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ORAL HYPOGLYCEMIC SIGNALING PATHWAYS: UNVEILING NOVEL THERAPEUTIC TARGETS IN DIABETIC NEPHROPATHY

by
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AN ABSTRACT OF THE THESIS OF

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Title: Oral Hypoglycemic Signaling Pathways: Unveiling Novel Therapeutic Targets in Diabetic Nephropathy

Background: Diabetic nephropathy (DN) is a major chronic diabetic complication that arises from persistent hyperglycemia. It is characterised by a gradual loss of glomerular filtration surface area and capillary volume as well as expansion of mesangial matrix due to the excessive production and deposition of extracellular matrix. One unifying mechanism that is shown through extensive research to mediate DN injury is oxidative stress. However, the mechanisms have yet to be elucidated. Previous work in our lab has implicated the NADPH-oxidase (NOX) enzymes in the pathologies of DN. However, to our knowledge, no studies have examined the role of NOXs on autophagy.

Aim: The following study aimed to investigate the impact of selected Type 2 diabetes pharmacological drugs on Type 1-induced DN, as well as to dissect novel mechanisms associated with NADPH-induced oxidative stress on autophagy in DN.

Methods: Sprague-Dawley male rats were used for this study. RT-PCR was used to assess mRNA levels of PPAR-γ, Fibronectin, COL IV, Nephrin, DUOX1, DUOX2, AMPK, mTOR, and autophagy markers, LC3A, and LC3B. NOX activity was assessed using the NADPH Oxidase Activity Assay. High Performance Liquid Chromatography (HPLC) was used to assess oxidative status and ROS production in kidney tissue. Blood samples were also harvested for creatinine level analysis. Finally, histological studies were performed to illustrate pathological changes using Periodic Acid Schiff and Masson Trichrome staining.

Results: Both monotherapy and combination therapy showed no effect on the blood glucose levels. However, a restoration of excreted urinary albumin levels, glomerulosclerosis and tubulointerstitial fibrosis were demonstrated. Diabetes-induced oxidative stress was reflected via HPLC concomitant with increased NADPH oxidase activity. The administration of the drugs or their combinations were shown to reverse NOX-induced ROS production. The expression of PPARγ, Fibronectin, COL IV, Nephrin, DUOX1, DUOX2, AMPK, mTOR, and autophagy associated proteins markers LC3A and LC3B were partially reversed in the treated animals compared to non-treated STZ-induced Type 1 diabetic animals. Such data were indicative of a restoration of autophagy.

Conclusion: These preliminary findings highlight the potential benefit of specific combination therapies. This helps shed light on the common molecular mechanisms between Type 1 and Type 2 diabetes, and emphasizes their role in restoration of autophagy, a reno-protective mechanism in DN. Future studies are necessary to extend this investigation *in vitro* (cultured rat and human podocyte cells) as well as *in vivo* in additional animal models of diabetes, in order to better investigate the signaling mechanisms and any possible cross talk with other pathways of DN pathophysiology.
Key words: Diabetic Nephropathy, Autophagy, PPARγ, AMPK pathway, mTOR pathway, Metformin, Liraglutide, Pioglitazone.

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ABBREVIATIONS

AGE-RAGE:	Advanced-glycated end Products
AICAR:	5-aminoimidazole-4-carboxamide riboside
AMPK:	AMP-activated Protein Kinase
ANG II:	Angiotensin II
AUBMC	American University of Beirut Medical Centre
BUN	Blood Urea Nitrogen
C	Control
CKD:	Chronic Kidney Disease
CMA:	Chaperone mediating Autophagy
COL IV:	Collagen IV
D	Diabetic
DM:	Diabetes Mellitus
DM	Diabetic Metformin
DML	Diabetic Metformin Liraglutide
DMP	Diabetic Metformin Pioglitazone
DN:	Diabetic Nephropathy
DL	Diabetic Liraglutide
DP	Diabetic Pioglitazone
DPP-4:	Dipeptidyl peptidase-4
DPL	Diabetic Pioglitazone Liraglutide
DPML	Diabetic Pioglitazone Metformin Liraglutide
ESKD:	End Stage Kidney Disease
ESRD:	End Stage Renal Disease
GBM:	Glomerular Basement Membrane
GFB:	Glomerular Filtration Barrier
GFR:	Glomerular Filtration Rate
GIP:	Glucose-dependent insulinotropic polypeptide
GLP-1:	Glucagon-Like Peptide-1
GLUT2:	Glucose Transporter 2
HG:	High Glucose
HPLC	High Performance Liquid Chromatography
MT	Masson Trichrome

mTOR:	Mammalian Target of Rapamycin
NADPH-Oxidase:	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NF-κB:	Nuclear Factor Kappa B
NIH	National Institute of Health
PAS	Periodic Acid Schiff
PPAR:	Peroxisome Proliferator Activated Receptor
REDOX:	Renal NADPH Oxidase
ROS:	Reactive Oxygen Species
RT-PCR	Real-Time Polymerase Chain Reaction
SRNS:	Steroid-resistant Nephrotic Syndrome
STZ:	Streptozotocin
TGF-β:	Transforming Growth Factor
TZD:	Thiazolidinediones

CHAPTER I
INTRODUCTION

A. Diabetes Mellitus

Diabetes Mellitus (DM) is a chronic metabolic disorder characterized by the disruption of glucose homeostasis leading to persistent hyperglycaemia. DM is subcategorized into Type 1 and Type 2, also known as insulin-dependent and insulin-independent diabetes respectively. Secreted by pancreatic beta cells (β-cells) of the islets of Langerhans in the pancreas, insulin is a hormone that takes part in multiple roles in metabolism through regulating blood glucose levels and the storage of fat. The common ground between the two types of diabetes is an impairment in the body's response to insulin, which in turns leads to the disruption of normoglycemia (The National Institute of Diabetes and Digestive and Kidney Diseases Health Information Center, 2017). Type 1 DM is an autoimmune disorder characterised by insufficient insulin production. According to the International Diabetes Federation, statistics done in 2014 showed that approximately 382 million people worldwide lived with diabetes of which 5-10% having Type 1, while the remaining cases being Type 2 DM (You & Henneberg, 2016). Even though both types of DM may be regulated by a modified diet along with regular exercise and insulin therapy if necessary, DM is a chronic condition that is often associated with multiple lethal complications (Ergun-Longmire & Maclaren, 2000).

Both types of DM lead to various complications affecting both the small and large blood vessels which in turn lead to macrovascular and microvascular and complications (American Diabetes Association, 2017). Macrovascular complications are due to

damage of large blood vessels. Such damage results in cardiovascular complications including angina, myocardial infarction, transient ischemic attacks and strokes (Forbes, 2013). On the other hand, microvascular complications are due to damage of the small blood vessels. Such complications include retinopathy, neuropathy, and nephropathy, all referred to as the diabetic triopathy. Diabetic nephropathy (DN) is one of the most dangerous yet most commonly occurring complication that happens during the later stages of diabetes, affecting 20-40% of both Type 1 and Type 2 diabetic patients (Reutens & Atkins, 2011).

B. Diabetic Kidney and Nephropathy

1. Renal Glomerular Physiology

The kidney is a vital organ of the urinary system that is largely responsible for maintaining the body's homeostasis through the filtration of blood, regulation of water and electrolytes and keeping the acid-base balance in check (Mukhi, Nishad, Menon, & Pasupulati, 2017). The smallest functional unit of the kidneys that is responsible for removing waste products is the nephron, ensuring protein-free filtered urine (Anil Kumar, Welsh, Saleem, & Menon, 2014). Each kidney contains millions of interconnected nephrons, each of which is made up of two main structures, a glomerulus, and renal tubules. At the level of the glomerulus, blood infiltrates from the capillaries, through the glomerular filtration barrier (GFB), and then into the urinary space of the nephron. The GFB is composed of three important layers, the glomerular endothelial cells, glomerular basement membrane (GBM) and a layer of highly differentiated cells called podocytes (Smoyer & Mundel, 1998).

The glomerular endothelial cells are the innermost layer of the GFB. These specialised epithelial cells line the inner surface of the glomerular capillaries, contributing to the tight barrier between the blood and glomerular urinary space (Arif & Nihalani, 2013). Their central function is allowing the passage of plasma fluid and small solutes into the bowman's space. This is mediated by their unique fenestrated morphological characteristics (Satchell & Braet, 2009).

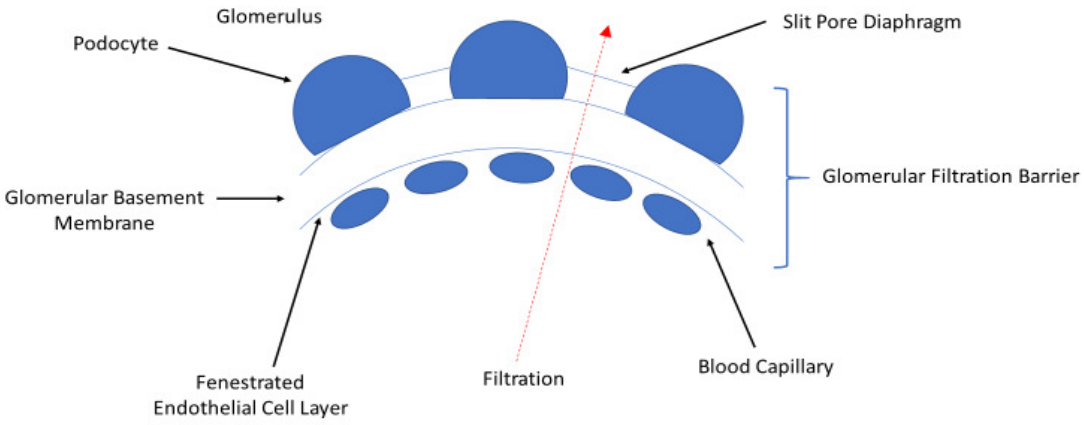


Figure 1. Schematic Diagram of the Glomerular Filtration Barrier

The GBM on the other hand is a selectively permeable barrier that sits between the endothelial cells and podocytes and functions to separate the circulating blood in the capillaries from the urinary space (Miner, 2012). It minimises the entrance of plasma proteins such as albumin into the ultrafiltrate (Haraldsson & Jeansson, 2009). The intact GBM is composed of several structural proteins that are significant for maintaining its integrity such as fibronectin, Collagen IV (COL IV), laminin, Nidogen and heparin sulphate proteoglycans (Mukhi et al., 2017) (Arif & Nihalani, 2013).

Many cell types produce fibronectin, including the glomerular mesangial cells. Fibronectin is known to be involved in in the regulation of cell survival, proliferation, and differentiation as well as numerous other cellular functions (Bentmann et al., 2010; Manabe, Oh-e, & Sekiguchi, 1999; Sens et al., 2017; von Au et al., 2013). For example, several studies have shown that the mesangial cells themselves require fibronectin to deposit collagen into the extracellular matrix (Miller, Pozzi, Zent, & Schwarzbauer, 2014; Sottile & Hocking, 2002). Such effects are facilitated by the presence of several isoform, many of which appear to be affecting cell surface receptors (Johansson & Hook, 1980). In DN, fibronectin is found to accumulate in the expanded mesangial matrix (Assad, Schwartz, Virtanen, & Gould, 1993). Collagen IV, on the other hand, is a trimeric extracellular matrix protein consisting of Gly-X-Y amino acid triplet repeats. Alterations in specific subunits of COL IV causes defects in the GBM (Arif & Nihalani, 2013). Interestingly, mutations in specific genes encoding for COL IV have been shown to trigger the severe autosomal recessive Alport syndrome, a basement membrane disease leading to kidney failure, hearing loss and eye abnormalities. Alport patients usually present with haematuria, an indication of kidney dysfunction (Miner, 2012) (Gubler, 2008) (Arif & Nihalani, 2013) (Poschl et al., 2004). Laminin, another protein which is also part of the GBM, is secreted as αβγ heterotrimers (Arif & Nihalani, 2013). The main form of laminin found in the GBM is LM-521; however, during glomerulogenesis, laminin trimer depositions are altered (Miner et al., 1997). Unfortunately, an insufficient concentration of laminin trimers can lead to damage in the GBM (Smyth et al., 1999) and may eventually result in glomerular proteinuria, haematuria, polycystic kidneys and renal failure within months (Shannon, Patton, Harvey, & Miner, 2006).

Finally, the outermost layer of the glomerulus that plays an important role in sustaining the GFB and contributes substantially to the development of glomerular pathologies constitutes the podocytes (Burford et al., 2017). Podocytes are terminally differentiated cells with a limited capability to reproduce and are mainly characterised by their complex cytoskeleton. Their cell bodies, from which primary extensions arise, adhere to the GBM through numerous foot processes (Ahola et al., 2003). These processes continuously crosslink between neighbouring podocytes, forming the slit pores just above the GBM (Khurana, Bruggeman, & Kao, 2012). The main function of the podocytes is to provide an epithelial protective layer on the surface of the glomerular capillaries. They accomplish this through the interaction between two main proteins: nephrin and podocin.

Within the kidneys, the molecular protein nephrin is predominantly found at the foot processes of the glomerulus. In 1998, the integral transmembrane protein nephrin was the first protein to be identified as part of the slit pore diaphragm (Kestila et al., 1998). It mainly functions to preserve a normal slit pore diaphragm and facilitates vital cell signalling pathways within podocytes (Li & He, 2015). Nephrin has demonstrated its ability to form disulfide bridges between nephrin molecules or between other slit pore diaphragm proteins such as podocin (Kestila et al., 1998; Tryggvason, 1999). Damage to nephrin however is not an indication of loss of the podocytes viability. This therefore suggests that nephrin may be compensated for by other proteins throughout glomerular development (Done et al., 2008).

Like nephrin, podocin aids in the formation of the slit pore diaphragm (Mulukala et al., 2016). Studies have shown that mutations in the genes coding for podocin affect the structure of the slit pore diaphragm and disrupts the podocytes cytoskeleton through

hindering normal interactions with the extracellular matrix and vital cell signalling pathways (Gigante, Piemontese, Gesualdo, Iolascon, & Aucella, 2011). Indeed, mutations in podocin have been linked to severe renal disorders such as steroid-resistant nephrotic syndrome (SRNS), a disease that mainly occurs in children and is characterized by heavy proteinuria, hypoalbuminemia, and oedema. Unfortunately, SRNS has poor prognosis and may often lead to end-stage renal disease (ESRD) (Mulukala et al., 2016). Unfortunately, the gene expression of podocin *(NPHS2 gene)* is the most regularly mutated gene that leads to SRNS accounting for 18% of the total cases (Benoit, Machuca, & Antignac, 2010; Boute et al., 2000). Altogether, these findings show the importance of glomerular cellular arrangements, structure, and proteins in mediating the physiological role of renal filtration and maintaining kidney function and integrity. Hence, alterations in any of these proteins or layers may insult the renal system, leading to several renal pathologies.

2. Glomerulopathy and Diabetes

Extensive research has been done to show the implications of DM and associated hyperglycaemia on kidney structure and function but only a limited number of studies focused on elucidating the specific pathways involved. Unfortunately, the risk of developing DN has increased over the years with DN now accounting for 20-40% of the mortality from DM (Mukhi et al., 2017). DN begins with albuminuria that develops from microalbuminuria, an early indicator of kidney pathology, into macroalbuminuria (Battisti, Palmisano, & Keane, 2003). When proteins can no longer be efficiently prevented from entering the initial filtrate, proteinuria develops indicating damage to the GFB. In fact, proteinuria is the most common cause of chronic kidney failure and end stage kidney disease (ESKD), accounting for 40% of all cases of ESKD (Lim,

2014). Proteinuria is associated with a persistent loss of glomerular filtration surface area and capillary volume, which may be later followed by excessive production and deposition of extracellular matrix proteins and hence expansion of the mesangial matrix (Steffes, Osterby, Chavers, & Mauer, 1989). Moreover, DM has been shown to provoke changes within the complex cytoskeleton of the podocytes leading to foot processes effacement that would compromise the podocytes function thus resulting in proteinuria (Pavenstadt, Kriz, & Kretzler, 2003). Additional studies have shown that DM may cause a decrease in glomerular podocyte density, which is thought to lead to scar tissue formation and reduce the glomerular filtration rate (Kato & Susztak, 2012; Reidy, Kang, Hostetter, & Susztak, 2014).

