على التفاعل مع ميتالوبروتينيز-9 . تمت دراسة ليبوكالين-2 وتأثيره على الانسجة والخلايا في الفئران المصابة بداء السكري عن طريق الستربتوزوتوسين. باستخدام تقنيات البولوجيا المورفولوجية أظهرت الدراسة انه يتم تواجد الليبوكالين-2 مع الانسولين في خلايا البنكرياس الطبيعية وبنكرياس الفئران المصابة بالسكري. بالإضافة بعد ظهور مرض السكري العلاج عن طريق ليبوكالين-2 قادر بشكل كبير على خفض مستويات البلازما للكوليسترول، ترايقليسيرايد، ليبوبروتين منخفض الكثافة، بيليروبين، وانزيمات الكبد ك الانين امينوترانسفيرايز و اسبراتيت امونوترانسفيز. و أيضا من تحسين مستويات البروتين الكلي و تمكن أيضا من رفع مستويات الانسولين. بالإضافة الى ذلك العلاج مع ليبوكالين-2 أظهر نشاطا كبيرا كمضاد للأكسدة عن طريق رفع مستويات الانزيمات المضادة للأكسدة في البلازما وأنسجة البنكرياس. أظهرت دراساتنا المختبرية أن مصل الليبوكالين-2 تسبب بزيادات كبيره وهامة في افراز الانسولين من خط خلايا الانسولين بتركيز 12^-10 و 8^-10. في الختام يخفف ليبوكالين-2 المضاعفات الحادة والمزمنة لمرض السكري ويبدو انه علاج مساعد واعد في إدارة مرض السكري.

مفاهيم البحث الرئيسية: مرض السكري، ليبوكالين-2، الجرذان، الهرمونات، مؤشرات الايض، المجهر الاليكتروني.

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Special thanks go to my family and friends who helped me along the way.

Dedication

To my beloved parents and family

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List of Abbreviations

AST	Aspartate Aminotransferase
ALT	Alanine Aminotransferase
CAT	Catalase
CHOL	Cholesterol
DM	Diabetes Mellitus
DPP-4	Dipeptidyl Peptidase-4
EM	Electron Microscope
ERK	Extracellular Signal-regulated Kinase
FBG	Fasting Blood Glucose
GIP	Gastric Inhibitory Peptide
GLP-1	Glucagon Like Peptide 1
GSH red	Glutathione Reductase
GTT	Glucose Tolerance Test
HbA1C	Glycated Hemoglobin
HDL	High Density Lipoprotein
IHC	Immunohistochemistry
IL-6	Interlukein-6
LCN2	Lipocalin-2
LDL	Low Density Lipoprotein
MCP-1	Monocyte Chemoattractant Protein-1
NF-κB	Nuclear Factor Kappa B
PBS	Phosphate-buffered Saline
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase

STAT3	Signal Transducer and Activator of Transcription 3
STZ	Streptozotocin
T1DM	Type 1 Diabetes
T2DM	Type 2 Diabetes
TG	Triglycerides

Chapter 1: Introduction

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a complex and heterogeneous group of endocrine disorders. All types of diabetes mellitus share hyperglycemia as a common outcome and disturbances of glucose, lipid, and protein metabolism. Absolute or partial loss of insulin action and/or insulin secretion (Bradley et al., 1998), and depletion in functional β- cell mass are considered to be the key mechanisms that lead to the development of the disease (Cnop et al., 2005). The American Diabetes Association (ADA) defines type 1 diabetes mellitus (T1DM) as autoimmune β-cell destruction, usually leading to absolute insulin deficiency. ADA refers to type 2 diabetes mellitus (T2DM) as a progressive loss of β-cell insulin secretion, frequently occurring on the background of insulin resistance, and gestational diabetes mellitus (GDM) as diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation. Other types of diabetes include MODY, pancreatic diabetes, and drug- or chemical-induced diabetes. All types are diagnosed on the basis of hyperglycemia, however, each type has different diagnostic marker. In type 1, which results from autoimmune destruction of beta cells, the presence of pancreatic autoantibodies in the blood is an important diagnostic marker. MODY which is a monogenic type of diabetes caused by a single gene defects, sequencing is used as a diagnostic test to sequence the known monogenic diabetes genes to determine the pathological variant. Haemochromatosis, an inherited condition leading to iron overload and accumulation can damage multiple organs including the pancreas if not treated. This condition may lead to DM and can be diagnosed by ferritin and gene sequencing. Cushing syndrome, which is a hormonal disorder due to a long-term

exposure to high amounts of cortisol may induce hyperglycemia and this type of diabetes can be diagnosed using dexamethasone suppression test. However, in type 2 diabetes there is no specific diagnostic test to identify it other than the exclusion of the other causes. Classifying patients with diabetes mellitus into subgroups based on disease progression and risk of complications are being investigated (Ahlqvist et al., 2018; Pearson, 2019).

1.2 Epidemiology of DM

Based on the International Diabetes Federation, 9.3% of the adult population aged 20-79 years globally are considered diabetic which is equivalent to 463 million in which the majority 79.4% account for individuals from low to middle income regions. The prevalence of the disease is estimated to further increase to 578.4 million by 2030 and by 2045, 700.2 million adults aged 20 - 79 years, will suffer from diabetes (Atlas, 2019). Among all individuals diagnosed with diabetes, only 10 to 15% have T1DM, and the majority of the diagnosed population is considered type 2 diabetic which is the most common form. The prevalence of type 1 diabetes is increasing among all age groups specifically in children which is doubling every 25 years (Patterson et al., 2019; Pociot & Lernmark, 2016). It is estimated that approximately 90,000 children are diagnosed yearly. Recently, it has been shown that DM causes an average loss of 11 to 12 years of life expectancy (Livingstone et al., 2015). This loss increases in patients who developed the disease earlier in life. For example, patients who are diagnosed before the age of 15 years live 2.5 years less than those diagnosed after the age of 30 years.

1.3 DM Classification and Diagnosis

1.3.1 Type 1 Diabetes Mellitus

T1DM, also known as insulin dependent diabetes, juvenile-onset diabetes and ketosis-prone diabetes is caused by autoimmune-mediated β-cell destruction and apoptosis, leading to the loss of β-cell ability to produce insulin and leading to a lifelong need for treatment with exogenous insulin. The disease is the consequence of invading or resident macrophages and T cells, which secretes chemokines and cytokines in the islet microenvironment and deliver cell–cell pro-apoptotic signals, or by stressed, injured or dying β- cells that attract and activate immune cells to the islets (Eizirik et al., 2009; Gonzalez-Duque et al., 2018). The etiology of β-cell-targeted autoimmunity depends on the patient's genetic background, age and multiple environmental factors, including viral infections and diet. This, in majority of cases occurs years before the eventual development of hyperglycemia and the disease symptoms (DiMeglio et al., 2018; Ilonen et al., 2019). Pathogenic association between immune cells and β- cells leads to inflammation and increased β- cell destruction by apoptosis (Eizirik et al., 2009; DiMeglio et al., 2018; Todd, 2010). Several months to years before T1DM onset the presence of autoantibodies is detected, which acts as biomarkers for the development of autoimmunity. Those autoantibodies associated with the disease onset, targets most of the islet antigens including insulin and its precursor preproinsulin (PPI), 65 kDa glutamic acid decarboxylase (GAD65; also known as GAD2), insulinoma-associated protein 2 (IA-2) and zinc transporter 8 (ZNT8) (Gonzalez-Duque et al., 2018; Thomaidou et al., 2018; DiMeglio et al., 2018). Individuals with specific HLA genotypes (which encode MHC proteins) - that is, HLA-DR and HLA-DQ genotypes (HLADR-DQ) — have an increased risk of

developing two or more autoantibodies and T1DM (DiMeglio et al., 2018; Quispe-Tintaya, 2017). Anti-insulin or anti-GAD65 β-cell autoantibodies are the earliest autoantibodies to appear during childhood, which target insulin and GAD65. However, rarely IA-2 autoantibody or ZNT8 autoantibody are detected first (Thomaidou et al., 2018; DiMeglio et al., 2018). What influences the development of the first β-cell targeting autoantibodies is still investigated in different follow-up studies of children from birth (Gonzalez-Duque et al., 2018; Ilonen et al., 2019; Todd, 2010). A staging classification system for the pathogenesis of T1DM has been suggested based on the detection of autoantibodies, and the progression to β-cell destruction. The first stage recognized is the presence of β- cell autoantibodies in normoglycemic people, the second stage shows dysglycemia in the presence of β- cell autoimmunity, and the third stage as clinical T1DM due to the symptoms associated with hyperglycemia. Up to date T1DM cannot be prevented or completely cured via currently available therapeutic approaches (DiMeglio et al., 2018; Greenbaum et al., 2018). However a delay on disease establishment has been detected by approximately 2 years when using monoclonal antibody against CD3 which is present on the surface of CD8+ T cells that dominate the pancreatic immune infiltrated cells leading to β-cell destruction in T1DM in stage 2 patients (Herold et al., 2019).

1.3.2 Type 2 Diabetes Mellitus (T2DM)

T2DM accounts for the majority, more than 80% of all diagnosed diabetic patients. The pathogenesis of type 2 diabetes mellitus (T2DM) is caused by two key disturbances one is relative insulin deficiency due to β-cell dysfunction and the other is insulin resistance (Weyer et al., 1999; Lyssenko et al., 2005; Cnop et al., 2007). Multiple key risk factors lead to the rise in the global epidemic of type 2 diabetes

mellitus including obesity, aging, poor activity, sedentary lifestyle and energy dense diets (Zheng et al., 2018). These have resulted in four-fold rise in the number of patients during the last 40 years (Risk & Collaboration, 2016). T2DM is linked with significant morbidity and mortality. On average 6 years loss in life span is observed, and in cases of disease onset at younger age the loss in life expectancy elevates up to 12 years (Sattar et al., 2019). However, the management of the chronic complications has improved over time. No clear diagnostic criteria exist for T2DM which makes it an ill-defined form of the disease. Clustering approaches using age at diagnosis, BMI, HbA1c, HOMA estimates of β- cell function and insulin resistance, and glutamic acid decarboxylase autoantibodies have subtyped patients into moderate or severe forms of T2DM, with a predominance of insulin resistance or insulinopenia (Ahlqvist et al., 2018).

1.3.3 Islet Morphology

Knowledge on islet morphology and the pathophysiology of diabetes mellitus are obtained from restricted studies of human pancreas collected at necropsy and on murine models, such as non-obese diabetic (NOD) mice, ob/ob and db/db mouse models of obesity and T2DM, and the GK rat model of T2DM. Data from these models have established diabetic model for the human disease to a limited extent. The emerging of the Network for Pancreatic Donors with Diabetes (Pugliese et al., 2014) in addition, the systematic organization of the Exeter Archival Diabetes Biobank provided access to ~500 pancreas in good condition from patient with T1DM and non-diabetic individuals as controls from both necropsy and organ donors, representing variant ages of disease onset and duration (Rodriguez-Calvo et al., 2018). Various methods, including the integrated analysis of histology, physiology, mass cytometry,

genomics and immunology of T1DM organ donors (Kaestner et al., 2019), provides better ways to collect information from these samples.

1.3.4 Characteristics of Human B- Cells in T1DM and T2DM

Key findings obtained from histological studies conducted on T1DM patients, revealed elevated heterogeneity of inflammation and β- cell destruction. Some cell clusters contain islets lacking β-cells, and other clusters have almost normal-looking islets and/or β- cell content, with fewer infiltrating immune cells (Rodriguez-Calvo et al., 2018; Morgan & Richardson, 2018). Moreover, hyperexpression of class I HLA (Morgan & Richardson, 2018) and β- cell stress markers such as ER stress (Marhfour et al., 2012) were detected particularly in insulin-expressing islets. In T1DM patients who are diagnosed within 0–6 years after onset, β- cells are found to express markers of cellular aging, as CDKN1A and β-galactosidase, as well as IL-6 and serpine 1, suggesting an inflammatory ageing-associated secretory phenotype (Thompson et al., 2019). In a study conducted on T1DM mouse, clearance of senescent β- cells decreases the incidence of diabetes mellitus (Thompson et al., 2019). In another study of human T1DM, loss of the β-cell markers insulin, proinsulin and amylin, precedes β-cell death (Damond et al., 2020). Islets extracted from NOD mice exposed to severe insulitis are non-functional during extraction, but they become functional once freed from the infiltrating immune cells in 7 days in culture (Strandell et al., 1990). Moreover, islets extracted from patients with T1DM after 5–6 days in culture has regained their function (Krogvold et al., 2015; Marchetti et al., 2000). Different studies showed impairment of α cell gene expression and function in T1DM patients islets and cells (Brissova et al., 2018; Mastracci et al., 2018) in comparison with islets from non-diabetic individuals. Alpha cells are not targeted by the autoimmune destruction in

T1DM and shows resistance to stress stimulated by metabolic disorders (Brissova et al., 2018; Marroqui, Masini, et al., 2015) or virus infection (Marroqui, Lopes, et al., 2015) compared to β- cells. However, the causes of α-cell impairment and dysfunction require further investigations, but the change in islet morphology and loss of β- cell–α- cell contacts is believed to have an influence. Insulin secretion at low levels, is found in 80% T1DM patients with average of 21 years duration of the disease, as evaluated by urinary C-peptide. Moreover, T1DM patients with no C-peptide detection were still producing proinsulin (Sims et al., 2019). Recent studies suggest that other islet cells in specific α-cells begins to express low levels of insulin after β-cell destruction (Lam et al., 2019). This is supported by findings in murine study showing trans-differentiation of α-cells to β-cells after β- cell extraction (Thorel et al., 2010) or by inhibiting the α-cell transcription factor ARX (Courtney et al., 2013). The immune system plays key role in β- cell function and survival (Eizirik et al., 2009). The presence of cytokines at different phases of insulitis in the islet environment is significantly upregulated. For instance during the early stages of inflammation, type I interferons level is high, and at later stages, the blood concentration of IFNγ, IL-1β, tumor necrosis factor (TNF), and IL-17 rises (Eizirik et al., 2009). This triggers ER stress, changes in alternative splicing (Ortis et al., 2010; Eizirik et al., 2012) and elevation of HLA class I (Morgan & Richardson, 2018; Marroqui et al., 2017). All of these, in addition to elevated β-cell synthesis of chemokines and cell death, are the cause of increased β-cell neoantigens in response to infiltrating immune cells, leading to increase in the immune assault (Eizirik et al., 2009; Gonzalez-Duque et al., 2018).

In T2DM, pancreatic islets experience a loss of β-cell mass ranging from 25–60%, elevated β- cell apoptosis, decreased pancreatic insulin and higher amyloid deposition by islet amyloid polypeptide which in turn leads to ER stress (Raleigh et

al., 2017) compared with non-diabetic individual (Rahier et al., 2008; Del Guerra et al., 2005). Alpha cell mass overall is unaltered in T2DM (Henquin & Rahier, 2011) and β-cell mass loss is minimal in new onset cases and increases with longer duration of the disease (Rahier et al., 2008). However, β- cell function is ultimately impaired at T2DM onset, it is predicted to decline by ~80% (Cnop et al., 2007; Cnop et al., 2007; Utzschneider et al., 2009). These findings indicate that β-cell dysfunction is a primary player in T2DM pathogenesis and happens independently of β-cell loss. The causes for the impairment of β-cell function and β-cell survival in T1DM and T2DM differs. For instance, cytokines can stimulate β-cell dysfunction and death in T1DM, while free fatty acids (FFAs) could induce ER stress and cause β-cell dysfunction and destruction in T2DM. However, these effects could be exerted through similar downstream signal transduction pathways. Large body of evidence indicates that increased β-cell ageing arises in T2DM. β- cells are long lived cells with restricted neogenesis and replication. As demonstrated by (Tamura et al., 2016) adult α and β cells telomere shortening is highly detected before the age of 20 years and decreases thereafter, indicating replicative senescence of postmitotic adult β- cells. Increased telomere exhaustion was detected in β-cells in T2DM (Tamura et al., 2014). β- cells cellular aging is characterized by acid β- galactosidase and p16INK4A expression which in turn induces the secretion of pro-inflammatory cytokines and loss of β- cell markers. In type 2 diabetic patient islets, elevated β- galactosidase expression in cells were noticed and β-cell expressing β-galactosidase showed upregulation in p16INK4A, CCL4 and IL-6 expression (Cristina et al., 2019). However, cellular aging and senescence contribution to β-cell dysfunction in T2DM is still unclear.

1.4 Management of Diabetes Mellitus

1.4.1 Lifestyle Modification

The lifestyle of an individual plays an important role in the person's health, especially the type and quality of the food consumed. Ingestion of nutrients with low glycemic index in key to reducing diabesity (Brand-Miller et al., 2003). It has been reported that physical inactivity and/or a sedentary lifestyle are major risk factors for the development of obesity and insulin resistance. Physical activity coupled with dietary modification are beneficial to improving reducing obesity and increasing insulin sensitivity in target cells such as muscle and liver (Horton, 1988). A meta-analysis on the effects of low-carbohydrate diets on CVD risk factors showed a large and significant decrease in total body weight, BMI, abdominal circumference, blood pressure (systolic and diastolic), triglycerides, fasting glucose, HbA1C, and CRP. All of these were also associated with increased levels in the level of HDL, the good cholesterol (Santos et al., 2012). This beneficial effect of lifestyle modification and diet showed intense physical activity coupled with appropriate diet resulted in significant weight reduction and decreased levels of glycated hemoglobin (HbA1c).

1.4.2 Pharmacotherapy

A large number of both oral and injectable drug classes are available to control both types of DM. Hyperglycemia, the hallmark of the disease, is a result of different metabolic events. In type 2 diabetes, this include diminished insulin secretion and elevation in glucagon production by the pancreatic islet cells, increased hepatic output of glucose, impairment of GLP-1 and GIP secretion and/or action, elevation in insulin

resistance and decline in β-cell mass and function (Weyer et al., 1999; Dunning & Gerich, 2007; Toft-Nielsen et al., 2001).

1.4.2.1 Insulin

In T1DM, after meal ingestion, insulin secretion is not stimulated, however, glucagon secretion is maintained. Without proper exogenous insulin replacement, the endogenous glucose production will not be controlled, glycogen synthesis will be decreased with elevation in systemic glucose levels. Administration of exogenous insulin mimics the native insulin hormone and normalizes all these abnormalities. Glargine and detemir are two widely used long acting human insulin analogues. Detemir absorption is slow from the subcutaneous tissue and circulation due to the fatty acid chain, which binds to circulating albumin. Glargine on the other hand is injected as an acidic solution, in neutral PH crystals are formed and they are absorbed slowly. Both are used once either morning or evening to achieve normal glucose levels, with a decreased hypoglycemic risk. Mealtime insulin, which are short-acting insulin preparations include glulisine, aspart and lispro. These insulin preparations are administered at meals time. They have shown reduced hypo- and hyperglycemia states, however, several clinical trials showed a modest reduction on HbA1c by these agents (Kaufman, 2003).

1.4.2.2 Metformin

Metformin is an insulin sensitizer commonly used for the treatment of T2DM in adult and children. It is one of the most studied and prescribed drug in the global pharmaceutical market. Metformin can reduce weight and HbA1c when combined with lifestyle interventions (Meneghini et al., 2011; Domecq et al., 2015). Metformin is able to reduce weight because it blocks hepatic gluconeogenesis, reduces appetite,

stimulates and increases insulin sensitivity in all peripheral tissues. It is not completely clear how metformin exerts its effect, however, it has been reported that it inhibits mitochondrial respiratory chain and cAMP synthesis with a concomitant reduction in protein kinase A activity. The activity of mitochondrial glycerophosphate dehydrogenasea and adenosine monophosphate (AMP)-activated protein kinase (AMPK) are also modified accordingly (Zhou et al., 2001; Madiraju et al., 2014; Rena et al., 2013). A clinical trial conducted by the Diabetes Prevention Program showed that metformin reduced the incidence of type 2 diabetes by 31% in high risk obese patients who had grossly impaired glucose tolerance (Aroda et al., 2017). Metformin is also a useful drug in the treatment of a large variety of health problems associated with diabesity. Metformin, combined with lifestyle modification is used in the treatment polycystic ovary syndrome (PCOS) (Naderpoor et al., 2015), gestational diabetes mellitus (Jiang et al., 2015), non-alcoholic fatty liver disease (NAFLD) (Chalasani et al., 2012), and even cancer (Wu et al., 2015).

