UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Metformin alleviates neuronal and renal related stress signals in diabetic C57BL/6 mice

By

Taskeen Fathima Docrat

BMedSci (Hons) Medical Biochemistry

Master of Medical Science

211501473

Submitted in fulfilment for the degree of Doctor of Philosophy (Medical Biochemistry), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal

Supervisor

Professor Anil. A. Chuturgoon

2019

PREFACE AND DECLARATION

I, Taskeen Fathima Docrat, declare that:

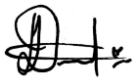
i) The research reported in this thesis (unless otherwise indicated) is my original work, has not been submitted for any degree or examination at any other university, does not contain other persons' data, diagrams, graphs, or other information, unless specifically being acknowledged as sourced from other persons, does not contain other persons' writings unless being acknowledged as being sourced from other researchers. Where other written sources have been quoted then:

a) Their work has been re-written, but the information sourced has been referenced to the authors

b) Where their exact words have been used, their writing has been placed within quotation marks and referenced

ii) Where I have reproduced a publication of which I am author, co-author, or editor, I have indicated in detail which part of the publication was written by myself alone and have properly referenced such publication

iii) This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged and the source being detailed in the thesis and reference section.

Signed:  Date: 13/02/2020

Taskeen Fathima Docrat

DEDICATION

This thesis is dedicated to my late father, my loving Mum and Husband.

ACKNOWLEDGEMENTS

These few words not do justice to the immense gratitude I feel to all of you that played integral roles in my PhD journey.

Professor A. A. Chuturgoon

To someone that has been more than just a supervisor, a guiding light, a role model, our Guru. You once told me that I had a "bulldog mentality", this has stuck with me throughout my PhD and helped me remember to stay internally strong, keep moving forward, be self- reliant and not dependent on factors outside of myself. Thank you for moulding me into the young scientist I am today and for teaching me how to be the best version of myself. I am truly blessed to call you my Professor. Your immense support and patient understanding is deeply appreciated. I only aspire to be half as incredible as you are someday. I will always remember these years working under you, they are the foundation of my career. You are a source of inspiration and motivation; I wish you endless happiness and success in every aspect of your life. God bless.

Dr. Savania Nagiah

From being the girl that purchased your medical physiology textbook in undergrad- to a doctoral scholar under your wing. You have helped me soar through the stormy weather of my PhD. Thank you for picking up the pieces and for always being there, as a supervisor, bagel buddy and a good friend. As J. K Rowling said - "there are some things you can't share without ending up liking each other and knocking out a twelve-foot mountain troll is one of them", my PhD being the mountain troll. May you continue to brighten the lives of future students.

My Family

Thank you for the support during the trying times throughout the years of my PhD. Having 5 siblings does have its perks, a caring family standing beside me with their love and unconditional support. Thanks mum, for playing the role of both parents, being there whenever I needed you, always pushing me forward, supporting me each day, and showing me the light when I lost my way- my wonder woman.

My heart felt regard goes to my father in law, and mother in law for their love and moral support.

My Husband

My truest friend, my better half, my rock. What started off as lab partners has blossomed into a wonderful marriage. I find it difficult to express my appreciation because it is so boundless. I would not be writing these acknowledgements today if it were not for your constant support along the way. I am indebted to your kindness, encouragement, generosity, thoughtfulness, time, and

love. You were always around when I thought it was impossible to continue, you helped me keep things in perspective- especially when I experienced “imposter syndrome”. Thank you for sharing this amazing journey with me and believing in me through it all. You have always showed me how proud you are. Thank you for being the highlight of everyday (especially with failed experiments), for the good times, the adventures, and all the foodscapes. Ps, I love you

Department of Medical Biochemistry

Writing a PhD can be a lonely task- To all my friends that were more like family along the way, soon to be Dr. Ghazi and Thilona Arumugam, thank you for your friendship, the intense vibrant discussions, the laughs, and all the hot chocolate socials. I will cherish the memories we have created. Mrs Narisha Needhi, thank you for all the lab assistance, the motherly advice, and sharing important life lessons with us. I wish you all nothing but success in the years to come.

The National Research Foundation and College of Health Science, UKZN

Scholarships and funding

I thank the Almighty for giving me the strength and patience to work through all these years so that today I can stand proudly with my head held high.

TABLE OF CONTENTS

PUBLICATIONS

1. Taskeen Fathima Docrat, Savania Nagiah, Nikita Naicker, Sooraj Baijnath, Sanil Singh, Anil A. Chuturgoon (2019) The protective effect of Metformin on mitochondrial dysfunction and endoplasmic reticulum stress in diabetic mice brain. *European Journal of pharmacology* (In review, European Journal of pharmacology, Ms. Ref. No.: EJP-52205)

2. Taskeen Fathima Docrat, Savania Nagiah, Anil A. Chuturgoon (2019) Metformin mediates neuroprotection by regulating miR-141 and dampening the NF-κB-mediated inflammasome pathway in a diabetic mouse model. *Biomedicine and pharmacotherapy* (In review, Ms. Ref. No.: BIOPHA-D-19-00128)

3. Taskeen Fathima Docrat, Savania Nagiah, Anil A. Chuturgoon (2019) Metformin regulates renal stress by Sirtuin 1 signalling axes and prevents intrinsic cell death in diabetic mice. *Journal of cellular Physiology* (In review, Ms. Ref. No.: JCP-19-5700)

PRESENTATIONS

1. The effects of Metformin, Fenugreek seed extract (FSE), and 4-hydroxyisoleucine (4-OH-Ile) in hyperglycaemic C57BL/6 black mice. Docrat, T. F., Nagiah, S., Chuturgoon, A.A. *JSS Medical college, Mysore, India* (21-23 February 2018). Oral Presentation

2. Metformin attenuates hyperglycaemia-induced neuronal oxidative stress by induction of mitochondrial maintenance proteins in C57BL/6 mice. Docrat, T. F., Nagiah, S., Naicker, N., Baijnath, S., Bester, L., Singh, S., Chuturgoon, A. A. *College of Health Sciences Research Symposium, University of KwaZulu-Natal, Durban, South Africa* (11-12 October 2018). 3rd place Oral Presentation – PhD category

3. Neuroprotective role of Metformin in diabetic mice (C57BL/6). Docrat, T. F., Nagiah, S., Chuturgoon, A.A. *University of Mauritius, ANDI Centre of Excellence for Biomedical and Biomaterials Research, MSIRI Building, Réduit, Mauritius* (22-23 April 2019) Oral Presentation

4. Metformin regulates renal stress by Sirtuin 1 signalling axes and prevents intrinsic cell death in diabetic mice. Docrat, T. F., Nagiah, S., Chuturgoon, A.A. *College of Health Sciences Research Symposium, University of KwaZulu-Natal, Durban, South Africa* (1 November 2019) 2nd place Oral Presentation – PhD category

LIST OF FIGURES

ADDENDUM C

LIST OF TABLES

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AGES	Advanced glycation end products
AIF	Apoptosis-inducing factor
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
AR	Aldose reductase
ARE	Antioxidant response element
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BAX	BCL2-Associated X Protein
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
CARD	Caspase-recruitment domain
CAT	Catalase
Ca^{2+}	Calcium
cDNA	complementary DNA
CHOP	C/EBP homologous protein
Cyt-c	Cytochrome c
DAMPS	Damage-associated molecular patterns
DM	Diabetes mellitus
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
ECL	Enhanced Chemiluminescence

Eif2	eukaryotic translation initiation factor 2
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GK	Glucokinase
GLUTs	Glucose transporters
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSK3β	glycogen synthase kinase-3β
GSTA4	Glutathione S-Transferase Alpha 4
H_2O_2	Hydrogen peroxide
HIF-1	Hypoxia-inducible factor 1
HNE	Hydroxy trans-2,3-nonenal
HRP	Horseradish peroxidase
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
IDDM	Insulin-dependent diabetes mellitus
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-18	Interleukin 18

IR	Insulin resistance
IRE1	Inositol-requiring protein 1
IRS	Insulin receptor substrate
Keap1	Kelch-like ECH-associated protein 1
kDa	Kilo Daltons
LonP1	Lon protease
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDM2	Mouse double minute 2
MF	Metformin
MG	Methylglyoxal
MiR	Micro RNA
MiRISC	miRNA-induced silencing complex
mRNA	Messenger RNA
mt	Mitochondrial
mtDNA	Mitochondrial DNA
NAD/NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NAM	Nicotinamide
NF-κB	Nuclear factor κB
NFT	Neurofibrillary tangles
NIDDM	Non-insulin dependent diabetes mellitus
NLRP3	Nod-like receptor protein 3
NRF	nuclear transcription factors
Nrf2	Nuclear respiratory factor 2
OCTs	Organic cation transporters
OGTT	Oral glucose tolerance test

OS	Oxidative stress
OxPhos	Oxidative phosphorylation
$O_2^{\bullet -}$	Superoxide anion radical
PAMPs	Pathogen-associated molecular patterns
PARP-1	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PC-PEPCK	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
PI3K	Phosphoinositide 3-kinase
PP2A	Protein phosphatase type 2A
PRRs	Pattern-recognition receptors
PTM	Post-translational modification
qPCR	Quantitative polymerase chain reaction
RFC	Relative fold change
RNA	Ribonucleic acid
RNU6	RNA U6 small nuclear
ROS	Reactive oxygen species
RT	Reverse transcriptase/ Real time
Ser	Serine
SGLT	Sodium-glucose co-transporter
Sir2	Silent information regulator 2
Sirt1	Sirtuin 1
Sirt3	Sirtuin 3
SOD2	Superoxide dismutase 2

STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA cycle	Tricarboxylic acid cycle
Thr	Threonine
TrkB	Tyrosine kinase receptor B
Tyr	Tyrosine
TNF-α	Tumour necrosis factor-α
TZDs	Thiazolidinediones
UPR	Unfolded protein response
UTR	Untranslated region

ABSTRACT

In recent years, diabetes has become more prevalent due to modern, demanding, and sedentary lifestyle patterns. This disease is characterised by insulin resistance and associated molecular complications. Metformin (MF) is a popular antidiabetic agent that has effects beyond glycaemic control such as regulation of metabolic molecular pathways. However, the exact mechanisms against hyperglycaemic induced end organ damage remain elusive. This study aimed to investigate the protective effects of MF in the brain and kidney *in vivo*, by exploring dysregulated pathways related to mitochondrial function, oxidative stress, ER stress, inflammation, and apoptosis.

This study established a diabetic mouse (C57BL/6) model (Ethics no: AREC/057/016) through intraperitoneal multiple low-dose STZ (50 mg/kg BW) injections (10 days). Blood sugar levels of 7-16mmol/L were considered diabetic, and the 15 day treatment period (MF, 20 mg/kg BW per day, oral gavage) was inducted thereafter. Fasting (12 hr) plasma OGTT revealed MF significantly lowered blood glucose levels in diabetic mice. All mice experiments were performed by Dr. N. Naicker. Post-sacrifice (isoflurane), the investigator (author) of the work in the presented in this thesis assisted in harvesting Whole brain and kidney tissue and performed all downstream protein and mRNA analyses. Diabetic mice exhibited heightened oxidative stress by protein carbonylation, and diminished antioxidant responses in both the brain and kidney compared to normoglycaemic mice. Metformin significantly reduced protein carbonylation, increased *GSTA4* expression in the brain; and *Nrf2* and *GPx* mRNA levels in the kidney, alleviating oxidative stress. Further, MF improved mt activity, and decreased the *HIF-1* expression in the kidney through upregulation of AMPK, and Sirt1 expression. In addition, MF induced epigenetic changes in mice brain through *miR-148a* repression and concomitant increases in PGC-1α, Sirt1, and Sirt3 protein and gene expressions, thus regulating mt biogenesis. Mitochondrial chaperone proteins HSP60, HSP70 and LonP1 in diabetic mice brain were upregulated through a MF-induced *miR-132* repression mechanism. Regulation of the UPR by PERK-eIF2α inhibition after MF-treatment attenuated ER stress in diabetic mice brain and kidney tissue. Moreover, renal injury associated with diabetes was attenuated by MF through decreased CHOP expression, downstream to ER stress. This finding was supplemented by inhibition of Bax, cyt-c, and ultimately the intrinsic apoptotic pathway.

MiR-141 modulates expression of PP2A, a phosphoesterase that regulates phosphorylation of tau protein. In hyperglycaemic mice there was increased *miR-141* expression with concomitant *PP2A* downregulation in the brain. Treatment with MF exerted epigenetic regulation by downregulating *miR-141* expression, concomitantly increasing PP2A and subsequent downregulation of tau protein phosphorylation at Ser^{396}. Additionally, MF inhibited proinflammatory NLRP3

inflammasome and related components by regulation of the PP2A/ NF-κB cascade. Neuroplasticity was increased by increased BDNF overexpression by MF in diabetic mice.

Herein we show that MF exerts protective mechanistic effects in the brain and kidney over an acute experimental period. We highlight that anti-oxidant and Sirt1 modulation are at the forefront of renal cell defence to metabolic stress. Neuroinflammatory and epigenetic therapeutic targets of MF are revealed through miRNA regulatory mechanisms, integrating the mechanisms of diabetic neuronal and renal damage.

CHAPTER 1

1. Introduction

1.1 Background

In recent years, a dramatic growth in the prevalence of diabetes has been documented in all regions of the world, with approximately 422 million people now living with the (Association, 2019). Without interventions, the number of diabetic people is expected to increase to 629 million in the next three decades (Atlas, 2017). This significantly impacts socio-economic conditions related to global healthcare as diabetic related complications negatively affect quality of life, health services and economic costs. The growth of diabetes incidence has led to population-based studies assessing diabetic complications that are concentrated in Europe, North America and other high-income countries, whilst low- and middle-income countries are neglected (Harding et al., 2019). This may partially be because diabetes was considered a burden in developed countries, but a huge increase has now been reported in developing countries. Third world countries within Africa often do not have the resources for the prevention, diagnosis, treatment and management of the disease, thereby contributing to the increase in sufferers. Diabetes is broadly categorized into two groups: Type 1 diabetes mellitus (T1DM) where insulin deficiency occurs through selective pancreatic β-cell destruction by the immune system (Simmons and Michels, 2015), and Type 2 diabetes mellitus (T2DM) where the body does not respond to normal insulin secretion, resulting in metabolic disturbances (Zaccardi et al., 2016).

The prominence in diabetes incidence since the 1980s, together with declining mortality among people with diabetes (likely due to medical intervention and awareness), has significantly increased the years of life spent with diabetes. Aside from the metabolic alterations characterised by hyperglycemia, observations of trends in 'emerging' diabetes complications have singled out macrovascular and microvascular complications, such as retinopathy, end-stage renal disease (nephropathy), and neuropathy as being responsible for much of the disease burden associated with this condition (Harris et al., 2012, Harding et al., 2019).

The liver and kidney are the only organs that contain sufficient glucose-6-phosphatase (G-6-P) enzymatic activity to maintain glucose levels in the blood through gluconeogenesis (*de novo synthesis* of glucose) (Meyer and Gerich, 2000). The release of glucose into circulation meets the demands of highly metabolic organs like the brain (Gerich et al., 2001, Gerich, 2010). Therefore, the liver and kidney are established gluconeogenic organs (Alsahli and Gerich, 2017), and their roles are dysregulated in patients with diabetes.

Many studies are beginning to show a link between diabetes and impaired cognitive processes, hastening the progression to dementia (Biessels and Despa, 2018, Campbell et al., 2013, Messier, 2005). The biochemical and physiological conditions produced by diabetes, such as advanced glycation end products (AGES) and aberrant metabolic processes, are increasingly being considered as risk factors for neurodegenerative diseases (Beeri et al., 2005).

At the cellular level both the brain and kidney have high mitochondrial content. Hyperglycaemia and disturbed glucose metabolism cause mitochondrial (mt) dysfunction, leading to an overproduction of reactive oxygen species (ROS) (Sivitz and Yorek, 2010). An imbalance between ROS production and the ability to detoxify the reactive intermediates induces oxidative stress. Excessive ROS damages macromolecules, including nucleic acids, lipids, and proteins, leading to a decline in physiological function. ROS attack on proteins may be reversible or irreversible, often leading to either a loss of function or protein aggregation (Haigis and Yankner, 2010). Protein modifications are well established in diabetes, with patients exhibiting high levels of protein carbonyl derivatives in blood. Furthermore, hyperglycaemia perturbs mt bioenergetics, thereby diminishing mt oxidative phosphorylation (OxPhos). The dramatic increase in cytosolic NADH is referred to as pseudohypoxia (Williamson et al., 1993), a phenomenon recently gaining significant attention. As a consequence, reductive stress occurs, followed by oxidative stress and eventual cell death and tissue dysfunction (Song et al., 2019).

Fluctuations in the ADP/ATP or the NAD/NADH ratios determine cellular energy status; energy sensing proteins monitor either the AMP/ATP or NAD/NADH ratios, or both (Hardie et al., 2012, Cantó and Auwerx, 2009). The enzymatic activity of AMP-activated protein kinase (AMPK) and Sirtuin 1 (Sirt1, a member of the Sirtuin family) can be classified as metabolic sensors. Sirt1, a NAD^+-dependent histone deacetylase, responds to high glucose levels alongside AMPK and mt dysfunction (Cantó and Auwerx, 2009, Reznick and Shulman, 2006). These metabolic sensors act as gatekeepers for mt turn over and are vital links in a regulatory network for metabolic homeostasis.

Non-canonical mechanisms that may not involve increases in AMP, ADP or NAD^+ can activate AMPK and Sirt1 such as elevated ROS (Hardie et al., 2012, Kao et al., 2010, Rabinovitch et al., 2017). Alterations in the oxidative environment causes oxidative and endoplasmic reticulum (ER) stress (Bhandary et al., 2013). These are hallmark features of DM and disturbances in the redox state interrupts disulphide bond formation and protein misfolding, further generating ROS (Cao and Kaufman, 2014). Within the ER, protein misfolding in the secretory pathway gives rise to the unfolded protein response (UPR) which lowers the load of misfolded proteins, and can initiate inflammation and apoptosis (Eizirik et al., 2013, Hasnain et al., 2012).

Diabetes triggers inflammatory processes that promote degenerative pathways in the brain. The inhibitory effect of PP2A on inflammatory mediators has been outlined (Sangodkar et al., 2016). The arbitrators of inflammation encompass cytokine production e.g., tumour necrosis factor-α (TNF-α) and interleukins (IL), resulting in activation of nuclear factor κB (NF-κB) signalling pathways (Kitada et al., 2019). In association with this is the mt regulation of the NLRP3 inflammasome, through production of ROS and damage-associated molecular patterns (DAMPs) (Shimada et al., 2012, Jo et al., 2016).

Chronic glucose overload overwhelms the cells defence mechanisms and may result in cellular suicide, more commonly known as apoptosis/ programmed cell death. Cells require apoptosis to maintain homeostasis, however dysregulation of this system develops through ROS production or hypoxic states (Fulda et al., 2010, Pallepati and Averill-Bates, 2012). The intrinsic pathway is triggered by mt and ER stress, wherein, activation of initiator caspase-9 occurs through formation of an apoptosome, followed by executioner caspase -3 and -7 (Orrenius et al., 2015). Aberrant biochemical parameters have important pathological consequences for oxidative stress-related diseases such as diabetes, and secondary neurological and nephropathic effects.