Taken together, a large body of data describes the GFB malfunction as the most prominent damage in DN. Current reports conclude that the development of DN is irreversible, with its outcome being either dialysis or kidney transplantation. Therefore, targeting diabetic related complications such as DN at molecular and cellular levels is a high priority. Nevertheless, this requires a clear understanding of the mechanisms through which hyperglycaemia induces kidney injury and the means by which this injury progresses. One possible explanation is that hyperglycaemia triggers a state of oxidative stress, characterized by the production of reactive oxygen species (ROS). Indeed, oxidative stress has now been established as the final common mediator of all diabetic complications and their etiologies.

C. Reactive Oxygen Species: Sources and Significance

Reactive oxygen species are small and highly reactive oxygen-containing molecules (Bedard & Krause, 2007) that may either be produced by direct sources or as a by-

product of several biochemical reactions (Thannickal & Fanburg, 2000) (Klebanoff, 1980). ROS are known to interact with countless organic and inorganic molecules in the body. Such interactions may lead to the destruction or modification of various cellular entities such as lipids and proteins. ROS also disrupts several homeostatic and regulatory processes in nearly all cells and tissue (Bedard & Krause, 2007) by interfering with molecules that are important for gene expression and signal transduction such as secondary messengers and mediators of cellular pathways (Turpaev, 2002). Thus, ROS generation is thought to be a major contributor to the damage in biological organisms (Harman, 1956) and is consequently tightly regulated by cellular antioxidant mechanisms (Turpaev, 2002).

The generation of ROS occurs within many cellular organelles including the mitochondria and peroxisomes (Bedard & Krause, 2007) (Balaban, Nemoto, & Finkel, 2005). Extensive research has identified numerous cellular sources of ROS which have been investigated in a disease-specific manner. These sources include advanced-glycated end products (AGE-RAGE), Cytochrome-P450s, Xanthine oxidases, cyclooxygenases and lipoxygenases, nitric oxide synthase, oxidative-phosphorylation mitochondrial enzymes and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidases/ NOXs) (Niedowicz & Daleke, 2005). Many of these sources are involved in the pathogenesis of diabetic complications such as cardiomyopathy, retinopathy, neuropathy and nephropathy (Lambeth, Krause, & Clark, 2008) (Maalouf et al., 2012).

1. Oxidative Stress in Diabetic Nephropathy

Oxidative stress is a fundamental imbalance between ROS production and the ability of the body to rid itself of the excess ROS through antioxidants (Betteridge,

2000). It is actually thought that cellular injury by oxidative stress is due to the imbalance in intracellular oxidation-reduction state, created by a decrease in intracellular transport of ROS (Root-Bernstein R, 2002). Several findings have suggested that oxidative stress plays a central role in the pathogenesis of DN (Vasavada & Agarwal, 2005) mainly by targeting specific structures in the kidney such as the glomerulus and the tubulointerstitium along with its vascularisation (Obrosova IG, 2003).

Low level ROS production has been shown to be involved in growth factor signalling, mitogenic responses, apoptosis and oxygen sensing (Geiszt M, 2000). Furthermore, ROS is thought to mediate extensive biological injury such as peroxidation of cell lipids, oxidation of proteins and mutation cleavage of DNA (Niedowicz & Daleke, 2005). ROS is also known to induce transcription factors such as hypoxia-inducible factor alpha and nuclear factor Kappa B (NF-κB), encouraging cellular proliferation and hypertrophy (Studer RK, 1997) as well as inducing renal vasoconstriction by the peroxidation of arachidonic acid (Montero A, 2000).

Multiple human studies demonstrated that patients with DM have elevated levels of superoxide dismutase, an enzyme that plays a critical role in the cellular REDOX (Renal NAPDH Oxidase) balance. However, such studies had contradictory outcomes revealing either an increase or a decrease in antioxidant activities in diabetic patients (Helmersson J, 2004) (Giugliano D, 1996). These findings therefore highlight just how complex oxidation-reduction reactions are in diabetic patients compared to normoglycemic patients. Additional studies conducted implied that patients with severe proteinuria experience higher levels of superoxide-induced DNA damage compared to those with microalbuminuria (Shimoike T, 2000).

D. Reactive Oxygen Species and NOX in Diabetic Nephropathy

Nicotinamide adenine dinucleotide phosphate-oxidase is an enzyme that catalyses the addition of an electron to oxygen, producing superoxide (Haugen E, 1999). Several pathways may be activated due to high glucose (HG) within the cell resulting in harmful outcomes, one of which is the NADPH pathway. NADPH is required in the polyol pathway, reducing glucose into sorbitol for the production of glutathione (Bernobich et al., 2004). Renal NADPH oxidase, a primary source of ROS in the kidney, is shown to be highly expressed in the proximal convoluted epithelial cells of the renal cortex (Niedowicz & Daleke, 2005) (Geiszt M, 2000).

Transmembrane proteins that are referred to as NOX, are members of the NADPH oxidase family. Such proteins function by transferring electrons across a biological membrane, reducing oxygen supply to superoxide. Therefore, the biological function of NOX enzymes is to generate ROS (Bedard & Krause, 2007). Over time, several subunits of NOX were identified. NOX2 was first identified as an enzyme component (Bedard & Krause, 2007). Later, homologs of NOX2 were discovered and referred to as NOX1 (Banfi et al., 2000; Bedard & Krause, 2007) which was then followed by NOX3 (Cheng, Cao, Xu, van Meir, & Lambeth, 2001; Kikuchi, Hikage, Miyashita, & Fukumoto, 2000), NOX4 (Geiszt, Kopp, Varnai, & Leto, 2000; Shiose et al., 2001), and NOX5 (Banfi et al., 2001; Cheng et al., 2001). Two large thyroid oxidases, DUOX1 and DUOX2, were also added as members of the NOX family (De Deken et al., 2000; Dupuy et al., 1999).

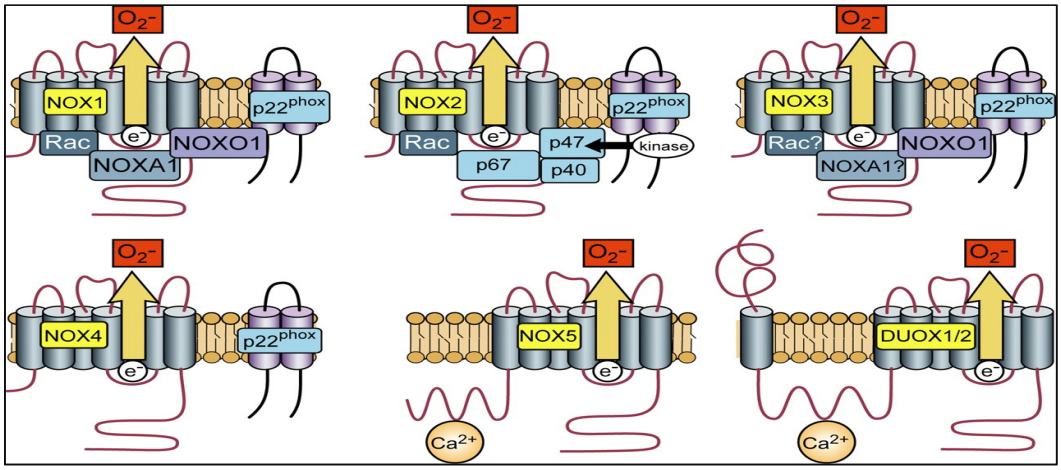


Figure 2. NADPH oxidase family of enzymes (Bedard & Krause, 2007)

There are several expressed isoforms of NOX in the kidney cortex such as NOX1, NOX2 and NOX4, with the latter being the most abundant (Bedard & Krause, 2007). NOX4 is mainly localised in renal tubule cells (Geiszt et al., 2000; Shiose et al., 2001) and to a lesser extent in the glomerular mesangial cells (Gorin et al., 2003). NOX4 overexpression in rat and mouse glomeruli has been shown to induce matrix expansion in diabetic kidney disease (A. A. Eid et al., 2009; Gorin et al., 2005). It comes as no surprise then that HG increases the expression of NOX4 and NADPH oxidase activity within the podocytes (A. A. Eid et al., 2009).

The main functions of NOX in the kidneys are characterised into three categories. NOX enzymes, a possible source of ROS, are involved in the regulation of renal blood flow (Lopez, Salom, Arregui, Valero, & Fenoy, 2003). They are also able to alter renal cell fate in multiple mechanism (Lodha et al., 2002; Rhyu et al., 2005) one of which is through the activation of ERK1/ERK2 pathway (Gorin et al., 2004).

Additionally, NOX-dependent oxidative activation of transcription factors, such as NF-κB, is said to enhance the regulation of renal gene expression (Dorsam et al., 2000).

NOX-derived ROS is greatly recognised to play a role in DN (Han, Lee, Park, Lee, & Taub, 2005; H. B. Lee, Yu, Yang, Jiang, & Ha, 2003). It has been debated that NADPH oxidase inhibitors may prevent renal damage in diabetic animals (Asaba et al., 2005), however it is still not confirmed what NOX isoforms participate in mediating the ROS-dependent tissue damage observed (Bedard & Krause, 2007). Several studies have shown that there is an increased production of NOX4 mRNA in DN and that treatment with NOX4 antisense RNA in diabetic animals decrease kidney damage (Gorin et al., 2005). However, more studies are needed to confirm the latter. Furthermore, under HG conditions, ROS has been shown to trigger podocyte apoptosis, reducing cell count, and contributing to DN (Susztak, Raff, Schiffer, & Bottinger, 2006). Even though the decrease in number of podocyte is a strong indicator of DN, the mechanism at which podocyte reduction occurs as well as its dysfunction remains to be defined.

E. AMP-activated protein kinase (AMPK) Pathway

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/ threonine kinase (Hardie & Carling, 1997; Sarbassov, Guertin, Ali, & Sabatini, 2005) that functions as an energy sensor and its activity is controlled by the levels of glucose (Long & Zierath, 2006). It is activated by the imbalance in AMP: ATP and ADP: ATP ratios in an attempt to restore energy homeostasis in the cell (Rena, Hardie, & Pearson, 2017). There are several subunits of AMPK that are all cell and tissue specific. The two α gene subunits (α1 and α2) for example are highly expressed in the kidney such as in the glomerular cells (Cammisotto & Bendayan, 2008), where phosphorylation of a

threonine residue (Thr^{172}) is needed for the activation of AMPK (Hawley et al., 1996). As the intracellular levels of ATP decrease, the cellular concentration of AMP increases leading to the activation of AMPK through several mechanisms (Hardie, 2007; Hardie & Hawley, 2001). Upon activation of the AMPK pathway, many other biological pathways are modulated such as protein synthesis (M. J. Lee et al., 2007), autophagy (Matsui et al., 2007; Meijer & Codogno, 2007) and apoptosis (Riboulet-Chavey, Diraison, Siew, Wong, & Rutter, 2008). In 2010, a study was performed demonstrating that the inactivation of AMPK due to HG levels increases the expression of Nox4, which would lead to podocyte apoptosis through the production of ROS (A. A. Eid et al., 2010).

Multiple metabolic stressors may activate AMPK such as hypoxia, ischemia, oxidative and hyperosmotic stresses, as well as glucose deprivation (Carling, 2004; Hardie, 2003, 2004). Upon activation of AMPK, catabolic pathways are triggered to produce ATP whereas anabolic pathways are switched off to avoid consuming ATP, therefore maintaining cellular energy storage (Hardie, 2003). The activation of AMPK may also be accomplished pharmacologically through treating cells with an artificial activator known as 5-aminoimidazole-4-carboxamide riboside (AICAR), an adenosine analogue taken up by the cells and then phosphorylated (Corton, Gillespie, Hawley, & Hardie, 1995).

F. mTOR Signalling Pathway

Cellular growth, survival and metabolism are controlled by the mammalian target of rapamycin (mTOR) signalling pathway (Wullschleger, Loewith, & Hall, 2006). mTOR, also a serine/threonine protein kinase, is characterised into two

complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Dann, Selvaraj, & Thomas, 2007). mTORC1 contains important elements that dictate cell size and mediate protein synthesis (Wullschleger et al., 2006) (S. Eid et al., 2016). Podocyte apoptosis induced either by Type 1 diabetes *in vivo* or due to HG concentrations *in vitro* has been recently linked to the activation of mTORC1 pathway and subsequent inactivation of the AMPK/tuberin pathway (A. A. Eid et al., 2013). Furthermore, the activation of mTORC1 enhances oxidative stress through upregulating Nox1 and Nox4 expressions and increasing NADPH oxidative activity (A. A. Eid et al., 2013) (A. A. Eid et al., 2010) (A. A. Eid et al., 2009). Indeed, inhibiting mTOR via rapamycin dramatically reduced glomerular epithelial cell apoptosis, thickening of the GBM and foot process effacement and attenuated mesangial expansion and albuminuria, all hallmarks of DN (A. A. Eid et al., 2013).

On the other hand, minimal data is available concerning the function of the rapamycin-insensitive complex mTORC2 with regards to glomerular maintenance and glomerulopathy (Godel et al., 2011) (Grahammer, Wanner, & Huber, 2014). Nonetheless, mTORC2 has been shown to be involved in controlling cell survival and cytoskeletal structure (Wullschleger et al., 2006).

G. AMPK/mTOR Pathways and Kidney Pathophysiology

AMPK is considered a critical player in the regulation of energy homeostasis in the kidney. In DM, AMPK activity is reduced but the mechanism by which this occurs is still unclear (Cammisotto, Londono, Gingras, & Bendayan, 2008) (M. J. Lee et al., 2007). The reduced activity of AMPK was associated with diabetes induced renal hypertrophy (M. J. Lee et al., 2007). Research has shown that there is a crosstalk

between both AMPK and mTOR signalling pathways. Upon its activation, AMPK inhibits specifically mTORC1 and its downstream signalling, although the mechanism through which this happens is still not clear.

The mTOR pathway may be activated by hyperglycaemia through the activation of the Akt/protein kinase B pathway and the inhibition of AMPK, therefore leading to features characteristic of DN including thickening of the GBM and accumulation of mesangial matrix (Lieberthal & Levine, 2009). Metformin, AICAR and other AMPK activators have been shown to increase AMPK phosphorylation, inhibit HG-induced protein synthesis and prevent HG-induced changes in the signalling pathway downstream of mTORC1 in cultured glomerular epithelial cells (M. J. Lee et al., 2007).

H. Autophagy

Derived from the Greek meaning 'self-eating', autophagy is mainly detected by its degradation of the mitochondria and other intracellular structures within the lysosomes of a rat liver perfused with glucagon (Deter & De Duve, 1967). In fact, autophagy was initially touched upon in 1962 by Ashford and Portein using liver cells but no breakthroughs were made. It was not until 1993 when autophagy related genes (Atg) were discovered in Saccharomycetes. Uncovering the molecular mechanism of autophagy is still in process and to date, there are thirty-two different autophagy related genes (Nakatogawa, Suzuki, Kamada, & Ohsumi, 2009).