1.4.2.3 α-Glucosidase Inhibitors

Acarbose, voglibose and miglitol are the three main drugs used to inhibit α-glucosidase found in the epithelium of the small intestine. This enzyme cleaves monosaccharides from polysaccharides to promote their absorption by the gut epithelium. α-glucosidase inhibitors thus prevent the absorption of carbohydrate from the small intestine. In a new meta-analysis study, α-glucosidase inhibitors significantly reduced HbA1c, 2-h postprandial glucose and bodyweight when compared to placebo. Unfortunately, the high prevalence of flatulence and diarrhea has prevented the wide use of this class of drugs (Gao et al., 2018).

1.4.2.4 Dipeptidyl Peptidase-4 (DPP-4) Inhibitors

Dipeptidyl Peptidase-4 (DPP-4) inhibitors increase the level of incretin by inhibiting DPP-4, the enzyme that degrades GLP-1 and glucose-dependent insulinotropic polypeptide (GIP). (Herman et al., 2006). GLP-1 and GIP enhance glucose-dependent pancreatic insulin response, suppress glucagon release, and increase satiety (Ahrén, 2013; Herman et al., 2006). Several clinical and pre-clinical studies have shown that the outcome of the signs and symptoms of DM is better after the use of DPP-4 inhibitors. DPP-4 inhibitors promote glycemic control, insulin secretion, and β-cell function in animal models (Pospisilik et al., 2002) and in subjects with T2DM (Herman et al., 2006). In patients with type 2 diabetes, DPP-4 inhibitors reduced postprandial glucose, fasting plasma glucose and HbA1c levels with low risk of hypoglycemia and weight gain. It also promote pancreatic beta cell function with no association with insulin resistance (Lyu et al., 2017). Currently available DPP-4 inhibitors include but not limited to sitagliptin, vildagliptin, saxagliptin, linagliptin, alogliptin, gemigliptin, trelagliptin, omarigliptin, evogliptin, gosogliptin, teneligliptin and anagliptin. With the use of this drug, no weight gain has been detected and an average HbA1c decrease of 0.5–1.0% has been reported (Pappachan, 2015).

1.4.2.5 GLP-1 Receptor Agonists

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are two gut secreted peptide hormones from L-cells and K-cells, respectively. They are released following a meal ingestion and play a key role in promoting insulin secretion and synthesis based on glucose levels. In addition, they suppress glucagon production (Baggio & Drucker, 2007) and both hormones have an additive affect. The GLP-1 agonists are structurally different from the endogenous GLP-1, and this change in the structure facilitate longer half-lives and resistance to the

DPP-4 activity, which increases the activity of these agonists. GLP-1 agonists have the same effect as the endogenous hormone, they lower blood glucose in a dose-dependent manner. In addition, they also affect fat accumulation and in part are associated with weight loss (Baggio & Drucker, 2007; Flint et al., 1998; Radford, 1990). Moreover, several clinical studies indicates that using GLP-1 receptor agonists lowers systolic blood pressure and exerts cardiovascular protection affect (Fonseca et al., 2014; Russell-Jones et al., 2009; Marre et al., 2009). Exenatide and liraglutide are the two drug forms of GLP-1 receptor agonists available in the market. Exenatide is the first GLP-1 receptor agonists to be approved in USA as therapeutic agent for the treatment of T2DM patients. It is a 39-amino acid synthetic peptide that is 53% homologous to human endogenous GLP-1 (Diego et al., 2006; Nielsen et al., 2004). While liraglutide is 97% homologous to the endogenous human GLP-1, with one amino acid substitution which increases its plasma half-life up to 13 hours (Colagiuri, 2010). Exenatide is injected two times daily one-hour prior to meals and liraglutide is administrated once-daily (Diego et al., 2006).

1.5 Peptides as Therapeutic Agents

Peptides are naturally occurring compounds which are made of short chain of amino acids that exert their function by their interaction with their specific receptors followed by different intracellular events (Vlieghe et al., 2010). The use of peptides as therapeutic agents has been promising for the treatment of several diseases including inflammatory diseases, cancer, cardiovascular diseases, and diabetes mellitus. Oxytocin is the first peptide to be synthesized in 1953 however, in 1982, recombinant human insulin was the first peptide to be approved for clinical use (Puttagunta & Toth, 1998). Currently about 60 peptide drugs are approved and available in the market. The

number of peptides investigated as therapeutic agents preclinically and entering clinical trials is increasing (Dinca et al., 2016). The challenges including the presentation of poor metabolic stability, membrane permeability and oral bioavailability associated with peptides was restricting their investigation and production by pharmaceutical companies in the past. However, recent modification in drug delivery systems, new formulations, and other fields involved have made it possible to overcome those disadvantages and make use of their advantages such as high potency and selectivity of their targets as well as the low toxicity, low accumulation in tissue and fast clearance, as seen in multiple natural peptide drugs such as insulin (Anderson & Delgado, 2008; Barabási et al., 2011).

1.6 Lipocalin-2

Lipocalin-2 (LCN2), a novel adipokine consisting of 198 amino acids, is also known as neutrophil gelatinase-associated lipocalin. Other names associated with LCN2 include, siderocalin, uterocalin, α1-microglobulin related protein, and 24p3. LCN2 belongs to large group of proteins that carry small and lipid soluble biological agents in blood circulation. These hrophobic molecule include steroids, fatty acids, retinoids, prostaglandins and hormones. (Flower, 1994; Chu et al., 1998). Two cell surface receptors has been proposed for this protein one is megalin/glycoprotein GP330 which binds human LCN2 (Hvidberg et al., 2005) and SLC22A17 or 24p3R which binds mouse Lcn2 protein (Devireddy et al., 2005). LCN2 is encoded by a gene located at chromosome locus 9q34.11. LCN2 gene produce at least five functional transcripts that translate into proteins, the most common of which encodes for a 198 amino acid secreted protein. LCN2 was initially isolated from neutrophil granules released at site of infection and inflammation in human (Flower, 1994; Kjeldsen et al.,

1993), and from mouse kidney cells. LCN2 is a bacteriostatic agent capable of iron depletion during antibacterial innate immune response. It does this by degrading the ferric siderophores enterobactin (Ent) of bacteria (Nairz et al., 2009; Holmes et al., 2005). It has also been shown that LCN2 has a protective role on the body during the process of infection, inflammation (Cowland & Borregaard, 1997), injury and tissue stress. In addition, it is able to interact with and stabilize matrix metalloproteinase-9 (MMP-9) in human neutrophils. The Lcn2 and MMP-9 complex, prevents MMP-9 auto-degradation and upregulate the activity of MMP-9 *in vitro*. MMP-9 is known for its ability to degrade the extracellular matrix and basement membranes. This LCN2/MMP-9 complex is suggested to aid in tumor progression, invasion and metastasis (Kobara et al., 2013; Triebel et al., 1992). In addition to its bacteriostatic properties, previous studies in animal models suggest important roles for Lcn2 in many physiological and pathological processes such as cell differentiation, apoptosis, organogenesis, inflammation, kidney damage and liver injury, Moreover, it is also suggested that Lcn2 has a role in cancer progression and metastasis (Candido et al., 2016). Recent studies have shown that the blood level LCN2 is increased after the onset of obesity and type 2 diabetes (Yan et al., 2007). Some reports have associated Lcn2 to insulin sensitivity and glucose metabolism, however, its role in the modulation of insulin sensitivity, glucose and lipid metabolisms has not been completely elucidated. Human lipocalin-2 protein is represented in upper case (LCN2), while the mouse and rat homologues are usually represented in lower case (Ngal or Lcn2) to differentiate between them (Kjeldsen et al., 2000).

1.6.1 Structure

Human LCN2 and it is homologues in mouse and rat, contain a 20-amino acid signal peptide at the N-terminal end of the protein, which is cleaved prior secretion, followed by the domain where lipocalins bind to their ligands, known as lipocalin domain. The highly conserved LCN2 structure is comprised of an eight stranded beta-barrel that forms a closing calyx in an antiparallel direction which represents the internal ligand-binding site allowing lipocalins to bind to their ligands. This binding cavity of LCN2 is significantly larger and polar than other lipocalin proteins and enables it to bind to cell surface receptors and form macromolecular complexes by binding to larger and less hydrophobic ligands as mammalian proteins, which in turn provides key functions in cell regulation, proliferation and differentiation (Goetz et al., 2000). LCN2 occurs in various molecular forms as a monomer (25-kDa), a disulfide-linked homodimer (46-kDa) and a disulfide-linked heterodimer with human neutrophil gelatinase B (135-kDa) (Kjeldsen et al., 1993). The amino acid sequence between NGAL homologues among different species shows that human NGAL have less identity similarity to the mouse and rat homologues (62% and 63% similarity respectively). This is very important to note because most of the studies on the function of LCN2 are done on murine. However, the mouse and human homologue are highly similar in the architecture domain (Figure 1) and three dimensional structure.

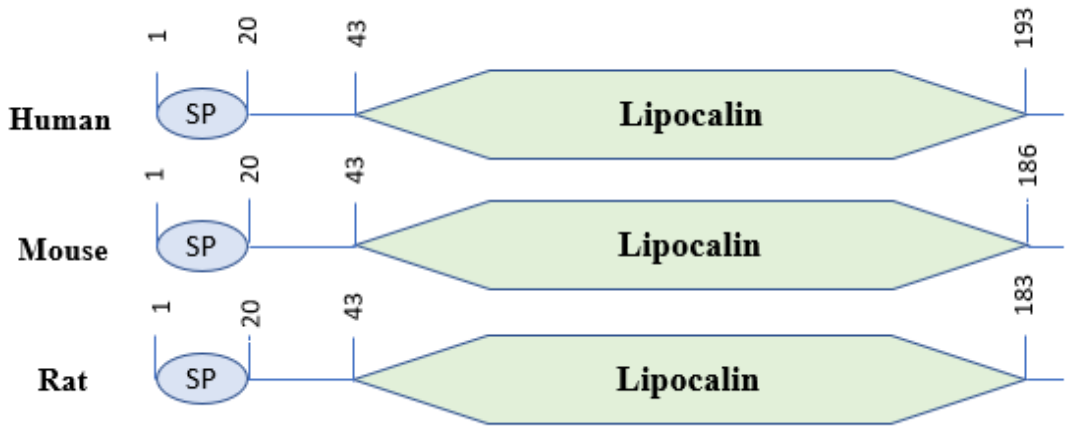

Figure 1: Human, mouse and rat LCN2 domain architecture showing high degree of similarity. Consisting of 20-amino acid signal peptide at the N-terminal end of the protein, which is cleaved prior to secretion. This is followed by the domain where lipocalins bind to their ligands, known as lipocalin domain or lipocalin fold, which makes up most of the length of the protein.

1.6.2 Tissue Distribution of Lipocalin 2

LCN2 was first identified and located in the azurophilic neutrophil granules (Kjeldsen et al., 1993). Following this, several researchs have been conducted to investigate the expression of LCN2 in various tissues. It has been identified that LCN2 is expressed in several normal tissues including liver, lung, kidney, bone marrow, adipose tissue, macrophages, thymus, non-neoplastic breast duct, prostate, small intestine and trachea where almost there is limited to no expression detected of LCN2 in the normal pancreas, endometrial glands, and peripheral blood leucocytes. LCN2 is however, completely absent from the normal brain, heart, skeletal muscle, spleen, testes, ovary and colon (Seth et al., 2002; Moreno-Navarrete et al., 2010; Cowland et al., 2003; Cowland & Borregaard, 1997; Furutani et al., 1998). In comparison to adult LCN2 is less investigated in infants. An investigation of LCN2 expression in several human fetal tissue has reported an expression of LCN2 in multiple tissues at different

weeks of gestation such as in the epidermis of the fetal skin approximately in the 20-24 week of gestational age. Afterwards, the expression concentrates toward lower layers of the skin and becomes progressively receded toward the hair follicles (Mallbris et al., 2002). In murine tissues the expression of LCN2 has been investigated intensively. In the embryo, LCN2 is expressed in the hypertrophic and perihypertrophic regions of the developing cartilage (Owen et al., 2008). It has been reported to be expressed in various tissues of the adult murine such as luminal epithelium and glands of the mouse uterus (Huang et al., 1999) bone marrow, liver, spleen, testis, lung and no expression was detected in the normal liver, heart, kidney, small bowel, thymus, brain or in the fetus liver (Aigner et al., 2007). The expression of LCN2 is strongly upregulated in the tissues as well as the body fluids in various pathological conditions, including inflammatory and metabolic disorders especially in several organs such as liver, heart, lungs, bone marrow, kidney, and spleen (Borkham-Kamphorst et al., 2011; Aigner et al., 2007).

1.6.2.1 Adipose Tissue

Several studies have reported LCN2 expression in adipose tissue (Lin et al., 2001; Kratchmarova et al., 2002; Wang et al., 2007). Reports have shown that the expression of LCN2 is elevated during adipoctyogenesis in 3T3-L1 cell line. A study by Yan et al., showed that white adipose tissue (WAT) was the major source of LCN2 expression and reported its absence in brown adipose tissue (BAT) in wild-type male mice. They suggested that obesity regulates its expression (Yan et al., 2007).

1.6.2.2 Liver

The liver is considered the main LCN2 source during infection or post-partial hepatectomy (PHx). The induction of more than 30-40 folds of LCN2 mRNA

expression was monitored in the liver as well as a significant increase in serum LCN2 levels after 24 and 36 hours post-bacterial challenge by the administration of *K. pneumoniae* (1×10^3 CFU) and *E. coli* (1×10^7 CFU). Moreover, surgical stress on the induction of LCN2 expression was investigated using the PHx model revealing that serum LCN2 protein and hepatic LCN2 mRNA levels were significantly increased post-PHx (Wazen et al., 2015).

1.6.2.3 Brain

Astrocytes are star-shaped glial cell of the central nervous system. They are responsible of the metabolic and trophic support to neurons and the modulation of synaptic activity. In response to a brain injury, astrocytes undergo reactive astrocytosis. LCN2 is an autocrine mediator of reactive astrocytosis performing multiple roles in regulating cell death, morphology, and migration of astrocytes. Lcn2 expression and secretion increased after inflammatory stimulation in cultured astrocytes. As shown by Western blot analysis in C6 glia cells and primary astrocytes, the expression and secretion of Lcn2 was markedly increased after the treatment with different inflammatory stimuli (Lee et al., 2009)

LCN2 expression is upregulated in patients with traumatic brain injury (TBI) in both, the injured tissue of the brain as well as the plasma serum of the patients. Furthermore, LCN2 expression is positively correlated with the severity of traumatic brain injury, suggesting that LCN2 can be used as a biomarker for the severity of TBI. Since this disease can cause serious complication to the patients leading to disability and mortality (Zhao et al., 2016). In the animal model LCN2 is expressed as well in endothelial cells, basal ganglia post ICH, corpus callosum after cerebral ischemia (Marques et al., 2012).

1.6.2.4 Lung

LCN2 mRNA expression has been identified in human lung and trachea where it is localized to goblet cells and ciliated epithelial cells in the normal bronchial and alveolear type II pneumocytes derived cell line A549. Moreover, a significant increase in LCN2 mRNA expression was detected in bronchial epithelial cells and alveolar type II pneumocytes during lung inflammation (Cowland et al., 2003). This was supported by another study which showed an elevation in LCN2 mRNA expression and serum levels monitored in the lung after 24 and 36 hours post-bacterial challenge by the administration of *K. pneumoniae* (1×10^3 CFU) and *E. coli* (1×10^7 CFU) (Wazen et al., 2015).

1.6.2.5 Kidney

LCN2 expression is upregulated in both acute and chronic kidney damage. LCN2 has been demonstrated as a potential early biomarker for kidney injuries in several studies (Bennett et al., 2008; Kümpers et al., 2010; Mishra et al., 2003). Human and mouse transplantation studies reveal that LCN2 expression is derived from the damaged nephrons (Ashraf et al., 2016). Another potential source of systemic LCN2 is neutrophils and macrophages (Hvidberg et al., 2005). Chronic kidney disease (CKD) in addition is also associated with marked upregulation in LCN2 levels in tissue and body fluids (blood and urine) (Stenvinkel, 2010; Viau et al., 2010).

1.6.2.6 Bone Marrow

Strong Expression of LCN2 mRNA is detected in human bone marrow cells (myelocytes and metamyelocytes) (Cowland & Borregaard, 1999). In cells of intermediate maturity, Costa et al., found that LCN2 was expressed during osteoblast differentiation (Costa et al., 2013).

1.6.2.7 Skin

The activated keratinocytes in the epidermis and infiltrated neutrophils in the dermis of psoriatic human patients and murine showed higher mRNA and protein expression levels of LCN2 compared to those in normal controls (Shao et al., 2016). Interestingly, treating psoriatic patients with calcipotriol for 14 days resulted in no change in LCN2 expression in these lesions. While once the lesions healed, the expression of LCN2 dropped by itself, suggesting that LCN2 expression is regulated by the disease process (Mallbris et al., 2002).

1.6.2.8 Neutrophils

LCN2 is constitutively expressed in human neutrophils and was initially isolated as a 25-kDa neutrophil protein. During inflammation LCN2 is secreted by prepackaged neutrophil granules (Kjeldsen et al., 1993)

1.6.2.9 Heart

Previous studies has proved that Lcn2 is upregulated in ischemia-reperfusion processes, coronary heart disease and myocardial infarction (Yndestad et al., 2009; Choi et al., 2008; Hemdahl et al., 2006; Aigner et al., 2007) suggesting that it is a promising and useful biomarker of the severity and mortality of heart diseases. Another study demonstrated that Lnc2 expression has been detected in the mouse cardiomyocytes. Moreover, hypoxia induced the mRNA expression and protein levels of LCN2 in HL-1 cells (Xiong et al., 2016). Furthermore, a study by Aigner and colleagues revealed that LCN2 mRNA expression and protein levels were significantly upregulated in heterotopic heart transplanted murine (Aigner et al., 2007).

1.6.2.10 Pancreas

LCN2 protein levels were markedly higher in pancreatic juice in patient with chronic pancreatitis and pancreatic cancer (Kaur et al., 2013). Moreover, ELISA analyses of LCN2 showed high levels in mice with mild acute pancreatitis (MAP) and severe acute pancreatitis (SAP) compared to control (Chakraborty et al., 2010). In another study they have tested different markers in the serum of KPC mice model to determine their expression. mouse CXCL16, mouse TIMP1, and mouse LCN2 ELISA analysis revealed a significant elevation in serum levels in mice with PanIN2/3 lesions or invasive PC compared to controls. However, LCN2 was the most promising serum biomarker for the pancreatic cancer in mice (Slater et al., 2013).

1.6.2.11 Spleen

LCN2 has a low expression in the normal spleen but after experimental autoimmune encephalomyelitis (EAE) induction in the mouse, LCN2 was significantly increased in the spleen, specifically in the neutrophils and dendritic cells (Nam et al., 2014).

1.6.2.12 Synovial fluid

LCN2 is highly expressed in the synovial fluid of patients with rheumatoid diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) (Gupta et al., 2007; Katano et al., 2009) compared to healthy control. However, synovial LCN2 levels were approximately 15 times higher than in plasma. Moreover, neutrophil LCN2 is positively correlated with granulocyte cells detected in the synovial fluid (Bläser et al., 1995) and LCN2 detected in OA synovial fluid up regulates cartilage breakdown by protecting MMP-9 against auto-degradation (Gupta et al., 2007), which supported and

suggested LCN2 as a biomarker for arthritic disease and inflammatory disorders (Wilson et al., 2008).

The below Table 1 summarizes the tissue distribution of LCN2 under various pathological conditions.

Table 1: Tissue distribution of altered lipocalin-2 expression in human, rat and mouse in organ disorders.