Fine tuning of cell defence mechanisms occur at the epigenetic level where microRNAs (miRNAs) modulate target gene expression through direct binding to 3'untranslated region (UTR) (Kadamkode and Banerjee, 2014). Neuronal cells are enriched with miRNAs that promote their function and survival, and aberrant expression profiles of miRNAs during hyperglycaemia and diabetes are well documented (Lukiw et al., 2013, Sempere et al., 2004). The limited information available on the mechanistic regulation of neuronal gene expression by miRNAs highlights the need to establish pharmacological outcomes.

Studies that aid in understanding the mechanisms and pathways dysregulated by hyperglycaemia could help identify and improve preventive and therapeutic strategies for associated damage. Anti-hyperglycaemic agents have effects on the brain and kidneys other than those that are involved in glucose regulation. Medicinal preparations intended to lower blood glucose differentially affect inflammation, cognitive function, and other brain and kidney indices. Metformin is a guanidine derivative of the French Lilac *Galega officinalis* plant and has been used in the treatment of diabetes dating back to the 1950s. This naturally derived drug was developed by Jean Sterne and named 'Glucophage', directly translating to 'glucose eater', as it efficiently lowers blood-glucose (Bailey and Day, 2004). It has become the most popular first-line oral treatment for diabetes due to its reduced toxicity. Metformin has been proven to lower all-cause mortality in diabetic patients as compared to those not utilising the drug (Roussel et al., 2010), displaying its beneficial role. Ingested MF primarily suppresses hepatic glucose production; however, it remains intact and accumulates in the brain, and is eliminated through the renal system. This drug rapidly passes the

blood-brain barrier, and strong evidence is provided showing its direct impact on the central nervous system (Beckmann, 1969, Chen et al., 2009, Lv et al., 2012, Wilcock and Bailey, 1994). Metformin is known to improve glucose uptake, and sensitivity to insulin by inhibiting gluconeogenesis through AMPK activation (Zhou et al., 2001). At the cellular level, MF targets mitochondrial respiratory chain complex 1, and is the mechanism by which it induces AMPK (Stephenne et al., 2011). Despite the definitive role of MF in decreasing glucose levels in blood, no unequivocal mechanisms exist, and several secondary molecular targets potentially comprise its beneficial effects.

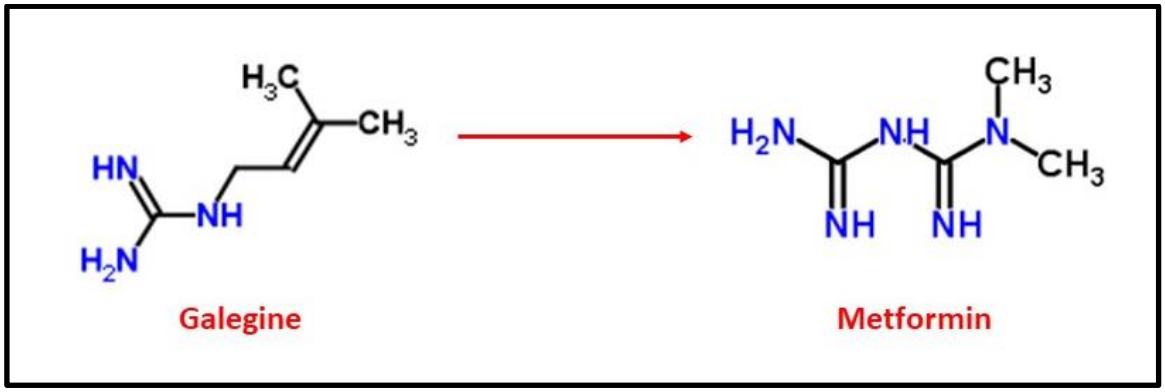


Figure 1: Metformin-a Galegine derivative (Prepared by author)

1.2 Problem statement

Research has provided the scientific basis for, and confirmed, the "traditional" but important role of MF as an anti-diabetic agent in the treatment of T2DM. Diabetic complications span across a multitude of platforms, including organ damage and the incidences of neuropathy and nephropathy. Yet, there remains a gap in knowledge on the potential of MF to alleviate organ damage at the molecular level through activation of cell defence mechanisms.

1.3 Hypothesis

It was hypothesised that MF promotes cytoprotective defences against diabetic oxidative stress to alleviate associated brain and kidney tissue damage in C57BL/6 mice by improving mt function and fine-tuning epigenetic regulation.

1.4 Aim and Research questions

This study aimed to investigate the neuronal and renal protective effects of MF on dysregulated pathways related to mt function, inflammation, oxidative stress, ER stress and apoptosis. The following research questions were posed:

- How does MF improve mt function?
- Will ER stress and unfolded protein response (UPR) be targeted by MF?
- Can MF improve diabetic induced inflammatory status?
- Does MF affect microRNA dysregulation in diabetes?

1.5 Objectives

The effects of MF were determined by measuring its effects on mRNA and protein expression in mice brain and kidney tissue homogenates. Specifically, the following objectives were investigated:

- Mt stress and biogenesis markers: AMPK, Sirt1, PGC-1α
- Assessment of protein carbonyl formation and antioxidant regulation as markers and mechanisms of oxidative stress alleviation
- ER stress sensors in UPR
- Tau hyperphosphorylation, PP2A and BDNF signalling in relation to neuroinflammation
- NF-κB regulation of related transcripts of the NLRP3 inflammasome
- MiRNA specific target regulation of mt biogenesis markers
- The apoptotic pathway and its mechanism in nephropathy

1.6 Experimental approach

Animal models are frequently used to assess the molecular pathways involved in diabetes (Kim et al., 2009). In this study, diabetes was induced by intraperitoneal administration of STZ, a naturally occurring agent that like glucose, enters the β-cells of the pancreas via the GLUT 2 receptor (Szkudelski, 2001). Its ability to prevent insulin production has been established through PARP-1 activation and subsequent depletion of the NAD^+ pool (Sandler and Swenne, 1983). The common trend of STZ administration involves multiple low doses or a single high dose which partially inhibits pancreatic function, resulting in a T2DM model. Based on previous studies, a range of STZ treatment concentrations (50 mg/kg, 100 mg/kg, 150 mg/kg) were selected and the glucose outcome was assessed (Table S1). The model was designed to administer multiple low doses over an acute time period, as it effectively increases blood glucose levels to the T2DM

range (between 7-16 mmol/L; or 150-300 mg/dL) (Sumner et al., 2003), and is known to induce insulitis in mice (Fang et al., 2019, Like and Rossini, 1976, Wang and Gleichmann, 1998). Initial concern regarding the use of STZ as a diabetic model existed as organ damage may be due to its toxicity and not the diabetic effect. However, substantial evidence is provided by a study showing transplantation of pancreatic islets reversed the hyperglycaemia induced by STZ and improved renal function (Palm et al., 2004). Zhuo and colleagues recently demonstrated that intraperitoneal administration of STZ alone is an efficient non-obese rodent model in comparison to a high-fat sugar/STZ rodent model, as both models had similar pathological traits of T2DM through effects on insulin and glucose levels (Zhuo et al., 2018). They further validated the non-obese T2DM model by assessing protein expression of insulin signalling and related inflammatory pathway. These findings indicate that administration with STZ alone is enough to establish the T2DM model, is more economical and efficient than combination treatment. It is important to note that the dosage administered in different species vary as different subgroups of a strain will have different STZ sensitivity. Chapters 3, 4 assess the molecular effects of antidiabetic drug, MF, on mt and epigenetic mechanisms in brain tissue, whilst chapter 5 focuses on the renal stress induced by STZ, and the role played by MF in nephrotoxic relief.

CHAPTER 2

2. Literature review

2.1 Diabetes

2.1.1 Definition and prevalence

Diabetes is characterised by high blood glucose as a result of relative or absolute lack of insulin caused by abnormal pancreatic function. Resulting hyperglycaemia disrupts the energy production required for optimal tissue function. The global burden of this disease is on the rise (Fig 2.1), with rapid increases in diabetic prevalence being proportional to urbanization and sedentary lifestyles (Blas and Kurup, 2010). With this comes the burden of diabetes-associated complications that have financial, social and developmental implications especially in third world countries.

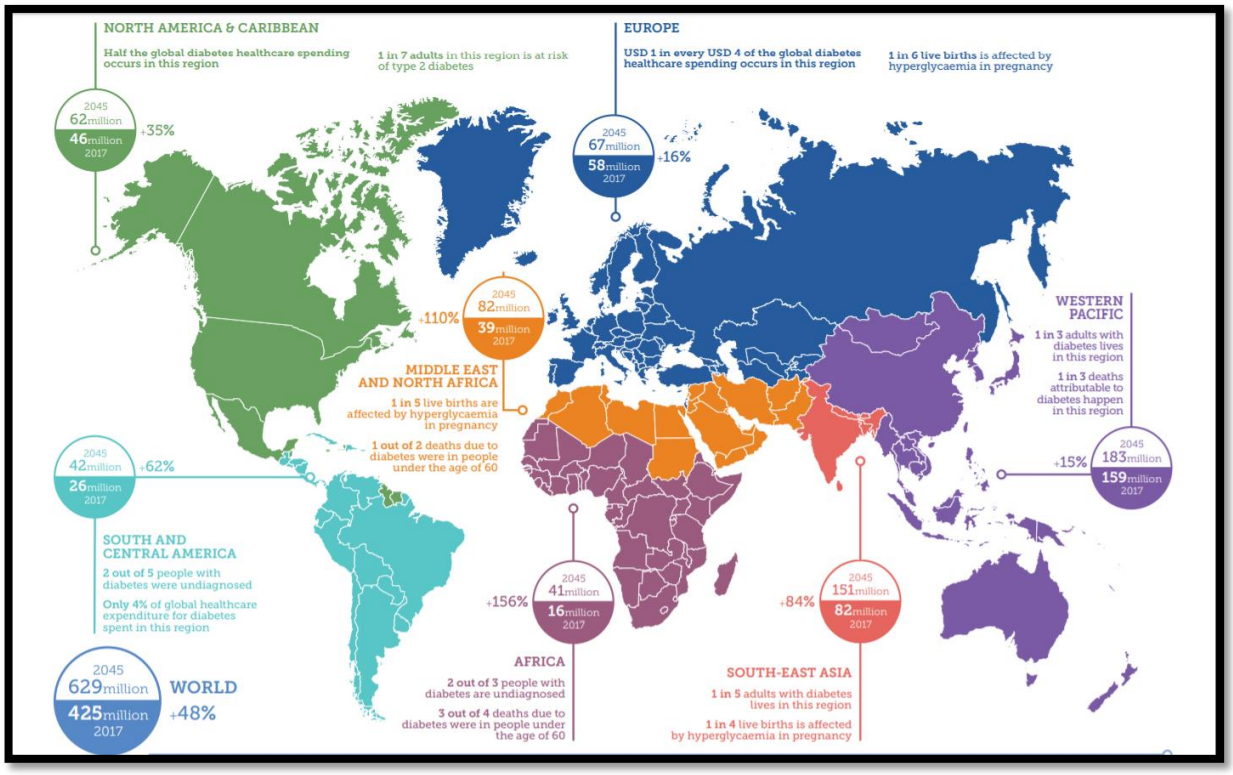


Figure 2.1: Global prevalence of diabetes (Atlas, 2017)

2.1.2 Types of diabetes mellitus

The proposed classifications include insulin-dependent diabetes mellitus (IDDM), commonly known as Type 1; and non-insulin dependent diabetes mellitus (NIDDM) referred to as Type 2. However, patients are categorized according to their treatment regime rather than pathogenesis (Goodpaster et al., 2010).

2.1.2.1 Type 1

Type 1 is an auto-immune disorder that ensues following destruction of insulin producing pancreatic β-cells and comprises of approximately 5-10% of all diabetics (DiMeglio et al., 2018). Although traditionally defined as juvenile onset, a considerable number of cases present in adulthood. This differs in the severity of autoimmune response and therapeutic efficacy (Thomas et al., 2018). Two subclasses exist, type A (more common) where serological autoimmune responses can be detected (Fig 2.2); and type B (uncommon), the idiopathic type, where humoral autoimmunity is undetectable (Pietropaolo et al., 2012). However, this distinction is not widely adopted. Treatment regimens for T1DM include regular subcutaneous injections of insulin as the body is unable to produce it on its own. The risk of hypoglycaemia is managed by closely monitoring blood glucose levels daily.

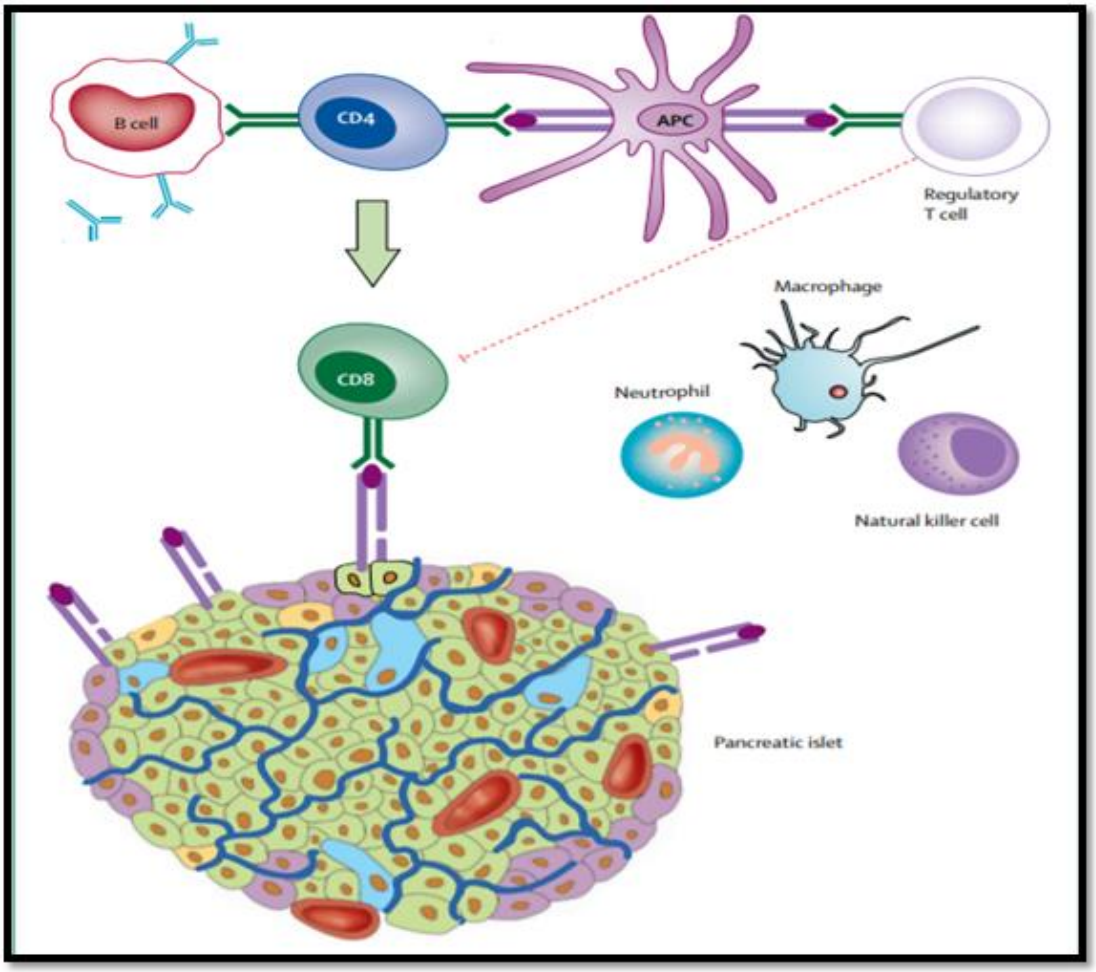


Figure 2.2: Interaction between the pancreatic β-cell and innate and adaptive immune systems (DiMeglio et al., 2018). Diabetes is initiated by the presentation of β-cell peptides by antigen-presenting cell (APCs). Autoantibodies against β-cell proteins produced by Activated T cells within the pancreatic lymph node can be measured in circulation and are considered a defining biomarker of type 1 diabetes

2.1.2.2 Type 2

The most common type of diabetes is the adult-onset / T2DM which develops through insufficient insulin production or insulin resistance (Paramithiotis et al., 2019). Several investigations have identified the risk factors associated with development of T2DM (Fig 2.3) leading to neuropathy, retinopathy, nephropathy and increased risk of cardiovascular disease (Cersosimo et al., 2018, DeFronzo, 2004, Stumvoll et al., 2005). In the initial stages, insulin resistance is compensated by further insulin secretion leading to hyperinsulinaemia and hyperglycaemia. This heterogenous disorder is common in obese patients and varies in treatment responses (Inzucchi et al., 2015, Pozzilli et al., 2010). Initial treatment for T2DM is lifestyle intervention, followed by various

pharmacological aids. These drugs include sulphonylureas, biguanides (more common), glucosidase inhibitors, and thiazolidinediones.

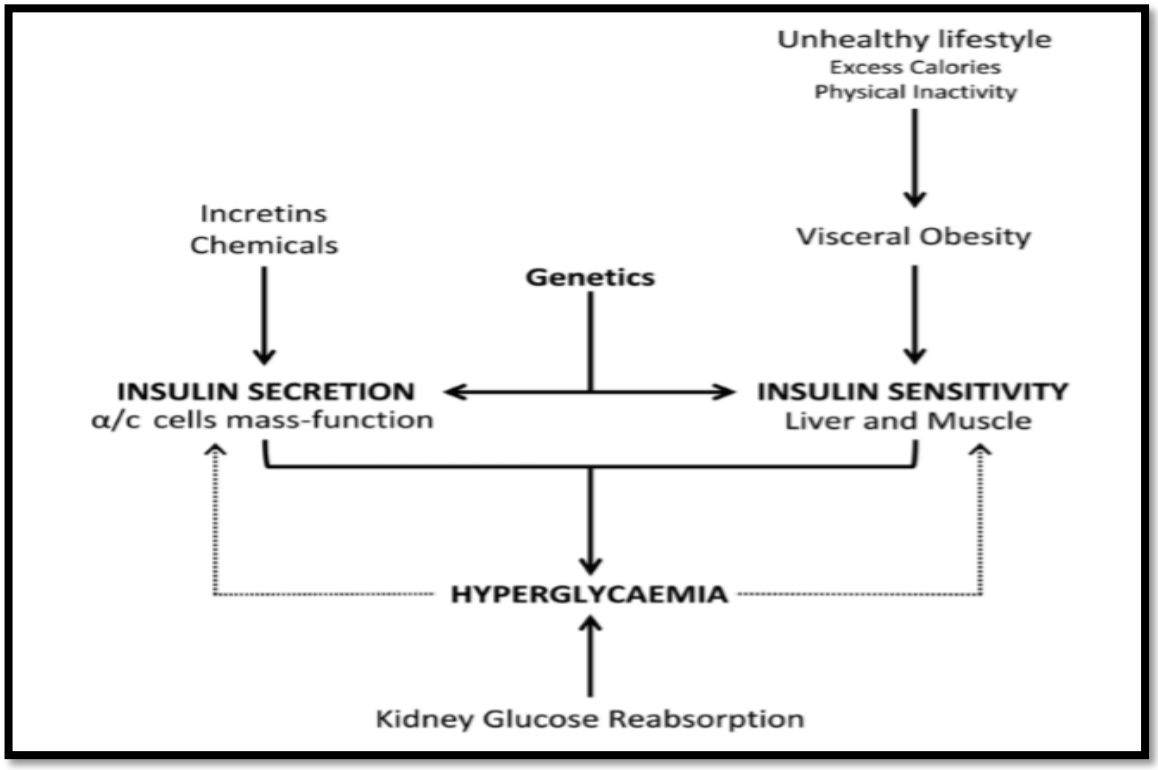


Figure 2.3: Pathophysiology associated with Type 2 DM (Zaccardi et al., 2016)

2.2 Glucose metabolism

Metabolism is broadly defined as the sum of biochemical processes in organisms that produce or consume energy. Core metabolism is simplified to encompass pathways involving the use of major nutrients that are essential for macromolecular synthesis and energy homeostasis. Several human diseases involve abnormal metabolic states that perturb normal physiology, leading to cell and organ dysfunction (DeBerardinis and Thompson, 2012). Numerous regulatory mechanisms linking cell signalling with the regulation of metabolic pathways have now been identified. The integration of cellular responses to hormones and their effects on core metabolic pathways is a good example of this (DeBerardinis and Thompson, 2012). In particular, the regulation of glucose metabolism by insulin is at the forefront of current research.