Autophagy starts as a phagophore, an isolation of the membrane, which then expands to engulf the intracellular components of interest such as protein aggregates, organelles, or ribosomes. This sequesters the intracellular components into a double-membraned autophagosome that in turn matures and fuses with a lysosome, leading to

the degradation of the autophagosomal components by the action of the lysosomal acid proteases. The lysosome then transports the amino acids and other by-products of the degradation out of the cytoplasm, ready to be recycled and reused during cellular metabolism (Mizushima, 2007).

Autophagy is classified into three types: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy is when an autophagosome fuses to a lysosome, forming an autolysosome. Microautophagy on the other hand happens when cytosolic components are taken up by a lysosome via invagination of the lysosomal membrane. The third classification is CMA. Here, proteins are translocated across a lysosomal membrane in a complex with a chaperone protein, such as Hsc-70, that is recognisable by the lysosomal receptor-associated membrane protein 2A (LAMP-2A), finally ending being degraded (Saftig, Beertsen, & Eskelinen, 2008).

This whole process is assembled from several key stages starting with phagophore formation, Atg5–Atg12 conjugation, interaction with Atg16L and multimerization at the phagophore, LC3 processing and insertion into the extending phagophore membrane, capturing of random or selective targets for degradation, fusion of the autophagosome with the lysosome and finally proteolytic degradation by lysosomal proteases of engulfed molecules (Glick, Barth, & Macleod, 2010).

1. Autophagy and Diabetic Nephropathy

As previously mentioned, DM is a metabolic dysfunction that induces cell stress in almost all body tissues. In addition, DM impairs autophagy by activating mTORC1 and decreasing the activity of AMPK (Kume, Koya, Uzu, & Maegawa, 2014). In fact,

recent research has suggested that this consequent impairment of autophagy is involved in the pathogenesis of many diabetic complications including DN (Tanaka et al., 2012). When renal cells are under stress due to conditions such as hyperglycaemia or proteinuria, the defence mechanisms of the cell such as autophagy are compromised. Consequently, autophagy may preserve podocytes among other renal cells including the mesangial, glomerular endothelial and tubular epithelial cells against the diabetes-associated stressors. Thus, regulating autophagy could possibly bear a therapeutic potential against DN (Kitada, Ogura, Monno, & Koya, 2017).

Normally, renal function declines with age and glomerular, vascular and interstitial scarring increases in the kidney (Coresh J, 2003). In DM patients, there are many additional unfavourable conditions that inevitably promote intracellular stressors such as oxidative stress which may further enhance and speed up the aging process of the kidneys. Thus, seizing the aging process may be beneficial for preserving renal function in DN (Jha JC, 2016).

2. PPARγ and Diabetic Nephropathy

Peroxisome proliferator activated receptors (PPARs) are part of a nuclear receptor superfamily that are described as ligand-activated transcription factors (Sher, Yi, McBride, & Gonzalez, 1993). There are three isotypes of PPARs identified: PPARα, PPARβ/δ and PPARγ (Dreyer et al., 1992; Issemann & Green, 1990). PPARγ, the main isoform discussed in this paper, is highly expressed in adipose tissue and to a lesser extent in the heart, colon, spleen, intestine, skeletal muscles and the kidneys (J. P. Berger, Akiyama, & Meinke, 2005). In addition to its well-known role in metabolic processes, PPARγ was shown to play a role in adipogenesis and insulin sensitivity (J.

Berger et al., 1999; Lehrke & Lazar, 2005). The target genes that are thought to be involved upon PPARγ activation include those related to glucose-sensing by the pancreatic β-cells in DM patients (Altan et al., 1997; Guan et al., 2002). For example, PPARγ is known to directly activate the glucose transporter 2 (GLUT2), a transmembrane protein carrier that allows glucose movement across the cell membrane and β-glucokinase (β-GK), an enzyme that functions as a glucose sensor (H. I. Kim & Ahn, 2004).

PPARγ agonists are a class of drugs that were developed based on the relationship between PPARγ and diabetes in animal models (Agarwal & Garg, 2006; Knouff & Auwerx, 2004; Savage et al., 2003). A group of thiazolidinediones (TZD) that are highly selective to PPARγ improves insulin sensitivity of adipocytes, muscles, and macrophages and hence, increases their glucose uptake. TZD also inhibit gluconeogenesis within the liver, thus resulting overall in lowering blood glucose levels (H. I. Kim & Ahn, 2004; Oberbach et al., 2006).

I. Types of Treatments Available for Patients with Diabetic Miletus

The therapeutic drugs used for Type 2 DM patients include metformin, TZD, sulphonylureas, meglitinides and insulin. Excluding TZDs, all the mentioned drugs need dose adjustments and sometimes immediate suspension in DM patients with a reduced glomerular filtration rate (GFR). Even though such drugs show beneficial effects, they may sometimes lead to several unfavourable conditions such as lactic acidosis by metformin or hypoglycaemia due to insulin (Di Lullo et al., 2017).

1. Metformin and Diabetic Nephropathy

Metformin is a drug derived from galegine, a natural product from the plant *Galega officinalis* (Rena et al., 2017). It is part of the biguanide family and is mainly described as an effective antihyperglycemic agent. Metformin decreases hepatic glucose production via inhibition of mitochondrial respiratory-chain complex 1, which in turn activates AMPK (Benoit Viollet, 2012). In 2001, a study reported that high levels of metformin for a short period were needed for the activation of AMPK in rat hepatocytes *in vivo,* however when the duration was prolonged, the amount of metformin required was significantly reduced (Zhou et al., 2001). Even though metformin has been on the market since the 1950's, its molecular mechanism of action is still debatable.

Since metformin is a well-known anti-hyperglycaemic drug, it is therefore an effective anti-diabetic agent for patients with Type 2 DM, lowering blood glucose levels through the activation of AMPK pathway (Ravindran, Kuruvilla, Wilbur, & Munusamy, 2017). It has also been thought that metformin may be used as a potential therapy in Type 1 DM, showing similar effects on glycaemia. Although the use of Metformin in Type 1 DM is not as frequent, it has indeed proved to enhance glucose control in individuals that are considered to be overweight or obese (Livingstone, Boyle, & Petrie, 2017).

2. Liraglutide and Diabetic Nephropathy

The metabolic hormone incretin acts by decreasing blood glucose levels when released into the blood after a meal. It achieves that through controlling the secretion of insulin or inhibiting glucagon release from the islets of Langerhans. Both intestinal

peptides glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are incretins and they happen to be inactivated by the same enzyme dipeptidyl peptidase-4 (DPP-4) (Drucker & Nauck, 2006).

Liraglutide is a drug derived from the metabolic hormone incretin, GLP-1. It acts as a receptor agonist that stimulates insulin secretion. Liraglutide is an injectable agent that works by lowering glucose for patients with Type 2 DM eventually reducing the development of macroalbuminuria via mechanisms that are still unclear (MacIsaac, Jerums, & Ekinci, 2017). There has also been evidence showing beneficial effects of liraglutide in STZ-induced Type 1 diabetic rats on both cardiovascular and renal levels however the exact mechanisms behind these effects are still not fully elucidated (Hendarto et al., 2012). Unfortunately, the GLP-1 peptide has a short half-life and is rapidly degraded by the enzyme DPP-4. Hence reducing the duration of action of Liraglutide. Several approaches have been taken to develop an improved GLP-1. One approach was developing a receptor that is resistant to the enzyme DPP-4, prolonging the half-life of GLP-1. Another attempt was to create inhibitors of the DPP-4 enzyme or alternatively increase plasma levels of GLP-1 (Lovshin & Drucker, 2009). In both cases, the treatment found to inhibit the sodium-hydrogen ion exchanger within the proximal tubules therefore increasing sodium excretion as well as generating a tubulo-glomerular feedback, constricting afferent glomerular arterioles. Such protective measures are likely due to the decrease in oxidative stress, inflammation, and glomerulosclerosis (Lytvyn, Bjornstad, Pun, & Cherney, 2016). However, the mechanism through which liraglutide reduces the progression of macroalbuminuria continues to be unidentified but is most likely associated with the improvement of

metabolic control and modulation of the inflammatory pathways (MacIsaac et al., 2017).

3. Pioglitazone and Diabetic Nephropathy

Pioglitazone is a drug that belongs to the TZD family. It is a synthetic ligand for PPARs that are used as anti-diabetic drugs to treat DM (Smith, 2001). It acts by altering protein synthesis and the transcription of genes that influence carbohydrate and lipid metabolism. Pioglitazone is also known to enhance glycaemic control in patients with Type 2 DM by increasing insulin sensitivity via its action at PPARγ-1 and PPARγ-2. Such interactions result in increasing glucose transporters 1 and 4, decreasing free fatty acids and remodel adipose tissue. Ultimately, this increases glucose uptake and its use in the peripheral organs and decreases gluconeogenesis in the liver, thus reducing insulin resistance as a final outcome (Smith, 2001). In advanced stages of chronic kidney disease (CKD), administration of pioglitazone does not have to be adjusted as it is nearly completely metabolised by the liver ("KDOQJ Clinical Practice Guidlines for Diabetes and CKD," 2012). In STZ-induced Type 1 diabetic rats, TZD compounds showed to prevent glomerular hyperfiltration, albuminuria and excessive production of extracellular matrix proteins in the glomeruli (Haneda, Koya, & Kikkawa, 2001). Data on pioglitazone as an administered drug is unfortunately limited. For example, there are no known side effects with patients undergoing haemodialysis. However, clinical studies have shown possible peripheral oedema both when pioglitazone is administered as either a monotherapy or as a combination with other drugs (Di Lullo et al., 2017).

J. Hypothesis and Aim of the Study

Diabetic complications such as DN are likely to lead to organ damage and homeostatic imbalances and therefore, controlling blood glucose levels is a high priority. However, strict control is somewhat difficult to achieve and is fraught with complications. Thus, it is crucial to discover and implement new ways of treatment that can, alongside the control that can be achieved, reverse, or at least halt these chronic complications. In the context of searching for new and effective treatments for DN, this study aims to identify the role of NADPH-induced oxidative stress and its crosstalk with different signalling molecules in autophagy. The method of investigation was mainly administering drugs that are already FDA approved for the treatment of Type 2 DM into STZ-induced Type 1 diabetic rat models and considering their effect on the kidneys. The purpose of this study was to elucidate some of the possible mechanistic roles through which these drugs might affect podocyte cell proliferation, extracellular matrix protein accumulation and oxidative stress induction in a diabetic setting. Indeed, identifying the mode of action of these drugs helps understand more their potentials and limitations and opens doors to new research insights that can be game changers in treating DN in both types of DM.

We hypothesized that diabetes induces AMPK inactivation, GLP-1, and PPAR-ϒ alteration, that are orchestrated by NADPH oxidases. The use of Metformin, Liraglutide, and Pioglitazone signals to reverse the seen alterations, reduce NADPH oxidase-induced ROS production, and reverses autophagy alteration that lead to kidney injury.

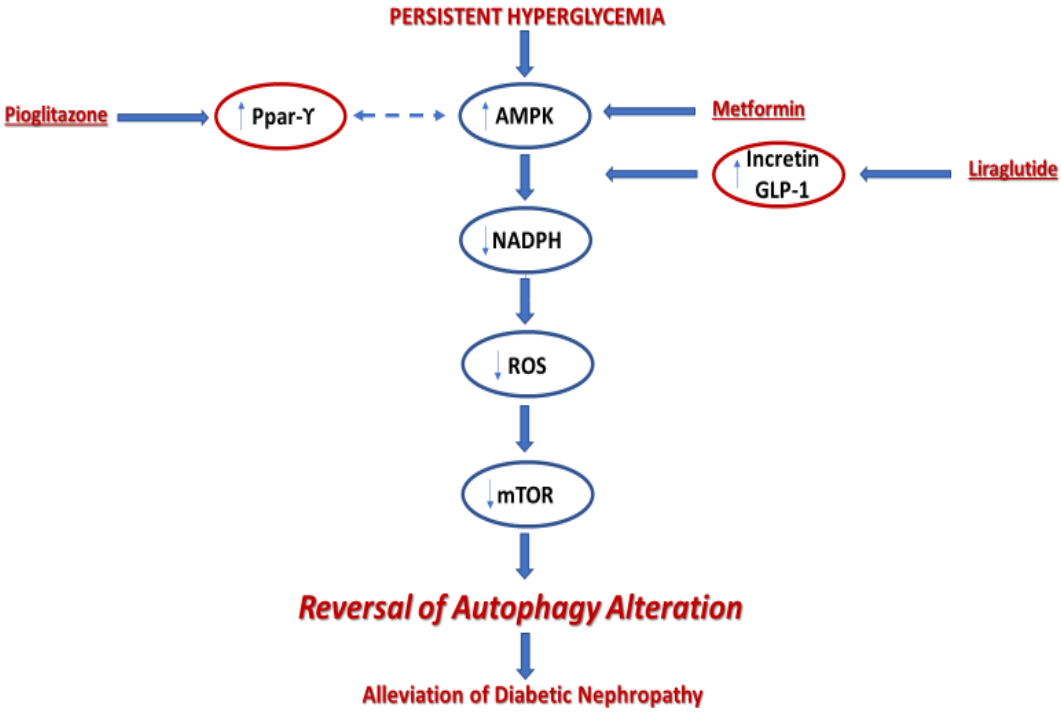


Figure 3. Hypothesis

CHAPTER II

METHODS AND MATERIALS

A. Animal Model

All animal procedures were directed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee (IACUC) at the American University of Beirut. The animal model of diabetes used for this study is Streptozotocin (STZ)-induced male Sprague-Dawley rats.

Male Sprague-Dawley rats weighing between 200-225g received a single 55 mg/kg body weight intravenous injection of STZ (Sigma-Aldrich, Steinheim, Germany) dissolved in sodium citrate buffer (0.01 M, pH 4.5) on day zero. Controls received similar injections of citrate buffer. Glucose measurements were taken three days after the STZ injection and blood was obtained via tail vein punctures and a glucometer (Accuchek, Roche). Rats with a fasting blood glucose of $\geq$ 250 mg/dl were considered diabetic. Blood glucose levels were monitored on regular basis and they were significantly different in diabetic animals relative to their control littermates. Five days after administration of STZ injection, different treatments were given to each group accordingly for a duration of fifty-seven days. On day fifty-seven, the rats were sacrificed, and the liver, pancreas, heart, aorta, sciatic nerve and kidneys were harvested and appropriately stored for later evaluation. For the focus of this study, the kidneys were processed for both histological and molecular assessment.

Animals were divided into nine groups each containing three rats: I, control rats (C); II, diabetic rats (D) and seven diabetic groups that were given the following treatments:

-Group III: 150 mg/kg body weight of Metformin, administered once daily by intraperitoneal injection (DM).

-Group IV: 5 mg/kg body weight of Pioglitazone, administered once daily by intraperitoneal injection (DP).

-Group V: 0.3 mg/kg body weight of Liraglutide, administered twice daily by subcutaneous injection (DL).

-Group VI: 150 mg/kg body weight of Metformin and 5 mg/kg body weight of Pioglitazone, both administered once daily by intraperitoneal injection (DMP).

-Group VII: 150 mg/kg body weight Metformin and 0.3 mg/kg body weight of Liraglutide, administered once daily by intraperitoneal injection and twice daily by subcutaneous injection respectively (DML).

-Group VIII: 5 mg/kg body weight of Pioglitazone and 0.3 mg/kg body weight of Liraglutide, administered once daily by intraperitoneal injection and twice daily by subcutaneous injection respectively (DPL).

-Group IX: 150 mg/kg body weight Metformin, 5 mg/kg body weight of Pioglitazone and 0.3 mg/kg body weight of Liraglutide. Both Metformin and Pioglitazone were administered once daily by intraperitoneal injection and Liraglutide was administered twice daily by subcutaneous injection (DPML).