Tissue/cell	Type of lipocalin-2	Species	Method of localization	Authors
Kidney	LCN2 protein LCN2 mRNA LCN2 protein LCN2 mRNA LCN2 protein LCN2 mRNA	Mouse/human Mouse Human 	qRT-PCR, WB, IHC ELISA, qRT-PCR ELISA 	(El Karoui et al., 2016) (Ashraf et al., 2016) (Belcher et al., 2014)
Heart	LCN2 protein LCN2 mRNA LCN2 protein LCN2 mRNA	Mouse Mouse	qRTPCR, WB RT-PCR, WB	(Xiong et al., 2016) (Aigner et al., 2007)
Brain	LCN2 protein LCN2 mRNA LCN2 protien	Human Mouse/human	qRT-PCR, ELISA, WB IF, IHC, ELISA, qRT-PCR	(Zhao et al., 2016) (Marques et al., 2012)
Pancreas	LCN2 protein LCN2 protein LCN2 protien	Mouse Human Mouse	ELISA ELISA ELISA	(Slater et al., 2013) (Kaur et al., 2013) (Chakraborty et al., 2010)
Lung	LCN2 mRNA LCN2 mRNA	Human Human	IHC,ELISA,NB qRT-PCR	(Cowland et al., 2003) (Kangelaris et al., 2015)
Synovial fluid	LCN2 protein LCN2 protein	Human Human	WB, Zymographic analysis ELISA	(Gupta et al., 2007) (Bläser et al., 1995)
Skin	LCN2 protein LCN2 mRNA LCN2 protein LCN2 mRNA	Human Human/mouse 	IHC, RT-PCR RT-PCR,ELISA,WB, IHC, Cell culture, Chemotaxis assay	(Mallbris et al., 2002) (Shao et al., 2016)

1.6.3 Inductors of Lipocalin-2 Expression

1.6.3.1 Proinflammatory Cytokines

1.6.3.1.1 Tumor Necrosis Factor Alpha (TNFα)

Several studies have shown that TNFα induces the expression and secretion of Lcn2 (Yan et al., 2007; Lin et al., 2001). The continuous treatment of 3T3-L1 adipocytes by TNFα for 24, 48, or 72 h have shown marked increase in LCN2 expression and secretion within 24 h, when compared to control. However, the effect of TNFα on LCN2 levels increased over time. The ability of TNFα to induce Lcn2 mRNA expression is dependent on NF-κB pathway. siRNA-mediated knockdown of P65 subunit of NF-κB has significantly attenuated the induction of TNFα on LCN2 expression and secretion (Zhao et al., 2014). Lee et al. also confirmed that treatment of primary astrocytes with by TNFα (100 ng/ml) significantly increased the expression of LCN2 (Lee et al., 2009).

1.6.3.1.2 Interferon γ (IFNγ)

Several studies have shown that IFNγ induce LCN2 expression and secretion in cultured murine and human adipocytes. Intraperitoneal injection of IFNγ have been shown to stimulate Lcn2 expression and secretion in mice epididymal WAT. Post 24 h treatment by IFNγ, the cellular levels of LCN2 were induced and remained high. The ability of IFNγ to induce Lcn2 mRNA expression is dependent on STAT1 pathway. siRNA-mediated knockdown of STAT1 has resulted in marked attenuation in the induction effect of IFNγ on LCN2 expression and secretion (Zhao et al., 2014).

1.6.3.1.3 Lipopolysaccharides (LPS)

Lipopolysaccharide is a strong inducer for lipocalin-2 expression in different tissues. The administration of peripheral LPS in mice for 24 h resulted in high detection of lipocalin-2 inducement by LPS with over a 140-fold induction. However, LCN2 was not detected in *Lcn2*$^{-/-}$ mice, treated with Phosphate buffered saline (Kang et al., 2018). Another study investigating reactive astrocytosis has also confirmed that treatment with by LPS (100 ng/ml) markedly increased the expression of LCN2 (Lee et al., 2009).

1.6.3.1.4 17-β-Estradiol (E2)

Treatment of subcutaneous adipose tissue from postmenopausal women with 17-β-estradiol (E2) resulted in significant increase in LCN2 gene expression by 4-fold and LCN2 protein expression by 2.7-fold. E2 effects on LCN2 is suggested to be regulated by ERβ pathway but not through ERα pathway. It has been identified that dexamethasone+E2 can mediate LCN2 expression by the inhibition of ERα and stimulation of ERβ suggesting it may result and play a role in the development of glucocorticoid-induced insulin resistance in human adipose tissue (Kamble et al., 2019).

1.6.3.1.5 Hyperglycemia

Lipocalin-2 expression is increased in diabetic/obese mice in various tissues as adipose and liver tissue. The use of microarray analysis for identification of genes expressed in *db/db* obese/diabetic mice and controls resulted in the expression of lipocalin-2 gene. This was significantly induced in the liver tissue of obese/diabetic mice, as well as human subjects with diabetes, where they showed higher mean serum

concentrations of lipocalin-2 than those who were not affected by the disease (Wang et al., 2007).

1.6.3.1.6 Gender

The measurement of lipocalin-2 concentrations in serum samples collected from 229 adult participants by Wang et al. has revealed that lipocalin-2 concentrations in obese and lean groups were markedly higher in males compared to females. They showed that circulating lipocalin-2 concentrations in smokers were much higher than those in nonsmokers (Wang et al., 2007). Another study has also demonstrated that in adipose tissue, men had higher LCN2 mRNA expression than women (Kamble et al., 2019).

1.6.3.1.7 Obesity

There is a positive correlation between serum lipocalin-2 concentrations and BMI (Wang et al., 2007). Western blot analysis, immunoprecipitation and in-house immunoassay measuring human lipocalin-2 showed that the affinity of lipocalin-2 antibody selectively were attracted to an ~25-kDa protein, which was confirmed by tandem mass spectrometry analysis to be human lipocalin-2, which demonstrated that lipocalin-2 concentration in obese subjects was markedly higher than those in lean ones. Moreover, the plasma concentration of lipocalin-2 were higher in obese than lean subjects in both human and murine (Wang et al., 2007). Another study has also revealed a significant upregulation of LCN2 expression and secretion in adipose tissue of obese (ob/ob) mice compared to lean controls (Yan et al., 2007).

1.6.3.1.8 Advanced Glycation End-Products (AGEs)

AGE treatment increases the mRNA and protein expression of LCN2. AGE stimulates LCN2 expression via RAGE-NADPH oxidase reactive oxygen species pathway, by the activation of PI3K-Akt and JNK signal transduction in human aortic smooth muscle cells (HASMCs). The activation of C/EBPβ is responsible for the transcriptional activation of LCN2 expression induced by AGE (Chung et al., 2013).

1.6.3.1.9 Insulin

In human omental adipose tissue explants, continuous infusion of insulin showed a significant dose-dependent increase in LCN2 expression and secretion. Insulin can also stimulate the expression of LCN2 via PI3K and MAPK signaling pathways (Tan et al., 2009).

1.6.3.1.10 Dexamethasone

Dexamethasone is a synthetic analog of glucocorticoid. It is a strong inducer of LCN2 expression and secretion. Treatment of 3T3-L1 adipocytes with dexamethasone resulted in the induction of Lcn2 mRNA by ~80-fold. Furthermore, a mixture of methylisobutylxanthine and dexamethasone had a significant effect on *LCN2* expression. However, dexamethasone was the dominant contributor to *LCN2* induction (Yan et al., 2007).

1.6.3.1.11 BAY16

The treatment of male wistar rats with BAY16 a hepatotoxic agent has resulted in upregulation of LCN2 mRNA levels after 24 hours of treatment which continuously increased by ~16-fold and 37-fold after 3 days and 12 days following repeated administrations of BAY16. LCN2 serum levels correlated with the severity of liver

injury. Increased expression of LCN2 was detected in hepatocytes, biliary epithelial cells and proximal tubular epithelial cells of the kidney (Adler et al., 2010).

1.6.3.1.12 NF-κB Signaling Pathway

LCN2 contains the binding site of NF-κB in its promoter region. The nuclear factor-κB (NF-κB) is a transcription factor that mediate key pathways. Originally NF-κB signaling pathway was identified in immune cells and has emerged as a key mediator of LCN2 gene expression. NF-κB is comprised of five transcription factors p50, p52, p65 (RelA), c-Rel and RelB. the N-terminal region of these proteins establishes a common domain, Rel homology domain (RHD), which is important for the NF-κB dimerization and DNA binding activity. The C-terminal region of RelA (p65), c-Rel and RelB have transcription activation domain (TAD) therefore they activate gene expression, whereby p50 and p52 have a transcription repression domain (TRD) which inhibits gene expression. p50 and p52 require coactivators to be associated with in order to induce gene expression. NF-κB proteins bind to a specific sequence in the promoter region of target genes where they activate or inhibit gene expression depending on the presence of TAD or TRD. In case of LCN2 which is positively regulated by agents that influence the signaling pathway of NF-κB as TNFα, IL-1β and LPS, which are potent inducers of insulin resistance. In normal state IκB blocks the activity of NF-κB by binding to it. Due to the influence of NF-κB signaling, IκB kinase (IKK) becomes activated this kinase phosphorylate the inhibitor of NF-κB (IκB) resulting in the dissociation of IκB from NF-κB, and IκB is then ubiquitinated and degraded by proteasome. The free NF-κB translocate to the nucleus and regulates the expression of target genes (Hayden & Ghosh, 2008) as shown in Figure 2.

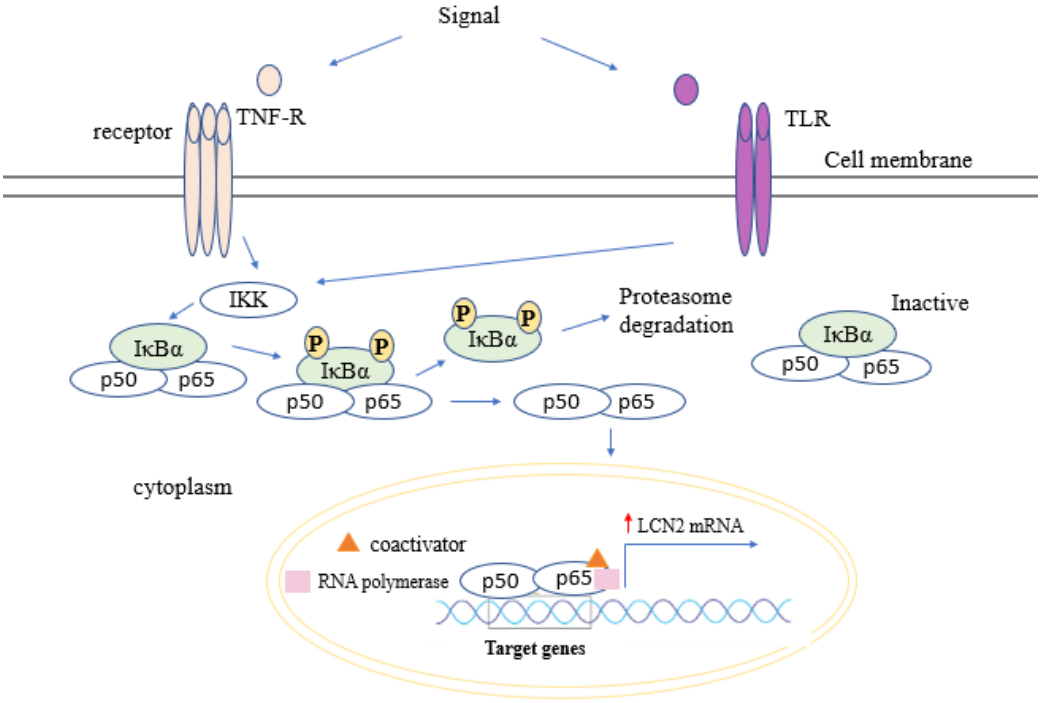

Figure 2: Regulation of LCN2 expression by NF-κB. NF-κB is present in the cytosol as heterodimer (Rel-A and p50) but it is kept inactive by the inhibitor of NF-κB (IκB). When the signaling molecule binds to the specific receptor that is present in the cell membrane, the receptor becomes activated and it phosphorylate IKK, which in turn phosphorylates IκB. The phosphorylated IκB gets ubiquitylated followed by subsequent degradation by proteasome, freeing the NF-κB complex, which translocates to the nucleus and binds with other coactivators and RNA polymerase. The complex in turn binds to the κB response element (κB RE) located at position −181/−171 on the LCN2 promoter. This leads to activation of transcription of LCN2 gene. Abbreviations: NF-κB, nuclear factor kappa B; IKK, IκB kinase; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha.

1.6.3.14 ERK Signaling Pathway

Extracellular signal-regulated kinase (ERK) is a member of the mitogen activated protein kinases (MAPKs) family. ERKs have a key role in regulating cell proliferation, cell growth and cell division. ERK signaling pathway begins with the binding of a ligand such as EGF to the extracellular portion of the membrane bound receptor of the family of receptor tyrosine kinase (RTK). EGF binding leads to the

dimerization of two subunit of the receptor tyrosine kinase, which catalyzes phosphorylation. The Grb2 binds to the phosphorylated RTK, then SOS binds to the Grb2. SOS activates Ras by substituting the GDP by GTP, while the activated Ras induces the activation of B-Raf. The activated Raf phosphorylates and activates MEK1/2, then, activated MEK1/2 phosphorylate and activate ERK1/2. ERKs are serine/threonine kinases. The kinase cascade leads to the activation of (jun) and (fos) transcription factors of the activator protein-1 (AP-1) family. Upon activation, these transcription factors translocate to the nucleus and bind to AP-1 motif of the DNA which leads to the expression of many genes encoding growth factors, cyclins and cytokines (Kim & Choi, 2010) as shown in Figure 3. A study by Zhao et al. have demonstrated that ERK signaling pathway regulates the capability of IFNγ and TNFα to induce LCN2 expression without effecting the nuclear translocation or DNA binding activity of STAT1 and NF-κB by IFNγ and TNFα. This suggests that ERK signaling pathway is vital for the maximum transcriptional activity of STAT1 and NF-κB (Zhao & Stephens, 2013b). Another study suggested that TNFα induces insulin resistance in 3T3-L1 adipocytes through suppressing the phosphorylation and expression of IR, IRS1/2 via the ERK signaling pathway (Fujishiro et al., 2003). A different study showed that the activation of the ERKs signaling pathway stimulates adipogenesis via regulating the gene expression of PPARγ and C/EBPα. These elements are critical for adipogenesis (Prusty et al., 2002). Treatment of murine 3T3-L1 adipocytes with IFNγ resulted in the degradation of PPARγ in adipocytes by IFNγ-induced ERKs activation which mediates phosphorylation of PPARγ at Serine112 (Floyd & Stephens, 2002).

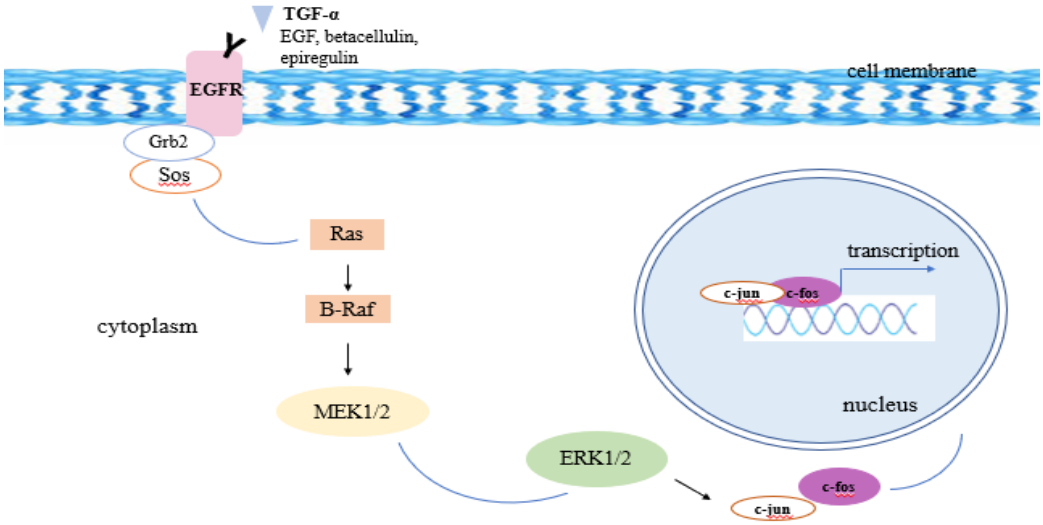

Figure 3: Regulation of LCN2 expression by ERK signaling pathway. Once EGF binds to the EGFR it gets activated and phosphorylated then it attaches to the Grb2 and Sos proteins in the cytosolic section. Grb2 and Sos activates Ras by substituting the GDP of Ras by GTP. Ras directly interacts with B-Raf and activates it. Raf then phosphorylates and activates MEK, which in turn phosphorylates and activates ERKs. ERKs lead to the activation of the transcription factors c-jun and c-fos.

1.6.3.1.15 JAK-STAT Signaling Pathway

The Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling pathway is used by hormone and cytokine receptors. It has been demonstrated that it up-regulates the expression of LCN2. There are four members of the JAK family: Jak1, Jak2, Jak3 and Tyk2, each of these contain a SH2 domain and FERM (four-point-one, ezrin, radixin, moesin) domain. and seven members of the STAT family: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. However, only STAT1, 3, 5A, 5B and 6 are expressed in mature adipocytes (Zhao & Stephens, 2013a). The pathway operates by activation of the receptor by the ligand (GM-CSF), the Tins as (EPO, Prolactin, Thrombopoetin and Leptin) and the Ters, which include interleukins and interferon. Activated JAK binds to the receptor and

phosphorylates the receptor on tyrosine residues. In the unstimulated cell, STAT is inactivated, while the activated JAK causes the recruitment of STAT and phosphorylates it. The phosphorylated STAT binds to another STAT and form homodimer or heterodimer with other STAT isoforms. STATs homodimer translocate to the nucleus and interact with promoters to modulate gene expression (Darnell, 1997) as illustrated in Figure 4. Previous studies have illustrated activators of JAK-STAT signaling pathways including cytokines IL-1 and IL-17 (Florin et al., 2004; Yang et al., 2008), Gp130 cytokines, including cardiotrophin-1 (Zvonic et al., 2004), IFNγ (Hogan & Stephens, 2003), interleukin-11 (Tenney et al., 2005) have been identified to utilize STAT1 and STAT3 signaling pathways in adipose cells. A cross talk between the JNK and NF-κB has been also suggested to act together to influence LCN2 expression.

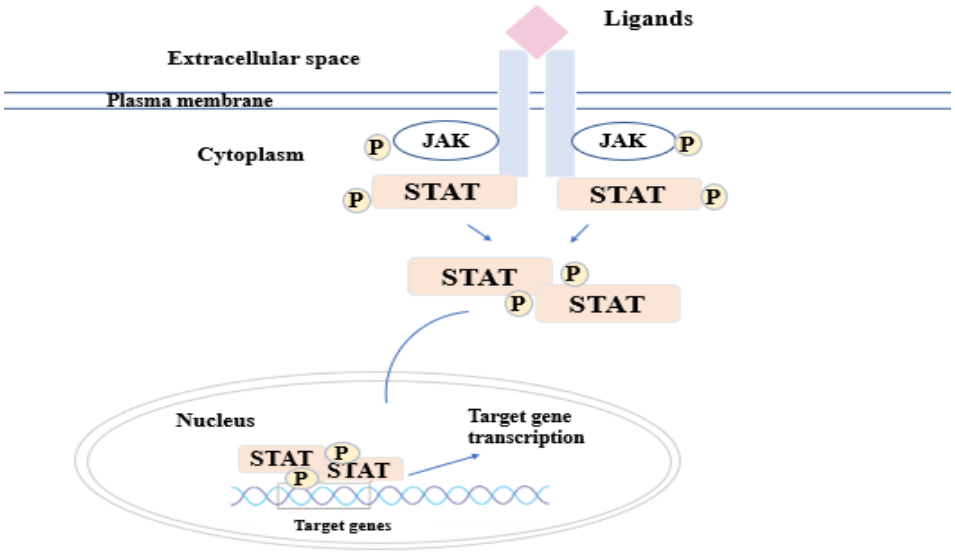

Figure 4: Regulation of LCN2 expression by JAK-STAT signaling pathway. The receptor gets activated by the binding of the specific ligand and activated JAK binds to the receptor and phosphorylates its own tyrosine component to activate its kinase function, which in turn phosphorylates STAT. Phosphorylated STAT binds to another STAT and forms a homodimer or heterodimer and then translocate to promote DNA transcription in the nucleus. Abbreviations: JAK, janus kinase; STAT, signal transducer and activator of transcription.

1.6.4 Inhibitor of Lipocalin-2 Expression

1.6.4.1 Rosiglitazone

Rosiglitazone is a drug with insulin-sensitizing and anti-inflammatory activities. The serum samples of T2DM patients treated with the peroxisome proliferator–activated receptor-γ agonist rosiglitazone, showed a significant decrease in circulating concentrations of lipocalin-2, and a marked decrease also has been detected in LCN2 expression in mice and circulating concentrations in both murine and humans. Moreover, rosiglitazone-mediated relative changes in serum lipocalin-2 concentrations correlated well with relative changes in insulin sensitivity and C-reactive protein (Yan et al., 2007; Wang et al., 2007).