The rate of glucose entering circulation is balanced by the rate of glucose removal from circulation (glucose disposal); these processes are integral for maintaining plasma glucose concentration and

homeostasis. Circulating glucose is derived from three sources: intestinal absorption during the fed state, glycogenolysis, and gluconeogenesis (Aronoff et al., 2004). The resultant energy substrates reach various tissues for adenosine triphosphate (ATP) production. Glucose metabolism (Fig 2.4) involves the conversion of glucose to glucose 6-phosphate (G-6-P), this phosphorylated form feeds into various anabolic processes including glycogen synthesis, the pentose phosphate pathway, fatty acid synthesis, hexosamine pathway, and ribose 5-phosphate for nucleotide synthesis. Excess glucose is either stored as glycogen and fatty acids; or shunted to form glycosylation reaction precursors and other oxidative routes that are damaging to cells (Jose et al., 2011).

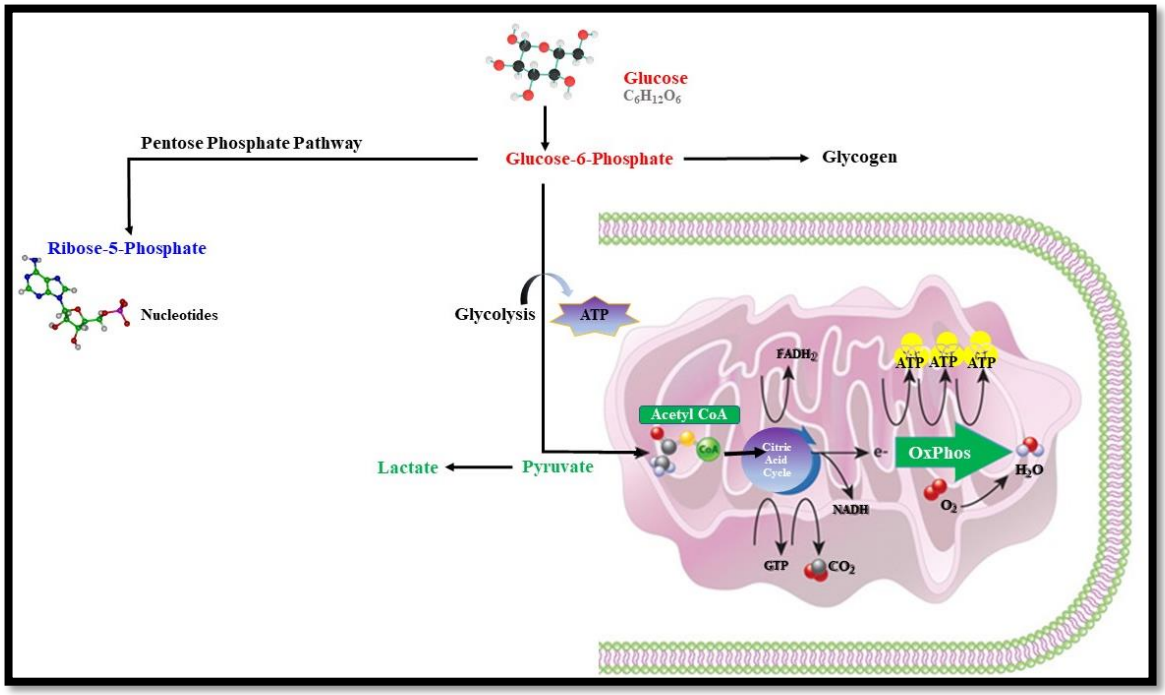


Figure 2.4: Fates of glucose. Glycolysis occurs in the cytoplasm whilst ATP is produced in the mitochondrion (Prepared by author)

2.3 Insulin signalling

The hormone insulin is secreted by the β-cells of the pancreas in response to hyperglycaemia, facilitating glucose uptake. Insulin binds to its receptors located on the cell membrane, increasing the number of glucose transporters (GLUTs) (Czech and Corvera, 1999). Glucose homeostasis is orchestrated by the insulin signalling pathway (Plum et al., 2006). Insulin binds to insulin receptor substrate (IRS), stimulating conformational changes by activating the phosphoinositide 3-kinase (PI3K) intracellular signalling pathway (Lee et al., 2014). The signalling cascade

stimulated by the binding of insulin to its receptor ultimately results in the recruitment of GLUT transport proteins at the cell surface, to facilitate glucose uptake from circulation (Chen et al., 2018). Insulin further promotes phosphorylation of glucose by transcription of glucokinase (GK), which plays an integral role in ATP production by glycolysis. Recent studies highlight the mechanisms involved in insulin's regulation of pathways (Fig 2.5) involved in mitochondrial (mt) metabolism (Cade, 2018, Yaribeygi et al., 2019). In mt rich tissue, pyruvate is transported into the mitochondrial matrix from the cytoplasm to produce acetyl-CoA, the substrate that feeds into the tricarboxylic acid cycle (TCA) cycle (Han et al., 2016). This cycle produces GTP (equivalent to ATP), nicotinamide adenine dinucleotide (NADH), and $FADH_2$; all of which operate in ATP production from oxidative phosphorylation (OxPhos). Insulin signalling is required for mt DNA and protein synthesis and stimulates mt oxidative capacity and ATP production. Although all organs rely on ATP as a potent source of energy, each has a unique metabolic profile that contributes to glucose homeostasis through utilisation and endogenous production of this monosaccharide. Insulin resistance is closely linked to metabolic disorders where the body doesn't respond to or produce insulin efficiently and glucose is prevented from entering the cell. The mechanisms involved in potentiating insulin resistance have been extensively researched and include lifestyle choices, genetics and environmental influences (Di Pino and DeFronzo, 2019, Gregor and Hotamisligil, 2011, Hotamisligil, 2010, Petersen and Shulman, 2018, Nakamura et al., 2010). Both the liver and kidney can provide glucose to the body on demand through glycogenolysis or gluconeogenesis (Azevedo et al., 2019) while the brain has no glucose stores, and relies solely on circulating plasma glucose.

Intracellular glucose may be reduced to sorbitol, transported back to extracellular fluid, or metabolised into G-6-P. Insulin signalling exerts effects beyond glucose metabolism such as repair and growth functions. Insulin receptor activation comprises a range of regulatory effects depicted in Fig 2.5, these include increasing glucose uptake through Akt signalling, control of gene expression by mitogen activated protein kinase (MAPK) signalling, mt regulation, protein synthesis, transcription of antioxidant genes, as well as autophagic and apoptotic inhibition (Carro and Torres-Aleman, 2004, Heras-Sandoval et al., 2014, Najem et al., 2014, Song et al., 2018).

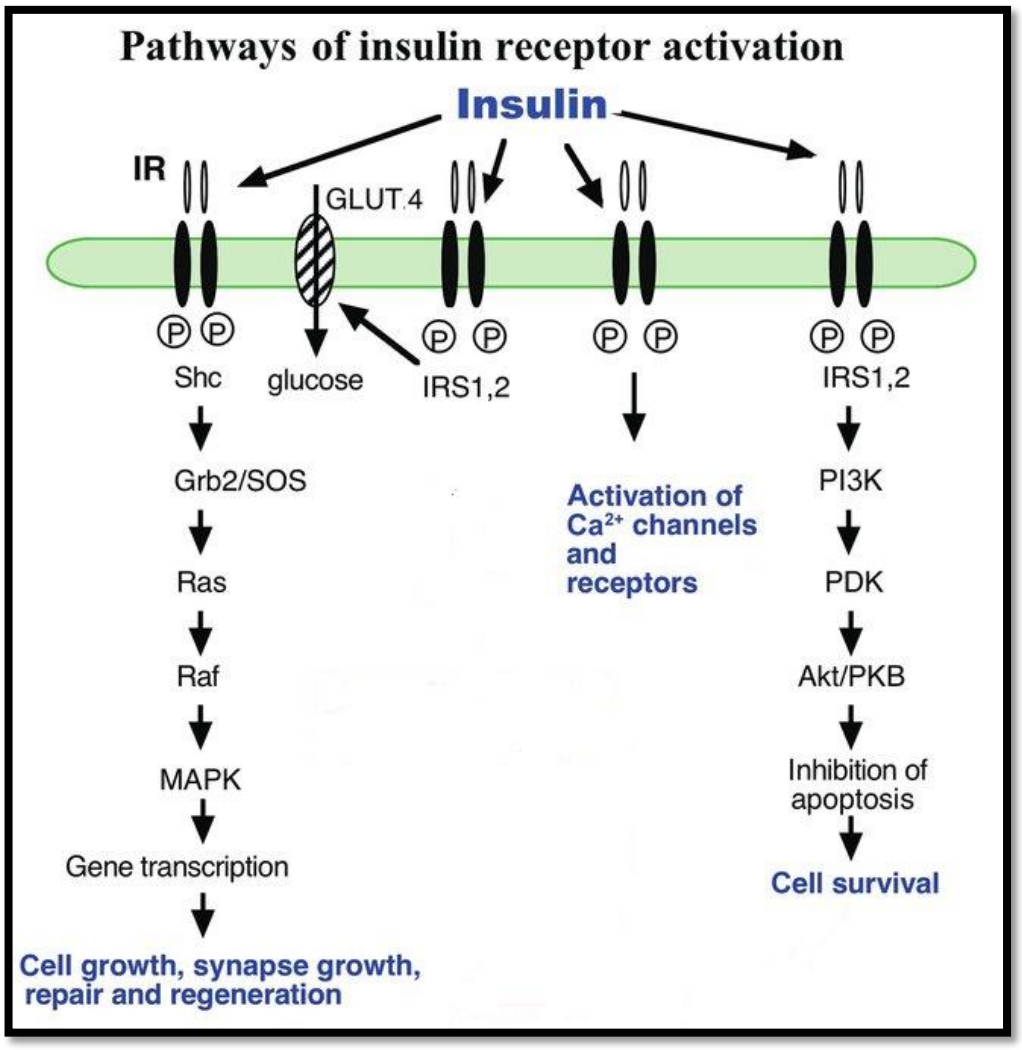


Figure 2.5: Insulin signalling and related pathways (Hölscher, 2014)

2.4 Diabetes associated complications

2.4.1 Organ damage

The adverse effects of hyperglycaemia affect the heart, eye, kidney, and brain (Barrière et al., 2018). Metabolic disturbances disrupt homeostasis by shunting excess glucose to alternative disposal pathways (Brownlee, 2001). These include the activation of the polyol pathway, formation of Advanced Glycation End products (AGEs) and products of the hexosamine pathway (Kajikawa et al., 2015, Vasconcelos-Dos-Santos et al., 2017). Among the various organs affected by high glucose, little attention has been given to the mechanisms of neuronal and renal damage.

For the purposes of this review diabetes-induced neuropathy and nephropathy will be discussed in depth.

2.4.1.1 Diabetic neuropathy

Damage to brain vasculature, functional impairment, and cerebral neuropathy are effects of diabetes and aberrant glucose metabolism (Mijnhout et al., 2006). “Passive” neurological responses to DM do not directly involve neuronal activity (Fig 2.6). This raises the question if neurons are just end-targets or if they are actively involved in the disease progression.

The brain requires a constant supply of glucose to meet its metabolic demands and sustain proper neuronal activity through the production of adenosine triphosphate (ATP) through oxidative metabolism (Harris et al., 2012). Glucose and insulin signalling pathways in the brain involve complex, interacting networks and counter-effects to regulate fasted and fed cycles (Nelson et al., 2009). Several isoforms of the glucose transporters facilitate glucose uptake in the brain (E González-Reyes et al., 2016). These transporters are responsible for the exchange of glucose to and from arterial blood and the brain. The majority of neuronal glucose is transported via GLUTs -3 and -4, whilst GLUT 1 facilitates transport in endothelial cells of the blood-brain barrier (BBB) and astrocytes (Ashrafi et al., 2017, Pearson-Leary and McNay, 2016, Simpson et al., 2007). Excessive glucose correlates with the passive metabolic effects in diabetes pathology. In the brain, this impacts maintenance and cellular repair mechanisms risking the development of neurodegenerative disorders. Similarly, active pathways involving neuronal gene alteration can further drive the pathogenic response to end-organ damage. High glucose promotes cognitive decline and increases the risk of dementia (Biessels and Kappelle, 2005). Evidence for a second mechanism of pathogenesis that involves active changes in gene expression in neurons of the CNS has been brought to light. These altered gene expression profiles result in molecular phenotypic and functional changes that can become detrimental over time. This is supported by a study showing impaired neuronal glucose metabolism in association with degenerative states through brain imaging (Cohen and Klunk, 2014).

There are several prevailing models underlying the aetiology of diabetic neuropathy, but its development is multifactorial. These include the polyol pathway, glycosylation end-product formation, and the oxidative stress (OS) theory. The polyol pathway involves the accumulation of sorbitol which leads to a decrease in the sodium-potassium adenosine triphosphatase activity and subsequent accumulation of axonal sodium accumulation. This leads to structural damage and impaired axonal transport. Hyperglycaemia or related OS induces oxidative glycation of free amino groups forming glycosylated end-products. Glycosylation of endothelial cell basement membrane leads to functional impairment, limiting vasodilation and increases the inflammatory

response. (Boulton et al., 2005, Shakher and Stevens, 2011). Considering that mt ATP provides the energy for neuronal function, DM induced impairments will lead to eventual neurodegeneration. The brain is highly susceptible to OS in hyperglycaemia due to its aerobic nature and relatively low antioxidant defence (El-kossi et al., 2011). This redox imbalance causes damage to brain microvasculature leading to BBB disruption, pericyte loss, and nervous system structure impairments. Glycaemic control early in diabetes can delay the onset of neuropathies and have prolonged effects (Shakher and Stevens, 2011), however the molecular events beyond glycaemic control are yet to be elucidated.

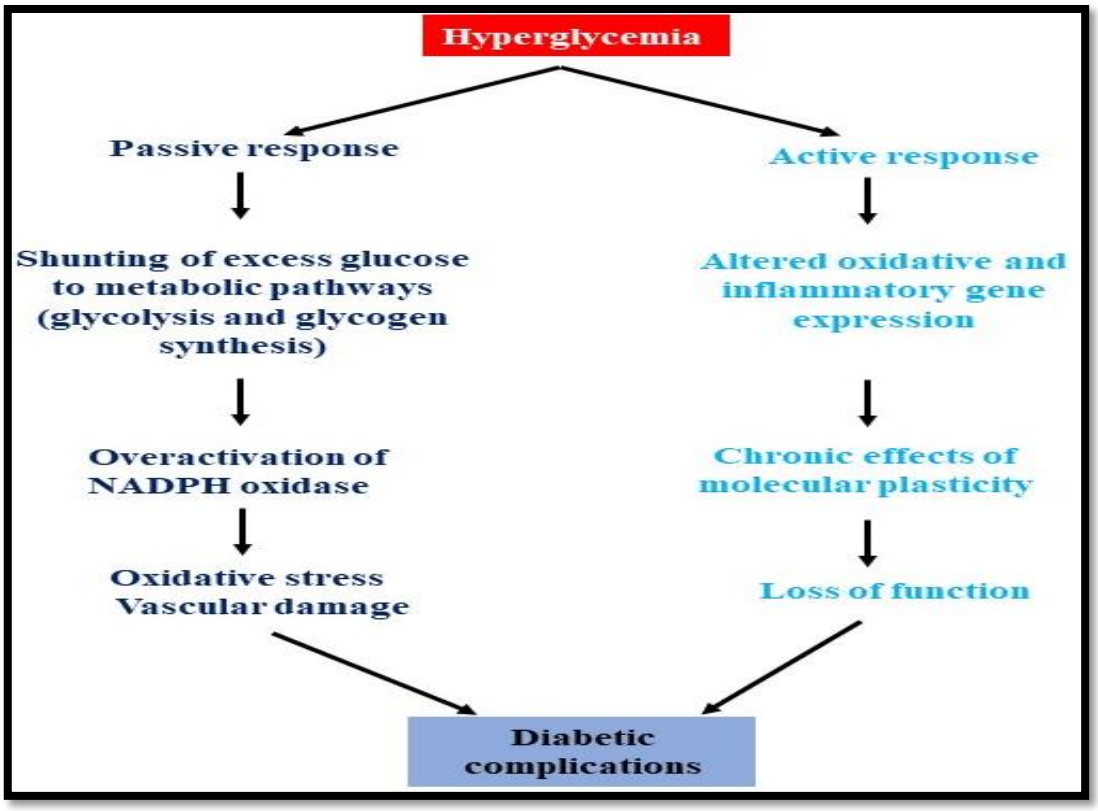


Figure 2.6: Neurological responses to hyperglycaemia can be active or passive (Prepared by author)

2.4.1.2 Diabetic nephropathy

Diabetic kidney disease or nephropathy develops when renal function deteriorates due to overwhelming glycaemic states. The increasing prevalence of diabetes combined with longer life spans is linked to glycaemic and blood pressure control (Braunwald, 2019). An increase in glucose serum concentration elevates serum osmolality. Hyperosmolality triggers both behavioural (polydipsia) and physiological (natriuresis, water retention) responses to maintain solute balance

and minimise fluid shifts between intracellular and extracellular environments (Klein and Waxman, 2003).

The functional role of the kidney is to produce urine, which serves as a means for excreting waste products and maintaining the osmolarity of body fluids. Most of the material filtered out of the blood is reabsorbed, including plasma glucose and water, to prevent wasteful loss. The kidneys require large amounts of energy to accomplish the reabsorption. Research indicates that the sympathetic nervous system may be responsible for this as it increases gluconeogenic precursors such as amino acids and lactate (Mitrakou, 2011). The hydrophilic nature of glucose promotes diffusion into the lipid bilayer, where entry is facilitated either by GLUTs (Mather and Pollock, 2011), or the sodium-glucose co-transporter (SGLT) family (Wright et al., 2007). Essentially, SGLTs support glucose reabsorption whilst GLUTs aid the release into circulation as shown in Fig 2.7 (Brown, 2000, Wright, 2001). Besides the conservation of glucose, the kidney possesses large concentrations of the G-6-P enzyme making it one of only two organs (the other being the liver) that contribute to gluconeogenesis. Interestingly, the kidney shows enhanced gluconeogenesis during the fed state, allowing the liver glycogen to be replenished through renal glucose release (Rowe et al., 2013). Metabolic alterations in renal glucose metabolism are associated with adverse effects and will be discussed later in this chapter.