Throughout the duration of the experiment, all animals were kept in a temperature-controlled room and on a 12/12-dark/light cycle and were provided access to standard chow and water.

B. Physical and Biochemical Analysis

Body weight as well as blood glucose levels were checked every 48 hours throughout the duration of the experiment. Blood samples were withdrawn from the tail vein and glucose levels were determined using the glucometer system Accu-check. The rats were initially considered diabetic when fasting glycaemia levels were ≥ 250mg/dL. At day fifty, rats were kept in metabolic cages for 24 hours. Urine samples were then collected, and the volume of water consumed was measured. Creatinine concentrations, Blood Urea Nitrogen (BUN) levels and (High Performance Liquid Chromatography) HPLC levels were determined by the medical laboratory at the American University of Beirut Medical Center (AUBMC).

C. Real Time-Polymerase Chain Reaction

mRNA levels in cells of the renal cortex were examined by real-time (RT-PCR) using the $\Delta\Delta C_t$ method. Total RNA was extracted from the renal cortex lysate using a TRIZOL reagent (Sigma Aldrich, Steinheim, Germany) and then converted into cDNA using the Revert First Strand cDNA Synthesis Kit according to the protocol. Quantification of cDNA was performed using RT-PCR Bio-Rad CFX96 with SYBR green dye and rat RT^2qPCR Primers (Integrated DNA Technologies, Inc., Coralville,

IA, USA) for fibronectin, Nephrin, LC3A, LC3B, AMPK, DUOX1, DUOX2 and mTOR. GAPDH was used as internal reference gene.

PRIMER	SEQUENCE
Fibronectin (rFn)	F: 5' -CGGGAACATCATCGGATCGT-3' R: 5' -GGAGAACCAGGAGAGCACAC-3'
Nephrin (rNph)	F: 5' -GTGACCTCAGTGATGACGCA-3' R: 5' -TAGGAGACACAAGCTCGGGA-3'
LC3A (LC3A)	F: 5' -ACCAGCCAGCATACCAAGTC-3' R: 5' -CCAGCACCCAAAAGAGCAAG-3'
LC3B (LC3B)	F: 5' -CGGGTTGAGGAGACACACAA-3' R: 5' -GAAGGTCTTCTCGGACGGC-3'
AMPK (AMPK-a1)	F: 5' -GCAGTTGCCTACCACCTCAT-3' R: 5' –GTACGCCTTGGTGTTTGGAT-3'
DUOX1 (DOUX-1)	F: 5' –AGGCACTGGTGGAAAACATC-3' R: 5' –GGAGAAAAGGTGCCTGAAAA-3'
DUOX2 (DOUX-2)	F: 5' –GAAGTCCACAGCAGCATCAA-3' R: 5' –CCACGGACATTGAAGAAACC-3'
mTOR	F: 5' –GCTGATTCGAGTAGCCATCC-3' R: 5' –GACGTTTCCTTCAGGGTCTG-3'
GAPDH	F: 5' -GGGGCTCTCTGCTCCTCCTG-3' R: 5' -CGGCCAAATCCGTTCACACCG-3’

Table1: List of Primers used in RT-PCR

D. Histochemistry Analysis

Kidney cortex tissues of the three rats from each group were fixed in a 4% formalin solution and embedded in paraffin block. The samples were then cut into 6 µm thick sections and placed on glass slides. The kidney sections were stained with Periodic acid Schiff (PAS) reagent to assess mesangial matrix accumulation and Masson Trichrome (MT) staining was used to evaluate the COL IV fiber deposition. A quantitative measurement of four randomly sampled glomeruli and four random samples from the proximal tubular areas was performed for three rats from each group by a blinded observer using Image J software.

E. NADPH Oxidase Analysis

NADPH oxidase activity was analysed and calculated in kidney cortex homogenates via the lucigenin-enhanced chemiluminescence method. The protein was extracted from the kidney cortex using a cooled mortar and pestle by smashing the frozen kidney tissue and suspending the remnants in lysis buffer. To commence the assay, 20 µg of each homogenate were added to 50 mmol/l phosphate buffer, pH 7.0, containing 1 mmol/l EGTA, 150 mmol/l sucrose, 5 µmol/l lucigenin, and 100 µmol/l NADPH. Photon emission expressed as relative light units were measured every thirty seconds for ten minutes in a luminometer. A buffer blank (<5% of the cell signal) was then subtracted from each reading. Superoxide production was expressed as relative light units per milligrams of protein.

F. Detection of Intracellular Superoxide in Kidney Cortex Using HPLC

Using kidney cortex tissue, cellular superoxide production was evaluated by HPLC analysis of dihydroethidium (DHE)-derived oxidation products. HPLC-based assay permits the separation of the superoxide-specific 2-hydroxyethidium (EOH) from the non-specific ethidium, as previously described (S. Eid et al., 2016). Homogenates from the kidney cortex tissue were briefly washed twice with Hanks' balanced salt solution (HBSS)-diethylenetriaminepentaacetic acid (DTPA) and incubated for 30 min with 50 μM DHE (Sigma-Aldrich) in HBSS–100 μM DTPA. Tissues were then harvested in acetonitrile and centrifuged (12,000 X g for 10 min at 4°C). The homogenate was then dried under a vacuum and analysed by HPLC with fluorescence detectors. Quantification of DHE, EOH, and ethidium concentrations were performed by comparison of integrated peak areas between the obtained and standard curves of each product under chromatographic conditions identical to those mentioned above. EOH and ethidium were monitored by fluorescence detection with excitation at 510 nm and emission at 595 nm, whereas DHE was monitored via UV absorption at 370 nm. Finally, the results are expressed as the amount of EOH produced (nmol) normalized for DHE consumed (i.e., initial minus remaining DHE in the sample; μmol).

G. Statistical Analysis

Statistical analysis was performed and assessed using student's unpaired *t*-test. All results were expressed as means ± standard errors (SE) (n=3) and the significance was determined as probability (p-value) equal to or less than 0.05 (≤ 0.05). When comparing control and diabetic, one asterisk (*) is used when $p \leq 0.05$ and two (**) is used when p

≤ 0.01. The (#) symbol is used when comparing untreated diabetic groups vs. treated diabetics at $p \leq 0.05$ and two (##) symbols when $p \leq 0.01$.

CHAPTER III

RESULTS

A. Body Weight and Blood glucose levels

Diabetes is associated with renal dysfunction that may be persistent even with light glucose control. In this study, we investigated the mechanism of action of known hypoglycemic drugs used in Type 2 diabetes to treat Type-1 induced renal damage.

Group	n	Glucose Levels Day 57 (mg/dL)	n	Body weight day 57 (g)	n	Kidney Weight day 57 (g)	n	Kidney Weight/ Body Weight day 57 (mg/g)
Control	3	108.8 ± 4.5	3	510. ± 10.9	3	1.85 ± 0.03	3	3.15 ± 0.1
Diabetic	3	600 ± 0.0	3	164 ± 3.1	3	1.18 ± 0.08	3	7.18 ± 0.4**↑
Diabetic + Metformin	3	518 ± 41.5	3	317 ± 14.4	3	1.75 ± 0.18	3	5.53 ± 0.6#↓
Diabetic + Pioglitazone	3	450 ± 138.0	3	394 ± 50.3	3	2.29 ± 0.12	3	5.80 ± 0.6#↓
Diabetic + Liraglutide	3	587.7 ± 12.3	3	252 ± 25.1	3	1.69 ± 0.15	3	6.70 ± 0.2↓
Diabetic + Metformin + Pioglitazone	3	590 ± 10.0	3	237.7 ± 8.8	3	1.64 ± 0.06	3	6.90 ± 0.2↓
Diabetic + Metformin + Liraglutide	3	528 ± 72.0	3	309.3 ± 17.9	3	1.67 ± 0.05	3	5.39 ± 0.4#↓
Diabetic + Pioglitazone + Liraglutide	3	569 ± 31	3	222.3 ± 23.8	3	1.52 ± 0.07	3	6.85 ± 0.6↓
Diabetic + Metformin + Pioglitazone + Liraglutide	3	532.3 ± 67.7	3	296.3 ± 30.4	3	1.58 ± 0.02	3	4.74 ± 0.4#↓

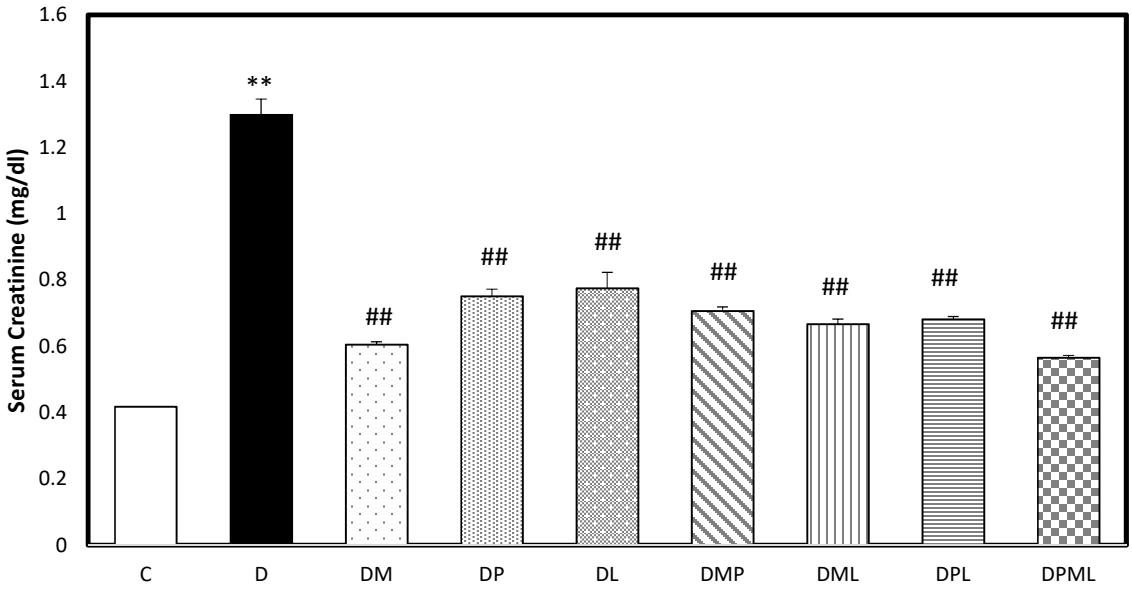
Table 2: Body weights, kidney weights, and blood glucose levels of C rats, D rats (Type 1-STZ-induced), DM, DP, DL, DMP, DML, DPL and DPML rats.
The results are means ± SEM from 3 different rats in each group. *p ≤ 0.05 or $^{**}P \leq 0.05$ vs control. $^{\#}P \leq 0.05$ or $^{\#\#}P \leq 0.01$ vs. diabetic.

Our results showed that administration of Metformin, Pioglitazone, Liraglutide as a monotherapy or as a combination treatment did not reduce glucose levels. Interestingly, these drugs reversed diabetes-induced renal hypertrophy as assessed by measuring kidney weight to body ratio. Results are expressed as mean ± S.E. (Table 2).

B. Blood Creatinine and Blood Urea Nitrogen Levels

With the onset of DM, renal function is disrupted which leads to the lowering of the GFR. With time, this in turn will induce renal failure. To confirm our suspicion that diabetes induces renal insufficiency, we evaluated the BUN and serum creatinine levels. Urea is in fact a byproduct of protein breakdown whereas serum creatinine is a metabolite of creatine mainly located in the skeletal muscle. In DN, such substances are not excreted normally, thus accumulating within the body causing their levels to rise (BA, 2007). Observing the results obtained in this study, both BUN and creatinine levels showed a significant increase in the untreated diabetic rats compared to their control littermates (Figure 4). The use of Metformin, Pioglitazone, Liraglutide, or in their double or triple combinations, showed significant decreased levels in both BUN and creatinine levels when compared to the STZ-induced Type 1 diabetic rats.

A



B

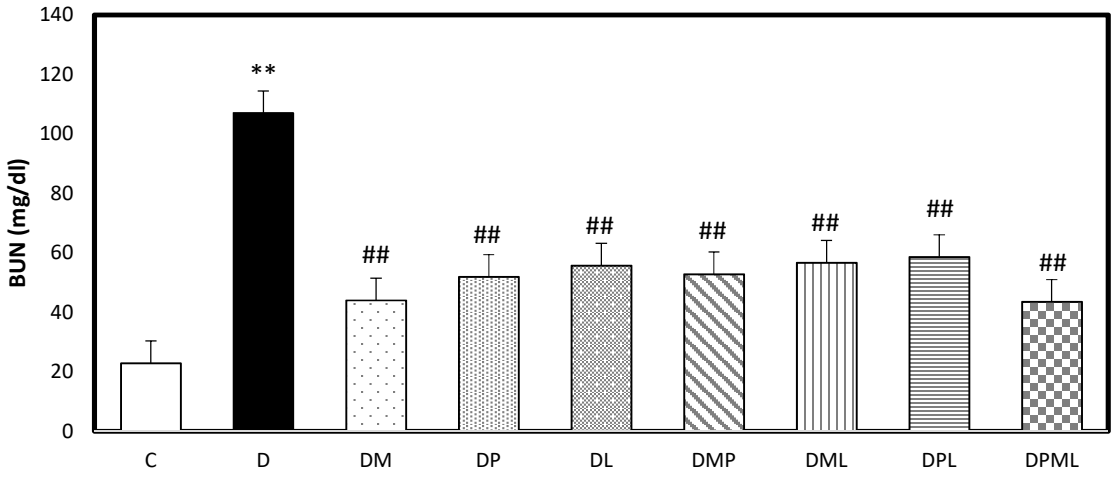


Figure 4: Treatments using Metformin, Pioglitazone and Liraglutide separately and in specific combinations reverse diabetes increased levels of blood creatinine and Blood Urea Nitrogen (BUN) levels.