1.6.5 Controversial Factors in LCN2 Expression

1.6.5.1 Epidermal Growth Factor (EGF)

Treatment of PDAC cell line AsPC-1 and BxPC-3 with EGF decreased the expression of LCN2 mRNA (by 48.4% in AsPC-1 and 44.7% in BxPC-3 cells) compared with untreated cells as detected by Q-PCR. Moreover, LCN2 protein was also markedly reduced by EGF treatment as detected by both Western blot analysis and immunofluorescence. This was accompanied with a marked decrease in the epithelial marker E-cadherin. EGF decreased LCN2 expression through the inhibition of the NF-κB pathway. However, in other cell types EGF showed different effect on LCN2 expression for example in renal tubular epithelial cells a treatment with EGF has resulted in upregulation of LCN2 expression via stabilization of HIF-1α (hypoxia inducible factor-1 α).

1.6.6 Lipocalin-2, Obesity and Insulin Resistance

LCN2 is a critical adipocytokine in the regulation of energy metabolism, glucose and lipid metabolism, body fat mass and insulin resistance. It has a regulatory importance in the activation of PPARγ, which mediates adipogenesis and lipogeneses in liver and adipose tissue (Zhang et al., 2008). However, the clear mechanism of LCN2 in the pathogenesis of obesity-related diseases is not clear yet. Both the mRNA expression and serum levels of LCN2 in adipose tissue and liver are markedly increased in obese and diabetic murine as well as in mice fed high fat diet (HFD) (Guo et al., 2010). Circulating LCN2 concentrations positively correlate with measures of adiposity, hyperglycemia, hypertriglyceridemia, and the insulin resistance index in humans (Wang et al., 2007). In obesity and related disorders, LCN2 expression and secretion is stimulated by proinflammatory cytokines as IL-6, IL-B, IFNγ and TNFα

in cultured human and mice adipose cells. Zhao et al. has demonstrated that IFNγ induces the expression of LCN2 via activating STAT1 and TNFα needs NF-kB signaling pathway in order to mediate LCN2 expression and secretion. This mechanism occurs in both human and murine (Zhao et al., 2014). LCN2 promoter contains the binding site of key transcription factors CCAAT/enhancer binding protein (C/EBP) and NF-kB (Shen et al., 2006) demonstrating that the transcriptional activation of LCN2 gene in the adipose tissue has a role in obesity, inflammation and metabolic disorders. In both preclinical and clinical research, a role for LCN2 has been demonstrated in obesity, insulin resistance and diabetes (Moreno-Navarrete et al., 2010; Wang et al., 2007; Yan et al., 2007). Recent studies suggest that LCN2 increases insulin resistance as law and colleagues showed that LCN2 knockout mice were protected from HFD-induced insulin resistance this effect was accompanied by attenuation in the expression of TNFα and 12-lipoxygenase in the tissues of the LCN2 deficient mice (Law et al., 2010). Another study showed that insulin sensitivity and glucose metabolism has been improved in LCN2 deficient cultured adipocytes(Yan et al., 2007). A preclinical study showed that a knockdown of LCN2 in mice resulted in increase in hepatic gluconeogenesis, decreased mitochondrial oxidative capacity, impaired lipid metabolism, and elevated inflammatory state under the high fiber diet condition in different organs. LCN2 deficient mice has showed an increase in insulin resistance, dyslipidemia, and fatty liver disease (Guo et al., 2010). Human study by (Huang et al., 2012) has showed that circulating levels of LCN2 was markedly higher in subjects with isolated impaired fasting glucose, isolated impaired glucose tolerance, combined impaired fasting glucose/impaired glucose tolerance and recently-diagnosed type 2 diabetes than normal subjects. This result suggests that LCN2 upregulation is in association with a higher risk for impaired glucose regulation and type 2 diabetes.

Insulin induction in human markedly increased serum LCN2 levels and its secretion in omental adipose tissue. This was modulated by phosphatidylinositol 3-kinase and mitogen-activated protein kinase (Tan et al., 2009). Another study conducted by (Liu et al., 2011) resulted in no correlation between cardiovascular risk factors and circulating levels of LCN2 and homeostasis model assessment of insulin resistance. Although some observations suggest a potentially metabolically protective role of LCN2, there are substantial inconsistent observations related to actions of LCN2 in relation to glucose tolerance and insulin sensitivity

1.6.7 LCN2 Gene and Diabetes

The cDNA sequence of human Lipocalin 2 is related to human chromosome 9 with a cytogenetic map 9q34.11. The promoter region of the Lcn2 gene contains the binding sites of several transcription factors, and nuclear receptor response elements, including nuclear factor-κB (NF-κB) (Shen et al., 2006; Zhao & Stephens, 2013b); CCAAT-enhancer binding protein (C/EBP) (Shen et al., 2006); glucocorticoid response element (Garay-rojas et al., 1996); retinoic acid response element (Shen et al., 2006; Guo et al., 2016); estrogen response element (Seth, al., 2002), and STAT1 (Zhao & Stephens, 2013b). All of this information indicates that the expression of LCN2 is important in diabetes and obesity, which represent the state of metabolic inflammation. Studies by different groups have indicated that diabetes which is accompanied by mild chronic subclinical inflammatory condition results in elevated levels of LCN2 in serum and different tissues (Bhusal et al., 2019; Lin et al., 2001). Induction of type 1 diabetes by streptozotocin in murine have shown increased levels of LCN2 in multiple tissues. In several studies, diabetic induced animals via streptozotocin injection resulted in elevation of LCN2 in body fluids as urine and

different body tissues as the kidney where it is usually used as a biomarker for acute and chronic kidney injury (Arellano-buendía et al., 2014; Brouwers et al., 2014; Korrapati et al., 2012; Liu et al., 2015). In a study conducted by (Soukas et al., 2000), upregulation of LCN2 was observed in the white adipose tissue of obese mice suffering from severe type 2 diabetes when compared with lean controls. Moreover, LCN2 expression is elevated in several murine obese and diabetic models as well as people (Yan et al., 2007; Eun et al., 2018; Tak et al., 2016; Kanaka-gantenbein et al., 2008). In addition, fatty liver disease human and murine models samples revealed elevation in LCN2 and its key association in lipid metabolism, inflammation and insulin resistance (Lambertz et al., 2017; Ye et al., 2016; Asimakopoulou et al., 2014; Semba et al., 2013).

Chapter 2: Aims, Hypothesis and Objectives

2.1 Hypothesis

The administration of lipocalin 2 would improve the metabolic complications of diabetes mellitus and enhance insulin secretion from the islet of Langerhans of diabetic rats.

2.2 Aim

To examine the effect of lipocalin 2 in the management of euglycemia, improving the secretion of insulin from β-cells of the pancreas and reducing oxidative stress in STZ-induced diabetic rat.

2.3 Objectives

1. Examine the effect of lipocalin 2 on total body weight and fasting blood glucose.
2. Determine how lipocalin 2 treatment would influence biochemical parameters, especially biomarkers of hepatic and renal functions.
3. Study the expression of lipocalin 2 in endocrine pancreas of normal and diabetic rats.
4. Determine whether lipocalin 2 co-localizes with endocrine hormones in the islet of Langerhans
5. To investigate how lipocalin 2 affects insulin release from normal and diabetic rat pancreas
6. Determine whether lipocalin 2 mitigate diabetes-induced oxidative stress in the pancreas and liver of diabetic rats

7. To examine the effect of lipocalin 2 treatment on molecules involved in insulin signaling.

Chapter 3: Materials and Methods

3.1 Animal Model

Three months of age male Wistar rats weighting approximately 250 g were utilized in this study. All animals were provided by the Animal Facility in the College of Medicine and Health Sciences, United Arab Emirates University. Animals were kept under specific pathogen free environment and maintained at 22-25°C with 12 hours light/dark cycle. They were housed in polycarbonate cages containing wood chips bedding in groups of three animals per cage. Cages were replaced every two days for the diabetic animals and twice weekly for normal animals. Animals were fed a standard rat chow diet and supplied with tap water *ad libitum*. The study was approved by Institutional Ethical Committee of the United Arab Emirates University, Al Ain, UAE (A5-14).

3.2 Establishing the Experimental Diabetes Model

The diabetic animal model was established by intraperitoneally injecting male wistar rats with a single dose of streptozotocin (STZ) using a dose of 60 mg/kg body weight. Streptozotocin were prepared freshly on the day of injection by dissolving it in citrate buffer (pH 4.5, 0.5 M) and was kept on ice at the time of injections. After 7 days, animals were fasted for 8 hours before checking FPG levels and only animals with readings > 250 mg/dl were considered diabetic. Animals with readings below 250 mg/dl were considered not diabetic and excluded from the study to maintain a standard of STZ-induced diabetes mellitus with a single dose.

3.3 Experimental Design

Animals were primarily divided into two groups, normal and diabetic. After that a further division were performed in each group into two sub-groups. One was injected with normal saline and the other with 8 µg/Kg of lipocalin-2 five-days/week for a period of 2 weeks.

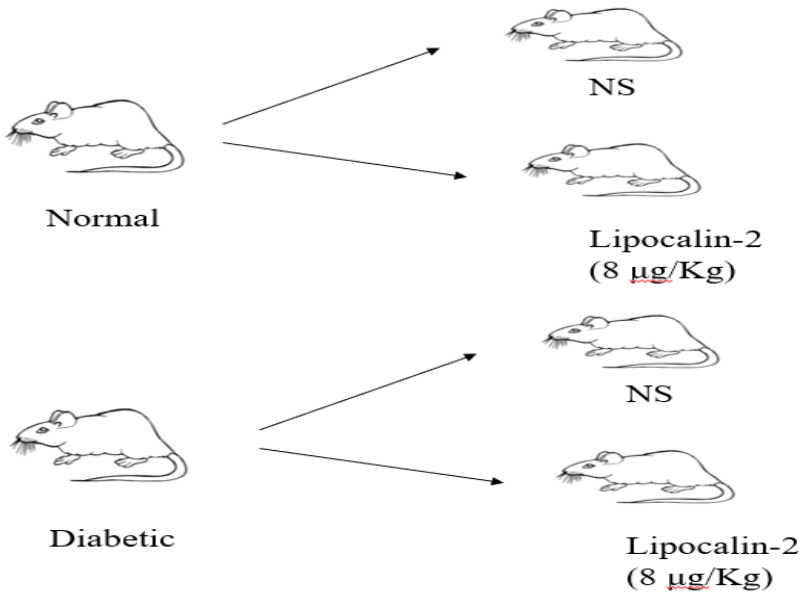

Figure 5: Schematic diagram of the animal groups in the study.

During the treatment period the animals body weight were checked once a week and their blood glucose levels were assessed from the tail vein using OneTouch® Ultra® glucometer (LifeScan, Inc., Milpitas, CA, USA) after fasting them for 8 hours. At the end of the treatment period of 2 weeks, intraperitoneal glucose tolerance test (IPGTT) was performed for all groups after 12 hours of fasting. Rats were injected with a glucose with the dose of 10 mg/kg intraperitoneally and blood samples were obtained from the tail vein at different time points 0, 30, 60, 90, and 120 minutes.

3.4 Blood/Tissue Collection and Tissue Processing

Prior to tissue collection IPGTT were performed, the animals then were anesthetized using a ketamine and xylazine cocktail (Albert Einstein Institute for Animal Studies, 2015). An incision was made through the abdominal wall. The blood was collected from the inferior vena cava using a 5-ml syringe. The pancreas was isolated and separated into three samples for immunohistochemistry (IHC), electron microscopy (EM), and molecular biology investigation. The liver was isolated for molecular biology investigation, and lastly, the gastrocnemius muscle was isolated for molecular biology investigation. The samples for the immunohistochemistry analysis were washed with PBS and embedded in Zamboni fixative, samples for electron microscopy (EM) analysis were washed and embedded in McDowell fixative and the samples for molecular biology analysis (WB) were snap frozen by liquid nitrogen then stored at -80^{o}C.

After one week of fixing the tissues, they were processed for further analysis. Samples that have been fixed in Zamboni for IHC, have been processed to make paraffin blocks by dehydrating the tissues through a series of ascending ethanol concentrations from 70% to 95% followed by two rounds in absolute ethanol. Then the tissues were dipped in xylene I and II for 2 hours each followed by three rounds in paraffin wax at 55°C. Then the tissues were embedded in paraffin blocks and transferred to the freezer to cool down and solidify. Tissues blocks were removed, trimmed, and sectioned into 3-5 μm thickness using a microtome. Sections were placed in a water bath with a temperature of 42°C, and later transferred onto coated slides. Slides were kept overnight to dry and for the sections to attach properly. Tissue samples kept in McDowell fixative for EM were cut into small pieces. Fat and

connective tissues were removed, then washed three times with 0.1 phosphate buffer each for 20 minutes. After that, tissues were incubated in 1% osmium tetroxide for 60 minutes followed with a dehydration step by immersing the tissues in a series of ascending concentrations of ethanol (30%, 50%, 70%, 95% and two changes of 100%) 20 minutes each. Tissues were then incubated at a ratio of 1:1 of LR White resin and 95% ethanol for one hour at room temperature on a rotator. Tissues were then infiltrated with pure LR White resin overnight at 4°C. On next day morning, samples were incubated with 100% fresh LR White for two hours at room temperature, specimens were embedded in pure resin in gelatin capsules. For polymerization gelatin capsules were placed in a holder under UV lamp (360-365 nm) in a chamber for 24-36 hours at room temperature. Resin blocks were removed from the gelatin capsules, and trimmed to expose the surface of the tissue for sectioning. Ultra-thin sections were obtained using diamond knife and they were put on nickel grids, which were placed on a filter paper to dry up.

3.5 Immunofluorescence Staining of Paraffin Sections

After generating tissue sections slides, sections were processed for immunohistochemical analysis. First dewaxing of the sections was achieved by immersing the slides by 2 washes with xylene each 5 minutes followed by rehydrating step achieved by incubating the slides for 3 minutes each in a series of descending concentrations of ethanol (two changes of 100%, 95%, 75%, and 50%). Then slides were kept in distilled water for 5 minutes and then they were transferred to a container with citrate buffer and placed in microwave for antigen retrieval. Slides have been cooled down for 20 minutes followed by 3 washes with PBS 5 minutes each. After that sections were marked with Pap-pen to determine the area of interest upon adding

antibodies. Blocking buffer then were added to the tissue sections for 45 minutes at room temperature to avoid any unspecific binding. After that, the primary antibody (Table 2 below) is added to the sections and left overnight at 4°C. The primary antibodies added were, insulin anti-guinea pig, lipocalin-2 anti-mouse, glucagon anti-rabbit, superoxide dismutase anti-rabbit, catalase anti-rabbit and glutathione reductase anti rabbit. On the next day, slides were washed with PBS for 3 times each for 5 minutes and then the secondary antibody was added and incubated for 1 hour at room temperature (FITC-guinea pig, Rhodamine-rabbit/mouse and FITC- rabbit). Finally, slides were washed three times with PBS, 5 minutes long each and then a mounting medium was added to the tissue sections (DakoCytomation Fluorescent mounting medium). Using fluorescence microscope and AxioCam HRc digital camera with AxioVision 3.0 software (Carl Zeiss, Oberkochen, Germany) fixed with z-place fluorescence, sections were investigated, and pictures were collected and adjusted using image J 1.47V.

Table 2: Primary and secondary antibodies used in IHC and their dilutions.

Primary antibody	**Dilution**	**Source**	**Secondary antibody**	**Dilution**	**Source**
Lipocalin-2 Mouse	1:100	Abcam	Rhodamine-Mouse	1:100	Jackson ImmunoResearch
Insulin Guinea Pig	1:2000	Dako	FITC-Guinea Pig	1:100	Jackson ImmunoResearch
Glucagon Rabbit	1:500	Dako	FITC- rabbit	1:100	Jackson ImmunoResearch
Superoxide dismutase Rabbit	1:500	Rockland	Rhodamine-Rabbit	1:100	Jackson ImmunoResearch
Catalase Rabbit	1:500	Abcam	Rhodamine-Rabbit	1:100	Jackson ImmunoResearch
Glutathione reductase Rabbit	1:500	Abcam	Rhodamine-Rabbit	1:100	Jackson ImmunoResearch

3.6 Immunoelectron Microscopy

Grids containing pancreatic sections were jet washed with deionized water then the grids were incubated with 10% H_2O_2 in water for 10 minutes. Followed by a wash with deionized water and then the grids containing pancreas sections were immersed for 20 minutes in 0.5 M NH4Cl in 0.01 M PBS (pH7.3). After that the grids were washed with washing buffer for 5 minutes then the tissues were blocked with washing buffer containing 20% normal goat serum (NGS) then the primary antibody (mouse anti-lipocalin-2) was added and incubated overnight at 4°C. On the next day, grids were kept at room temperature for an hour, washed with PBS for 3 times and incubated with blocking buffer for 20 minutes at room temperature. After that, secondary antibody (goat anti-mouse IgG coated with 10 nm gold particles) was added and incubated for 2 hours at room temperature. Grids were washed again with PBS for 3 times and the above steps were repeated for the same grids using a different primary antibody (rabbit anti-insulin) and incubated at room temperature for 2 hours. Grids were incubated in goat anti-rabbit IgG coated with 15 nm gold particles on the next day and grids were then fixed with glutaraldehyde (2.5% aqueous) for 5 minutes, washed with deionized water and allowed to dry overnight on a filter paper. On the following day, using uranyl acetate for 15 minutes and lead citrate for 7 minutes, grids were contrasted followed by a last wash with deionized water before viewing them with Philips TEM.

3.7 Markers of Oxidative Stress

Different oxidative stress markers were analysed including glutathione reductase (GSH), superoxide dismutase (SOD) and catalase levels in serum using

colorimetric analysis and in pancreas tissue using double-labeling immunofluorescence technique.

To measure glutathione reductase activity in serum, 25 µL of the serum was taken and 25 µL of 5% sulphosalicylic acid was added (1:1) ratio for deproteinization and to clear the turbidity of the serum. Next, serum in sulphosalicylic acid were centrifuged at 10000 rpm for 10 minutes at 4°C and the supernatant was transferred into a multi-well dish for colorimetric analysis. However, for Serum levels of SOD the serum was directly used in the colorimetric analysis using Cayman Chemical SOD assay kit (Cayman Chemical, Ann Arbor, Michigan, USA).

3.8 Biochemical and Peptide Hormone Analysis

3.8.1 Biochemical Analysis Using Cobas®

Biochemical analyzer was utilized to obtain clinical chemistry profiles using rat serum. Various markers have been investigated and quantified including lipid profile markers triglycerides (TG), cholesterol (CHOL), low density lipoprotein (LDL), high density lipoprotein (HDL), Kidney function markers such as, creatinine, urea, blood urea nitrogen (BUN), and liver function markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin (ALB) total protein and bilirubin.

3.8.2 Peptide Hormone Analysis Using Magpix®

The Magpix® Platform is a fluorescent-based detection system that can detect various biological molecules, such as proteins and nucleic acids, from the same sample. This technology depends on microspheric colourful beads that are coupled to reagents that can bind to the target analytes. Beads are added to a 96-well plate and

mixed with the sample being tested, beads will bind to the analyte based on antibody-protein reaction, the complex is biotinylated and finally the detection molecule is attached.

3.9 Cell culture, Treatment, and Insulin Secretion

INS-1 832/3 rat insulinoma cell line (obtained from Millipore #SCC208) were maintained in complete RPMI-1640 (Sigma Cat. No R0883) media supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.05 mM β-mercaptoethanol and 10% fatal bovine serum cultured at 37°C in a 5% CO_2 atmosphere. For glucose stimulated insulin secretion, cells were incubated in HBSS containing 114 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/LKh2PO_4, 1.16 mmol/L CaCl2. 25.5 mmol/L $NaHCO_3$, and 0.2% bovine serum albumin, pH 7.2. then cells were washed with HBSS + 2.5 mM glucose. After wash, cells were incubated for 1 h in HBSS with 2.5 mM glucose for basal insulin secretion and HBSS with 15 mM glucose for stimulated insulin secretion. After one hour, lipocalin-2 peptide was added to respective wells at 10^{-8} M, 10^{-10} M and 10^{-12} M and a control well was maintained with no peptide for 2 hours. After 2 hours the supernatant was removed to determine the insulin content using Mercodia high range rat insulin ELISA kit (Mercodia, Sylveniusgatan 8A, Uppsala, Sweden).