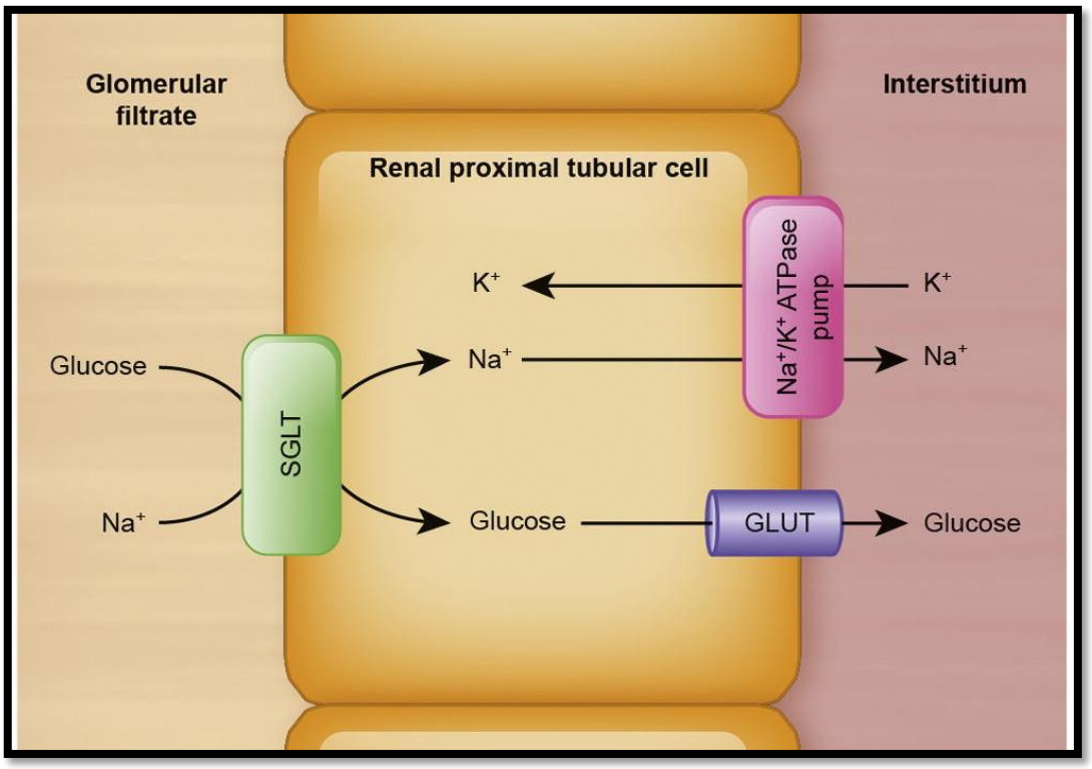


Figure 2.7: Glucose reabsorption in the kidney (Alsahli and Gerich, 2017)

In vivo studies and clinical trials have outlined the haemodynamic and metabolic changes in diabetes that promote ultrastructural alterations of the glomerular filtration barrier, including the glomerular basement membrane (GBM) thickening, mesangial extracellular matrix accumulation, and glomerulosclerosis (Gnudi et al., 2016). These alterations eventually cause microalbuminuria, indicating inefficient glomerular filtration rate (GFR) and nephropathic damage (Guimarães et al., 2007). Over the last two decades the incidence of chronic kidney disease has increased and factors like high blood pressure, autoimmune disease, cardiovascular disease, diabetes, and urinary tract infections contribute to its progression (Sulaiman, 2019).

In vivo studies demonstrate the renal changes associated with DM including glomerular hypertrophy, renal enlargement, and hyperfiltration (Grønbæk et al., 1998, Noda et al., 2001). Like the brain, the kidney requires high levels of ATP to maintain its function and is rich in mt content, making them susceptible to redox imbalances associated with hyperglycaemic conditions. High glucose-induced OS leads to inflammation and is associated with kidney injury (Dronavalli et al., 2008, Kanwar et al., 2011), whilst intensive glucose control improves renal function in diabetic patients (MacIsaac et al., 2017). However, the renoprotective effects of glucose lowering agents on the pathological mechanisms of nephropathy are not well established.

2.5 Metformin

A common treatment for T2DM includes oral anti-diabetic agents such as Biguanides (sensitizers) and Thiazolidinediones (TZDs). These drugs control glucose levels by reversing the effects of insulin resistance. Specifically, biguanides act on endogenous hepatic glucose production, whilst TZDs stimulate glucose disposal (Hotta, 2001). Initial treatment regimens involved three types of biguanides i.e.: metformin (MF), phenformin, and buformin. Although the effective dose of phenformin was considerably lower than MF, both buformin and phenformin have been associated with lactic acidosis and use has been terminated in most countries (Minamishima et al., 2019). Thus, MF is the only biguanide used in clinical practice, and is the present-day mainstay of T2DM management (Kamalta et al., 2018).

The origin of MF is a perennial herb, *Galega officinalis* (common name: French Lilac), known for centuries to reduce the symptoms of diabetes. Its active compound is a guanidine derivative, Galegine. Aside from glycaemic control, the full mechanistic potential of MF is unknown as its effects are independent to functional pancreatic β-cells. Given the relative safety and efficacy of the drug, the potential for repurposing is being explored (Gantois et al., 2019, Xourgia et al., 2019).

2.5.1 Pharmacokinetics of MF

Metformin is a positively charged molecule due to its guanidine-like structure (Fig 1). The generally administered form of MF, MF-hydrochloride, appears as a type of free base in biological fluids (Scheen, 1996). Following ingestion, MF is slowly absorbed by the small intestine with a bioavailability of 50-60% (Pentikäinen et al., 1979, Tucker et al., 1981). Although its hydrophilic nature prevents diffusion through cellular membranes, several organic cation transporters (OCTs) promote its uptake (Graham et al., 2011, Todd and Florez, 2014). It is important to note that MF is not completely absorbed by the intestine, is not metabolised by the liver, and is eliminated through the urinary system, thus it has direct effects on the kidney (Fig 2.8).

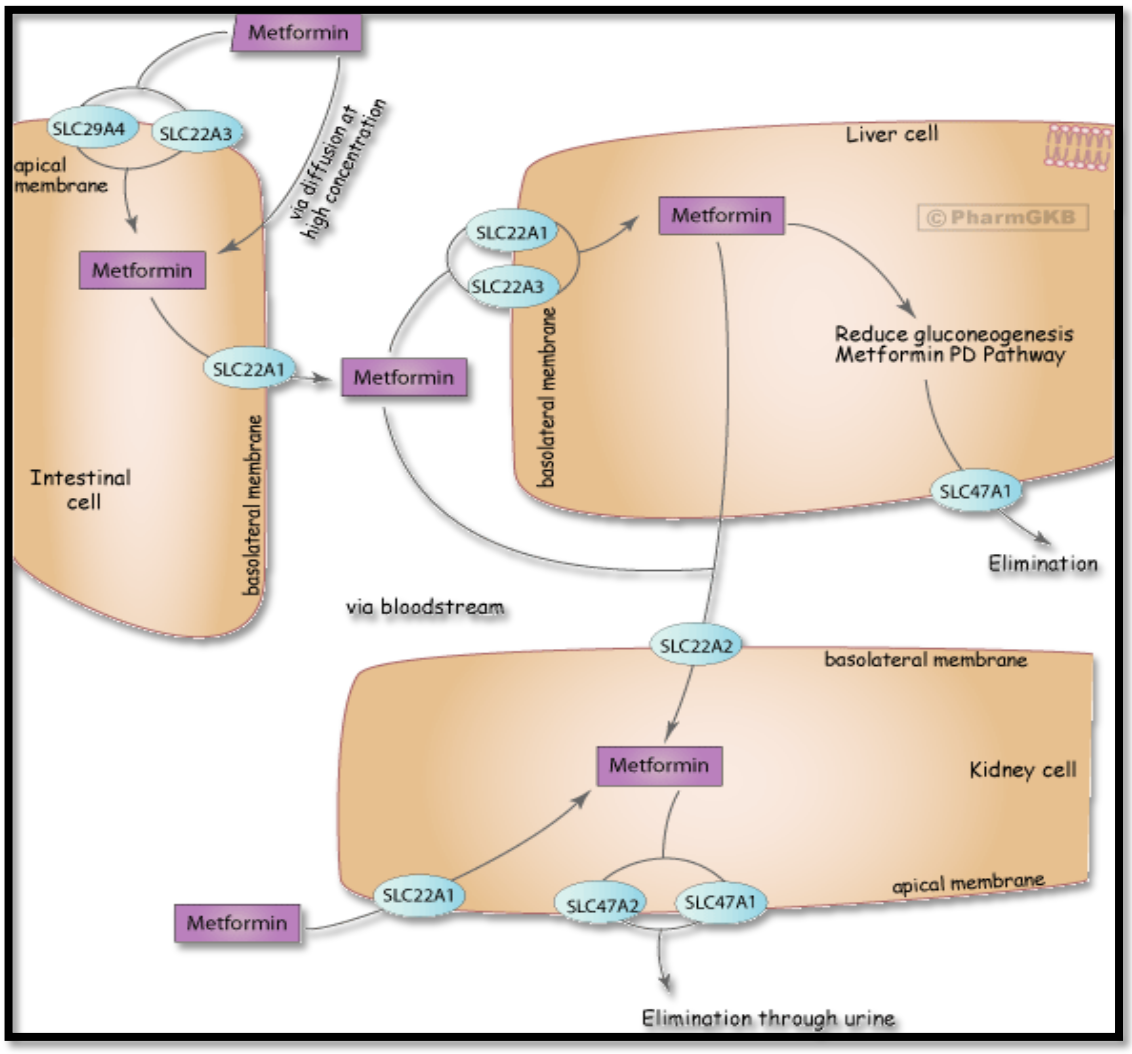


Figure 2.8: Pharmacokinetics of metformin transport (Gong et al., 2012)

2.5.2 Canonical mechanisms of action

Postprandially, MF exerts its glucose-lowering effects through a multitude of pathways linked to gluconeogenic inhibition and insulin signalling in the liver. Direct glycolytic stimulation decreased glucose absorption from the gut and plasma glucagon levels, as well as increased glucose-lactate conversion are involved in MF's proposed mechanism of action (Kartono et al., 2019). Metformin plays an integral role in maintaining cellular energy metabolism through regulation of adenosine monophosphate-activated protein kinase (AMPK) (Foretz et al., 2014). This enzyme is initiated upon decreased ATP/AMP ratios (Hardie, 2014). Further, MF inhibits mt complex 1 within the respiratory chain, decreasing intracellular ATP and activating hepatic AMPK in a liver kinase B1 (LKB-1)-dependent manner (Schäfer, 1983, Shaw et al., 2005). Metformin-induced AMPK activation facilitates recruitment of GLUT 4 in skeletal muscle, liver, and small intestine, promoting glucose uptake (Hundal et al., 2000, McCreight et al., 2016, Viollet et al., 2011). Physiologically relevant doses of MF trigger glycogen synthesis mediated by insulin action (Al-Khalili et al., 2005). Studies in STZ-induced hyperglycaemic rats reveal MFs modulation of gluconeogenic genes through decreased pyruvate carboxylase phosphoenolpyruvate carboxykinase (PC-PEPCK) and G-6-P promoters (Kim et al., 2008, Large and Beylot, 1999). The regulatory role of MF on gluconeogenic genes and amino acid-degrading enzymes have been demonstrated in an AMPK-dependent manner by PPAR-γ coactivator 1α (PGC-1α) activation (Takashima et al., 2010), and overall improvement of mt function (Iwabu et al., 2010). The involvement of PGC-1α in mt biogenesis has been shown by both gain and loss-of function studies (Lin et al., 2002). Furthermore, PGC-1α has important transcriptional co-activation roles, however direct activation may not be amenable. Hence, the complexity of glucose homeostasis is mediated by intricate signalling pathways (Fig 2.9), and activation of its upstream regulators like AMPK serves as a feasible target for pharmacological intervention. Cumulative discoveries involving MFs regulation of these pathways has uncovered its multiple targets in DM management.

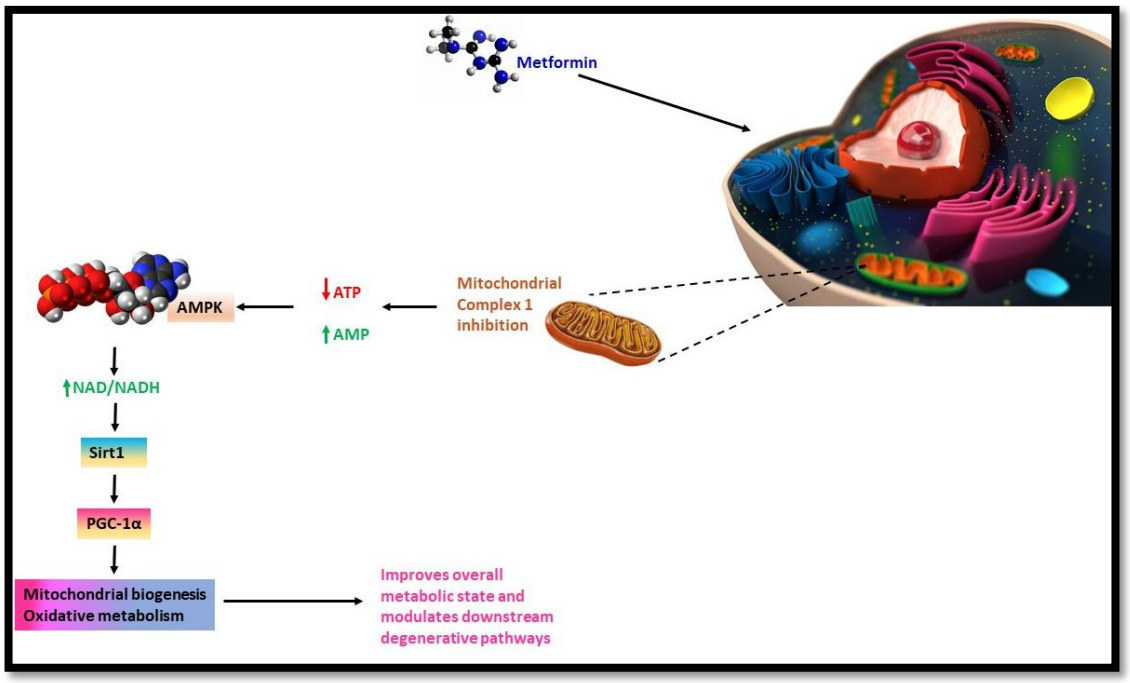


Figure 2.9: Metformin's mechanism of action (Prepared by author)

2.6 Molecular mechanisms underlying diabetic pathophysiology

Diabetic research has advanced tremendously with new knowledge at the cellular and molecular level, and how a diabetic ambiance promotes complications. Complex molecular mechanisms in diabetes attributes to the numerous metabolic derangements presented by the disease. Hyperglycaemia is known to impair cellular function by creating an imbalance in pro- and antioxidants (Selvaraju et al., 2012). The consequent generation of reactive oxygen species (ROS) and associated OS represents one of the hallmarks of DM and has received the most attention. The interaction between ROS and macromolecules within the cell explains the deterioration of lipids, proteins, and DNA (Schrauwen and Hesselink, 2004). This damage is responsible for the macro- and microvascular pathology of diabetes, as well as the biochemical pathways related to redox imbalances is discussed further below.

2.6.1 Oxidative stress (OS)

2.6.1.1 Mitochondrial dysfunction

Hyperglycaemia triggers OS primarily through the production of mt ROS (Masi et al., 2018), indicating that diabetics have increased redox imbalances than those of healthy individuals. Increased glucose substrates entering the TCA cycle under hyperglycaemic conditions consequently increases production of reducing equivalents feeding into the electron transport chain (ETC). As electrons are transported through the chain, a crucial threshold voltage in complex III of the ETC develops and electrons begin to accumulate (Fig 2.10). The energy derived from this voltage gradient generates ATP through its synthase activity (Wallace, 1992). As a result, free radicals containing one or more unpaired electrons including the hydroxyl radical ($OH^{\bullet}$), and superoxide anion radicals ($O_2^{\bullet -}$) are formed; which have deleterious effects on tissues by lipid peroxidation (Ichikawa et al., 1999). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mt uncoupling tend to aggravate the oxidative status (Eisner et al., 2018). Antioxidants combat OS and radicals. Enzymes that facilitate antioxidant production include catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) (Green et al., 2004). Diabetic conditions overwhelm the cellular scavenging of ROS contributing to the dysfunctional status.

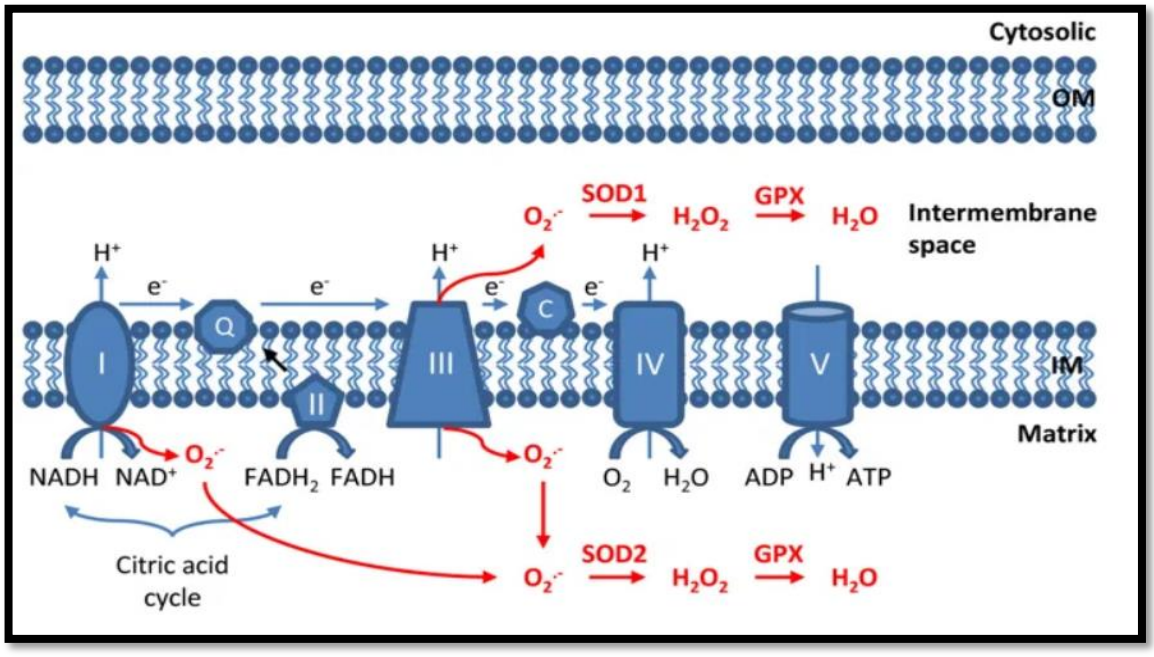


Figure 2.10: Redox cycling within the mitochondrion (Li et al., 2013)

2.6.1.2 Other sources of ROS

Additional sources of hyperglycaemic OS are nonenzymatic glycation (Mullarkey et al., 1990), glucose auto-oxidation (Wolff and Dean, 1987), interaction between glycated products and associated receptors (Nishikawa et al., 2000), and the polyol pathway (Chung et al., 2003). Enzymatic action in the polyol pathway induces sorbitol reduction and fructose production by aldose reductase (AR), and sorbitol dehydrogenase that require NADPH and nicotinamide adenine dinucleotide (NAD^+) respectively (Fig 2.11). This promotes OS as NADH is the substrate for NADH oxidase to generate ROS within the cell (Morre et al., 2000), and leads to further depletion of antioxidants like glutathione (GSH). Targeting the polyol pathway prevents the development of diabetic complications including neuropathy, cataracts, and nephropathy (Oates and Mylari, 1999).