(A) Blood creatinine levels taken from control, STZ-induce Type 1 diabetic rats, and diabetic treated groups. (B) Blood Urea Nitrogen (BUN) levels taken from control, STZ-induce Type 1 diabetic rats, and diabetic treated groups. The results are means ±

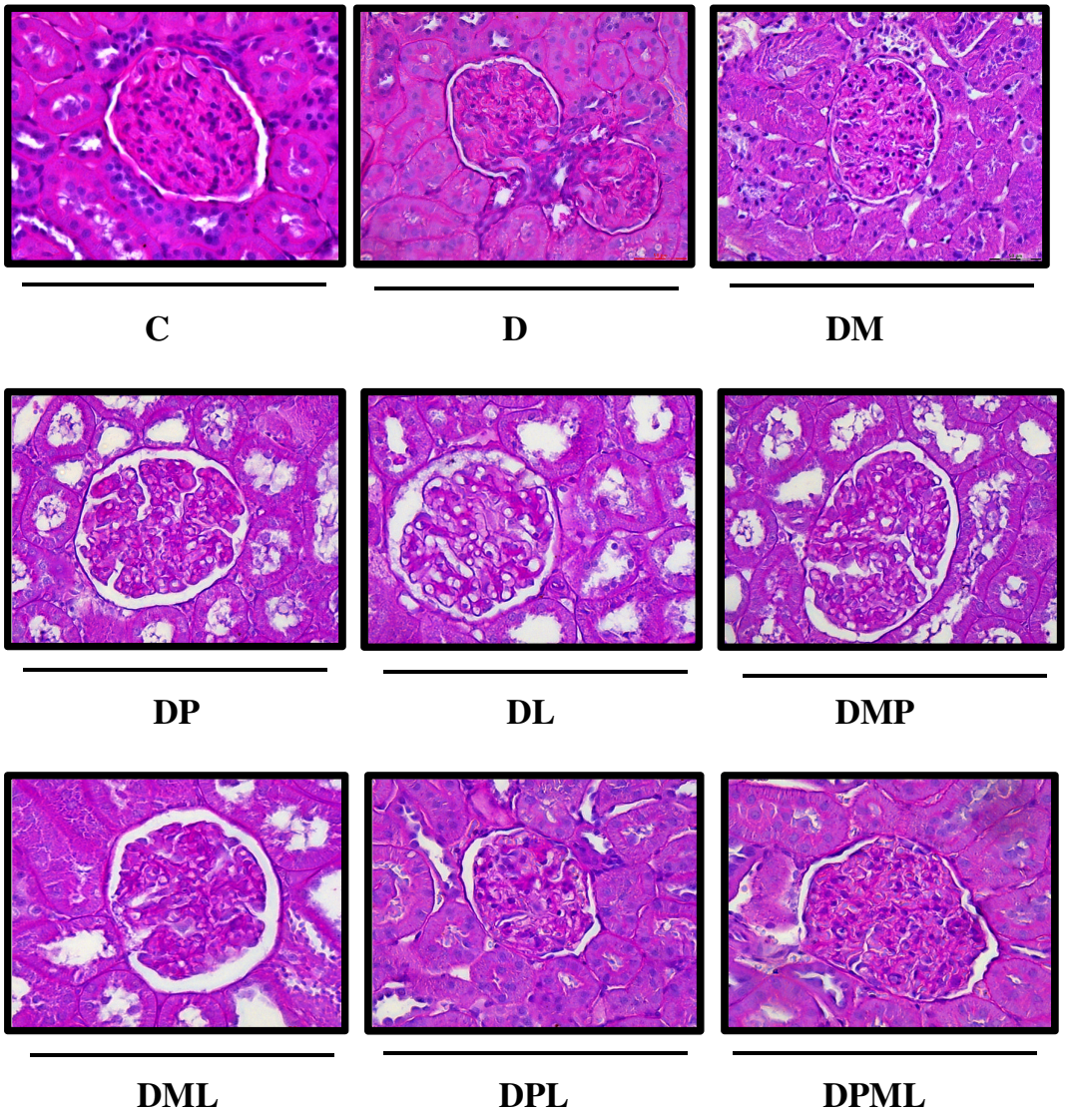
SEM from 3 different rats in each group. *p ≤ 0.05 or $^{**}P \leq 0.05$ vs control. $^{\#}P \leq 0.05$ or $^{\#\#}P \leq 0.01$ vs. diabetic.

C. Treatments using Metformin, Pioglitazone and Liraglutide both as monotherapy or as a combination attenuates glomerular and cortical tubular injury

Histological studies were conducted to evaluate the extent of renal cellular damage. Assessment of fibrosis and the accumulation of matrix protein were achieved on renal cortices of the different groups of rats. Applying PAS stain illustrated that there was an increase in extracellular matrix protein accumulation in the glomerulus and fibrosis in the proximal tubules in the kidney cortices taken from diabetic animals compared to the control ones (Figure 5). Treatment with Metformin, Pioglitazone, or Liraglutide, as well as in both their double and triple combinations, partially ameliorated the observed injury in the diabetic rats. In addition, the drugs used also displayed a decrease of tubular carbohydrate accumulation when compared to the untreated diabetic group.

Masson Trichrome stain which assesses COL IV deposition was used and our data obtained revealed an increased expression in COL IV fibers of both the glomerulus and proximal tubules taken from the diabetic rat groups when compared to the control group. This was partially reversed when the diabetic rat groups were treated with Metformin, Pioglitazone, or Liraglutide as monotherapies or as a double or triple combination, showing a decrease in COL IV fiber expression in the cortex when compared to the untreated diabetic group (Figure 6).

A.



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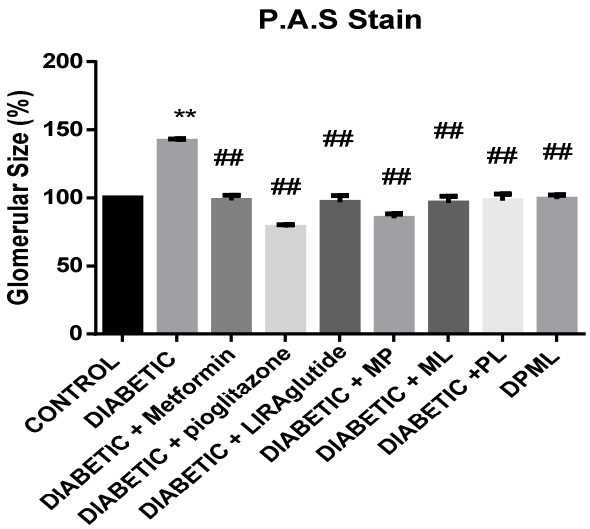
P.A.S Stain
Glomerular Size (%)
200
150
100
50
0
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##
##
##
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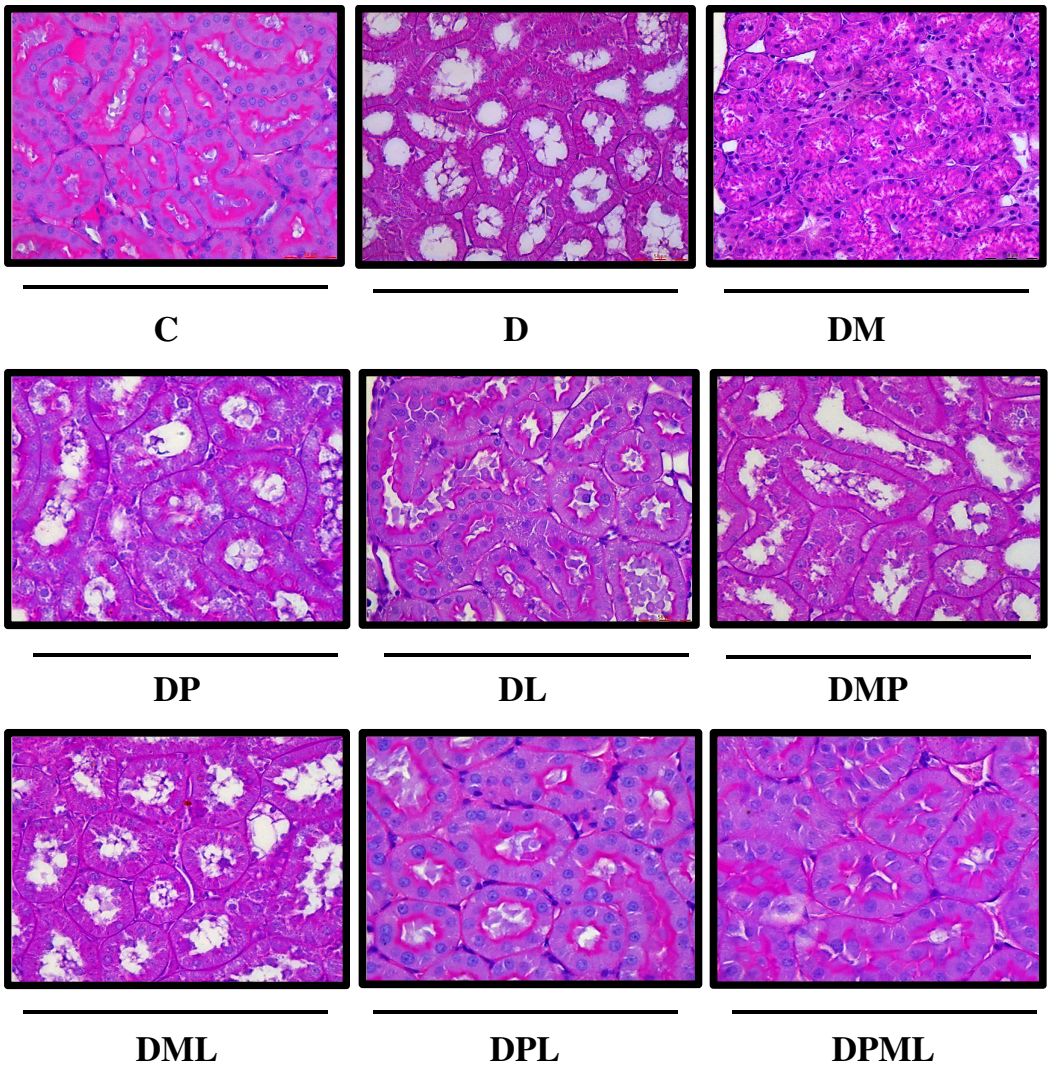
##
CONTROL
DIABETIC
DIABETIC + Metformin
DIABETIC + pioglitazone
DIABETIC + LIRAglutide
DIABETIC + MP
DIABETIC + ML
DIABETIC +PL
DPML

C.



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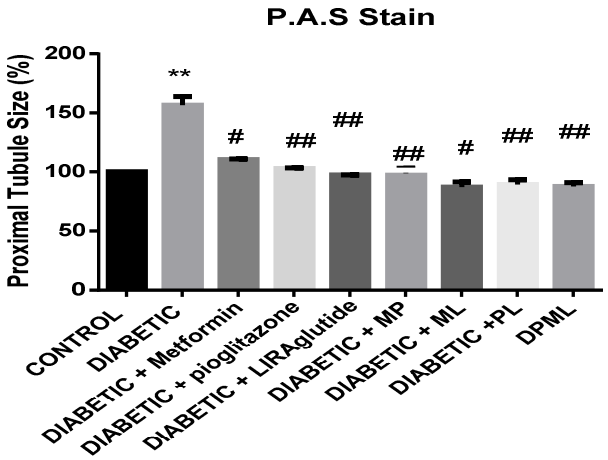
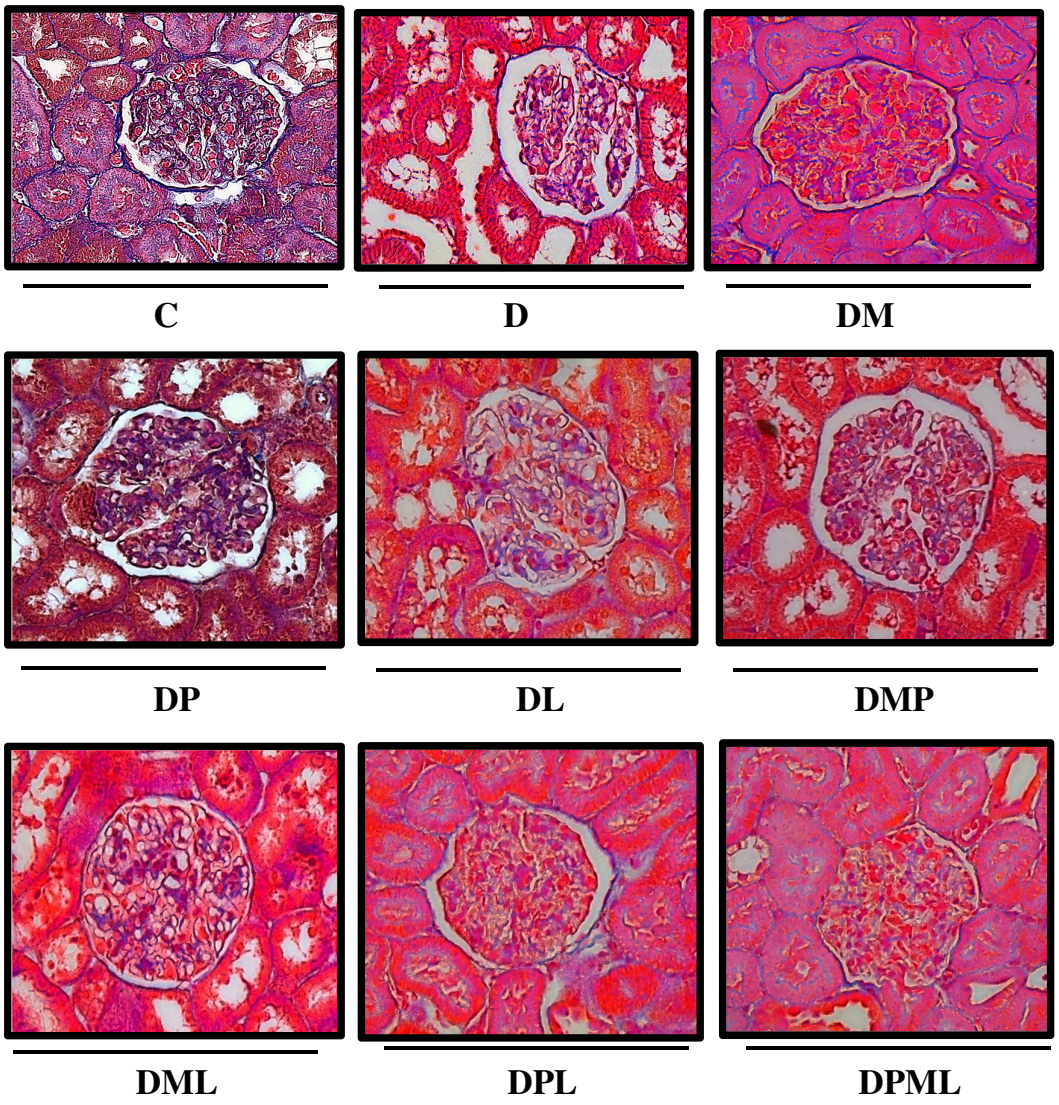


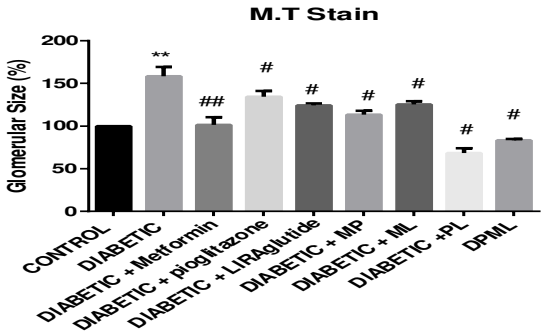
Figure 5: Treatments using Metformin, Pioglitazone and Liraglutide separately and in specific combinations ameliorates STZ-induced diabetes glomerulosclerosis and tubular fibrosis.

Kidney cortices stained with PAS to measure mesangial matrix deposition in the different groups of rats. (A) Representative histological analysis of glomeruli with PAS staining from the different groups of rats. (B) Histogram showing the quantitative analysis of PAS staining in the glomeruli using ImageJ software. (C) Representative histological analysis of proximal tubules with PAS staining from the different groups of rats. (D) histogram showing the quantitative analysis of PAS staining in the proximal tubules. The results are means ± SEM of several glomeruli and several proximal tubular areas from 3 different rats in each group. *p ≤ 0.05 or $^{**}P \leq 0.05$ vs control. $^{\#}P \leq 0.05$ or $^{\#\#}P \leq 0.01$ vs. diabetic.

A.



A.