3.10 SDS-PAGE and Western Blot Analysis

Cell lysates were prepared using RIPA buffer containing Pierce Protease inhibitor mini tablets. Equal amounts of cellular protein extracts or supernatants were diluted with LDS containing 10% mercaptoethanol as reducing agent, heated at 95°C for 5 minutes, and separated in 10% or 8% gels, using TGS running buffers. Proteins

were transferred into 0.2 µm Pvdf membranes (Thermo Scientific, Germany) and equal loading was shown in Ponceau S stain. Non-specific binding sites were blocked in Tris-buffered saline (TBS) containing Tween 20 and 3% non-fat milk powder. Appropriate primary antibodies (Table 3) were diluted in 3% blocking buffer (non-fat milk powder in TBST). And they were visualized by probing the membrane with the corresponding horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG (BIO-RAD, USA) and the reactive bands were visualized using ECL plus substrate (Thermo scientific, USA).

Table 3: Primary and secondary antibodies used in WB analysis and their dilutions.

Primary antibody	**Dilution**	**Source**	**Secondary antibody**	**Dilution**	**Source**
GAPDH	1:5000	Cell signaling	horseradish peroxidase conjugated anti-rabbit IgG	1:20,000	BIO-RAD
AKT	1:1,000	Cell signaling	horseradish peroxidase conjugated anti-rabbit IgG	1:20,000	BIO-RAD
p-AKT	1:1,000	Cell signaling	horseradish peroxidase conjugated anti-rabbit IgG	1:20,000	BIO-RAD
PI3K	1:1,000	Abcam	horseradish peroxidase conjugated anti-mouse IgG	1:20,000	BIO-RAD
LCN2	1:1,000	Abcam	horseradish peroxidase conjugated anti-mouse IgG	1:20,000	BIO-RAD

3.11 Statistical Analysis

Data samples were expressed as mean ± standard error of the mean. Statistical differences were analyzed using one-way ANOVA test on GraphPad software and the

p-value were obtained using tukey karmer post hoc test (GraphPad Software, San Diego, CA, USA). Values for differences with $p < 0.05$ were considered significant.

Chapter 4: Results

4.1 Metabolic Parameters

The below Figures (6) and (7) show the time courses of changes in body weight (g) and blood glucose level (mg/dl) in LCN2-treated (8 µg/Kg) and untreated diabetic and age-matched control rats over the 3 weeks period of the study. The presented results show that untreated diabetic rats suffered from significant weight loss when compared with age-matched normal controls. STZ-induced diabetic rats treated with LCN2 showed significant increase in body weight in comparison with saline-treated diabetic group. Moreover, diabetic rats had significantly elevated blood glucose levels compared with non-diabetic rats. LCN2 treatment of diabetic rats caused a slight improvement in glucose levels after one week and two weeks when compared with the diabetic control rats however, no significant reduction were observed.

4.1.1 Body Weight

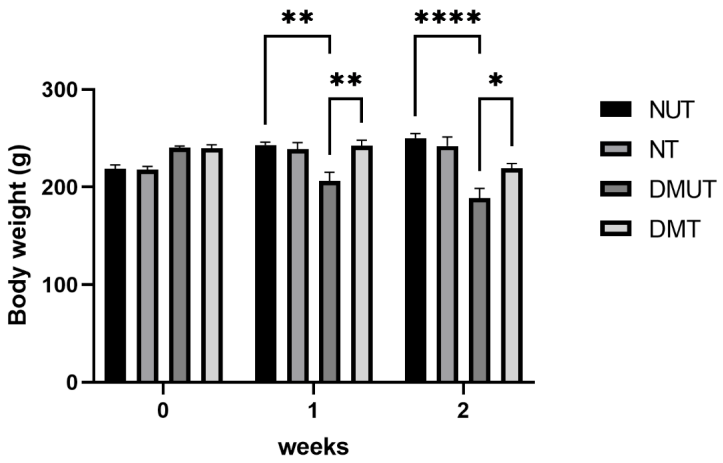

Figure 6: Effects of LCN2 treatment on weight in normal and diabetic animals. Note significant differences in body weight between DMUT and DMT. Values represented as mean ± SEM, n=6

4.1.2 Glucose Level

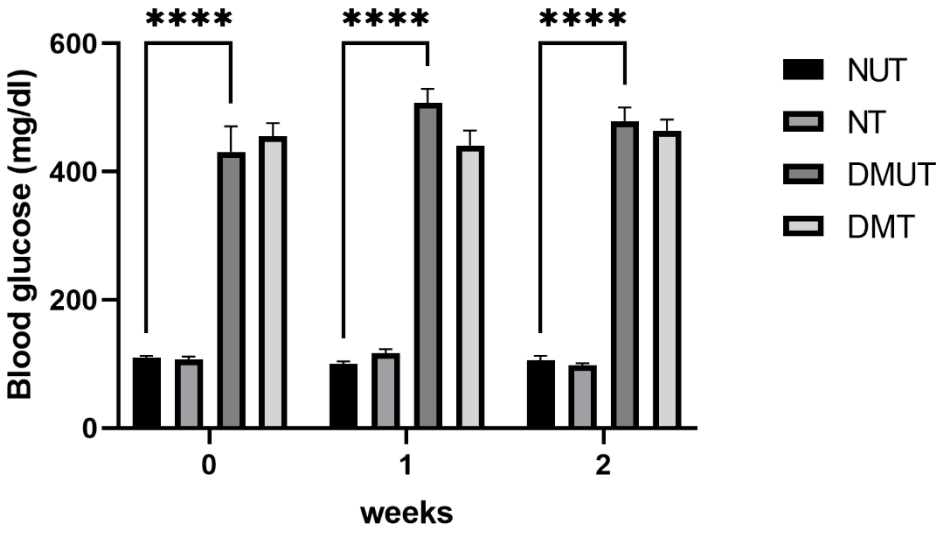

Figure 7: Effects of LCN2 on fasting plasma glucose levels in normal and diabetic animals. Values represented as mean ± SEM, n=6 Time course graphs representing the effect of LCN2 treatment on (a) body weight and (b) blood glucose levels in normal saline-administered, normal LCN2-administered, saline-administered diabetic and LCN2-administered diabetic rats. Values represented as mean± SEM, n=6.

4.1.3 IPGTT

To investigate the blood glucose homeostasis in the diabetic animals, all the experimental rat's glucose tolerance capacity was assessed. As shown in Figure (8), the diabetic animals showed significantly elevated glucose levels than the normal animals prior to glucose administration ($P < 0.05$). After intraperitoneal glucose administration, the blood glucose readings of the diabetic and normal rats reached the peak within thirty-minutes however, in the normal animals, lipocalin-2 caused a reduction in the FPG levels after 30 minutes of glucose challenge. Whereas both LCN2 and saline-treated normal control animals gradually returned to the basal levels after 120 minutes. In the diabetic group, lipocalin-2 treated diabetic animals exhibited

improvements in response to the glucose challenge when compared to normal-saline treated diabetic animals and lipocalin-2 caused a significant reduction in glucose levels after 60 minutes.

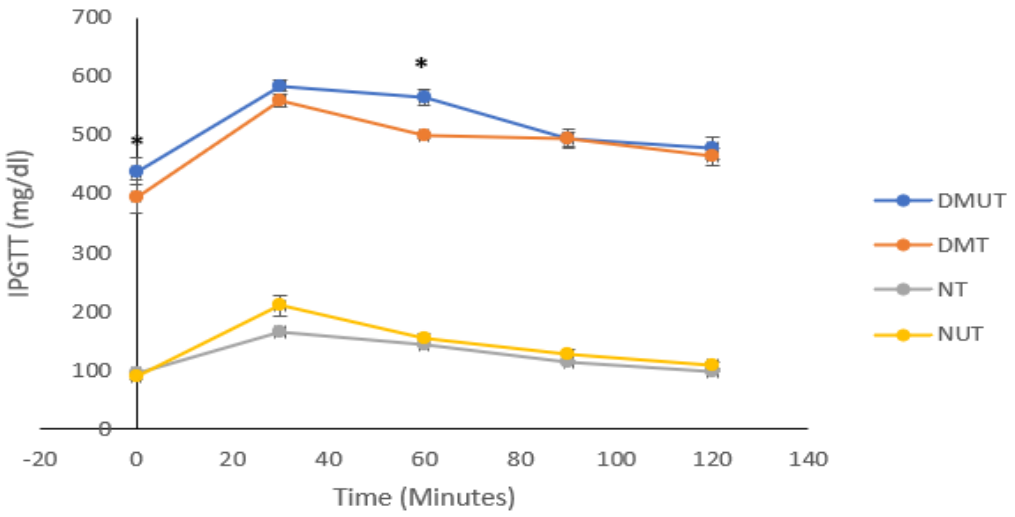

Figure 8. Effects of lipocalin-2 on IPGTT after 2 weeks of treatment of normal and diabetic animals. Note that the diabetic animals had significant higher glucose levels when compared with the normal animals prior to glucose administration and the glucose levels of the diabetic animals treated with LCN2 were significantly lower at 60 min after glucose administration compared to DMUT. Values represented as mean± SEM, n=6

4.2 Insulin Release

Figure 9 shows the effect of LCN2 in stimulating insulin release from rat insulinoma cell line.

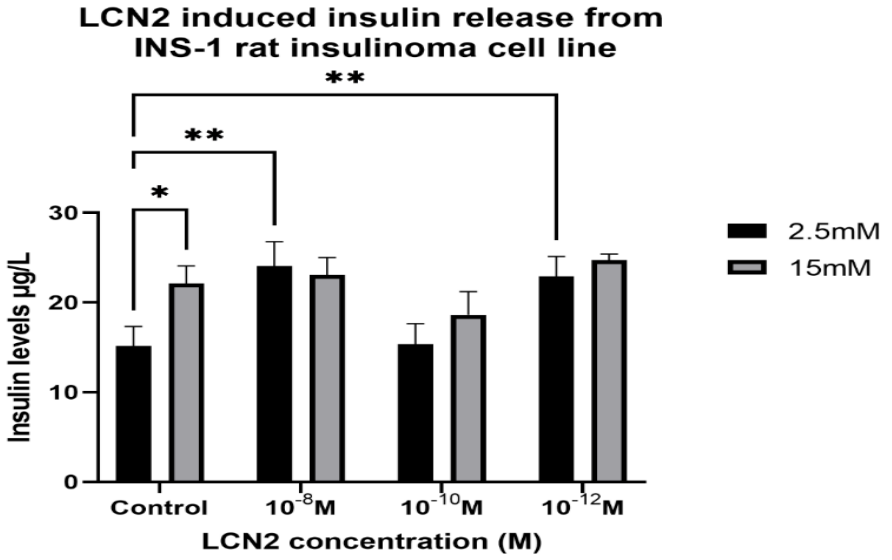

Figure 9: The effect of different concentrations of LCN2 in insulin secretion in INS-1 rat insulinoma cell line. Values represented as mean ± SEM, n=6 Figure (9) shows the results of the *in vitro* insulin secreting properties of LCN2 in cell culture in two different concentrations of glucose at 2.5 mM and 15 mM. Different LCN2 concentrations have been utilized in order to determine a dose-response curve for LCN2-induced insulin secretion effects. LCN2 at 10^{-8} M and 10^{-12} M stimulated the highest secretion of insulin in the lower glucose concentration while 10^{-10} M showed less secretion. However, with the higher glucose concentration no significant effect on insulin secretion with LCN2 was detected.

4.3 Immunohistochemical Localization of LCN2

4.3.1 Co-localization Analysis of LCN2 with Insulin in Pancreas

Double-labeling immunofluorescence technique were used to investigate whether LCN2 co-localizes with insulin in islets of Langerhans of normal and diabetic Wistar rats. This study demonstrates that LCN2 co-localized with insulin in the pancreatic islets of Langerhans as shown in Figure 10.

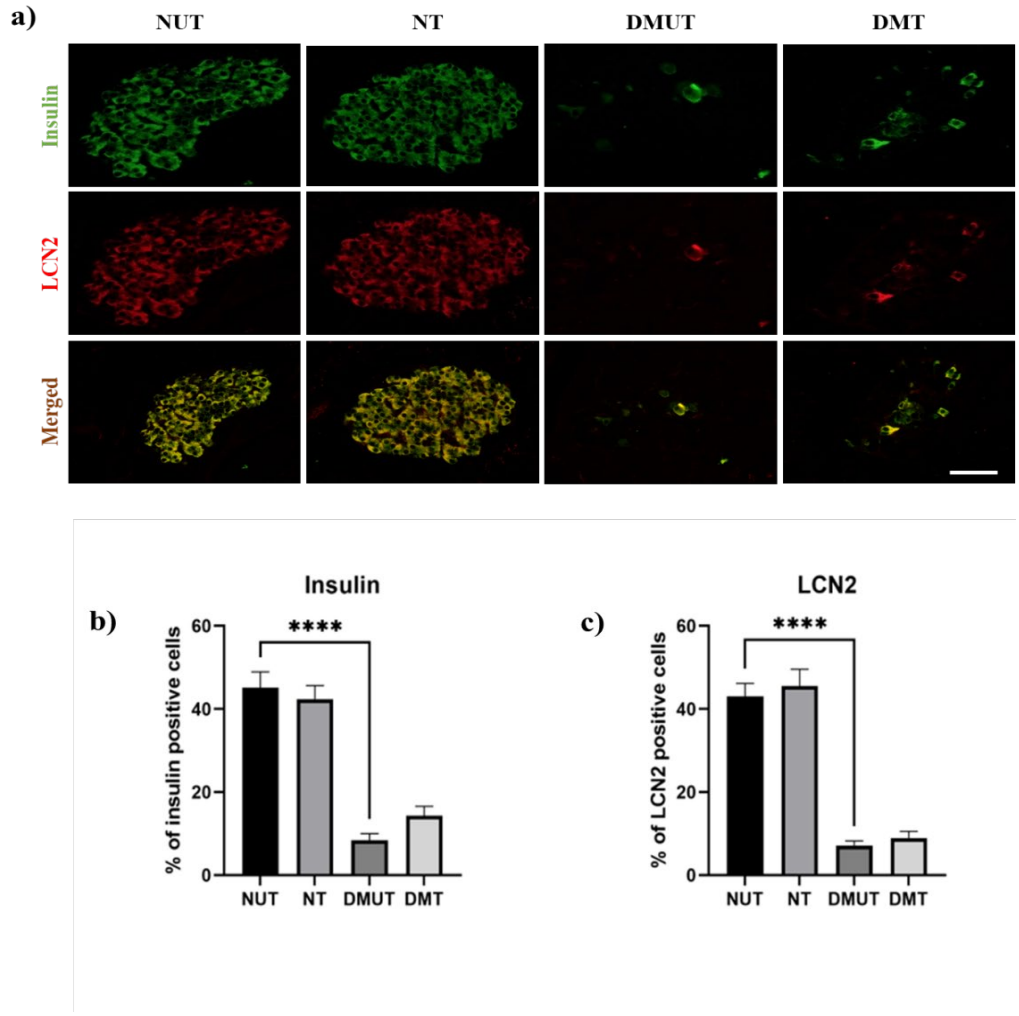

Figure 10: Co-localization of LCN2 with insulin in pancreatic islet cells of normal and diabetic rats. (a) Insulin positive cells are shown by green fluorescence while LCN2-positive cells are shown in red. The yellow color demonstrates cells that express both LCN2 and insulin. (b) and (c) Morphometric analysis. Note that LCN2 positive cells contain both LCN2 and insulin. The micrographs are representative of 6 such animals. Scale bar = 25 µm.

4.3.2 Co-localization Analysis of LCN2 with Glucagon in Pancreas

In order to investigate whether LCN2 and glucagon are expressed in the same cell, double labeling immunofluorescence was utilized using antibodies against LCN2 and glucagon. Glucagon was in the peripheral region of the pancreatic islets while

LCN2 was seen in cells that dominate the middle part of the pancreatic islets of Langerhans.

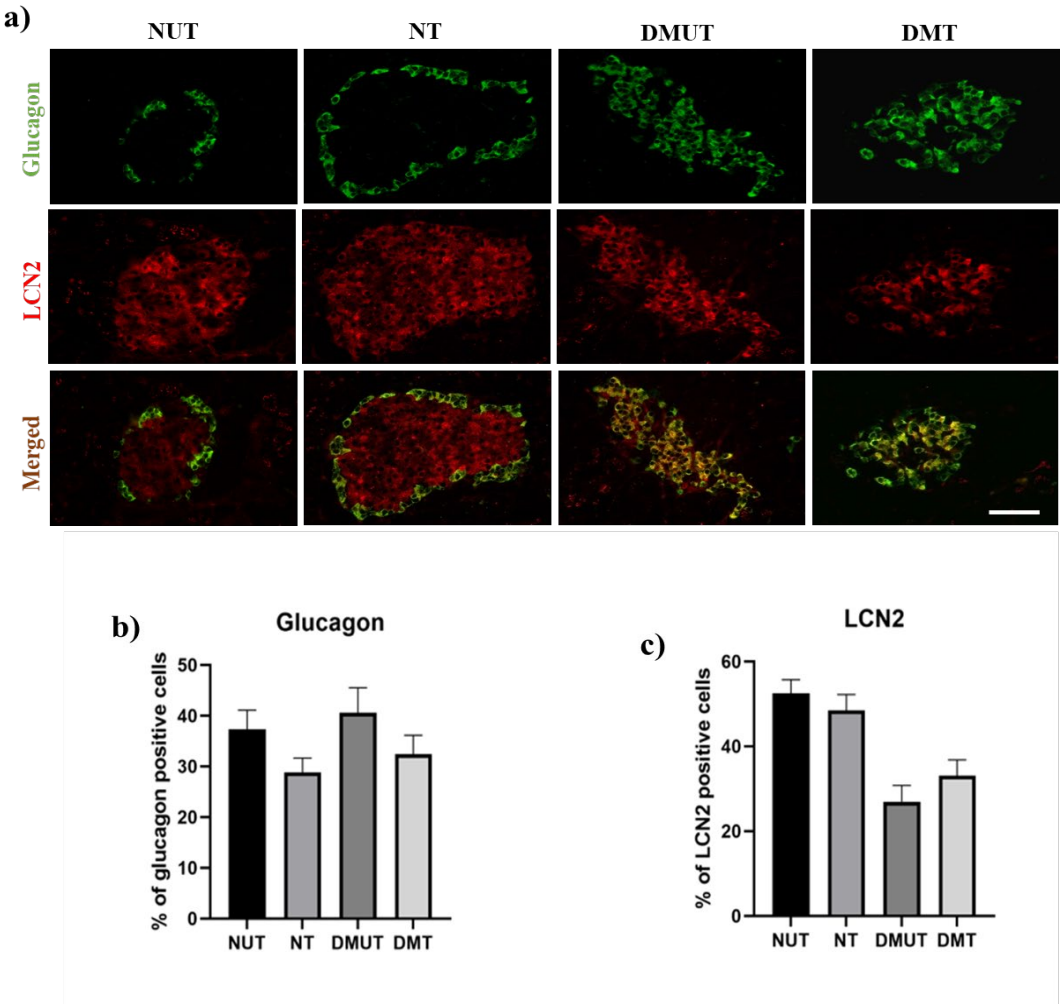

Figure 11: Co-localization of LCN2 with glucagon in pancreatic islet cells of normal and diabetic rats. In (a) Glucagon positive cells are shown by green fluorescence while LCN2-positive cells are shown in red. The yellow color demonstrates cells that express both LCN2 and glucagon. (b) and (c) represents the morphometric analysis. The micrographs are representative of 6 such animals. Scale bar = 25 µm.

4.4 Electron Microscopy

In Figures 12 and 13, show that insulin and LCN2 co-localized in the pancreatic beta-cells. 15 nm rabbit anti-insulin and 10nm mouse anti-LCN2 gold particles

conjugated IgG, were detected to co-exist in granules of pancreatic beta-cells. The number of insulin secretory granules in normal animals was higher compared to diabetic animals. Furthermore, less co-localization is seen in diabetic animals due to derangements in granules structure and numbers as seen in Figure (13).