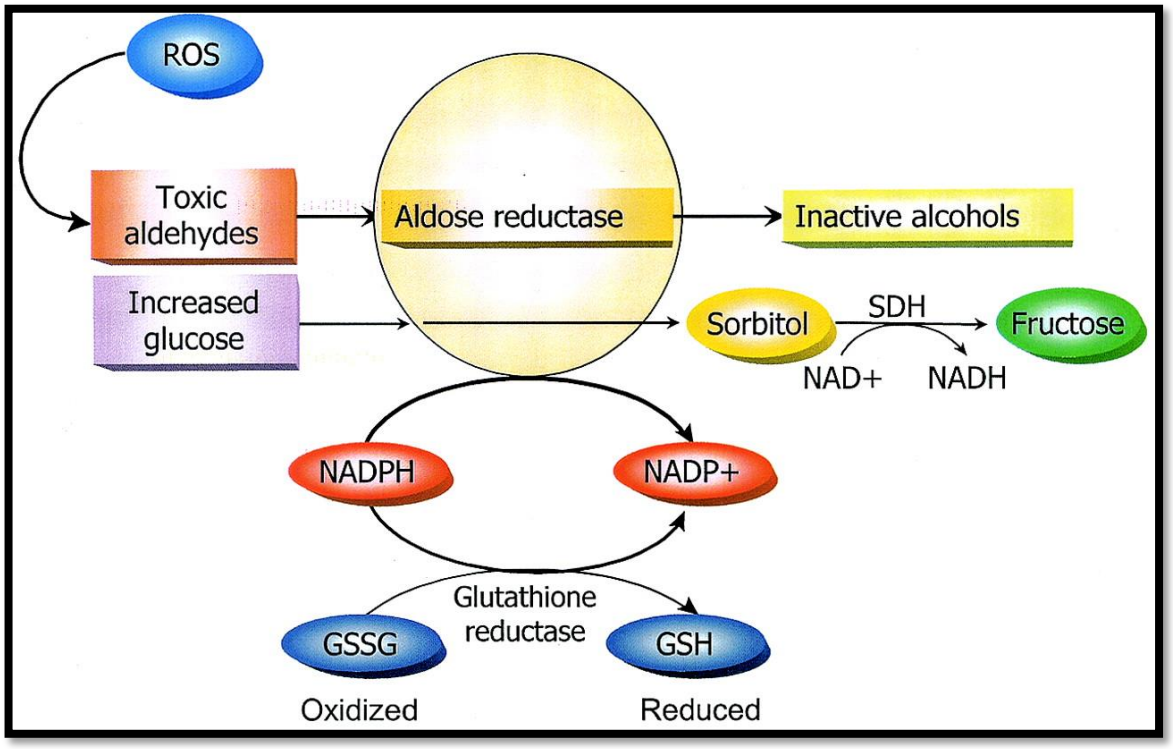


Figure 2.11: Diabetes increases flux through the polyol pathway (Brownlee, 2001)

2.6.2 Advanced glycation end products

Advanced glycation is significantly increased under chronic hyperglycaemic conditions. This process involves the binding of a reduced glucose molecule to free amino groups, affecting protein, lipids, and DNA (Brownlee et al., 1988), and serves as an indicator of many abnormal conditions such as OS (Fu et al., 1996). The addition of carbonyl groups to proteins is a non-enzymatic post-translational modification (PTM) that occurs through direct (oxidative) or indirect (non-oxidative) mechanisms. Certain ROS (lipid hydroperoxides and hydrogen peroxide) trigger oxidation of specific amino acids through a direct iron-catalysed mechanism (Cattaruzza and Hecker, 2008). The process is considered self-enhancing as selective carbonylation at protein targets (arginine, lysine, proline or threonine) tend to encourage neighbouring carbonylatable sites (Maisonneuve et al., 2009). The first line defence against ROS involves SODs and GPx, which catalyse the reaction of superoxide anion to hydrogen peroxide, followed by the conversion to H_2O and O_2 (Curtis et al., 2010). The interaction of hydroxyl radicals with the abovementioned amino acids is catalysed by free iron (II) (Fig 2.12). Alternatively, indirect formation of carbonyl groups derived from radical-mediated oxidation of lipids (malondialdehyde (MDA), 4-hydroxy trans-2,3-nonenal (4-HNE), acrolein) and autoxidation of carbohydrates [glyoxal, methylglyoxal (MG)] may occur through indirect adduction (Adams et al., 2001). Detoxification of reactive lipid aldehydes through phase I (aldo-keto reductases, aldehyde dehydrogenases, and alkenal/one oxidoreductase) or phase II (enzymatic glutathionylation) metabolism is carried out by oxidation and reduction reactions (Frohnert and Bernlohr, 2013). Further, the Maillard reaction of glucose (Oya et al., 1999) triggers a MG intermediate that produces advanced glycation end-products (AGEs) and disrupts enzyme secondary protein structures. The associated loss of function engages proteolytic degradation (Díaz-Villanueva et al., 2015). The sources for AGE formation include autoxidation and Schiff base formation (Fig 2.12). The accumulation of AGEs occurs in diseases like uraemia, diabetes, neuropathies, nephropathies and other inflammatory conditions (Ahmad et al., 2016, Ahmed, 2005, Jono et al., 2002, Yamamoto et al., 2005). Pharmacological intervention highlights the role of AGE-induced apoptosis in diabetic nephropathy (Ishibashi et al., 2012). Furthermore, a key role is played by AGEs in the pathogenesis of sensory neuron damage (Jack and Wright, 2012). A study demonstrated decreased viability in rat Schwann cells by AGE precursors, and resultant diabetic neuropathy (Sato et al., 2013). It is evident that glycosylation and eventual AGE formation is an important marker for OS and associated diabetic degeneration, making it a prime therapeutic target.

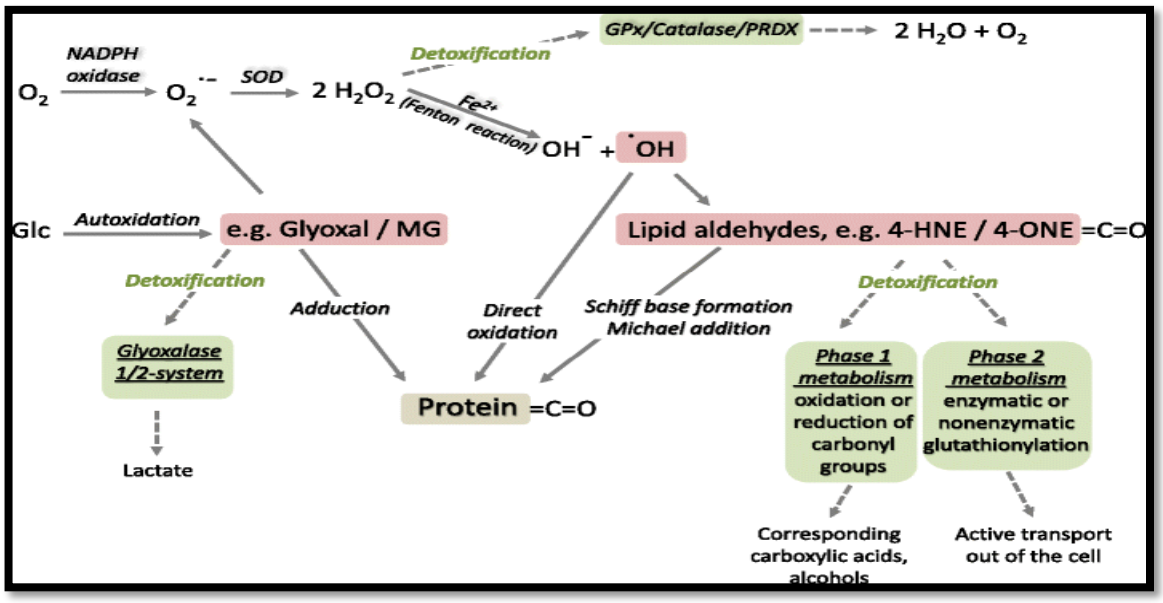


Figure 2.12: Biochemistry of protein carbonylation (Hecker and Wagner, 2018)

An instrumental role is played by these PTMs implicating them in metabolically altered diseased states including insulin resistance neurodegeneration, nephropathies, and aging (Albrecht et al., 2017, Curtis et al., 2012, dos Santos Mello et al., 2015, Sharma et al., 2016). In contrast to normal blood glucose, OS and associated protein carbonylation in hyperglycaemia causes proteasomal degradation, aggregation, and accumulation of carbonylated proteins (Fig 2.13).

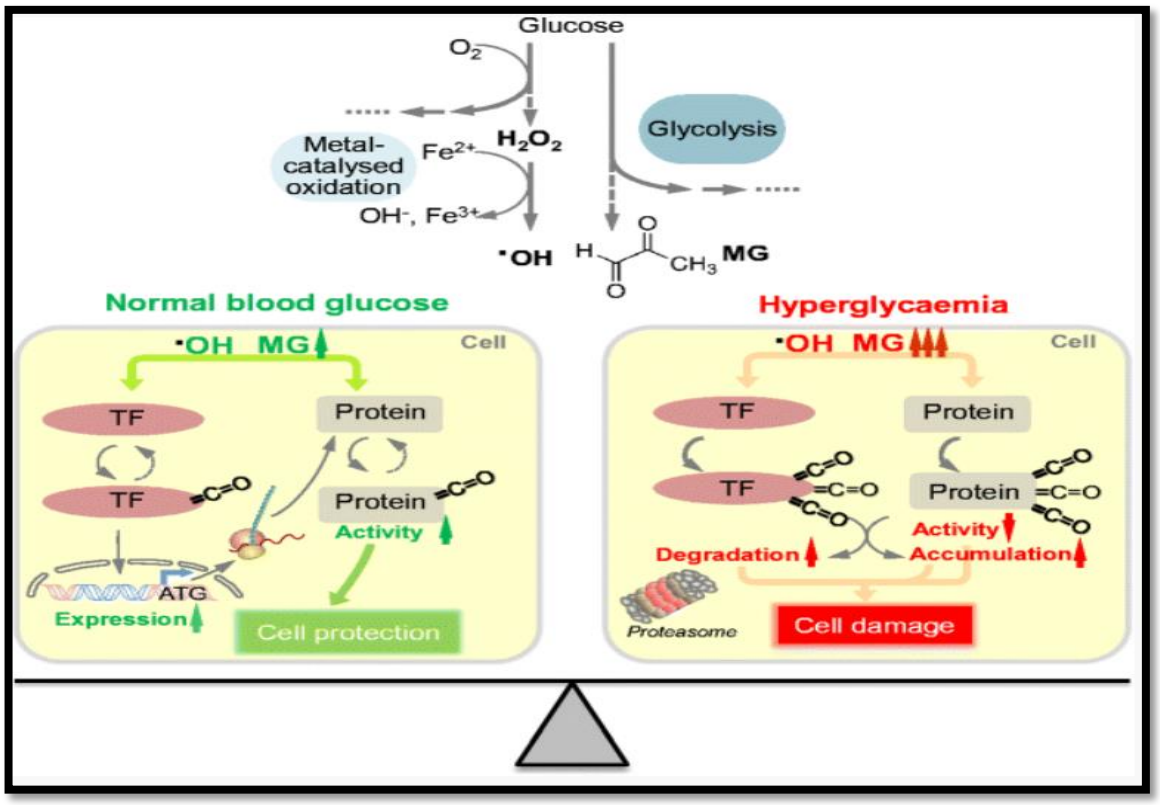


Figure 2.13: Balance of the redox status is crucial in cellular homeostasis (Hecker and Wagner, 2018).

2.6.3 Sirtuins (Sirts)

The mammalian family of sirtuins originate from *Saccharomyces cerevisiae* gene silent information regulator 2 (Sir2) (North and Verdin, 2004). These cellular energy sensors depend on NAD^+ for their enzymatic activity, directly linking them to metabolism. They have deacetylase activity which aids removal of acetyl groups from target proteins with acetylated lysine residues, including transcription factors and histones. Of the seven Sirts, Sirt-1 and -3 are the most extensively studied. Sirt1 is found in the nucleus and plays a key role in energy homeostasis (Li, 2013), whilst Sirt3 functions in mt energy regulation (Ahn et al., 2008), and both influence metabolically active tissues. Regulation of these Sirts extends further than cellular stressors and the NAD^+/NADH ratio, and can be controlled by endogenous proteins, and microRNAs (Choi and Kemper, 2013). Deacetylation of AMPK, transcription factors like PGC-1α and NF-κB by Sirts plays an important role in diabetic regulation (Akude et al., 2011, Cantó and Auwerx, 2009, Chong et al., 2012, Chowdhury et al., 2011, Hardie, 2008, Rodgers et al., 2005) Ubiquitous expression of Sirt1 and -3 have been found in mice brain, and is decreased in neurodegeneration (Jin et al., 2009, Zakhary et al., 2010). Additionally, chronic metabolic stress, oxidative stress, and hypoxic states decrease the expression of Sirt1 and -3 and have been demonstrated in diabetic kidney

disease (Kume et al., 2010, Wakino et al., 2015) as well as diabetic neuropathy (Fernyhough, 2015). A recent *in silico* study providing evidence of MF's direct effect on Sirt1 through molecular docking and experimental validation (Cuyàs et al., 2018), highlights its subsequent protective function. Therefore, agents like MF that regulate Sirts may have favourable impacts on slowing the progression of diabetes-induced organ damage.

2.6.4 Hypoxia and pseudohypoxia

In 1993, Williamson and colleagues devised the concept of pseudohypoxia and its relation to diabetes (Williamson et al., 1993). This phenomenon occurs when the cell is unable to use oxygen due to redox imbalances with decreased NAD^+, and NADH accumulation (Luo et al., 2015, Luo et al., 2016, Yan, 2018). This results in damage to cellular components like lipids, proteins, and DNA (Yan, 2014), culminating in cellular death and tissue dysfunction (Fig 2.14) (Bandeira et al., 2013, Shah et al., 2007).

Figure 2.14: Schematic diagram of hyperglycaemic stress (Prepared by author)

Hyperglycaemic activation of the polyol pathway drives NADH overproduction. Associated DNA damage activates poly (ADP ribose) polymerases (PARPs) and depletes the NAD^+ pool as it is required for its activity (Pacher et al., 2002). Consequently, NAD^+ availability for Sirt1, a known

AMPK activator, is depleted (Nishikawa et al., 2015). Furthermore, the hypoxia inducible factor-1 (HIF-1) complex is activated by hyperglycaemia, hypoxia, nitric oxide as well as ROS. Under normoxic conditions HIF-1 is regulated by prolyl 4-hydroxylases hydroxylate proteosomal E3 ubiquitin ligase degradation (Chen and Sang, 2016). Under hypoxia or pseudohypoxia, downregulated Sirts cause a HIF-1-mediated switch from OxPhos to glycolysis by inhibiting pyruvate dehydrogenase and subsequent prevention of acetyl coA entry to the TCA cycle (Fig 2.15) (Chen and Sang, 2010).

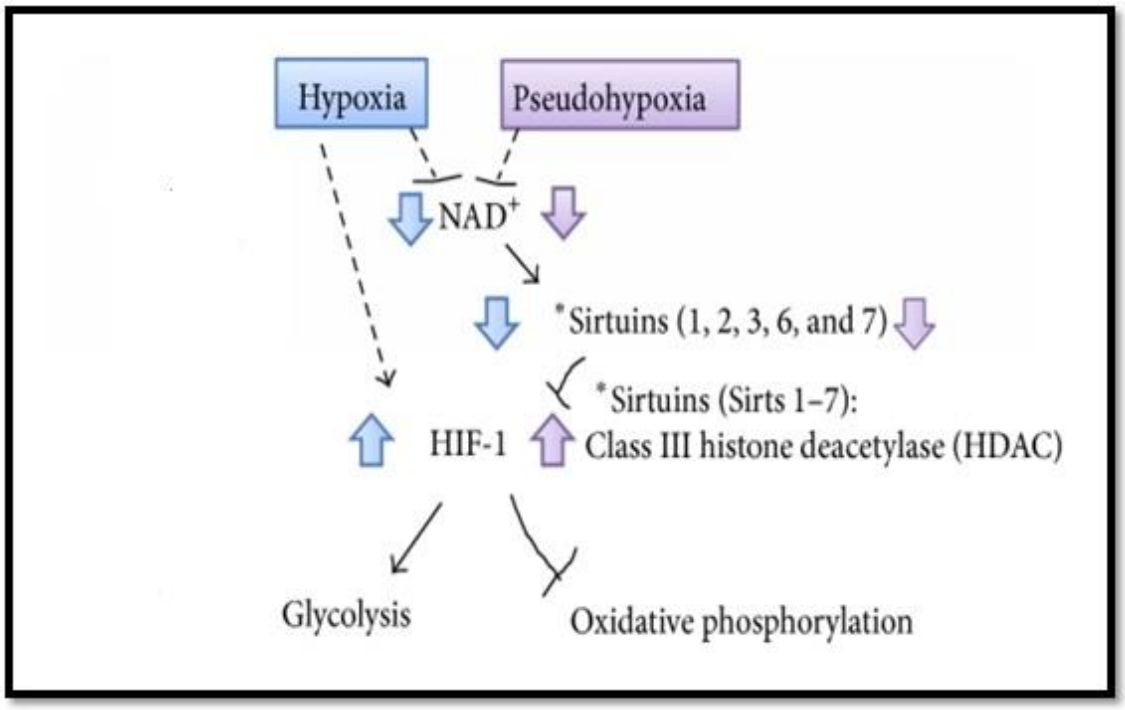


Figure 2.15: Sirtuin/HIF-1 axis and their effect on glycolysis-OxPhos switch (Takiyama and Haneda, 2014)

2.6.5 Endoplasmic reticulum (ER) stress

Cellular versatility comes from finely tuned performance by organelles like the endoplasmic reticulum (ER) (Chang et al., 2006). It functions in biosynthesis, post-translational modifications including glycosylation, and protein folding (Hampton, 2002). Secretory and trans-membrane proteins are tailored by the ER before translocation to the appropriate organelle. Homeostasis is maintained by sequential actions of the unfolded protein response (UPR) following ER stress (Gardner and Walter, 2011). These include three ER-proximal sensors that are in the transmembrane: 1) protein kinase RNA (PKR)-like ER kinase (PERK), 2) inositol-requiring protein 1 (IRE1), and 3) activating transcription factor 6 (ATF6); which are present in all cell

types (Fig 2.16). The predominant pathway involved in this stress response is activation of PERK which leads to eukaryotic initiation factor 2 (eIF2α) phosphorylation and activation (Cullinan and Diehl, 2004). The pathogenesis of hyperglycaemia is exacerbated by ER stress-induced ROS production, which is primed by the activation of CHOP and p38 MAPK (Zhong et al., 2015). Further, calcium cycling in ER stress conditions encourages ROS generation in mitochondria as seen in Fig 2.17, and this leads to intrinsic apoptosis (Li et al., 2009). Reduced insulin signalling mediated by PI3K/Akt and phospho- glycogen synthase kinase 3β (GSK3β) induces ER-stress apoptosis through CHOP (Srinivasan et al., 2005). Diabetic mouse models exhibiting CHOP deletions reduce OS, promote cell survival, and improve β-cell function (Song et al., 2008). Furthermore, mice with PERK deficiencies represent dysfunction pancreas function (Harding et al., 2001), indicating the involvement of ER stress in diabetes. Investigating the potential of pharmacological agents in ER stress will provide new mechanistic insights and developing novel targets disorders related to ER-stress.