M.T Stain
Glomerular Size (%)
200
150
100
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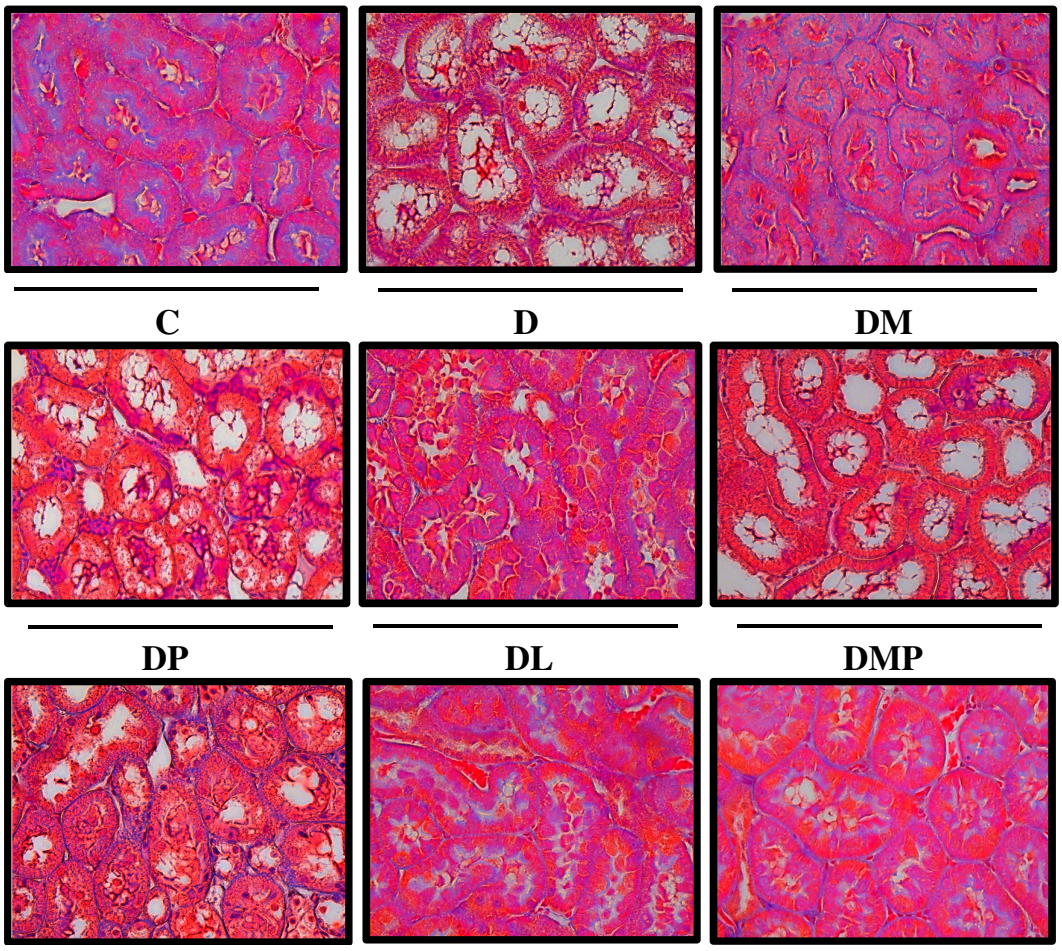
CONTROL
DIABETIC
DIABETIC + Metformin
DIABETIC + pioglitazone
DIABETIC + LIRAglutide
DIABETIC + MP
DIABETIC + ML
DIABETIC +PL
DPML

B.


C
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DML **DPL** **DPML**

C.

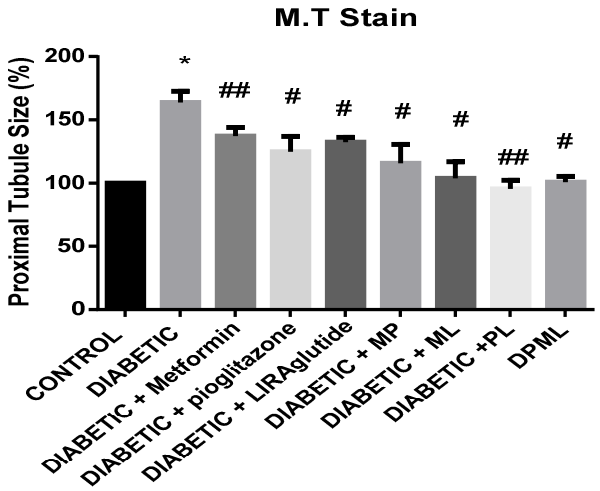


Figure 6: Treatments using Metformin, Pioglitazone and Liraglutide separately and in specific combinations ameliorates STZ-induced diabetes glomerulosclerosis and tubular fibrosis.

Kidney cortices stained with Masson Trichrome to measure COL IV fibers expression in the nine different groups of rats. (A) Representative histological analysis of glomeruli with Masson Trichrome staining (fibrosis: blue color) from the different groups of rats. (B) Histogram showing the quantitative analysis of trichrome staining in the glomeruli using ImageJ software. (C) Representative histological analysis of proximal tubules with Masson Trichrome staining (fibrosis: blue color) from the different groups of rats. (D) Histogram showing the quantitative analysis of trichrome staining in the proximal tubules using ImageJ software. The results are means ± SEM of several glomeruli and several proximal tubular areas from 3 different rats in each group. *p ≤ 0.05 or $^{**}P \leq 0.05$ vs control. $^{\#}P \leq 0.05$ or $^{\#\#}P \leq 0.01$ vs diabetic.

D. Treatments using Metformin, Pioglitazone and Liraglutide both as monotherapy or as a combination attenuates tubulointerstitial changes

When proteinuria is found in Type 1 DM, a well-defined group of renal structural abnormalities are expected to occur alongside, such as mesangial expansion

and tubulointerstitial changes (Mauer, 1994). Quantifying the expression of fibronectin, a well-known marker of extracellular matrix expansion and tubular interstitial fibrosis, through measuring mRNA levels revealed a trend of increase of fibronectin mRNA levels in the untreated diabetic animals compared to control ones (Figure 7). Rats treated with Metformin, Pioglitazone, or Liraglutide, or Liraglutide as well as the double and triple combinations showed a decreased level of mRNA expression of Fibronectin. Liraglutide decreased fibronectin mRNA levels significantly alongside with the same significant decrease where Pioglitazone and Liraglutide are combined. The use of Metformin and Pioglitazone and Metformin and Liraglutide and the combination treatment of the three drugs showed a slight decrease in fibronectin mRNA levels. Taken together, the data suggests that diabetes increases extracellular matrix expression and tubular interstitial fibrosis as it is significantly seen in the histological cuts, but the significant molecular alteration has not been reached, and this may be explained by the small number of animals used in this study.

A

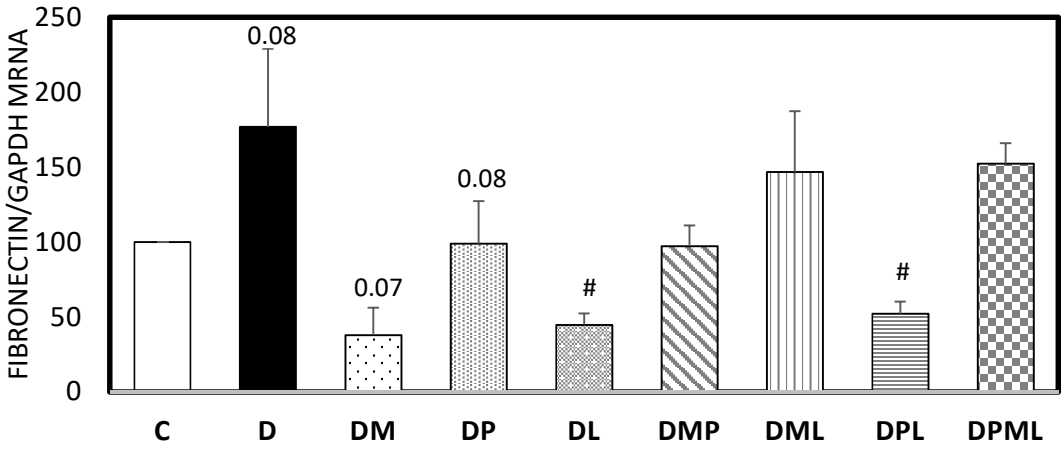


Figure 7: Treatments using Metformin, Pioglitazone and Liraglutide separately and in specific combinations ameliorate STZ-induced diabetic kidney injury.

Fibronectin mRNA expressions were assessed using RT-PCR. (A) Histogram showing fibronectin mRNA levels with respect to GAPDH mRNA levels. The results are means ± SEM from 3 different rats in each group. *$p \leq 0.05$ or ** $p \leq 0.01$ vs. control. #$p \leq 0.05$ or ##$p \leq 0.01$ vs. diabetic.

E. Downregulation of nephrin mRNA expression and its potential prevention in both monotherapy and in combination therapy attenuates kidney injury

In DN, albuminuria is mainly due to renal endothelial and epithelial cells dysfunction and alterations in the morphology of the slit-pore diaphragm (Miner, 2012). After 8 weeks of STZ-induced hyperglycemia, the expression of glomerular nephrin was significantly reduced in the diabetic group. Our data proposes that Metformin, Pioglitazone, and Liraglutide monotherapies as well as double and triple combination therapies of the drugs used in this study tend to restore the mRNA nephrin expression and reverse the GFB dysfunction.

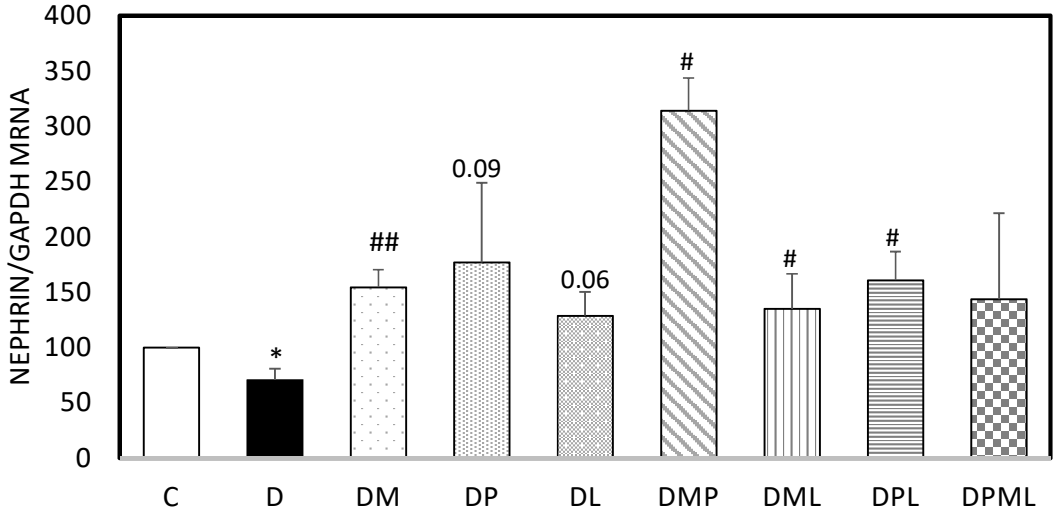


Figure 8: RT-PCR of nephrin mRNA expression.

Nephrin mRNA expressions were assessed using RT-PCR. (A) Histogram showing nephrin levels with respect to GAPDH mRNA levels. The results are means ± SEM from 3 different rats in each group. *$p \leq 0.05$ or ** $p \leq 0.01$ vs. control. #$p \leq 0.05$ or ##$p \leq 0.01$ vs. diabetic.

F. ROS production induced due to hyperglycaemia via NADPH oxidase dependent mechanisms, causing Diabetic Nephropathy (HPLC)

Thought to be the final common pathway of many known cellular stressors, ROS production is involved in hyperglycaemia-induced diabetic pathophysiology (A. A. Eid et al., 2013). In this study, the assessment of ROS superoxide anion production was performed using HPLC as shown in Figure 9. Our results showed that ROS production was significantly increased in the untreated STZ-induced Type 1 diabetic rats when compared to the control littermates. However, when comparing the treated diabetic groups, Metformin, Pioglitazone, Liraglutide, or their double or triple combinations, we see a significant decrease in ROS production when compared to the untreated STZ rats (Figure 9).

A.

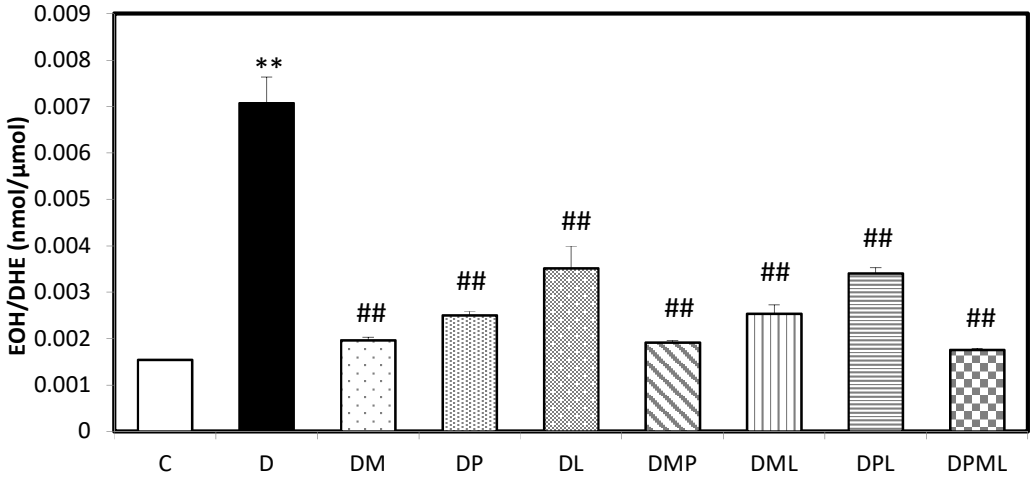


Figure 9. **Hyperglycemia upregulates superoxide production in kidney tissue of Type 1 diabetic rats.**

(A) Superoxide generation evaluated using DHE and HPLC in Control, Diabetic and diabetic treated animals (Diabetic plus metformin/Pioglitazone/Liraglutide/Metformin plus Pioglitazone/Metformin plus Liraglutide/Pioglitazone plus Liraglutide/ Metformin plus Pioglitazone plus Liraglutide). The results are means ± SEM of different cortical areas from 3 different rats in each group. *$p \leq 0.05$ or ** $p \leq 0.01$ vs. control. #$p \leq 0.05$ or ##$p \leq 0.01$ vs. diabetic.

G. NADPH oxidase mediates ROS production and induces renal injury

Identifying the sources of ROS that are increased in diabetes is an important step in understanding the exact mechanism of alteration that is reduced and will help in designing adjunctive therapy to treat diabetic complications. Our results obtained here demonstrated that NADPH-dependent superoxide generation was increased in the kidneys of the diabetic animals when compared to the controls. This increase was inhibited when rats were treated with either Metformin, Pioglitazone, Liraglutide, or with the double or triple combination treatment groups as shown in Figure 10. These results suggest that AMPK, GLP1, and PPAR-ϒ crosstalk and regulate NADPH oxidases, that in turn is responsible for the overproduction of ROS.

A.