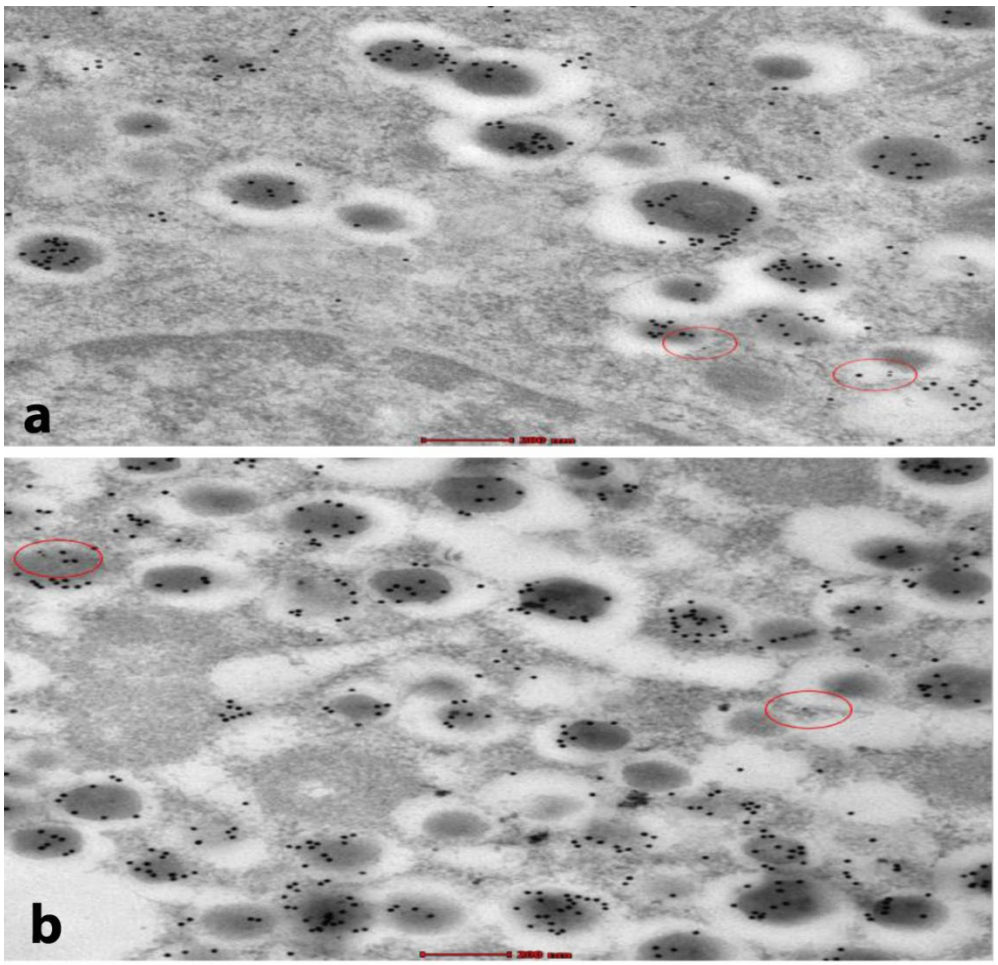

Figure 12: Electron micrograph showing (a) β-cell of normal rat treated with saline with intact secretory granules and (b) β-cell of normal rat treated with LCN2 Circled areas show the co-localization between insulin (15 nm) and LCN2 (10 nm). The electron micrographs are typical for 6 different animals in each group. (43 k)

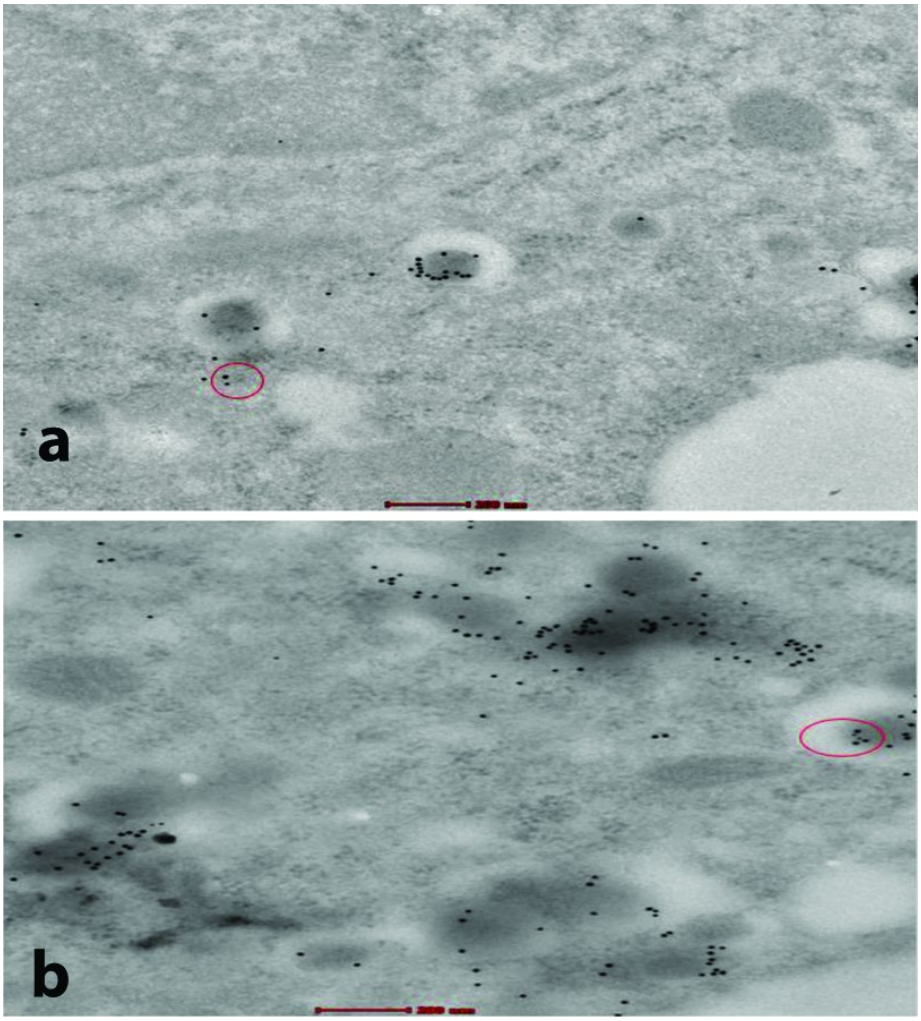

Figure 13: Electron micrograph showing (a) β-cell of diabetic rat treated with saline with deformed secretory granules and (b) β-cell of diabetic rat treated with LCN2 Circled areas show the co-localization between insulin (15 nm) and LCN2 (10 nm) within the same granule. The electron micrographs are typical for 6 different animals in each group. (43 k)

4.5 Markers of Oxidative Stress

The antioxidant activity of lipocalin-2 has been tested in the serum and the pancreas tissue using colorimetric analysis and immunofluorescence analysis respectively. Superoxide dismutase (SOD) and Glutathione reductase (GSH-red) levels were markedly decreased in serum after the induction of DM. As shown in

Figure (14), (15), (16) and (17) significant decline of antioxidant enzymes activities was observed in the STZ-induced diabetic animals. However, significant increase in the antioxidant enzymes SOD and GSH-reductase were found in serum of the LCN2 treated STZ-induced diabetic animals when compared to the diabetic control. showing improved ability of scavenging ROS. Moreover, all of the three target the enzyme's activities in the LCN2 treated diabetic animals showed almost normal levels and the LCN2-treated control animals showed higher GSH-reductase and SOD activity than the saline treated control animals.

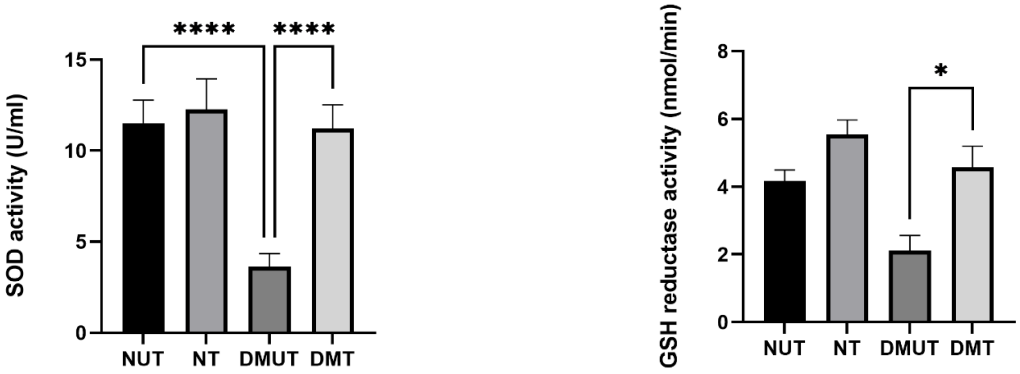

Figure 14: Effects of LCN2 on SOD and GSH reductase in serum. Values represented as mean ± SEM, n=6. Note the significant reduction in SOD and GSH-reductase levels after the induction of DM. LCN2 caused a significant increase in SOD and GSH reductase levels in serum.

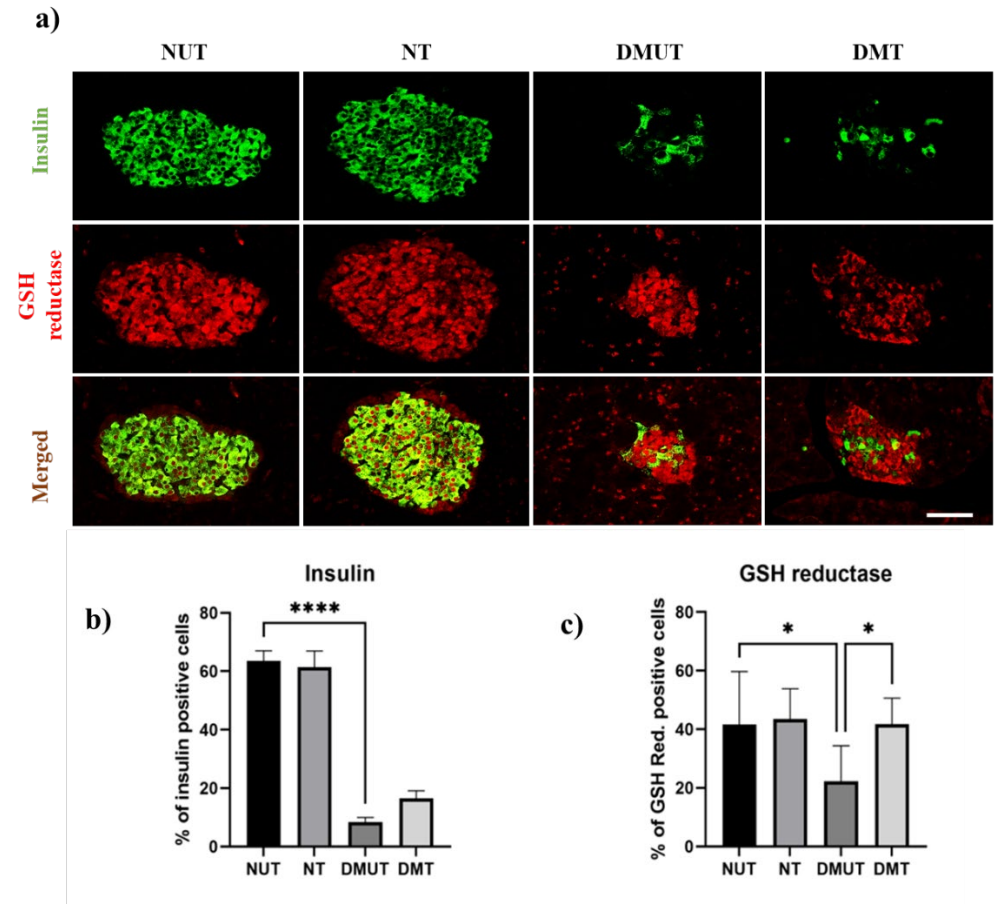

Figure 15: Effects of LCN2 on GSH reductase in pancreas of normal and diabetic rats. Note the significant reduction in GSH reductase levels after the induction of DM. LCN2 caused a significant increase in GSH reductase levels in the pancreas. The micrographs are representative of 6 such animals. Values represented as mean ± SEM. Scale bar = 25 µm.

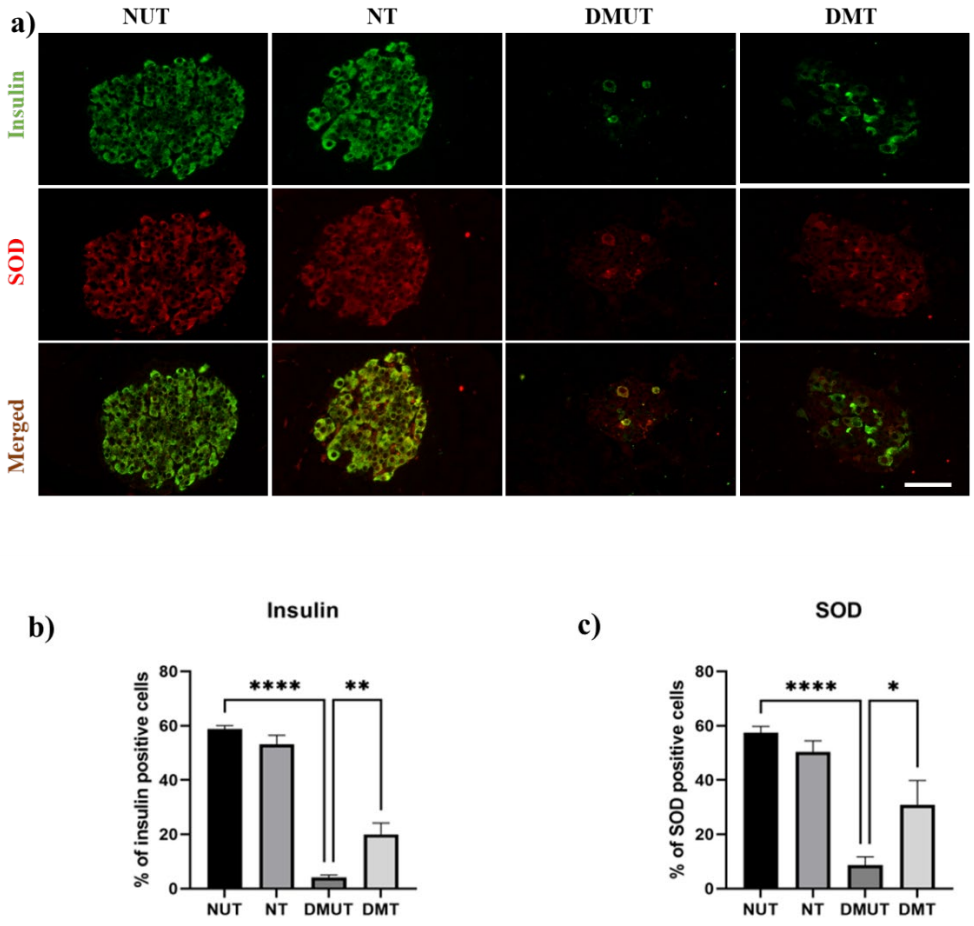

Figure 16: Effects of LCN2 on SOD in pancreas of normal and diabetic rats. Note the significant reduction in SOD levels after the induction of DM. LCN2 caused a significant increase in SOD levels in the pancreas. The micrographs are representative of 6 such animals. Values represented as mean ± SEM. Scale bar = 25 µm.

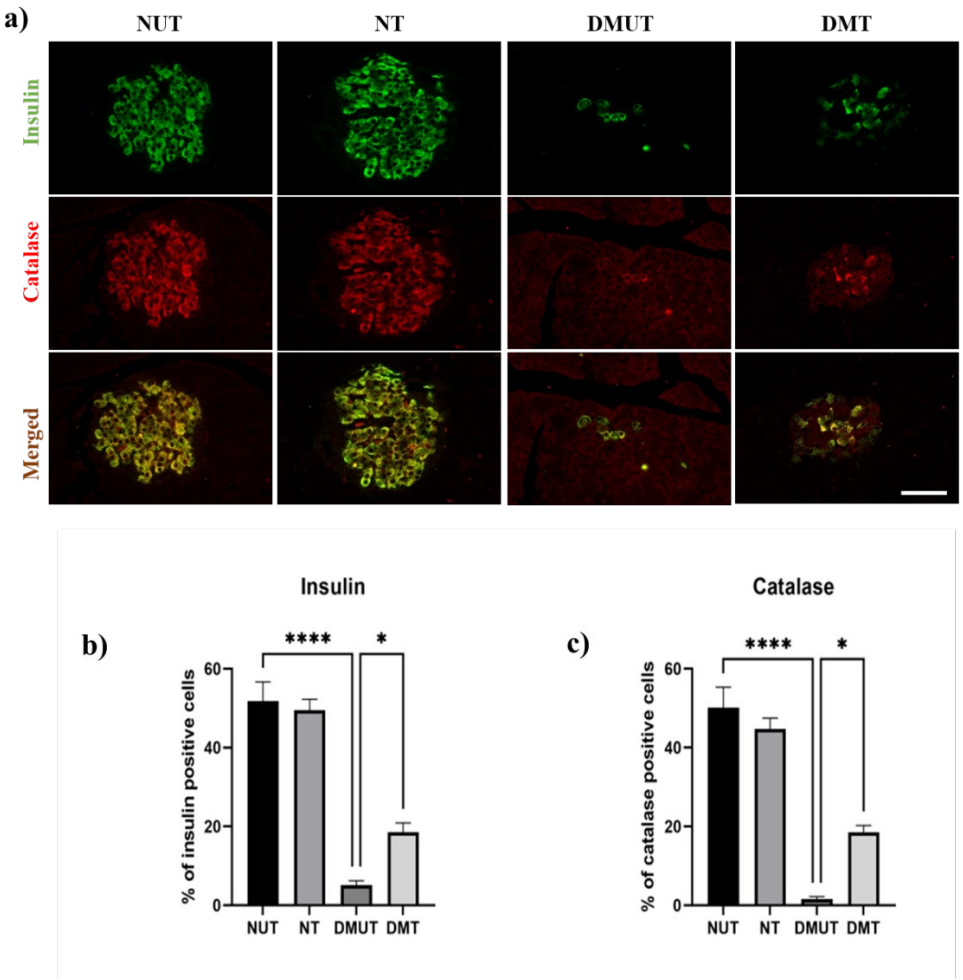

Figure 17: Effects of LCN2 on catalase in pancreas of normal and diabetic rats. Note the significant reduction in catalase levels after the induction of DM. LCN2 caused a significant increase in catalase levels in the pancreas. The micrographs are representative of 6 such animals. Values represented as mean ± SEM. Scale bar = 25 µm.

4.6 Effects of LCN2 on Biochemical Parameters

4.6.1 Liver Function Test

Figure 18: shows a significant increase in AST and ALT levels in diabetic control group compared to LCN2 treated and normal animals. Normal saline-animals in addition, showed higher levels in both AST and ALT when compared to LCN2-

treated controls, whereas there AST levels were also higher than the LCN2 received diabetic group.

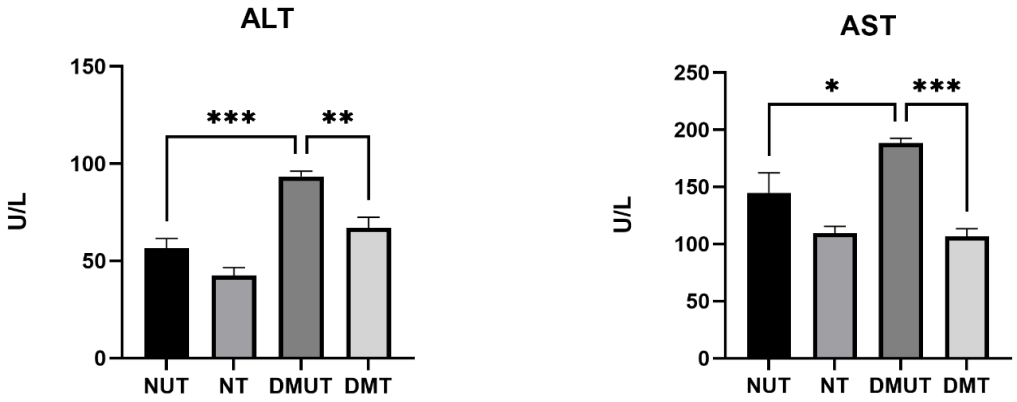

Figure 18: LCN2 effect on AST and ALT. Values represented as mean± SEM, n=6. Note the significant elevation in AST and ALT levels in diabetic animals and the significant effect of LCN2 in reducing both enzymes levels in the diabetic animals.