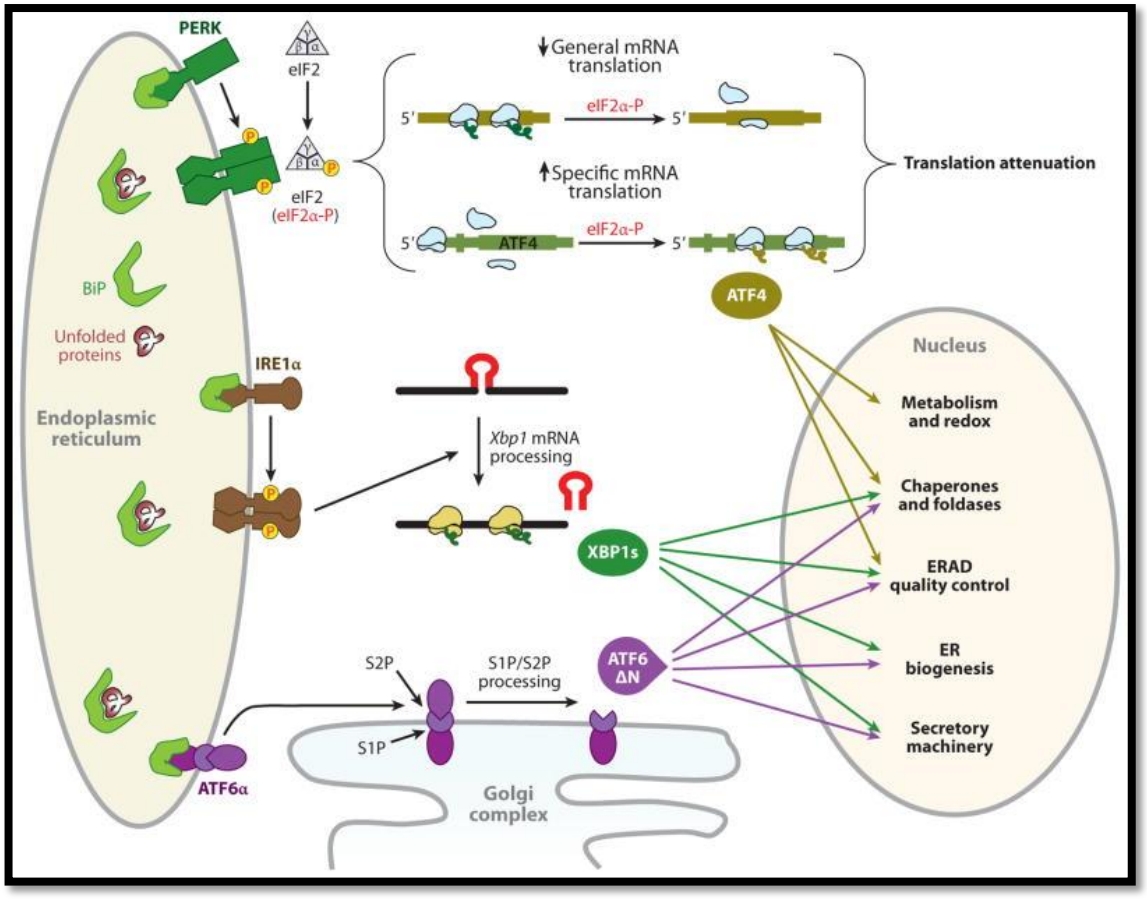


Figure 2.16: The adaptive UPR response to ER stress (Back and Kaufman, 2012)

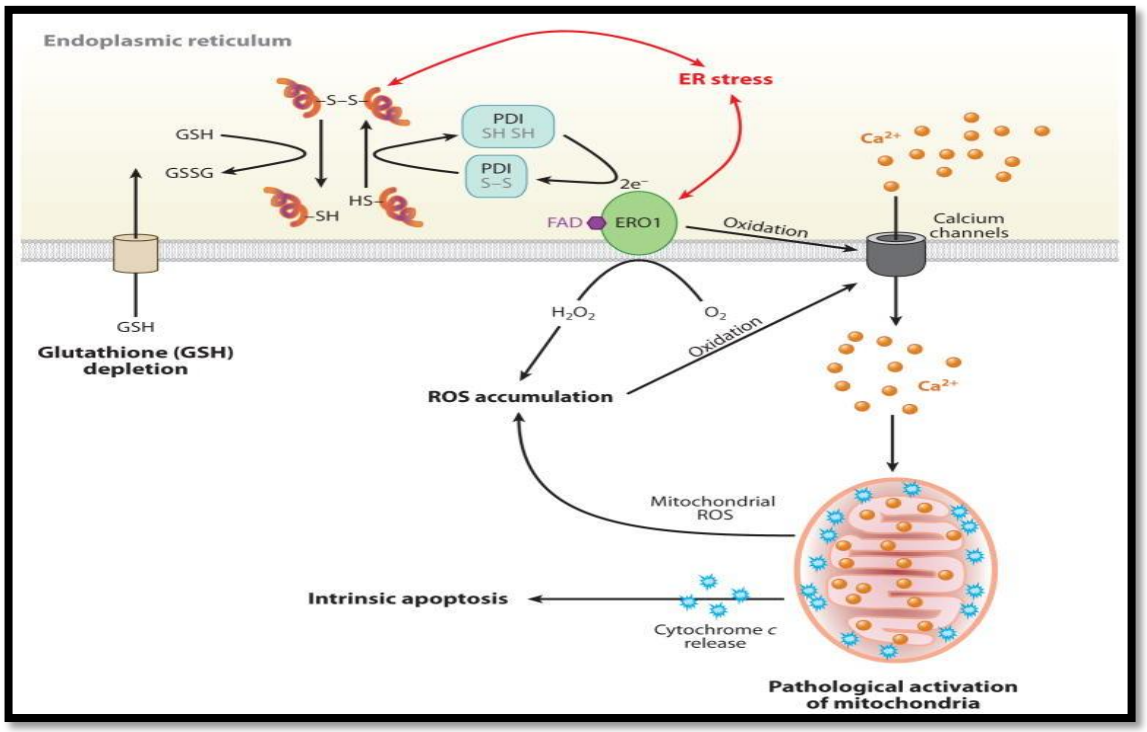


Figure 2.17: Mechanism of diabetic ER stress-mediated cell death (Back and Kaufman, 2012)

2.6.6 Inflammatory response

Immune and metabolic pathways are interdependent and are cardinal symptoms of diabetic patients. It is known that OS promotes the activation of inflammatory signalling pathways such as p38 mitogen-activated protein kinase (MAPK) and disrupts homeostasis (Rains and Jain, 2011). Additional to the pathways altered by mt redox imbalances is activation of the inflammatory transcription factor nuclear factor kappa B (NF-κB) (Yamagishi et al., 2012). This response unites the inflammatory and metabolic responses and is triggered by proinflammatory cytokines such as interleukin-6 (IL-6) and TNFα (Nisr et al., 2019). To exert their DNA-binding function, members of the NF-κB family require dimerisation. The RelA/p65 subunit is one of the multiple forms of NF-κB and is regulated by the IκB -kinase (IKK) complex (Ghosh and Karin, 2002). This complex leads to IκB-α phosphorylation and degradation through ubiquitin/proteasome system. Phosphorylation of the inhibitory components is essential for NF-κB activation. It is well established that chronic low-grade inflammation plays a role in metabolic disorders like diabetes (Baker et al., 2011).

In T1DM the autoimmune attack mediated by cytokines leads to NF-κB activation and promotes cellular dysfunction and death (Cardozo et al., 2001). Inhibition of the inflammatory response has

been shown to protect against apoptosis in the pancreas during multiple low dose STZ-induced diabetes (Eldor et al., 2006). The role of NF-κB involvement in T2DM was first established when the anti-inflammatory salicylate drug (aspirin) ameliorated hyperglycaemia through NF-κB inhibition and preventing IκB-α degradation (Kopp and Ghosh, 1994, Senftleben et al., 2001, Yin et al., 1998, Yuan et al., 2001). Increased translocation of NF-κB and induction of pro-inflammatory cytokines is associated with diabetic nephropathy (Cohen et al., 2002, Sakai et al., 2005), and neuropathies (Ametoy et al., 2003, Haak et al., 1999, Sandireddy et al., 2016). However, its specific contribution to diabetic pathogenesis requires further examination.

Damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) are triggered by endogenous stress (Robbins et al., 2014), and are recognised by pattern-recognition receptors (PRRs). The sensory units of the inflammasome recruit the caspase-recruitment domain (CARD) that dimerises in the inflammasome complex to promote pro-caspase 1 cleavage into caspase-1 (Mariathasan et al., 2004, Strowig et al., 2012). This stimulates pro-inflammatory IL-1β and IL-18 cytokine activation by caspase-1 cleavage as seen in Fig 2.18. In T2DM, activation of the NLRP3 inflammasome pathway by NF-κB orchestrates the inflammatory reaction (Arkan et al., 2005, Vandanmagsar et al., 2011). Dysfunctional mt processes and ROS are closely linked to NLRP3 inflammasome activation further implicating it in disease progression. Studies supporting inhibition of the NF-κB pathway by pharmacological inhibition leads to reduced formation of microvascular disease (Benzler et al., 2015, Chiazza et al., 2015). Knockdown of NLRP3 through IL-β and IL-18-dependent mechanisms have been demonstrated in myeloid cells of T2DM patients (Lee et al., 2013). It is evident that activation of NLRP3 inflammasome in diabetes leads to a pro-inflammatory ambiance. However, specific immune-metabolic mechanisms need to be identified in order to understand and prevent diabetic associated organ damage.

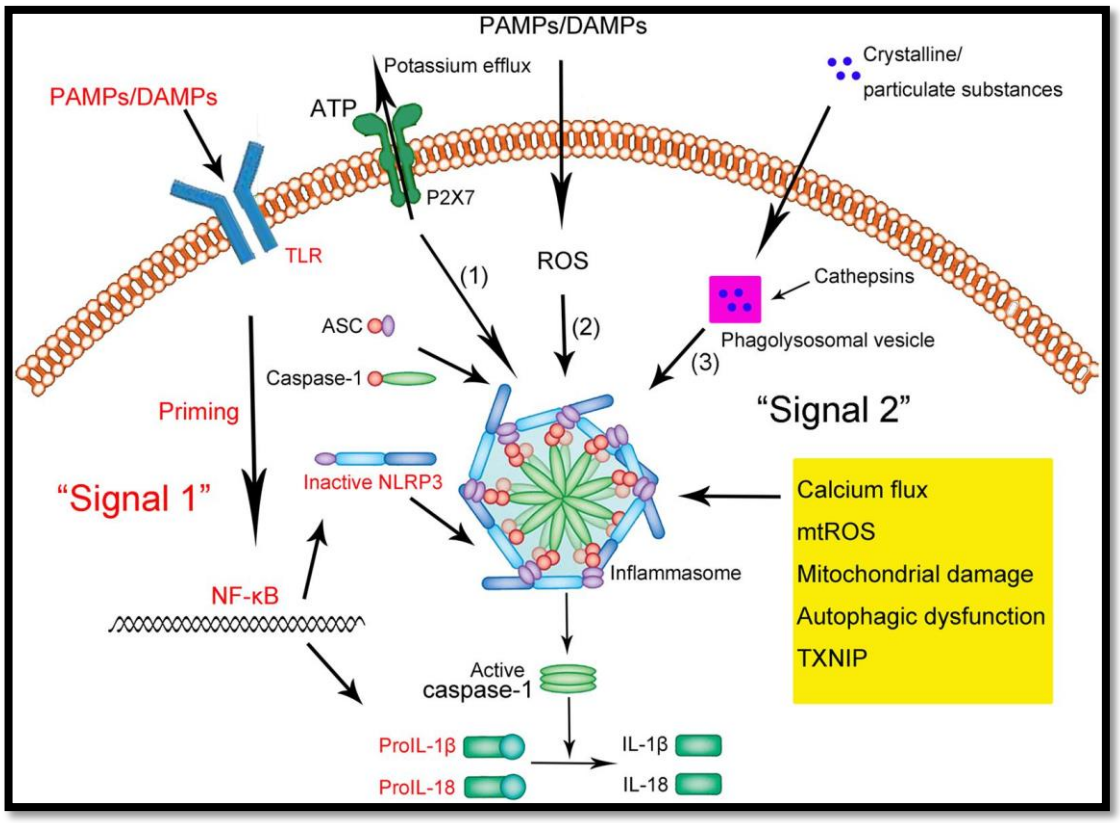


Figure 2.18: inflammasome activation in Diabetes Mellitus (Shao et al., 2015)

2.6.7 Neuroplasticity

Neuroplasticity refers to the ability of the brain to acclimatise to environmental stressors by neuronal remodelling. A key player in neuroplasticity is the neurotrophin brain-derived neurotrophic factor (BDNF) and its receptor TrkB (Bramham and Messaoudi, 2005, Wang et al., 2019b). In the brain, BDNF is maintained at basal levels, however elevated plasma glucose decreases BDNF levels (Krabbe et al., 2007). Administration of exogenous BDNF in diabetic mice promotes glucose homeostasis (Ono et al., 1997, Nakagawa et al., 2000). A recent study demonstrates circulating BDNF deficiencies upon glycaemic elevation in the brain in infant cord blood (Guzzardi et al., 2018). The kinase, GSK 3β is a critical feature of neuronal development and survival (Frame and Cohen, 2001a), and its inhibition has been found to regulate BDNF-dependent TrkB endocytosis (Liu et al., 2015). Mechanistic insights on BDNF regulation in the brain are limited and require further attention.

Neuronal function is further maintained by microtubules which are stabilised by tau protein (Vossel et al., 2010). This protein is regulated by the equilibrium of tau kinase and phosphatase activities. Diabetic disruptions of these regulatory factors will initiate abnormal tau

phosphorylation, and formation of tau aggregates. A common neurodegenerative disorder associated with DM is Alzheimer's Disease (AD), where hyperphosphorylation of tau, aggregation of Amyloid-β protein or neurofibrillary tangles occur (Reilly et al., 2017). Recent evidence draws the link between tau hyperphosphorylation and protein phosphatase 2A (PP2A) inhibition in Streptozotocin (STZ)-diabetic models and neuropathological hallmarks of Alzheimer's disease (AD) (Gratuze et al., 2017, Qu et al., 2011). Approximately 42 tau phosphorylation sites are maintained by GSK 3β, of which 29 have been shown in AD (Sergeant et al., 2008, Hanger et al., 2009). There are five phosphoserine/phosphothreonine protein phosphatases (PP), of which PP2A is highly expressed in the brain (Gong et al., 2005). Tau stability can be regained by protein phosphatase 2A (PP2A) function, however this phosphoesterase is depleted in diabetes (Zhang et al., 2016b). Tau hyperphosphorylation has also been proven in STZ-induced diabetic mice (Clodfelder-Miller et al., 2006). Furthermore, microglial and astrocyte activation are drivers of the pro-inflammatory response in the brain. Oxidative stress and related inflammatory pathways cause microglial production of cytokines and further phosphorylation of tau (Li et al., 2003). Structural collapse of microtubules following tau malfunction causes buckling of the neuron and neurofibrillary tangles, also known as the "Swiss cheese" effect. Taken together hyperphosphorylated tau and microglial activation is essential to the development of the diabetic associated neurodegeneration (Fig 2.19).

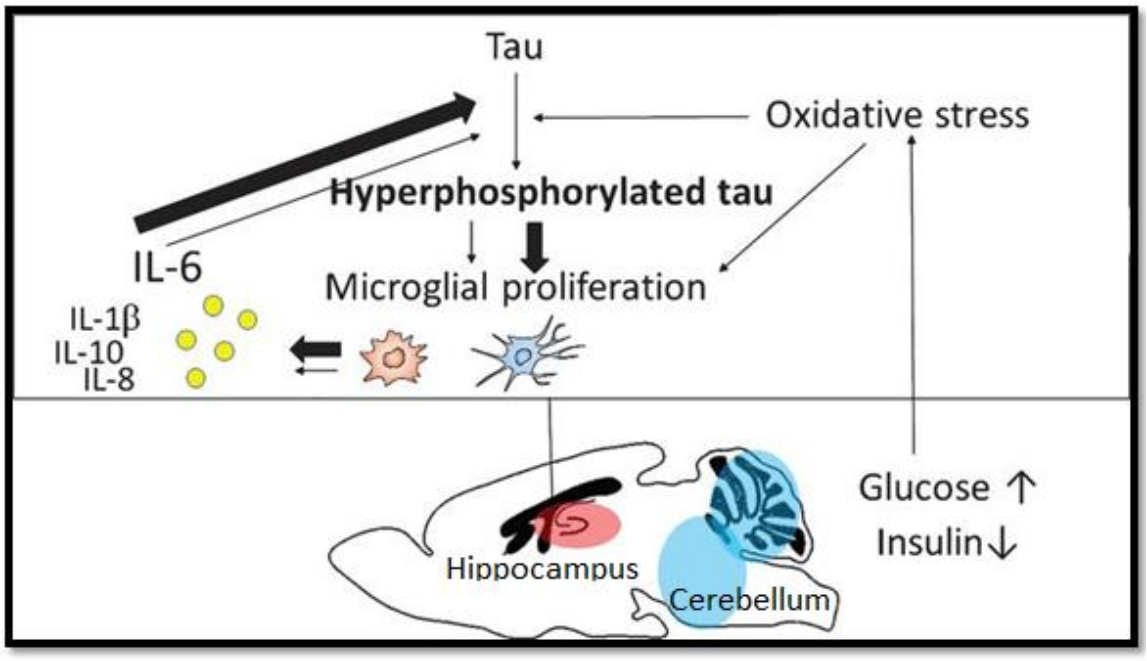


Figure 2.19: Mechanism of tau phosphorylation and inflammation linked to high glucose levels occurring in the hippocampus (Elahi et al., 2016)

2.6.8 Metabolic dysfunction and cell death

A multitude of signals experienced in metabolic stress leads to activation of tumour suppressor protein, p53. To name a few, nutrient, oxygen or growth factor deprivation, and OS are transmitted by metabolic sensors such as AMPK and eIF2α (Humpton and Vousden, 2016). Whilst normal conditions hold p53 levels in check, extreme activation in hyperglycaemic conditions is deleterious to the cell. E3 ubiquitin ligase, murine double minute 2 (Mdm2) upholds p53 protein levels by constant degradation via ubiquitination (Hock and Vousden, 2014). However, depleted Mdm2 following diabetic stressors like hypoxia and mt ROS, induces p53. Damage signals promote relocation of p53 to the mitochondria and influences binding to pro-apoptotic mediator, Bax (Castrogiovanni et al., 2018). This leads to the intrinsic pathway through mt cytochrome-c release. Subsequent apoptosome formation leads to caspase-9, and caspase-3 activation, leading to cell death (Fig 2.20). Furthermore, p53 targets p21 causing cellular senescence (Rufini et al., 2013).