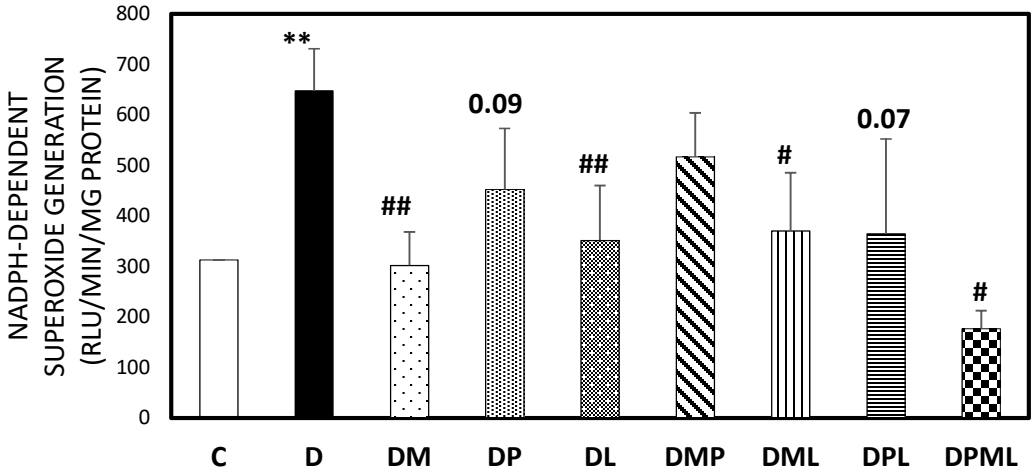


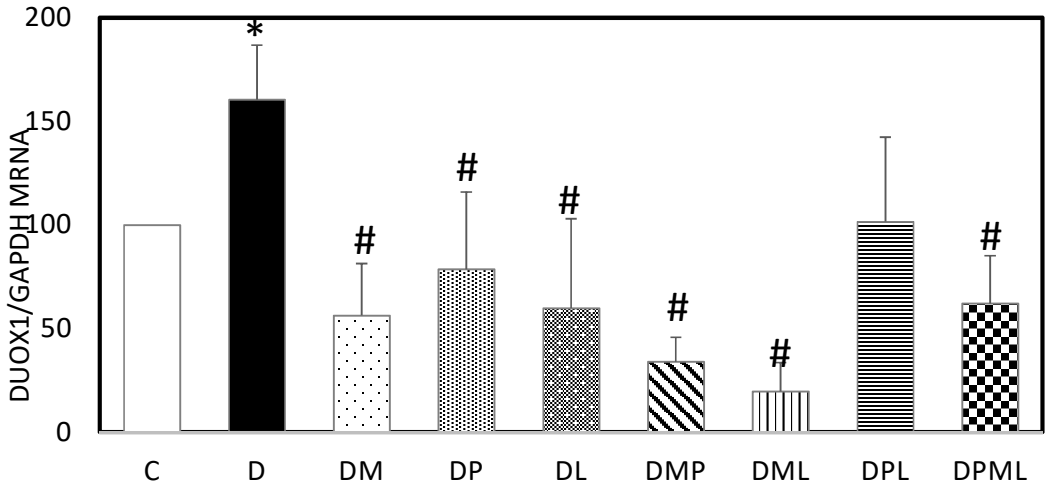
Figure 10: Both monotherapies and combination therapies attenuate kidney injury by reducing NADPH-derived ROS production.

NADPH oxidase activity in cortical homogenates. NADPH-dependent superoxide production was expressed as RLU/min/mg protein. The results are means ± SEM of different cortical areas from 3 different rats in each group. *$p \leq 0.05$ or ** $p \leq 0.01$ vs. control. #$p \leq 0.05$ or ##$p \leq 0.01$ vs. diabetic.

H. NADPH dual oxidases DUOX1 and DUOX2 mRNA expression and its potential involvement in Diabetic Nephropathy and autophagy

NADPH oxidases plays a fundamental role in both the development and progression of renal injury in animal models of Type 1 and Type 2 DN. Unfortunately, studies on DUOX oxidases in the context of DN are very limited, hence their physiological and pathophysiological functions specifically in this retrospect and their effect on autophagy need to be addressed in future investigations. In this study we showed through RT-PCR that both DUOX1 and DUOX2 mRNA levels were significantly decreased or showed a trend of decrease upon treatment with Metformin, Pioglitazone or Liraglutide as well as in their double or triple combination therapy.

A.



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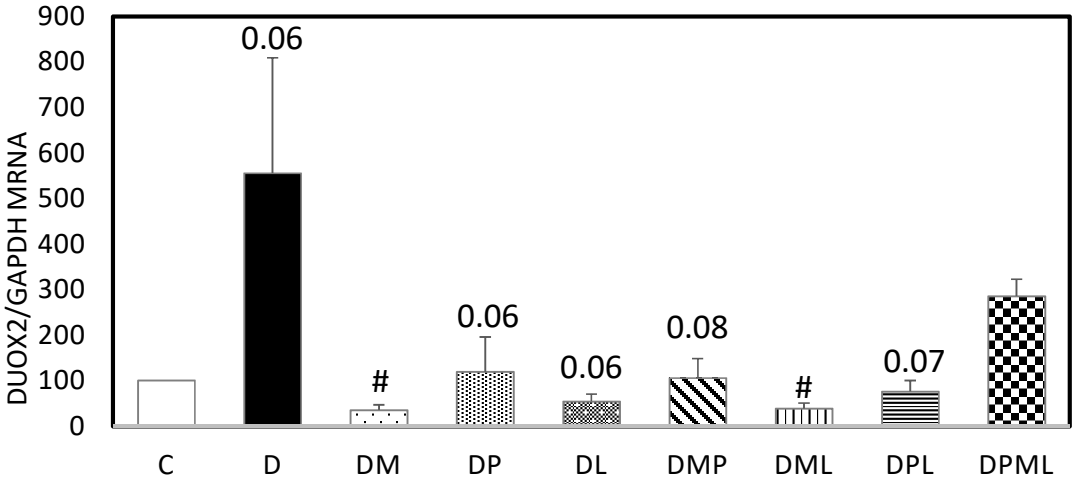


Figure 11: RT-PCR of DUOX1 and DUOX2 mRNA expression.

Both DUOX1 and DUOX2 mRNA expressions were assessed using RT-PCR. (A) Histogram showing DUOX1 levels with respect to GAPDH mRNA levels. (B) Histogram showing DUOX2 mRNA levels with respect to GAPDH mRNA levels. The results are means ± SEM from 3 different rats in each group. *$p \leq 0.05$ or ** $p \leq 0.01$ vs. control. #$p \leq 0.05$ or ##$p \leq 0.01$ vs. diabetic.

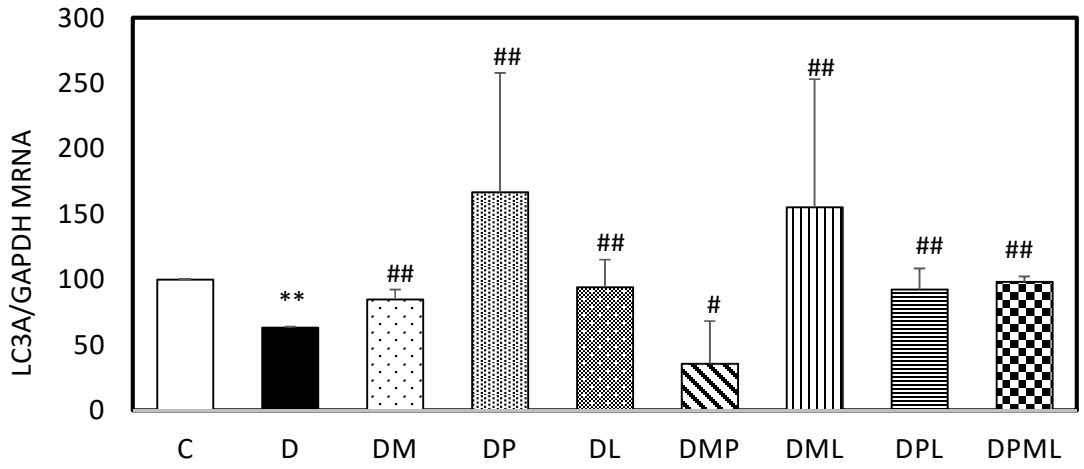
Taken together, these results suggest that DOUX1 and DUOX2 isoforms alongside with other NOX's (which should be identified and studied), play a major role in NADPH-induces ROS production, leading to diabetic renal injury.

I. Autophagy as a potential target in alleviating kidney injury

Autophagy functions to remove protein aggregates as well as damaged or excess organelles to maintain the cell's integrity and intracellular homeostasis. It thus shows renoprotective effect in several animal models of aging and acute kidney injury, especially at the level of the glomeruli. This study demonstrated by RT-PCR decreased mRNA levels of both LC3A and LC3B genes, which are considered as autophagy markers, in diabetic groups when compared to their control littermates (Figure 12).

Treatment with Metformin, Pioglitazone, or Liraglutide or in their combinations, reversed the alteration seen in the autophagy markers.

A.



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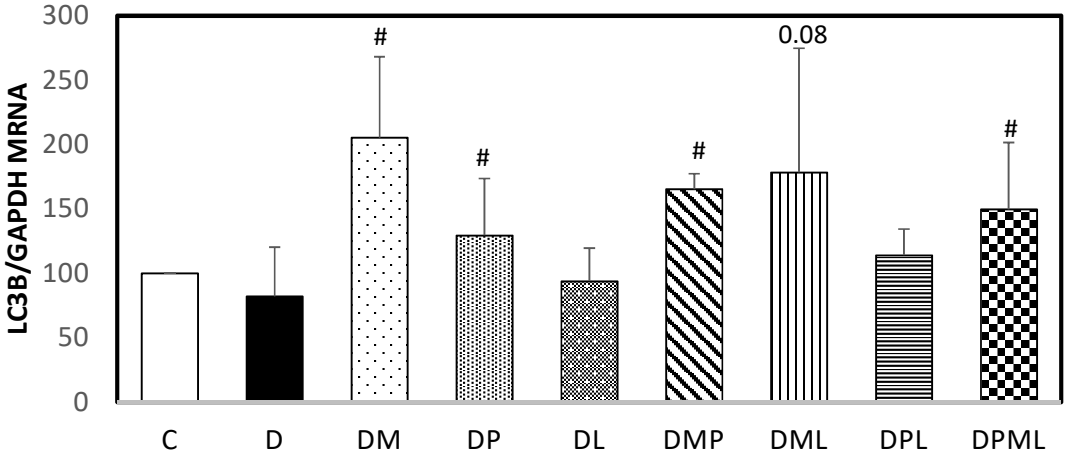


Figure 12: Markers of autophagy plays a role in STZ-induced diabetes kidney injury in both monotherapies and combination treatments.

Both autophagy markers LC3A and LC3B mRNA expressions were assessed using RT-PCR. (A) Histogram showing LC3A mRNA levels with respect to GAPDH mRNA levels. (B) Histogram showing LC3B mRNA levels with respect to GAPDH mRNA levels. The results are means ± SEM from 3 different rats in each group. *$p \leq 0.05$ or ** $p \leq 0.01$ vs. control. #$p \leq 0.05$ or ##$p \leq 0.01$ vs. diabetic.

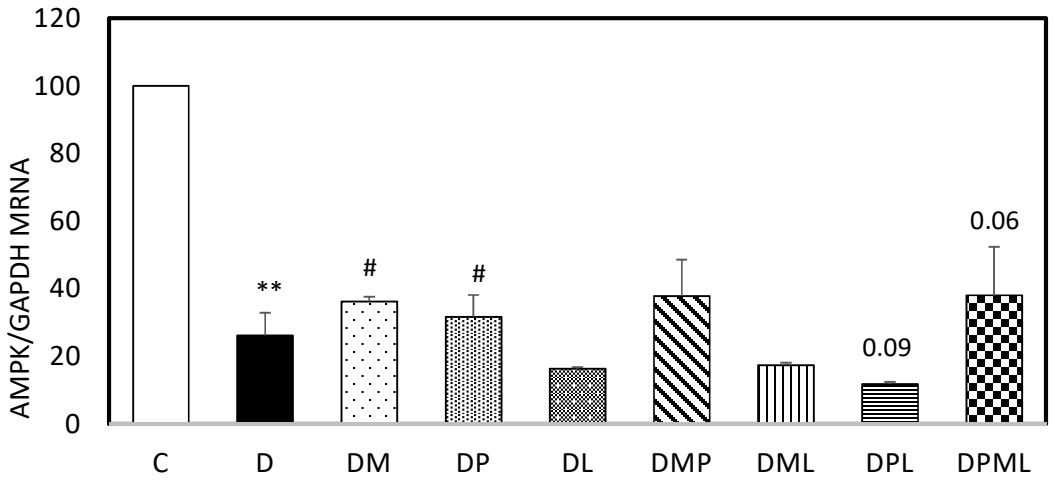
J. Possible renoprotective mechanisms in diabetic nephropathy via AMPK and mTOR pathways

AMPK is highly expressed in the kidneys, such as in podocyte function and diabetic renal hypertrophy.

AMPK and mTOR mRNA levels were evaluated in this study. As expected, Metformin activated AMPK and thus reversed functional and histological patterns as seen in diabetes suggesting that the PPAR-ϒ controls the AMPK function. On the other hand, Liraglutide had no effect on AMPK activity suggesting that the GLP-1 and incretin separately are downstream of the AMPK pathway. The combination treatment of Metformin and Pioglitazone tend to reactivate the AMPK activity as well as the

combination treatment of Metformin, Pioglitazone and Liraglutide. The use of Liraglutide in combination with Metformin or Pioglitazone did not show any effect on the AMPK activation.

A.



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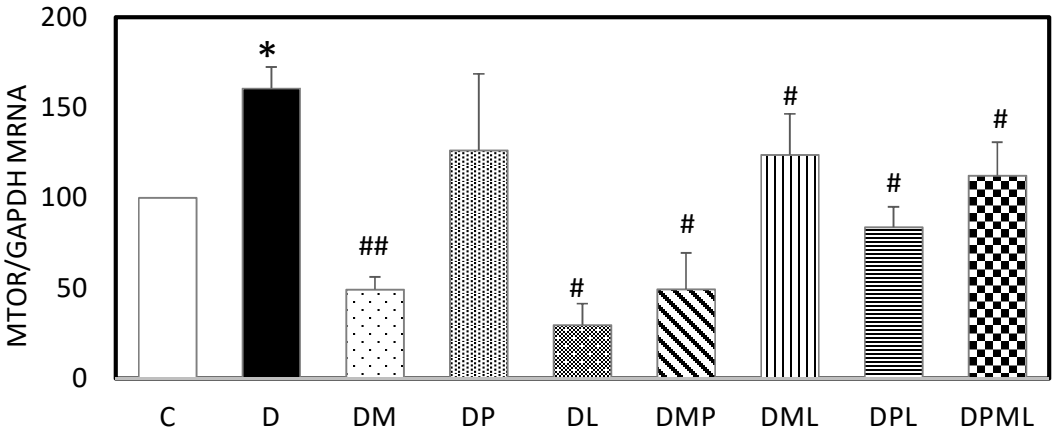

Figure 13: Potential link between nutrient sensing pathways mTOR and AMPK and regulating autophagy and kidney injury.

Both mTOR and AMPK mRNA expressions were assessed using RT-PCR. (A) Histogram showing AMPK mRNA levels with respect to GAPDH mRNA levels. (B) Histogram showing mTOR mRNA levels with respect to GAPDH mRNA levels. The

results are means ± SEM from 3 different rats in each group. *$p \leq 0.05$ or ** $p \leq 0.01$ vs. control. #$p \leq 0.05$ or ##$p \leq 0.01$ vs. diabetic.

AMPK mRNA levels in Figure 13A showed a significant decrease in the diabetic groups when compared to control group. Figure 13B shows mTOR mRNA levels, measured by RT-PCR. The untreated diabetic group shows a significant increase in mTOR expression compared to the control group. However, mTOR was generally downregulated in all the treatment groups with significant decrease in expression levels in the DM, DL and DMP groups.

As for the mTOR pathway, activation of AMPK using Metformin or activating the GLP-1 pathway, or activating PPAR-ϒ shows a significant reversal in diabetes induced mTOR activation. These results also were seen with a dual or triple combination of the drugs that were used.

CHAPTER IV
DISCUSSION

Diabetes mellitus has been linked to a wide range of acute and chronic complications. According to the International Diabetes Federation, 5-10% of diabetic people worldwide are diagnosed with Type 1 diabetes (Federation, 2014). Maintaining strict glycaemic and blood pressure control by means of therapeutic regimens is indeed significantly beneficial and has proved to be effective in reducing morbidity and mortality rates. Nevertheless, such firm regimens are not always possible and if so, may not fully alleviate the progression of diabetic complications that start early on such as DN. (Khavandi, Amer, Ibrahim, & Brownrigg, 2013). This work identified adjunctive therapies which may have a great benefit on reducing the onset and development of complications associated with diabetes.