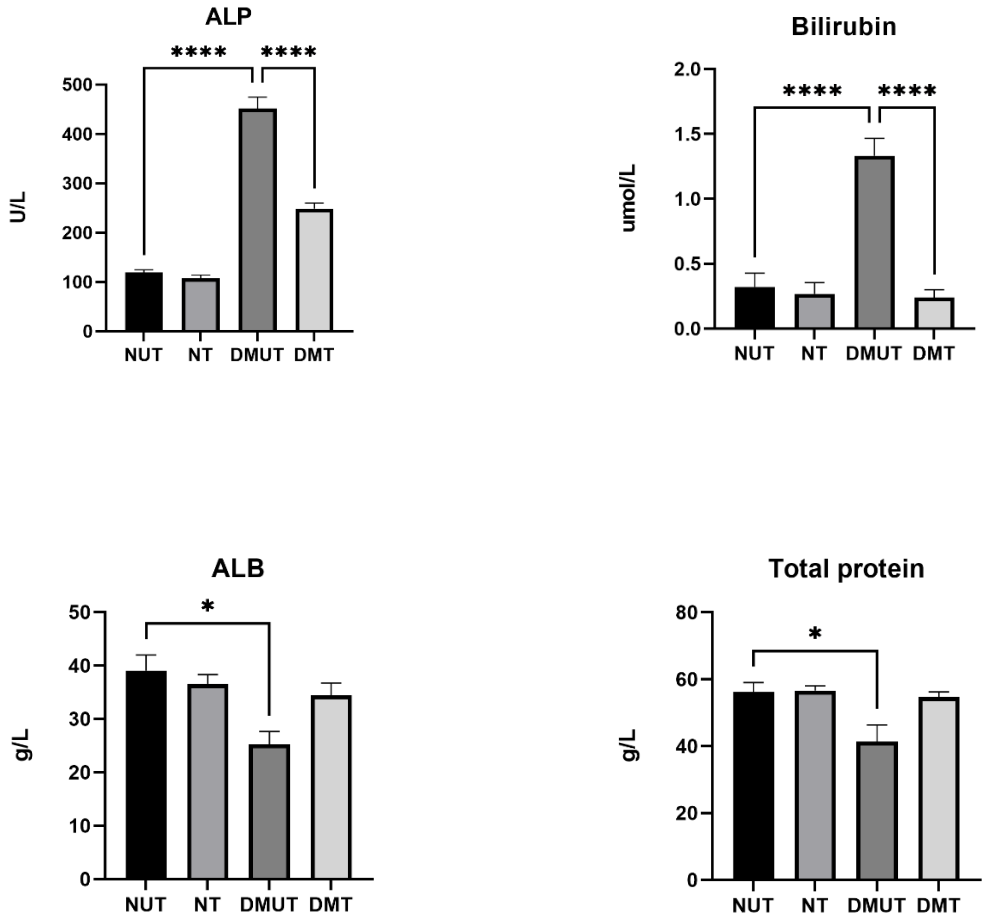

Figure 19: shows a significant increase in ALP and Bilirubin levels in diabetic control group compared to LCN2 treated and normal animals. In addition, ALB and total protein were significantly down regulated in diabetic control animals when compared to the normal controls, and an improvement in ALB and total protein levels were detected in LCN2 treated diabetic animals.

4.6.2 Kidney Function Test

Figure (20) shows kidney function test parameters of urea, blood urea nitrogen (BUN) and creatinine. A significant increase in urea and blood urea nitrogen levels in diabetic control group compared to normal animals were found. LCN2 treatment resulted in a reduction in both levels of urea and blood urea nitrogen. However, no

significant difference was seen in creatinine levels between normal and diabetic animals with or without the treatment.

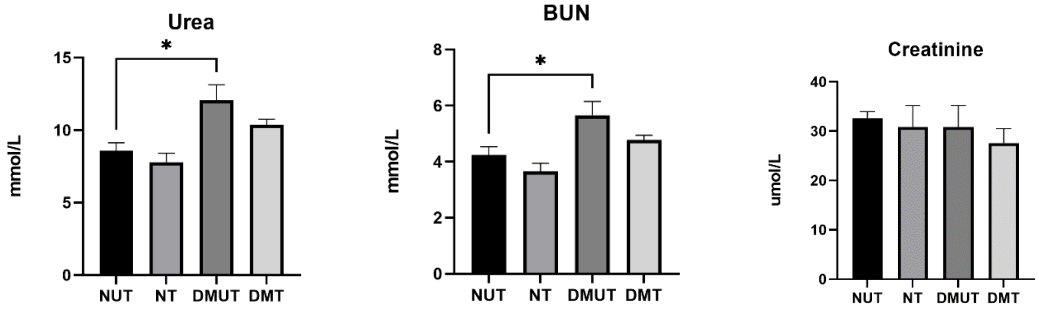

Figure 20. Effects of LCN2 on urea, BUN and creatinine. Values represented as mean ± SEM, n=6. Note the significant elevation in urea and BUN levels after the induction of DM and the LCN2-induced decrease in both molecules. On the other hand there is no noticeable difference in the levels of creatinine in all of the experimental animals with or without the treatment.

4.6.3 Lipid Profile

Strategies for the prevention of dyslipidemia are essential for the management and treatment of DM. in general, dyslipidemia is marked with increased TC, TG, LDL-C levels and reduced HDL-C level in serum (Dixit & Kar, 2010). Lipid profile for all the experimental groups is shown in Figure 21 The result shows that LCN2 caused a significant reduction in triglycerides, LDL and total cholesterol levels in both normal and diabetic treated groups compared to saline-treated littermates. Hepatic lipase was upregulated after the induction of DM, however, it was decreased with LCN2 treatment. High-density lipoprotein levels decreased after the induction of DM and were improved to normal levels after the treatment with LCN2.

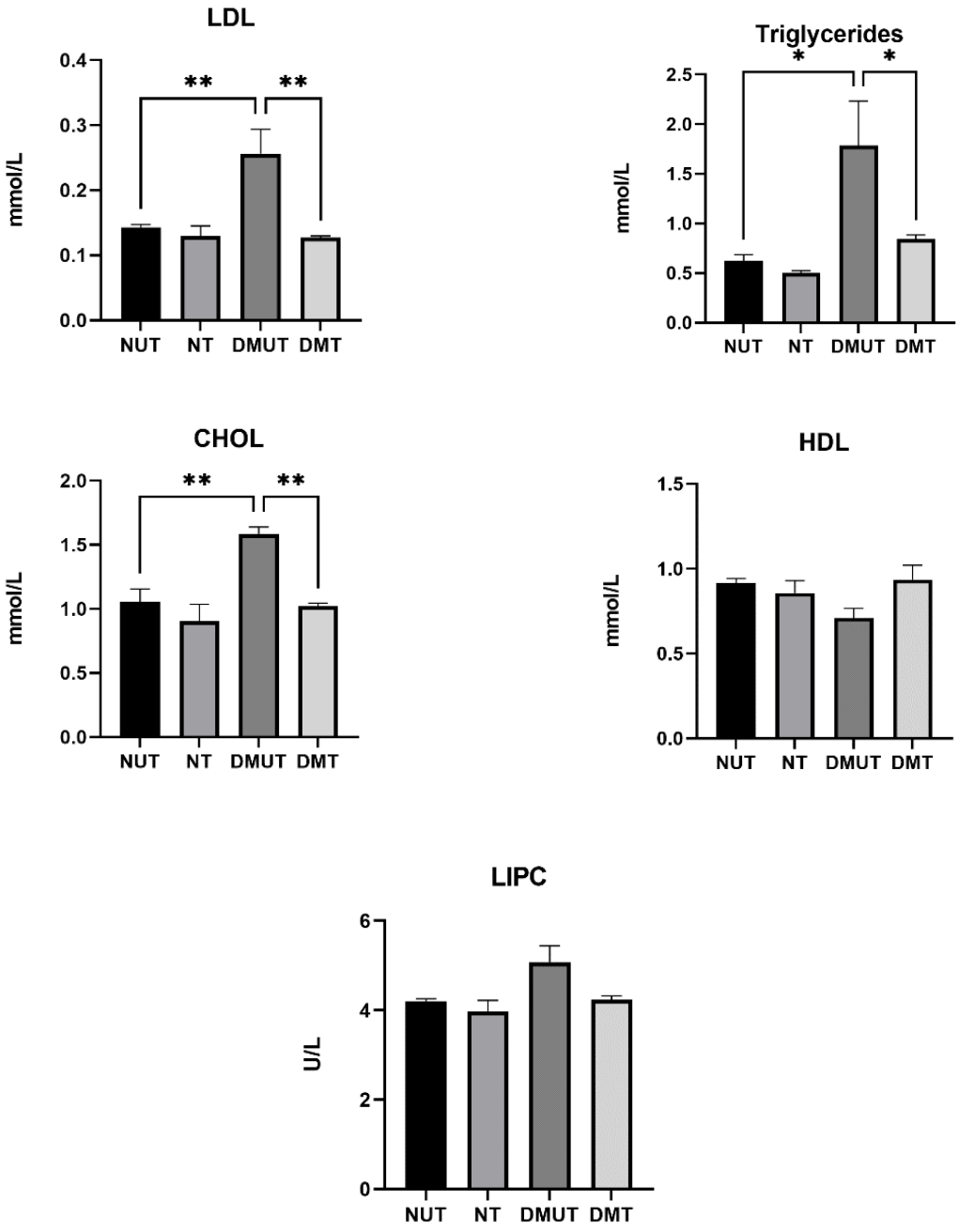

Figure 21: Effects of LCN2 on LDL, TG, TC, HDL and LIPC Values represented as mean ± SEM, n=6. Note the significant reduction in LDL, TG, and TC and the decrease in HDL and LIPC levels after the treatment by LCN2.

4.7 Peptide Hormones Analysis

Figure (23) shows the levels of insulin in all groups. A significant reduction in the levels of insulin was seen in diabetic animals compared to the normal control. LCN2 caused a significant increase in the insulin levels in diabetic animals while it had no effect in non-diabetic animals. LCN2 also caused a significant increase in the levels of Amylin in diabetic treated group when compared to the diabetic control animals. Figure (22), shows the effects of LCN2 treatment on GIP and GLP-1. LCN2 treatment caused a significant elevation in GIP levels in both normal and diabetic group. On the other hand, diabetes mellitus induction caused a significant upregulation in GLP-1 levels, which in turn was decreased after LCN2 treatment in diabetic animals. LCN2 effects on leptin levels have been shown in Figure (25) were the induction of DM caused marked reduction in leptin levels however, slight improvement in leptin levels with LCN2 treatment was detected in the diabetic animals.

Furthermore, the data shows that there was a significant reduction in C-peptide in diabetic control group compared to normal control animals. LCN2 treatment significantly elevated the levels of C-peptide in diabetic animals. Figure (26) shows the effect of LCN2 on IL-6 and MCP1. DM induction caused a significant increase in the levels of IL-6 and MCP1 and the treatment with LCN2 resulted in a significant decrease in both levels of IL-6 and MCP1.

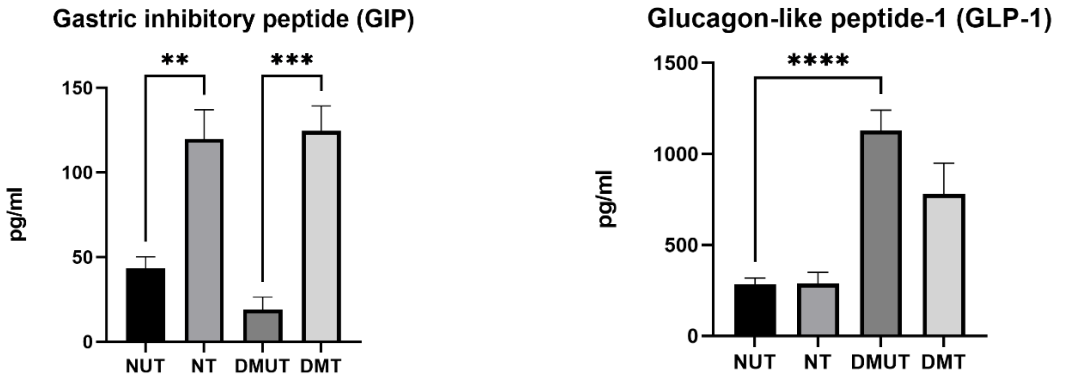

Figure 22: Effects of LCN2 on GIP and GLP-1 Values represented as mean ± SEM, n=6. Note that LCN2 significantly increased the levels of GIP in diabetic and normal animals and decreased GLP-1 levels in diabetic animals.

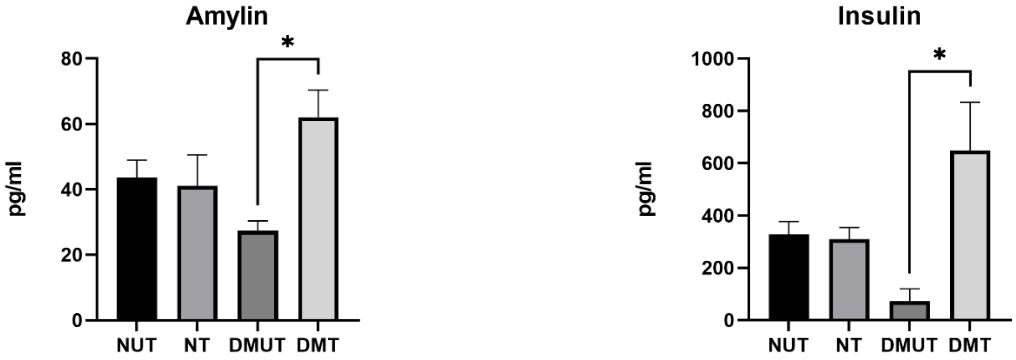

Figure 23: Effects of LCN2 on Amylin and Insulin Values represented as mean ± SEM, n=6. Note that LCN2 caused a significant increase in the levels of Amylin and insulin in the diabetic treated group when compared to the diabetic control group.

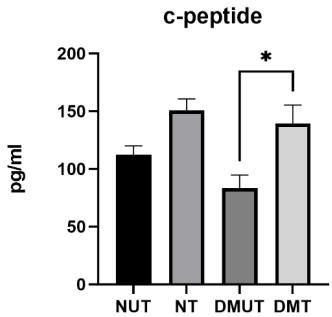

Figure 24: Effects of LCN2 on C-peptide. Values represented as mean ± SEM, n=6. Note that levels of c-peptide were decreased after induction of DM and LCN2 caused a significant increase in its levels when compared with the untreated diabetic animals.

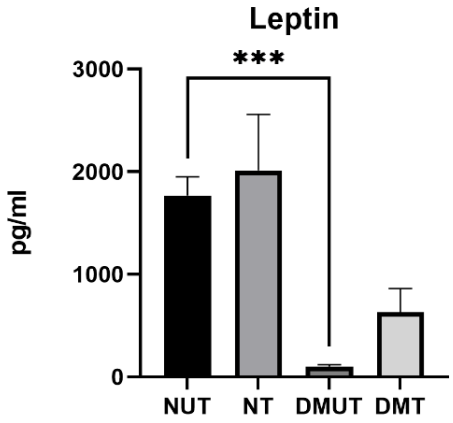

Figure 25: Effects of LCN2 on Leptin Values represented as mean ± SEM, n=6. Note that LCN2 improved leptin levels in diabetic animals compared to diabetic control.

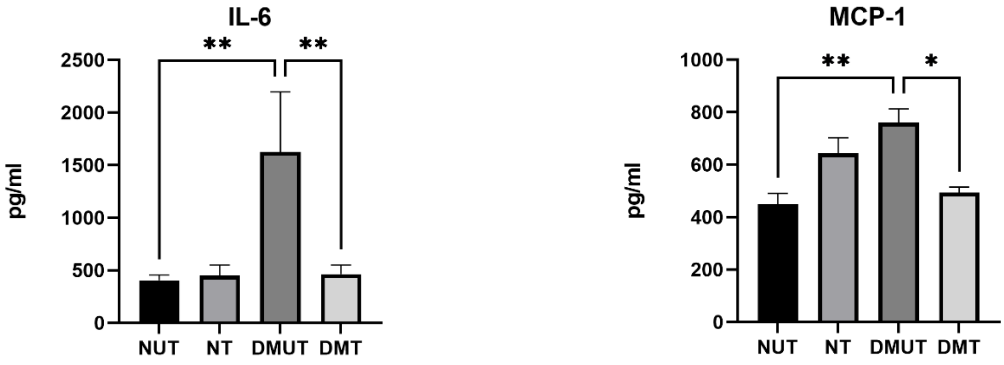

Figure 26: Effects of LCN2 on IL-6 and MCP-1 Values represented as mean ± SEM, n= 6. Note that LCN2 significantly decreased both levels of IL-6 and MCP-1 when compared with the diabetic control animals.

4.8 Insulin Signaling Pathway

PI3k/Akt pathway provides a survival signal that preserve cells from cell death due to various stress stimuli, different important molecules downstream the insulin signaling pathways including p-Akt at Ser473 and total Akt were examined to determine whether the PI3K/Akt signal network was affected by LCN2 treatment in

STZ induced diabetic rats. As shown in Figure 27, there is a decrease in phosphorylation of Akt at Ser473 amino acid in the STZ-induced diabetic animals with almost no change in total Akt protein expression in the three examined tissues, however treatment with LCN2 enhanced the phosphorylation of Akt at Ser 473.

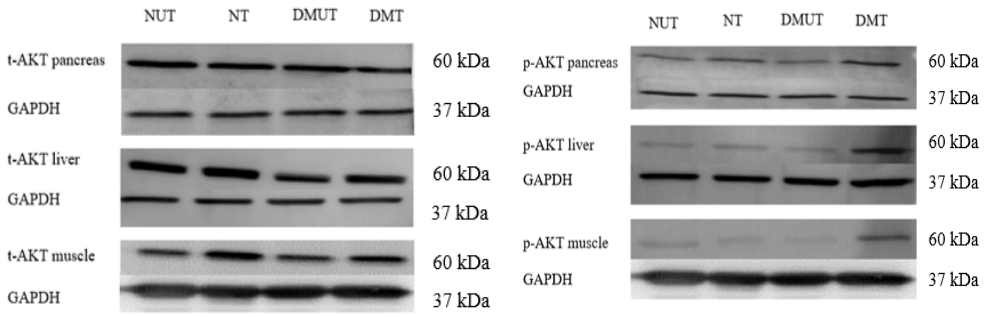

Figure 27: Effect of LCN2 on downstream targets of PI3K/Akt pathway in pancreas, liver and gastrocnemius muscle of LCN2-STZ treated diabetic rats. Animals were starved for 12 h and treated with or without STZ (60 mg/kg) body weight followed by stimulation with 8 µg/Kg of LCN2 for 5 consecutive days/week for two weeks. Total cell lysates were prepared and 50 µg proteins ran on SDS-PAGE followed by western blot analysis and reactive bands were detected using ECL plus substrate. Equal loading of protein was confirmed by probing the house keeping gene antibody GAPDH on the same western blot membrane.

Chapter 5: Discussion

5.1 Metabolic Parameters

5.1.1 Body Weight

All animals were maintained under similar conditions with access to water and food for the full duration of the study. The presented results show that untreated diabetic rats suffered from significant weight loss when compared with age-matched normal controls. STZ-induced diabetic rats treated with LCN2 showed significant increase in body weight and were able to maintain their normal body weight in comparison with saline-treated diabetic group. Although LCN2 is shown to inhibit food intake and acts as satiety signal via crossing the blood brain barrier and activating MC4R appetite suppressing pathway (Huang et al., 2012; Petropoulou et al., 2020). No significant weight change in the body weight of the non-diabetic LCN2 treated group were found in comparison with the saline treated normal group. Moreover, LCN2 reversed the weight loss associated with diabetic induction by STZ.

5.1.2 Glucose Levels

In normal animals treated with LCN2 and untreated similar fasting plasma glucose levels were found. on the other hand, the diabetic groups showed significantly higher glucose levels and treatment with LCN2 was not able to significantly decrease the plasma glucose levels. although LCN2 has been reported to improve glucose metabolism via inducing insulin secretion by various studies (Huang et al., 2012). It is important to note that the dosage used in this study and the treatment period are considered to be small compared to those studies. As well as the animal models and strains could also be another factor influencing the result.