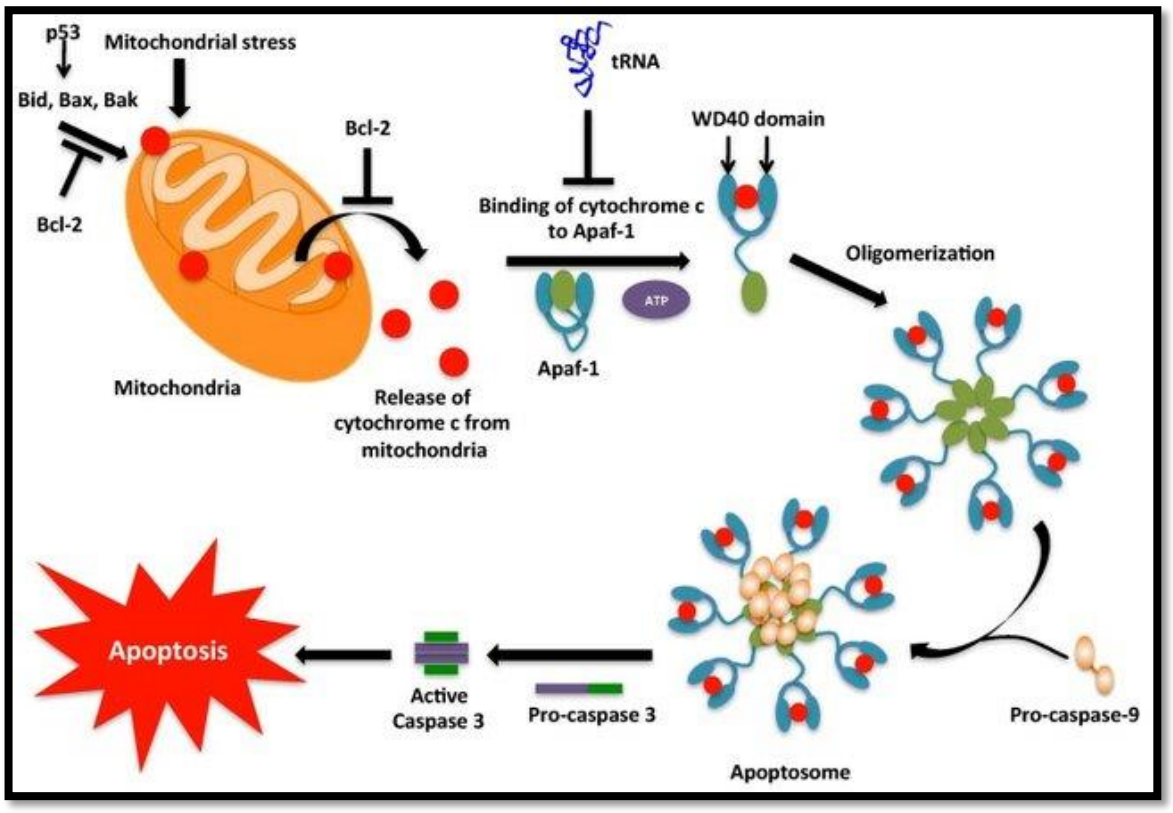


Figure 2.20: Intrinsic apoptotic pathway (Raina and Ibba, 2014)

2.7 Cell defence mechanisms

2.7.1 Antioxidant defences

Mitochondria promote multiple functions, including ROS and ATP production; $Ca2^{+}$ regulation, and subsequent cellular homeostasis. As previously discussed, OS and mt dysfunction lead to metabolic and neurodegenerative diseases, making them important targets for treatment. The upregulation of cellular antioxidant defences by pharmacological intervention counteracts OS induced by diabetes. Regulation of AMPK positively affects peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and mt biogenesis (Jäger et al., 2007). Additionally, Sirt -1 and -3 are promoted by AMPK, and regulate PGC-1α (Rodgers et al., 2005). These Sirts have a highly conserved catalytic core associated with a NAD^{+}-binding domain and deacetylase activity (Michan and Sinclair, 2007). Sirtuins are abundant in the brain, liver, kidney, skeletal muscle, pancreas, and adipose tissues, where they deacetylate histone and nonhistone targets in conditions of OS. Sirtuin 1 has added roles on inflammation by modulating RelA/p65 NF-κB signalling through deacetylation (Salminen et al., 2008). Another major Sirt1-mediated antioxidant response involves transcription factor Nrf2 which is maintained in the cytoplasm through Kelch-like ECH-associated protein 1 (Keap1) ubiquitinated degradation (Kensler et al., 2007). Redox imbalances disrupt the Keap1 system. This allows the release of Nrf2 and translocation to the nucleus where it binds to the antioxidant response element (ARE) and transcription of antioxidant genes (glutathione S-transferase, NADPH quinone oxidoreductase, heme oxygenase-1, and γ-glutamylcysteine synthetase) are stimulated (Chan et al., 2001).

2.7.2 Mitochondrial maintenance and chaperone proteins

Mitochondrial homeostasis is further maintained by a nuclear encoded protease, LonP1, and is abundant in all organisms. With its serine peptidase ATP-dependent nature it degrades oxidatively-modified and misfolded proteins in the mitochondrion (Pinti et al., 2016). Additional positive effects on mt functionality include cell support during hypoxia and ER stress coupled with normalising mtDNA metabolism through mt transcription factor A (TFAM). Further mt surveillance is provided by chaperones: heat shock protein (HSP) 60 and mt-HSP70. LonP1 maintains the complex formed by Hsp60 and mtHsp70 (Wadhwa et al., 2005), which has essential

functions for mt biogenesis like import and folding of client proteins. Activation of LonP1 improves cellular functionality and survival by inhibiting protein carbonylation, whilst LonP1 silencing leads to dysfunctional mitochondria (Ngo and Davies, 2009a). Additionally, Hsp70 mediates the antioxidant defence thereby inhibiting oxidative injury (Polla et al., 1996). Paradoxically lower HSP levels accentuate the damage incurred by hyperglycaemia, thus permitting organ injury as depicted in Fig 2.21. However, the role of Hsp70 in alleviating diabetes-associated neurodegenerative damage is highlighted (Calabrese et al., 2003, Kavanagh et al., 2011, Mancuso et al., 2007).

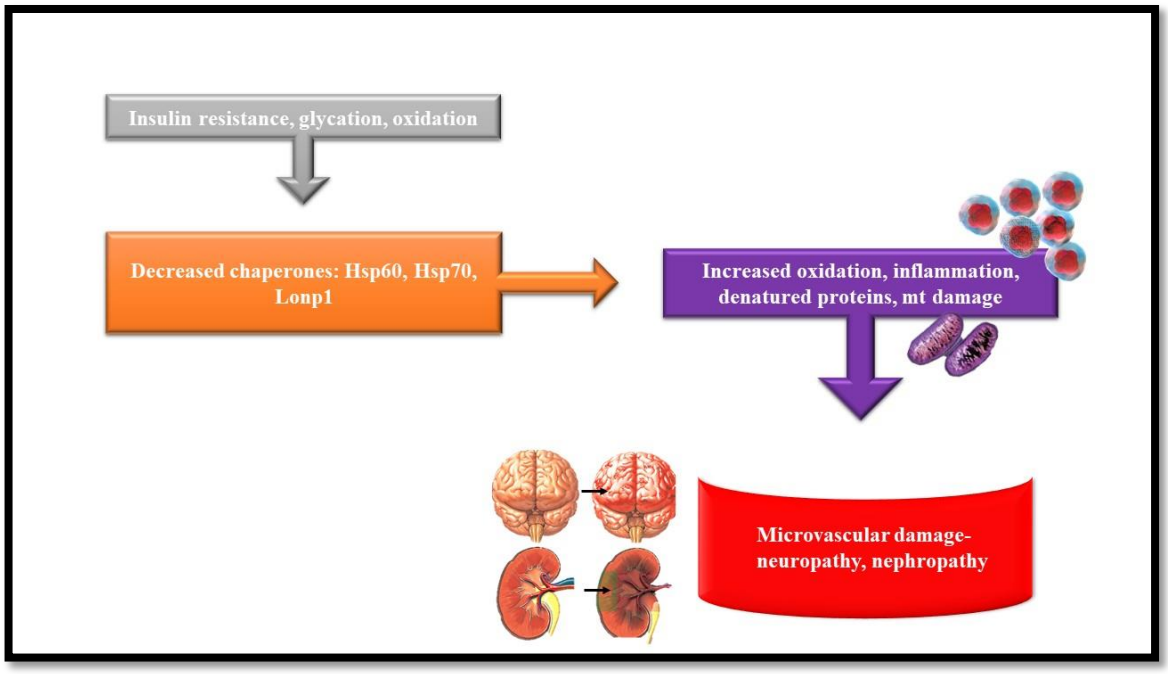


Figure 2.21: HSPs stabilise misfolded and denatured proteins (Prepared by author)

2.7.3 Epigenetics –role of microRNAs

The human transcriptome is governed by small non-coding RNAs that regulate protein coding genes. One of these categories include microRNAs (miRNAs) that function by mRNA inhibition of the 3′-untranslated region (3′-UTR) via the seed sequence region at the 5′ end of the miRNA (Chen et al., 2012) depicted by Fig 2.22. More than 1000 miRNAs are encoded by the human genome and have been indexed and annotated (Bartel, 2004, Berezikov et al., 2005). MicroRNAs may either control multiple genes, or a single gene can be regulated by more than one miRNA (Care et al., 2007, Pandey et al., 2009). They act upstream and downstream to various transcription

factors that mediate stress signals, cancer, cardiovascular diseases and diabetes (Kumar et al., 2011, Mishra et al., 2009, Zampetaki et al., 2010).

The process of miRNA biogenesis occurs in the nucleus, where genomic transcription of primary miRNA (pri-miRNA) sequences is followed by capping, splicing, and polyadenylation (Cai et al., 2004). Transcription of most miRNAs is carried out by RNA polymerase II, whilst others are transcribed by RNA polymerase III (Dieci et al., 2007). A complex known as microprocessor, consists of the nuclear RNase III enzyme (Drosha), which is essential for pri-miRNA processing into hairpin-shaped (~70-nucleotide (nt)-long) premature-miRNA (pre-miRNA) which are translocated to the cytoplasm (Muhonen and Holthofer, 2009). Here, the RNase III enzyme Dicer and its cofactor: transactivation-responsive RNA-binding protein (TRBP), cleave pre-miRNAs to release a short, ~22-base-pair (bp) RNA duplex. The miRNA-induced silencing complex (miRISC) is responsible for selection of miRNA strands and acts as the effector of the miRNA pathway, where messenger RNA (mRNA) translational inhibition or degradation occurs (Fig 2.22).

Dysregulation of miRNAs in diabetes hamper physiological and pathological processes, makes these small RNA molecules attractive targets for therapeutic intervention (Cuperus et al., 2011). Profound roles of miRNA dysregulation are highlighted in glucose metabolism (Dey et al., 2011). Identifying miRNA binding site targets through computational prediction facilitates understanding of their reciprocal nature (Pasquinelli, 2012). Hence, the epigenetic mechanism of miRNA gene regulation may lead to a variety of phenotypes including intricate diseases like diabetes and associated neurological disorders (Mattick and Makunin, 2006).

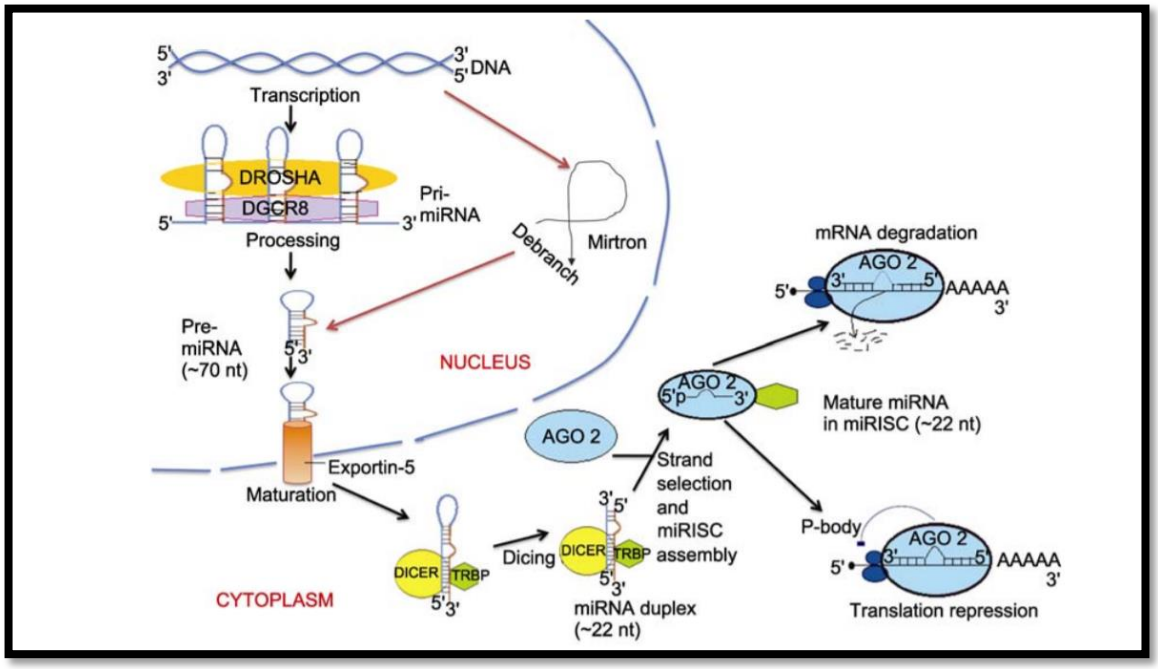


Figure 2.22: Mechanism of miRNA biogenesis and gene regulation (Kumar et al., 2012)

Among the various miRNAs dysregulated in the diabetes spectrum, this study focused on miR-132, -148a and -141. The role of miR-132 on metabolic and inflammatory targets has been demonstrated through nutritional availability *in vitro* (Strum et al., 2009), and its upregulation is further highlighted in prediabetic and diabetic mice (Nesca et al., 2013). MiR-148a expression significantly changes under hyperglycaemic conditions and has been associated with measures of pancreatic islet β cell function and glycaemic control (Lopez et al., 2017). Diabetic kidney fibrosis is alleviated through regulation of miR-141 and is associated with the PTEN/Akt/mTOR pathway (Li et al., 2017), however its modulation in diabetic neuropathy is unknown.

2.7.4 Streptozotocin model of T2DM

Diabetes represents an array of complexities involving different bodily systems. Animal models provide a platform for optimization, validation, and discovery of new therapeutics for human use. The various T2DM animal models range from nonhuman primates to nonmammalian models, each providing their own limitations and advantages (Fig 2.23). More commonly used, is the chemically induced diabetes mellitus (DM) rodent model for experimental studies, due to its simplicity and relative cost-effectiveness. This study used streptozotocin (STZ) as it is known to induce diabetes through partial pancreatic β-cell damage (Fig 2.24). Streptozotocin is an antibiotic obtained from *Streptomyces achromogenes* with structural glucosamine properties similar to that of nitrosourea (Srinivasan and Ramarao, 2007). β-cell toxicity upon STZ administration is attributed to its nitrosourea property, while its deoxyglucose nature promotes cellular entry across all membranes. Streptozotocin diabetogenicity is associated with free radical generation creating an imbalance in endogenous scavenging abilities. Additionally, it mimics DNA damage that is common to diabetics (Szkudelski, 2012). Severe decreases in β-cell mass, hyperinsulinaemia, and glucose intolerance develops with multiple low dose intraperitoneal injections of STZ (Fig 2.25), resembling the slow onset of T2DM that occurs in humans but within a shorter time period (Skovso, 2014).

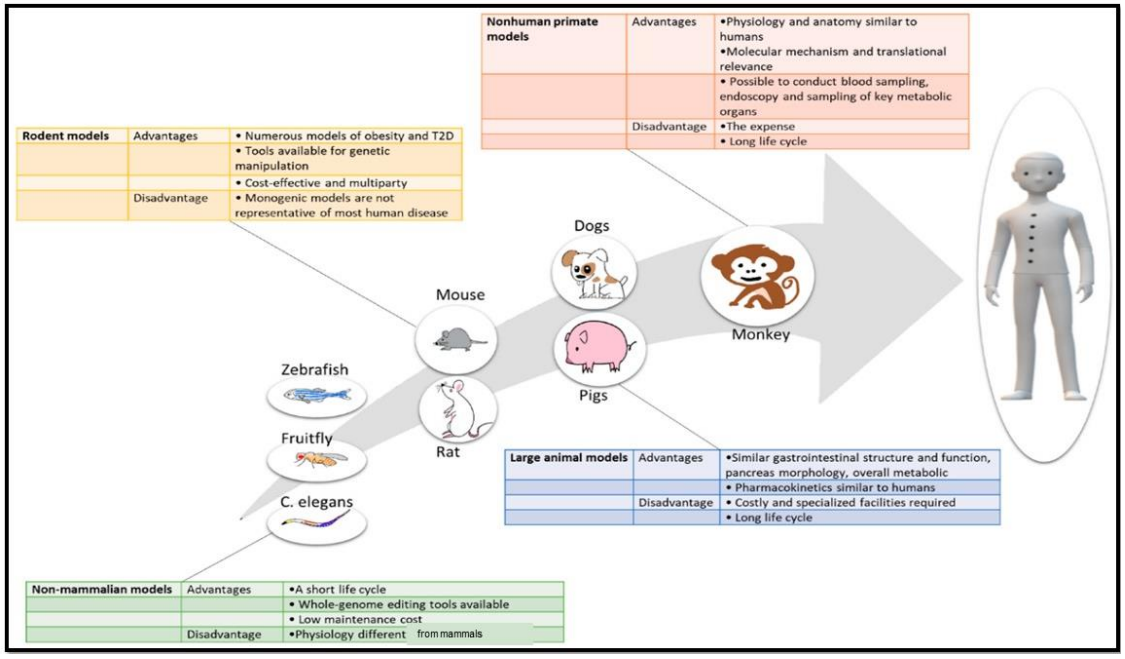


Figure 2.23: The pros and cons of different models used in diabetes research (Fang et al., 2019)

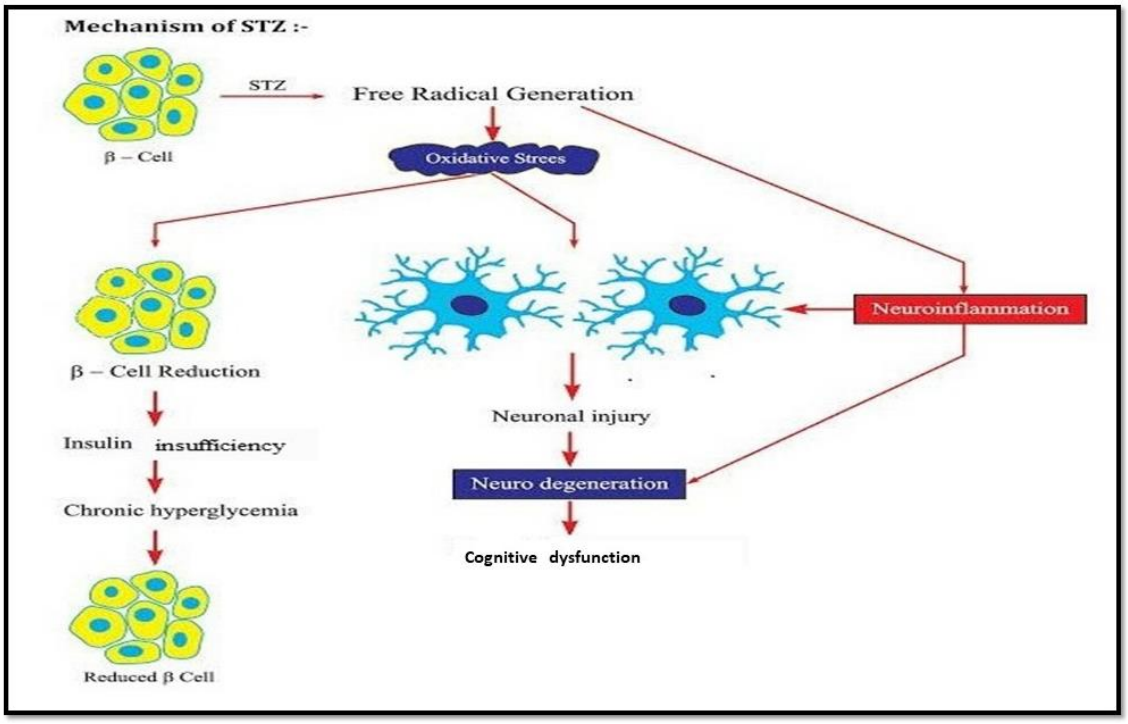


Figure 2.24: Mechanism of streptozotocin action (Dewangan et al., 2017)