As mentioned previously, upon DN diagnosis, substances such as creatinine and BUN are not excreted in a normal manner causing their accumulation within the blood to increase (Dabla, 2010). Our data are in line with these findings, suggesting that our experimental model shows a renal disfunction, mimicking the human alteration. More importantly, in our study, the use of oral Type 2 hypoglycemic drugs such as Metformin, Pioglitazone and Liraglutide partially reverse the renal damage caused by Type 1 diabetes, without affecting their glucose levels, suggesting that the role of such drugs used in this study goes beyond their simple action in glycaemic control. As such,

we can conclude that Metformin, Pioglitazone, or Liraglutide may play a role in reversing some of the known signalling pathways that induce renal damage.

In this study, we were able to show by histological studies that the integrity of glomerular cells, the glomerulus and the renal proximal tubules were nearly restored to normal when Metformin, Pioglitazone, and Liraglutide were used. In other words, these treatments reduced the histopathological features of DN including glomerulosclerosis and tubulointerstitial fibrosis. In addition, the treatments administered allowed the restoration of almost normal expression levels of nephrin in the kidneys thus showing a potential reversal of the GFB injury.

The activation of several pathophysiological mechanisms such as the metabolic polyol pathway, increased synthesis of TGF-β1, intracellular production of advanced glycation end-products, and the increase production of ROS can be mainly due to increased intracellular glucose levels. Such effects typically contribute to the expansion of the mesangial matrix and increased synthesis of fibrogenesis components such as fibronectin, collagen, and laminin, resulting in glomerulosclerosis and tubulointerstitial fibrosis. Furthermore, in DN, the functions of the podocytes and the slit-pore diaphragm are compromised, ultimately playing a key role in proteinuria. Concomitantly, our data shows that diabetes induced ROS production increase could be orchestrating the kidney damage observed.

It has been demonstrated that persistent levels of HG lead to increase in ROS generation (Fakhruddin, Alanazi, & Jackson, 2017; Yu, Robotham, & Yoon, 2006). Indeed, oxidative stress is a possible common factor that links the diverse mechanisms proposed for the pathogenesis of diabetic complications (Baynes, 1991). It usually

happens by the activation of multiple enzymatic and non-enzymatic sources of ROS (Lv et al., 2014) (Satoh et al., 2005); however, given that there are many possible sources of increased levels of oxidative stress in DM, the primary source may be tremendously difficult to determine. In the kidneys, we and others have shown that NADPH oxidases are highly expressed and that Nox enzymes contribute to ROS generation in conditions of HG (A. A. Eid et al., 2009) (Sedeek, Nasrallah, Touyz, & Hebert, 2013). Nox 4, the main Nox isoform expressed in the kidneys, was found to be the most involved in renal ROS production. It was also shown that the increase in the Nox4-derived ROS leads to oxidative stress and mediates renal hypertrophy and fibronectin overexpression. (S. Eid et al., 2013; Lv et al., 2014)). Yet the role of the other NADPH oxidase isoform in DN is not well studied. In this study, we demonstrated that ROS production was increased and paralleled by an increase in NADPH oxidase activity and that, in both monotherapy treatment groups with Metformin, Pioglitazone, and Liraglutide and their combination treatment groups decreased NAPDH oxidative stress, especially their biological effect on renal damage, despite the sustained hyperglycemia. These results suggest that AMPK, GLP-1 and PPAR-ϒ crosstalk with the NADPH oxidase enzymes under Type 1 DM conditions, subsequently decrease ROS production and oxidative stress.

Moreover, and to our knowledge, this is one of the first studies describing the involvement of DUOX1 and DUOX2 in renal injury induced by diabetes. Both dual oxidase isoforms DUOX1 and DUOX2 are part of the NADPH oxidase family. They are mainly found in the thyroid gland and in lung epithelial cells of adults. Both DUOX1 and DUOX2 are thought to be involved in hormones biosynthesis as well as in host defence by the production of H_2O_2. Even though the pathophysiological function of each Nox isoform is not fully understood, there are indications that they play a role in

angiogenesis, inflammation, cell growth and fibrosis (Ago, Kuroda, Kamouchi, Sadoshima, & Kitazono, 2011; Bedard, Lardy, & Krause, 2007), all of which are relevant to the development of DN. Because studies on the DUOX isoforms are limited, there is little information about their potential involvement in disease conditions, especially in DN and their role in the autophagy pathway in this respect. However, in this study we tested the role of DUOX1 and DUOX2 in DN. Our data show that both dual oxidases DUOX1 and DUOX2 were upregulated in DN and their mRNA levels were decreased upon AMPK, GLP-1, and PPAR-ϒ pharmacological activation.

Autophagy is one of the basic mechanisms through which tissues and cells deal with stress. It is nothing more than an intracellular degrading system that is extremely dependent on lysosomes. It is described in literature as a highly conserved mechanism that maintains intracellular stability (Liu, Shi, & Zhuang, 2016). It functions by eliminating impaired and senescent organelles and biological macromolecules through its adaptive mechanism. In the kidneys, autophagy is activated under stressful conditions such as oxidative stress and hypoxia in proximal tubular cells and is usually active even under normal conditions in podocytes.

Current research has revealed a link between autophagy, kidney aging and the occurrence of several kidney diseases such as DN (Huber et al., 2012). Within the kidneys, autophagy is a major player that deals with stress, specifically in podocytes. Alterations in the mechanism of autophagy have been shown to impair this critical function of podocytes. Autophagy has a renoprotective role by maintaining the homeostasis of podocytes in DN. It has been demonstrated that podocyte specific expression of autophagy related genes such as LC3A/B results in an increase in the basal level of autophagy in podocytes (Tagawa et al., 2016). Yet, under HG levels,

autophagy in podocytes becomes defective, causing podocyte injury. This is further supported by the fact that the expression of autophagy genes such as LC3A/B, Beclin-1 as well as Atg5-Atg12 decreased in STZ-induced diabetic mice (Tagawa et al., 2016). Another study showed that in both diabetic rats and patients, there was a defect or decrease in autophagy, suggesting that autophagy plays a role in the pathogenesis of DN (Yamahara et al., 2013). Such data have led to the conclusion that autophagy is involved in the pathogenesis of DN (Kume & Koya, 2015). However, the exact mechanism of podocyte injury, autophagy induced by DM, the role of ROS, its crosstalk with AMPK, GLP-1, PPAR-ϒ and mTOR are still vague. Therefore, it is vital to understand the regulation of autophagy, specifically within the podocytes.

In DM, autophagy has been shown to maintain cellular energy by providing nutrients during fasting conditions but also eliminating damaged organelles, lipids and miss-folded proteins. Furthermore, autophagy has been shown to play role in pancreatic β-cell dysfunction and insulin resistance (J. S. Yang et al., 2017). Several signalling pathways have been shown to alter autophagy in pathophysiology of disease, including the AMPK signalling pathways as well as the mTOR pathway. Our results show that, LC3A and LC3B, known markers of autophagy, homeostasis is altered in a diabetic milieu and this alteration is partially corrected. The AMPK, GLP-1, and PPAR-ϒ alteration are corrected with the use of Metformin, Pioglitazone and Liraglutide. These results are also correlated by a reversal of diabetes-induced mTOR activation, highlighting the key role of mTOR in DN and its possible function in regulating autophagy driven DN.

In the past decade, mTORC pathway has gained high attention where it has been correlated with several pathologies. In both Type 1 and Type 2 DM, mTORC1

signalling pathway is increased, which plays a protective role in kidney functioning (Sakaguchi et al., 2006; Y. Yang et al., 2007). In a previous study, the excessive activation of mTORC1 proved to be one of the major causes of glomerular damage (Inoki et al., 2011). In fact, the dysregulation of mTORC1 activity along with glomerular hypertrophy and enlargement of the podocytes are typical features for early development of DN (Godel et al., 2011). Although the exact mechanism of how the dysregulation of mTORC1 activity affects cellular integrity is still unclear, several studies have proposed that mTORC1 hyperactivation is linked to the activation of the Notch pathway, thus leading to further damage of podocytes (Bielesz et al., 2010; Niranjan et al., 2008; Sharma, Sirin, & Susztak, 2011). It has also been shown that the upregulation of mTORC1 inhibits autophagy. (Hartleben et al., 2010); (Laplante & Sabatini, 2009).

Previous studies have shown that the mTOR pathway acts as an inhibitor of the autophagy pathway (Ganley et al., 2009) (Kapuy, Vinod, & Banhegyi, 2014). Upon the availability of sufficient nutrients in the microenvironment of a cell, mTOR is activated which in turn blocks the regulation of autophagy. More specifically, it inhibits the expression of autophagy-related genes and hence the formation of the protein complexes necessary for autophagy (Liu et al., 2016).

Furthermore, activation of the AMPK pathway in turn inhibits the activity of mTOR by directly phosphorylating the complex, ultimately enhancing autophagy (Ding et al., 2010). As mentioned earlier, metformin is a well-known anti-hyperglycaemic drug and is mainly used as an effective antidiabetic agent for patients with Type 2 DM. Over the years, metformin has demonstrated multiple times its ability to reduce albuminuria in Type 2 diabetic patients as well as in rat models of Type 2 diabetes

(Amador-Licona, Guizar-Mendoza, Vargas, Sanchez-Camargo, & Zamora-Mata, 2000; J. Kim, Shon, Kim, & Kim, 2012). Recent studies have indicated that the potential therapeutic effect of Metformin may be mediated by the activation of AMPK (Rogacka, Piwkowska, Audzeyenka, Angielski, & Jankowski, 2014; Xu et al., 2014). Indeed, as discussed earlier, high levels of glucose reduce AMPK phosphorylation which then leads to the induction of mTOR activation in podocytes. This outcome is eliminated by pretreatment with Metformin. The use of Metformin also showed to reduce podocyte apoptosis induced by HG levels. Nevertheless, the exact cellular function and intracellular signalling of Metformin under diabetic conditions is still not fully understood. Langer et al showed for the first time that human podocyte cells express an uptake transporter of Metformin known as organic cation transporter 1 (oct1). Even though oct1 is mainly found in hepatic tissue, it is also expressed in the kidneys (Komazawa et al., 2013). This finding led to the assumption that the uptake of Metformin is possible by both human and rat podocytes. Langer et al., among other research groups, also showed that Metformin could influence mTOR activation. Metformin showed to reduce the activation of mTOR under both normal and HG conditions in human podocytes (Langer, Kreutz, & Eisenreich, 2016). Furthermore, phosphorylation of mTOR was shown to be significantly reduced by Metformin in renal tubular epithelial cells (Takiyama et al., 2011). In our study, we managed to demonstrate through the experiments conducted that the use of Metformin can ameliorate the effects of Type 1 DM on kidneys, however further investigation should be performed to elucidate its mechanism of action.

The second drug used in this study alone and in combination with several others is liraglutide. Liraglutide has been shown to lower blood pressure as well as prevent

kidney damage by decreasing renal oxidative stress in rat models of DN (Fujita et al., 2014). In a non-randomised study, treatment using Liraglutide showed to lower albuminuria (von Scholten, Lajer, Goetze, Persson, & Rossing, 2015). Another randomised clinical trial also using Liraglutide as a treatment showed an 18% reduction in urinary albumin-to-creatinine ratio in Type 2 diabetic patients (Davies et al., 2015). In addition, Hendarto et al. showed that Liraglutide inhibits oxidative stress and albuminuria in STZ-induced Type I DM rats via protein kinase-A mediated inhibition of NADPH oxidases (Hendarto et al., 2012). A randomised treatment with Liraglutide performed on diabetic rats over a period of four weeks showed to normalise urinary albumin excretion and oxidative stress markers, including the expression of NADPH oxidase components, TGF-β1 and fibronectin within renal tissues. It did not show any effect however on plasma glucose levels or body weight of the STZ-induced rat animal models (Hendarto et al., 2012). The results obtained in our study revealed a potential benefit of the administration of Liraglutide, both alone and with several combinations of other Type 2 diabetic drugs. In our study, histological analyses showed that Liraglutide attenuated tubulointerstitial changes in both the glomerular and proximal tubules. In addition, HPLC analyses showed a reduced production of ROS in the treated groups; however, the administration of Liraglutide and its mechanism of action on STZ-induced Type 1 diabetes must be further investigated.

Another previous study explored whether Liraglutide protected pancreatic β-cells via AMPK and mTOR signalling pathways. Both on a cellular and molecular level, Liraglutide showed to increase β-cell viability. Liraglutide showed to activate mTOR and its downstream effectors. Such effects were decreased by pathway blockers, AMPK activator AICAR and mTOR inhibitor rapamycin. Moreover, the effect of Liraglutide

protected β-cell proliferation using AMPK activators, suggesting that the enhancement of β-cell proliferation by Liraglutide is mediated in part by AMPK and mTOR signalling pathways (Miao et al., 2013).

An additional study previously performed showed that HG-induced ROS overproduction was dulled upon treatment using Liraglutide or Metformin. Such decrease was shown to be more adamant when using a combination treatment of both Liraglutide and Metformin. Both drugs, whether used alone or in combination also showed to inhibited NADPH-oxidase activation, and prevented HG-induced alterations of phosphorylation of AMPK. This study showed that both Liraglutide and Metformin treatments ameliorates HG-induced oxidative stress by inhibiting NADPH-oxidase pathway. In addition, when using the combination treatment of Liraglutide and Metformin, amplified protective effects (Batchuluun et al., 2014). This study alongside our study further suggests that such combination treatments may serve clinical usefulness in the prevention of diabetic complications.

The final drug used for this study was Pioglitazone, a drug of the TZD family that has shown to have renal protective effects. TZD's have in fact shown in past studies a potential role in relieving glomerular hyperfiltration in early stages of DN (Tanimoto et al., 2004). Further studies revealed that PPAR-γ, a TZD agonist, PPARγ, not only contributes to decreasing hyperglycaemia but also shows renal protective effects, including reduced albuminuria and reduced glomerulopathy (Ohga et al., 2007). Our results indicated a potential beneficial role of Pioglitazone in improving the renal injury sustained in this study via its renoprotective effects, however its method and dosage of administration must be optimised to improve on its potential effect in ameliorating kidney injury in Type 1 DM.

Pioglitazone has been previously shown to improve glycaemic control in Type 2 diabetes via insulin-sensitizing action. It was demonstrated that by using Pioglitazone on vascular smooth-muscle cells, to activate AMPK, therefore partially contributing to the inhibition of key proliferative signalling events. In addition, at a certain concentration of administration of Pioglitazone it activates AMPK which in turn would inhibit the mTOR activity (Osman & Segar, 2016). Inhibition of mTOR via rapamycin in turn increases hepatic glucose production, and reduces adipose tissue PPAR-ϒ activity. This study attempted to demonstrate that pharmacological Ppar-ϒ activation would attenuate diabetic complications associated with mTOR inhibition. This study showed that treatment using PPAR-ϒ activation ameliorates few of the disruptions in glucose homeostasis (Festuccia et al., 2014).

Taken together, our preliminary data demonstrated that both the monotherapy and combination treatments with Metformin, Pioglitazone, and Liraglutide show potential benefits for treating renal injury, a major complication of diabetes. This study also demonstrates the potential usage of Type 2 diabetes drugs on Type 1 DM and the role of autophagy in the pathogenesis of DN. Our preliminary data may be considered as novel insights and observations, contributing to the understanding of autophagy and its potential regulation through the drugs used. It thus sheds light on autophagy as a potential new therapeutic target for the suppression of DN. Future studies are certainly needed for more conclusive evidence.

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