5.1.3 Intraperitoneal Glucose Tolerance Test (IPGTT)

Plasma glucose levels before glucose administration were markedly higher in the diabetic group compared to control group. This study demonstrated that saline treatment was not able to reverse the sudden increase in plasma glucose levels after 30 minutes of the glucose load in control animals, however, lipocalin-2 treatment caused a reduction in plasma glucose levels after 30 minutes of glucose administration. On the other hand, all the diabetic groups had similar glucose readings after 30, 90 and 120 minutes of the glucose load. However, lipocalin-2 treated group showed a significant decrease in glucose level after 60 minutes of the glucose administration.

5.2 Effects of LCN2 on Insulin Secretion In Vitro

The data show that LCN2 has a profound secretagogue effect on insulin secretion at a dose of 10-8 M and 10-12 M in the established cell culture. LCN2, at a dose of 10-8 M and 10-12 M resulted in a significant elevation in the levels of insulin compared to control in the presence of 2.5 mM glucose. In the higher concentration of glucose (15 mM), the same dose of LCN2 did not result in a significant elevation in the insulin levels, however 10-10 M dose decreased the levels of insulin in both the lower (2.5 mM) and the higher (15 mM) concentration of glucose.

5.3 Immunohistochemical Localization of LCN2

The number of islet cells containing LCN2 was significantly lower in the pancreatic islet of diabetic rats when compared to normal rats. Double-labeling immunofluorescence technique were used to investigate whether LCN2 co-localizes with insulin in islets of Langerhans of normal and diabetic Wistar rats. This study demonstrates that LCN2 co-localized with insulin. The percentage distribution of

LCN2 and insulin-positive cells was close in both normal and diabetic rats, indicating high degree of co-localization. The number of LCN2 and insulin expressing cells declined significantly in STZ-induced diabetic rats in comparison to normal rats. The pattern of distribution of insulin and LCN2 has been altered after DM onset.

In order to determine whether LCN2 and glucagon are found in the same cell, double labeling immunofluorescence was utilized using antibodies against LCN2 and glucagon. Glucagon was in the peripheral region of the pancreatic islets while LCN2 was seen in cells that dominate the middle part of the pancreatic islets of Langerhans. There was no evidence of co-localization of LCN2 with glucagon in pancreatic islet alpha cells in the normal rats. However, the pattern of distribution of glucagon containing cells changed from peripheral dominant cells to center located islet cells in diabetic rats. The number of glucagon expressing cells elevated in the islets of diabetic rats and some degree of co-localization were found.

5.4 Distribution of Lipocalin-2 (LCN2) in Cytoplasmic Organelles of Islet Cells EM

Since LCN2 was found to co-localize with insulin using double-labeling immunofluorescence. Immunoelectron microscopy was conducted to determine the intracellular localization of LCN2 in pancreatic islet cells and to further confirm what was found at the light microscopy level. Multiple sizes of gold particles conjugated to IgG were assessed to study the level of co-localization of LCN2 and insulin in the cytoplasmic organelles of the beta cells. ultrathin sections processed for immunoelectron staining showed that pancreatic beta cells definitely contain LCN2. Gold particles conjugated IgG (10 nm) against LCN2 were observed on the secretory granules of islet beta cells accompanied by anti-insulin antibodies conjugated to 15 nm

gold particles. The number of 15 nm gold particles against insulin and the 10 nm gold particles conjugated to LCN2 antibody markedly decreased after the onset of DM.

Although several researches have shown the pattern of LCN2 expression, this is the first time to use electron microscope to localize LCN2 in the insulin-producing beta-cells. Furthermore, the findings also demonstrate the pronounced cellular organelle deformation and the morphological changes in the β-cells of the diabetic animals, nuclear envelope destruction and nucleus shrinkage, enlargement of the endoplasmic reticulum and the mitochondria and diminished secretory granules. These structural deformities are responsible for the functional disorders in beta-cells which mainly affect the expression and release of insulin. Moreover, the EM investigation confirmed the immunofluorescence findings that showed the co-localization of insulin and LCN2 in pancreatic β-cells in both normal and diabetic animals. This co-localization can explain a possible modulatory role of LCN2 in insulin-producing β-cells (Huang et al., 2012).

5.5 Effects of LCN2 on Antioxidant Enzymes Activities in Serum

Oxidative stress is a common hallmark of both T1DM and T2DM; increased biomarkers of oxidative stress are found in blood, urine and tissues including pancreas of diabetic patients (Wang & Wang, 2017). In chronic hyperglycemia state, due to several biochemical pathways such as nonenzymatic glycation of proteins, lipid peroxidation and DNA oxidation increased autooxidation and generation of free radicals is observed (Singh et al., 2005). The elevation in the reactive oxygen species and the decline in the antioxidant defense mechanisms results in cellular damage and the pathogenesis of vascular complications (Meenatchi et al., 2017). The common anti-oxidant enzymes in mammals superoxide dismutase, glutathione reductase and

catalase have a key roles in reducing and alleviating the oxidative stress in vivo (H. Zhao et al., 2016). Decrease in the antioxidants pool has been commonly observed in the diabetic rats (Ananthan et al., 2004). Therefore, increased antioxidant enzymes activities are the hallmark of anti-hyperglycemia. The antioxidant function of lipocalin-2 have been previously studied in different stress conditions (Roudkenar et al., 2007; Roudkenar et al., 2011; Yamada et al., 2016). And it has been shown to exert a cytoprotective effect against oxidative stress (Roudkenar et al., 2008; Halabian et al., 2013; Roudkenar et al., 2009). This result supports and extends the previous knowledge of the role of LCN2 in protecting against oxidative stress by increasing the antioxidant pool. As shown in Figure 14, significant decline of antioxidant enzymes activities was observed in the STZ-induced diabetic animals. However, significant increase in the antioxidant enzymes SOD and GSH-reductase were found in serum and the pancreas tissue of the LCN2 treated STZ-induced diabetic animals when compared to the diabetic control showing improved ability of scavenging ROS. Moreover, all of the target enzyme's activities in the LCN2 treated diabetic animals showed almost normal levels and the LCN2-treated control animals showed higher SOD and GSH-reductase activity than the saline treated control animals. The results suggests that LCN2 significantly reduces and alleviate oxidative stress induced by the hyperglycemia.

5.6 Effect of LCN2 on Biochemical Parameters

5.6.1 Liver and Kidney Function

In this study, significant increase in aspartate aminotransferase (AST) and alanine amino transferase (ALT) were observed post DM onset. Treatment with LCN2 lead to a significant reduction in AST and ALT in both diabetic and control animals. This decrease in AST and ALT levels suggests a protective effect of LCN2 on the liver function in DM. AST and ALT are liver enzymes that their levels are used as an indicator for liver function. AST is present in the cytoplasm and mitochondria of multiple body organs other than the liver including the heart, kidney, muscle, and RBCs. Liver damage results in the release of AST into the bloodstream and the diabetic animal model used in this study is characterized by upregulation in liver enzymes. In-addition lipid peroxidation and oxidative stress are also marked by an increase in AST which why the antioxidative stress role of LCN2 was further assessed in this study. In the liver, the precise role of LCN2 administration has not been investigated yet, however $LCN2^{-/-}$ mice were used in multiple studies to investigate the functional roles of endogenous LCN2 in multiple experimental liver injury models. In one study, the challenge with acute toxic chemicals or the establishment of mechanical induced cholestasis, had a significant liver damage in $LCN2^{-/-}$ mice as evidenced by elevated liver enzymes, AST and ALT post CCl_4, ConA and LPS application. This suggests that rapid LCN2 induction after direct toxic insult have a protective effect on hepatic cells (Borkham-Kamphorst et al., 2013).

Alkaline phosphatase (ALP) is an enzyme found to be primarily expressed by liver and bone, it has been shown to be increased in diabetes patients in comparison with non-diabetic controls. This result comes in line with another study by (Chen et

al., 2017) were they showed that Plasma ALP is an independent risk factor for the incident of diabetes after adjusting other liver-related factors. And in this study, STZ administration caused a significant increase in plasma ALP levels. However, treatment with LCN2 caused a marked reduction in ALP levels.

Elevation in bilirubin levels is a marker for severe liver injury which is seen in diabetic state (Zeng & Liu, 2019). The result further proved this observation, as significant increase in bilirubin levels were found after the induction of DM, however, treatment with LCN2 caused marked reduction in bilirubin levels.

In the body, serum albumin makes up major portion of the circulating proteins. It is well known that in diabetes state, the total protein level decreases due to protein breakdown as a source for energy. The results showed a significant decrease in albumin levels after the induction of diabetes. However, treatment with LCN2 improved serum albumin levels.

In this study, the kidney function were assessed through measuring creatinine, urea and blood urea nitrogen levels in the serum. DM induction resulted in significant increase in the levels of both urea and blood urea nitrogen in serum. LCN2 was able to reduce both molecules' levels in diabetic and normal LCN2 treated animals. However, creatinine had a minor elevation to no change after DM induction. In literature, different research groups have investigated the role of LCN2, and kidney injury and they have all agreed that LCN2 is a good predictor of acute kidney injury and biomarker for chronic kidney diseases (Tang et al., 2010) since the levels of endogenous LCN2 were higher in patients suffering from kidney disease.

Decreased total protein levels could indicate liver and/or kidney disorders while elevated protein levels than normal are suggestive of dehydration or cancer. As

the aim was to prove the protective role of LCN2 in diabetes mellitus, the increase and improvement of total protein levels by LCN2 suggests the capability of the peptide in reversing diabetic induced liver or kidney damage, in addition to reversing protein breakdown as reported for DM in several studies. It has been reported that protein is catabolized in severe diabetic conditions where cells are deprived of glucose (Charlton & Nair, 1998).

5.6.2 Lipid Profile

This study showed that LCN2 significantly reduced TG levels in both diabetic and non-diabetic animals. Moreover, there was a decline in HDL levels after the induction of DM however, an improvement in HDL level was observed after LCN2 treatment. Significant decrease was detected in LDL-C levels with the treatment of LCN2. No food and water restrictions were implied on all the experimental animal groups showing that food uptake had no effect on serum TG levels. LCN2 ability to reverse hypertriglyceridemia and its critical role in lipid metabolism has been investigated earlier. LCN2 deficiency in mice has been shown to potentiates HFD-induced obesity, dyslipidemia, fatty liver, glucose intolerance, and insulin insensitivity (Guo et al., 2010; Asimakopoulou et al., 2014).

5.7 Effects of LCN2 Treatment on Peptide Hormones of Normal and Diabetic Animals

In the study, the effects of LCN2 on various other peptides that play a critical role in metabolism and metabolic diseases such as diabetes and obesity have been studied. Glucose balance can be maintained directly via regulating glucose synthesis and storage or indirectly by regulating peptide hormones that could influence glucose

levels like insulin and glucagon, based on this the effect of LCN2 treatment on insulin hormone were investigated.

The pancreas plays a key role in body metabolism by synthesizing and secreting multiple peptide hormones like insulin, glucagon and amylin. The results showed that STZ administration caused a marked decrease in insulin levels in diabetic animals compared to normal animals. Treatment of normal animals with LCN2 had no effect in insulin levels when compared to saline-treated animals, however, treatment with LCN2 caused a significant increase in insulin levels in diabetic treated animals in comparison with the saline-treated animals. This upregulation in serum insulin levels was detected only in the diabetic group which mean that enhancement of insulin secretion could be glucose-dependent as this was not found in normal animals. This result is consistent with those of (Mosialou et al., 2020) which was conducted to investigate whether LCN2 is need it for β-cell adaptation to higher metabolic load via placing murine on HFD. LCN2 stimulated and increased the secretion of insulin approximately two folds higher in the WT mice in comparison to the Lcn2-/- which was unable to elevate insulin levels to meet the elevated requirement of the metabolic demand. This insulinotropic activity of LCN2 can be by stimulating β-cell proliferation and mass or by upregulating insulin gene expression. The actual molecular mechanism remains unknown. To verify these findings, C-peptide levels were measured as well. C-peptide levels can offer a measure of pancreatic cell activity that is preferable to insulin levels (Bell & Ovalle, 2000;Bulboacă et al., 2019) and values showed similar trend to the serum insulin levels, LCN2 caused a marked elevation in C-peptide in diabetic and normal animals. The significant improvement of C-peptide shows that beta-cell function can be partially preserved by LCN2 treatment. This can constitute a valuable strategy in diabetes treatment.

Amylin, another hormone synthesized and secreted by the pancreas and has a function in glucose homeostasis through assisting insulin action in addition to its function as a satiety agent (Ping Caoa, Peter Mareka, Harris Noora, Vadim Patsalob, Ling-Hsien Tua, Hui Wanga, Andisheh Abedinic, 2013). There was a downregulation in amylin levels after STZ injection and the data shows that LCN2 resulted in significant elevation in amylin levels in diabetic group. Amylin is co-produced with insulin in the pancreatic β-cells, the effects of LCN2 on amylin had the same trend to its effect on insulin. Different other peptide hormones including GIP, GLP-1 and leptin were investigated and the findings demonstrate that LCN2 significantly elevated serum GIP levels in diabetic and normal animals. In addition, LCN2 effects on GLP-1 which also belongs to the incretins family has been studied and the result showed that diabetes mellitus induction resulted in a marked elevation in GLP-1 levels compared to normal animals. LCN2 treatment caused a decrease in GLP-1 levels in diabetic animals while no change was detected in normal animals. This result may be found opposing since GLP-1 and its agonists are used clinically in the management of diabetes mellitus and glucose levels. Having said that, these findings can be supported by the fact that GLP-1 resistances can develop due to hyperglycemia and genetic alterations in diabetic risk genes TCF7L2 and WFS1 (Heni et al., 2012)

This proposed mechanism is confirmed by multiple different studies that showed a decrease of GLP-1 (Nauck et al., 1993) stimulates efficacy in patients with diabetes mellitus. Limited knowledge is known about the correlation between LCN2 and incretins.

Leptin was also investigated as it has an important role in energy homeostasis. Diabetes mellitus induction resulted in a significant decrease in leptin serum

levels.LCN2 treatment improved leptin levels in normal and diabetic animals. Previous researches demonstrated that leptin levels elevated in normal and diabetic animals after leptin infusion inverted hyperglycemia and dyslipidemia in STZ-induced diabetes mellitus and high fat diet induced diabetes mellitus (Denroche et al., 2011). As a result, the hypoglycemic effects of LCN2 may be exerted through leptin levels improvement as the data showed in the diabetic and normal rats. Mice deficient in LCN2 showed more weight gain when placed on high fat diet (HFD), severe insulin resistance, and impaired thermogenesis (Guo et al., 2010). Moreover, osteoblast-specific knockout of Lcn2 in murine resulted in glucose intolerance, insulin resistance, and pancreatic β-cell dysfunction that caused a reduction in insulin secretion post glucose challenge (Mosialou et al., 2020).

5.8 Effects of LCN2 Treatment on Inflammatory Markers of Normal and Diabetic Animals

Inflammation could be a cause and/or a result of diabetes mellitus and it is responsible for many complications that rise as a result of diabetes such as cardiovascular, renal, retinal and neuronal complications. Inflammatory markers and mediators such as IL-6 family and other chemokines like MCP-1 are suggested to be implicated in the pathogenesis of T1DM and T2DM (Kristiansen & Mandrup-Poulsen, 2004; Padgett et al., 2013) reported that pro inflammatory cytokines are involved in pancreatic islet cells death by triggering apoptotic or necrotic processes. IL-6 ability as a molecule to regulate the glucose metabolism has also been suggested by acting directly on the pancreatic cells and other different types of cells like the skeletal muscle cells, fat cells, hepatic cells, and neuroendocrine cells (Kristiansen & Mandrup-Poulsen, 2004. Whereas MCP-1, In addition to its role as a chemoattractant protein that is secreted by inflammatory cells, it could induce cellular death by oxidative stress

pathways (Kolattukudy & Niu, 2012). Beta cells possesses a limited ability to recover from cytokine insult, through repairing DNA damage, and enhancing insulin secretion in case the cytokine action is shorter than 24 hours (Scarim et al., 1997). However, irreversible cell destruction will result after 24 hours (Scarim et al., 1997). The pro-inflammatory cytokines induce an early necrotic process of islet cells, and with prolonged cytokine action that can be turned into late apoptosis (Hughes et al., 2009). DM induced by Streptozotocin (STZ) administration is a common experimental model to investigate diabetes mellitus-associated with inflammation stimulated by the cytotoxic effect of STZ on pancreatic beta cells. Therefore, rapid mediation of pro-inflammatory cytokines is considered major contributors to decrease beta-cells destruction, caused by inflammatory process. Due to that the possible anti-inflammatory role of LCN2 was investigated in the diabetic model used. LCN2 treatment had a marked effect on IL-6 where it caused a significant reduction in the serum levels of IL-6. Moreover, MCP-1 was significantly increased after the induction of DM and LCN2 treatment caused a significant decrease. This highlights the anti-inflammatory action of LCN2.

5.9 Insulin Signaling Pathway

It has been demonstrated that PI3K/Akt signaling pathway have a crucial role in insulin signal transduction (Li et al., 2015). In this study, the expressions of key proteins in glucose metabolism pathway including p-Akt (ser473) and total Akt were studied to determine whether LCN2 treatment in STZ-induced diabetic rat had an effect on the PI3K/Akt signaling pathway. As shown in Figure (27), the levels of total Akt in the three different tissues across all of the groups remained unchanged however,

the study demonstrated a noticeable increase in the Akt phosphorylation in the three examined tissues compared to the diabetic control.

Chapter 6: Conclusion

This research provided evidence about the co-localization of LCN2 with insulin and glucagon in normal and diabetic pancreatic β-cells. In addition, LCN2 significantly decreased levels of AST, ALT, ALP, bilirubin, CHOL, TG, LDL and improved the levels of total protein, ALB and HDL and decreased levels of BUN, Urea and LIPC in diabetic animals. Moreover, the levels of insulin, c-peptide, amylin and GIP were markedly increased while GLP-1 levels were reduced and leptin levels were improved in diabetic animals treated with LCN2. Furthermore, LCN2 showed a significant antioxidant activity by increasing GSH, SOD and CAT levels in pancreas in normal and diabetic animals, as well as their levels in serum. Finally, the in vitro observations show that at a concentration of 10^{-8} M and 10^{-12} M LCN2 caused the most significant effect on insulin secretion from the INS-1 rat insulinoma cell line.

Chapter 7: Limitations

Some limitations for this study can be highlighted in this section including the model of DM used in the study, which is by STZ-induced model of DM. STZ is a toxin that is known to be highly selective for pancreatic β-cells, however, it is unknown whether STZ can affect other organs and biological functions or not. furthermore, the dose and the period of the treatment used were relatively short compared to doses and durations of treatment where LCN2 was reported to increase insulin sensitivity and weight reducing effects in literature.

In literature, LCN2 role in metabolic syndromes and energy metabolism has been studied in a knockout model however, this is the first time to use exogenous LCN2 in an in vivo study of metabolic syndrome.

Chapter 8: Advancement in the Field

Management of type 1 diabetes (T1D) is currently based entirely on insulin replacement therapy. However, there is a requirement for better glycemic control, lower hypoglycemia rates, weight management, and additional reduction of cardiovascular risk in T1D patients.

The peptide therapy approaches are leading the way in pre-clinical and clinical development for both T1D and other diseases. They are entering an exciting phase of drug development.

At the moment peptide immunotherapy for T1D is extensively researched and it is very promising since peptide immunotherapy offers the ability to re-establish immune homeostasis by elevating regulatory T-cell population and decreasing/deleting the pathogenic T-cell pool. This is the optimal solution for T1D since it triggers the primary cause of the beta cell destruction and depletion and preserve the remaining cells to protect the patient's endogenous insulin secretion. However, a key challenge in this approach is that by the time of diagnosis substantial loss of beta cells has already taken place. So, in this case some patients optimal solution will include insulin therapies with an adjunct therapy.

the study shows that peptides like Lipocalin-2 can be used as adjunctive therapy to insulin therapies for diabetes mellitus to protect from further progression of the disease and the onset of the various complications via optimizing metabolic control, weight management, limiting and reducing the glycemic variability in patients with T1D and ameliorating the signs and outcome of DM.

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