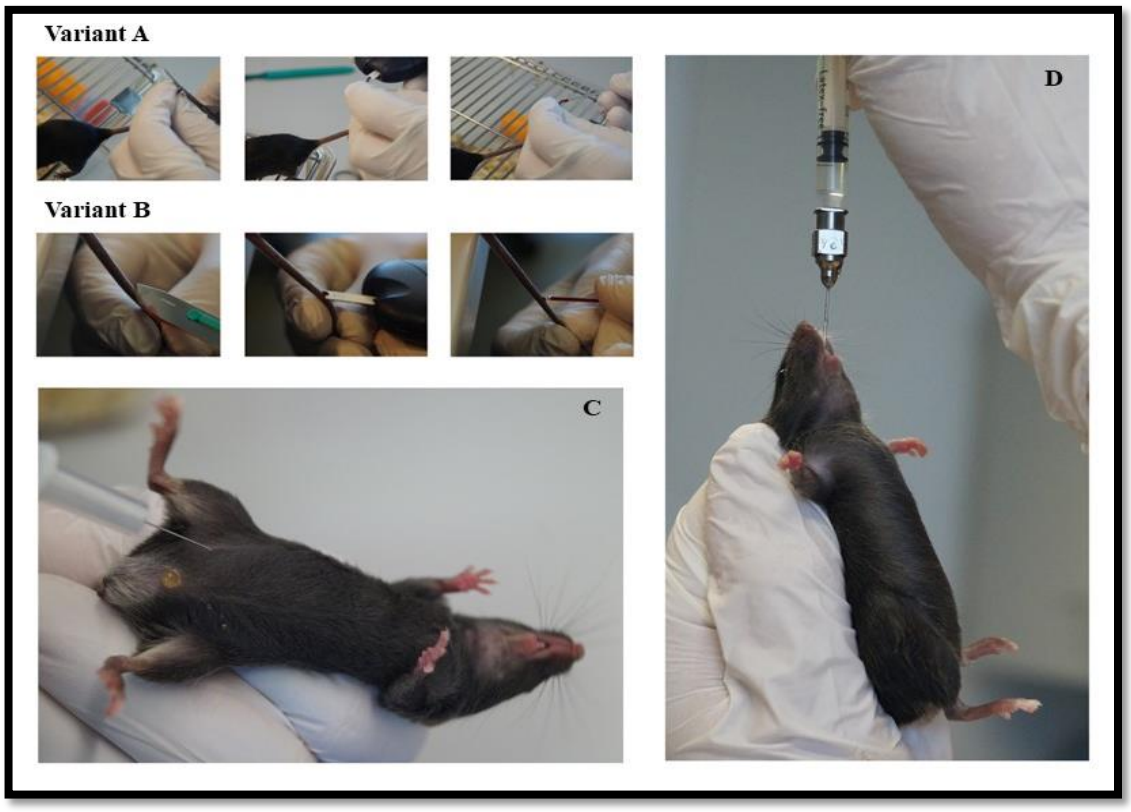


Figure 2.25: Variant A and B depict method of sampling for metabolic experiments. Image C illustrates the intraperitoneal injection of STZ. Image D portrays oral gavage used in treatment of diabetic mice (Nagy and Einwallner, 2018)

Evidence for the protective effects of MF exist; however, the data is limited for the metabolic effects of MF and diabetic organ damage *in vivo*. We aimed to outline the complex molecular mechanisms involved in hyperglycaemic neurodegeneration and renal damage by integration of oxidative and ER stress, cytoprotective markers, inflammation, cell death mechanisms and their epigenetic regulation by MF.

2.8 References

ADAMS, S., GREEN, P., CLAXTON, R., SIMCOX, S., WILLIAMS, M. V., WALSH, K. & LEEUWENBURGH, C. 2001. Reactive carbonyl formation by oxidative and non-oxidative pathways. *Front Biosci,* 6**,** A17-A24.

AHMAD, R., SAH, A. K. & AHSAN, H. 2016. Biochemistry and pathophysiology of glycation of DNA: Implications in diabetes. *Archives of Clinical and Biomedical Research,* 1**,** 32-47.

AHMED, N. 2005. Advanced glycation endproducts—role in pathology of diabetic complications. *Diabetes research and clinical practice,* 67**,** 3-21.

AHN, B.-H., KIM, H.-S., SONG, S., LEE, I. H., LIU, J., VASSILOPOULOS, A., DENG, C.-X. & FINKEL, T. 2008. A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. *Proceedings of the National Academy of Sciences,* 105**,** 14447-14452.

AKUDE, E., ZHEREBITSKAYA, E., CHOWDHURY, S. K. R., SMITH, D. R., DOBROWSKY, R. T. & FERNYHOUGH, P. 2011. Diminished superoxide generation is associated with respiratory chain dysfunction and changes in the mitochondrial proteome of sensory neurons from diabetic rats. *Diabetes,* 60**,** 288-297.

AL-KHALILI, L., FORSGREN, M., KANNISTO, K., ZIERATH, J., LÖNNQVIST, F. & KROOK, A. 2005. Enhanced insulin-stimulated glycogen synthesis in response to insulin, metformin or rosiglitazone is associated with increased mRNA expression of GLUT4 and peroxisomal proliferator activator receptor gamma co-activator 1. *Diabetologia,* 48**,** 1173-1179.

ALBRECHT, T., SCHILPEROORT, M., ZHANG, S., BRAUN, J. D., QIU, J., RODRIGUEZ, A., PASTENE, D. O., KRÄMER, B. K., KÖPPEL, H. & BAELDE, H. 2017. Carnosine attenuates the development of both type 2 diabetes and diabetic nephropathy in BTBR ob/ob mice. *Scientific reports,* 7**,** 44492.

ALSAHLI, M. & GERICH, J. E. 2017. Renal glucose metabolism in normal physiological conditions and in diabetes. *Diabetes research and clinical practice,* 133**,** 1-9.

AMERICAN DIABETES ASSOCIATION, 2019. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes—2019. *Diabetes Care*, 42(Supplement 1), pp.S13-S28.

AMETOY, A. S., BARINOV, A., DYCK, P. J., HERMANN, R., KOZLOVA, N., LITCHY, W. J., LOW, P. A., NEHRDICH, D., NOVOSADOVA, M. & O'BRIEN, P. C. 2003. The sensory symptoms of diabetic polyneuropathy are improved with [alpha]-lipoic acid: the SYDNEY trial.(Original Article: Emerging Treatments and Technologies). *Diabetes Care,* 26**,** 770-777.

ARKAN, M. C., HEVENER, A. L., GRETEN, F. R., MAEDA, S., LI, Z.-W., LONG, J. M., WYNSHAW-BORIS, A., POLI, G., OLEFSKY, J. & KARIN, M. 2005. IKK-β links inflammation to obesity-induced insulin resistance. *Nature medicine,* 11**,** 191.

ARONOFF, S. L., BERKOWITZ, K., SHREINER, B. & WANT, L. 2004. Glucose metabolism and regulation: beyond insulin and glucagon. *Diabetes spectrum,* 17**,** 183-190.

ASHRAFI, G., WU, Z., FARRELL, R. J. & RYAN, T. A. 2017. GLUT4 Mobilization Supports Energetic Demands of Active Synapses. *Neuron,* 93**,** 606-615.e3.

ATLAS, D. 2017. International Diabetes Federation. IDF Diabetes Atlas, 8th edn. Brussels, Belgium: International Diabetes Federation, 2017.

AZEVEDO, S. C. S., DA ROSA, C. V. D., WUNDERLICH, A. L. M., YAMADA, L. A., MARIANO, I. R., BATAGLINI, C., BRANQUINHO, N. T., RAPOSO, S. R., BAZOTTE, R. B. & BARONI, E. A. 2019. Glutamine or Glutamine Dipeptide Supplementation Improves Gluconeogenesis and Liver Glycogenosis in Type 1 Diabetic Rats. *Journal of Pharmacy and Pharmacology,* 7**,** 398-407.

BACK, S. H. & KAUFMAN, R. J. 2012. Endoplasmic reticulum stress and type 2 diabetes. *Annual review of biochemistry,* 81**,** 767-793.

BAILEY, C. J. & DAY, C. 2004. Metformin: its botanical background. *Practical Diabetes International,* 21**,** 115-117.

BAKER, R. G., HAYDEN, M. S. & GHOSH, S. 2011. NF-κB, inflammation, and metabolic disease. *Cell metabolism,* 13**,** 11-22.

BANDEIRA, D. M., DA FONSECA, L. J. S., GUEDES, D. S., RABELO, L. A., GOULART, M. O. & VASCONCELOS, S. M. L. 2013. Oxidative stress as an underlying contributor in the development of chronic complications in diabetes mellitus. *International journal of molecular sciences,* 14**,** 3265-3284.

BARRIÈRE, D. A., NOLL, C., ROUSSY, G., LIZOTTE, F., KESSAI, A., KIRBY, K., BELLEVILLE, K., BEAUDET, N., LONGPRÉ, J.-M. & CARPENTIER, A. C. 2018. Combination of high-fat/high-fructose diet and low-dose streptozotocin to model long-term type-2 diabetes complications. *Scientific reports,* 8**,** 424.

BARTEL, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell,* 116**,** 281-297.

BECKMANN, R. 1969. Absorption, distribution in the organism and elimination of metformin. *Diabetologia,* 5**,** 318-324.

BEERI, M. S., SILVERMAN, J. M., DAVIS, K. L., MARIN, D., GROSSMAN, H. Z., SCHMEIDLER, J., PUROHIT, D. P., PERL, D. P., DAVIDSON, M. & MOHS, R. C. 2005. Type 2 diabetes is negatively associated with Alzheimer's disease neuropathology. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 60**,** 471-475.

BENZLER, J., GANJAM, G. K., PRETZ, D., OELKRUG, R., KOCH, C. E., LEGLER, K., STÖHR, S., CULMSEE, C., WILLIAMS, L. M. & TUPS, A. 2015. Central inhibition of IKKβ/NF-κB signaling attenuates high-fat diet–induced obesity and glucose intolerance. *Diabetes,* 64.

BEREZIKOV, E., GURYEV, V., VAN DE BELT, J., WIENHOLDS, E., PLASTERK, R. H. & CUPPEN, E. 2005. Phylogenetic shadowing and computational identification of human microRNA genes. *Cell,* 120**,** 21-24.

BHANDARY, B., MARAHATTA, A., KIM, H.-R. & CHAE, H.-J. 2013. An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. *International journal of molecular sciences,* 14**,** 434-456.

BIESSELS, G. J. & DESPA, F. 2018. Cognitive decline and dementia in diabetes mellitus: mechanisms and clinical implications. *Nature Reviews Endocrinology,* 14**,** 591.

BIESSELS, G. J. & KAPPELLE, L. J. 2005. Increased risk of Alzheimer's disease in type II diabetes: Insulin resistance of the brain or insulin-induced amyloid pathology? *Biochemical Society Transactions,* 33**,** 1041-1044.

BLAS, E. & KURUP, A. S. 2010. *Equity, social determinants and public health programmes*, World Health Organization.

BOULTON, A. J., VINIK, A. I., AREZZO, J. C., BRIL, V., FELDMAN, E. L., FREEMAN, R., MALIK, R. A., MASER, R. E., SOSENKO, J. M. & ZIEGLER, D. 2005. Diabetic neuropathies: a statement by the American Diabetes Association. *Diabetes care,* 28**,** 956-962.

BRAMHAM, C. R. & MESSAOUDI, E. 2005. BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Progress in neurobiology,* 76**,** 99-125.

BRAUNWALD, E. 2019. Diabetes, heart failure, and renal dysfunction: The vicious circles. *Progress in cardiovascular diseases,* 62, 298-302.

BROWN, G. 2000. Glucose transporters: structure, function and consequences of deficiency. *Journal of inherited metabolic disease,* 23**,** 237-246.

BROWNLEE, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature,* 414**,** 813.

BROWNLEE, M., CERAMI, A. & VLASSARA, H. 1988. Advanced products of nonenzymatic glycosylation and the pathogenesis of diabetic vascular disease. *Diabetes/metabolism reviews,* 4**,** 437-451.

CADE, W. T. 2018. The manifold role of the mitochondria in skeletal muscle insulin resistance. *Current Opinion in Clinical Nutrition & Metabolic Care,* 21**,** 267-272.

CAI, X., HAGEDORN, C. H. & CULLEN, B. R. 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA,* 10**,** 1957-1966.

CALABRESE, V., SCAPAGNINI, G., COLOMBRITA, C., RAVAGNA, A., PENNISI, G., STELLA, A. G., GALLI, F. & BUTTERFIELD, D. 2003. Redox regulation of heat shock protein expression in aging and neurodegenerative disorders associated with oxidative stress: a nutritional approach. *Amino acids,* 25**,** 437-444.

CAMPBELL, N. L., UNVERZAGT, F., LAMANTIA, M. A., KHAN, B. A. & BOUSTANI, M. A. 2013. Risk factors for the progression of mild cognitive impairment to dementia. *Clinics in geriatric medicine,* 29**,** 873-893.

CANTÓ, C. & AUWERX, J. 2009. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Current opinion in lipidology,* 20**,** 98.

CAO, S. & KAUFMAN, R. 2014. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxidants & Redox Signaling,* 21**,** 396-413.

CARDOZO, A. K., HEIMBERG, H., HEREMANS, Y., LEEMAN, R., KUTLU, B., KRUHØFFER, M., ØRNTOFT, T. & EIZIRIK, D. L. 2001. A comprehensive analysis of cytokine-induced and nuclear factor-κB-dependent genes in primary rat pancreatic β-cells. *Journal of Biological Chemistry,* 276**,** 48879-48886.

CARE, A., CATALUCCI, D., FELICETTI, F., BONCI, D., ADDARIO, A., GALLO, P., BANG, M.-L., SEGNALINI, P., GU, Y. & DALTON, N. D. 2007. MicroRNA-133 controls cardiac hypertrophy. *Nature medicine,* 13**,** 613.

CARRO, E. & TORRES-ALEMAN, I. 2004. The role of insulin and insulin-like growth factor I in the molecular and cellular mechanisms underlying the pathology of Alzheimer's disease. *European journal of pharmacology,* 490**,** 127-133.

CASTROGIOVANNI, C., WATERSCHOOT, B., DE BACKER, O. & DUMONT, P. 2018. Serine 392 phosphorylation modulates p53 mitochondrial translocation and transcription-independent apoptosis. *Cell death and differentiation,* 25**,** 190.

CATTARUZZA, M. & HECKER, M. 2008. Protein Carbonylation and Decarboylation. *Circ. Res,* 102**,** 273-274.

CERSOSIMO, E., TRIPLITT, C., SOLIS-HERRERA, C., MANDARINO, L. J. & DEFRONZO, R. A. 2018. Pathogenesis of type 2 diabetes mellitus. *Endotext [Internet]*. MDText. com, Inc.

CHAN, K., HAN, X.-D. & KAN, Y. W. 2001. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proceedings of the National Academy of Sciences,* 98**,** 4611-4616.

CHANG, T.-Y., CHANG, C. C., OHGAMI, N. & YAMAUCHI, Y. 2006. Cholesterol sensing, trafficking, and esterification. *Annu. Rev. Cell Dev. Biol.,* 22**,** 129-157.

CHEN, L., MAGLIANO, D. J. & ZIMMET, P. Z. 2012. The worldwide epidemiology of type 2 diabetes mellitus—present and future perspectives. *Nature reviews endocrinology,* 8**,** 228.

CHEN, S. & SANG, N. 2010. Histone deacetylase inhibitors: the epigenetic therapeutics that repress hypoxia-inducible factors. *BioMed Research International,* 2011.

CHEN, S. & SANG, N. 2016. Hypoxia-inducible factor-1: a critical player in the survival strategy of stressed cells. *Journal of cellular biochemistry,* 117**,** 267-278.

CHEN, S., ZHAO, Z., KE, L., LI, Z., LI, W., ZHANG, Z., ZHOU, Y., FENG, X. & ZHU, W. 2018. Resveratrol improves glucose uptake in insulin-resistant adipocytes via Sirt1. *The Journal of nutritional biochemistry,* 55**,** 209-218.

CHEN, Y., ZHOU, K., WANG, R., LIU, Y., KWAK, Y.-D., MA, T., THOMPSON, R. C., ZHAO, Y., SMITH, L. & GASPARINI, L. 2009. Antidiabetic drug metformin (GlucophageR) increases

biogenesis of Alzheimer's amyloid peptides via up-regulating BACE1 transcription. *Proceedings of the National Academy of Sciences,* 106**,** 3907-3912.

CHIAZZA, F., COUTURIER-MAILLARD, A., BENETTI, E., MASTROCOLA, R., NIGRO, D., CUTRIN, J. C., SERPE, L., ARAGNO, M., FANTOZZI, R. & RYFFEL, B. 2015. Targeting the NLRP3 inflammasome to reduce diet-induced metabolic abnormalities in mice. *Molecular Medicine,* 21**,** 1025-1037.

CHO, N., SHAW, J., KARURANGA, S., HUANG, Y., DA ROCHA FERNANDES, J., OHLROGGE, A. & MALANDA, B. 2018. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes research and clinical practice,* 138**,** 271-281.

CHOI, S.-E. & KEMPER, J. K. 2013. Regulation of SIRT1 by microRNAs. *Molecules and cells,* 36**,** 385-392.

CHONG, Z. Z., SHANG, Y. C., WANG, S. & MAIESE, K. 2012. SIRT1: New avenues of discovery for disorders of oxidative stress. *Expert opinion on therapeutic targets,* 16**,** 167-178.

CHOWDHURY, S. K. R., DOBROWSKY, R. T. & FERNYHOUGH, P. 2011. Nutrient excess and altered mitochondrial proteome and function contribute to neurodegeneration in diabetes. *Mitochondrion,* 11**,** 845-854.

CHUNG, S. S., HO, E. C., LAM, K. S. & CHUNG, S. K. 2003. Contribution of polyol pathway to diabetes-induced oxidative stress. *Journal of the American Society of Nephrology,* 14**,** S233-S236.

CLODFELDER-MILLER, B. J., ZMIJEWSKA, A. A., JOHNSON, G. V. & JOPE, R. S. 2006. Tau is hyperphosphorylated at multiple sites in mouse brain in vivo after streptozotocin-induced insulin deficiency. *Diabetes,* 55**,** 3320-3325.

COHEN, A. D. & KLUNK, W. E. 2014. Early detection of Alzheimer's disease using PiB and FDG PET. *Neurobiology of Disease,* 72**,** 117-122.

COHEN, C. D., FRACH, K., SCHLÖNDORFF, D. & KRETZLER, M. 2002. Quantitative gene expression analysis in renal biopsies: a novel protocol for a high-throughput multicenter application. *Kidney international,* 61**,** 133-140.

CULLINAN, S. B. & DIEHL, J. A. 2004. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *Journal of Biological Chemistry,* 279**,** 20108-20117.

CUPERUS, J. T., FAHLGREN, N. & CARRINGTON, J. C. 2011. Evolution and functional diversification of MIRNA genes. *The Plant Cell,* 23**,** 431-442.

CURTIS, J. M., GRIMSRUD, P. A., WRIGHT, W. S., XU, X., FONCEA, R. E., GRAHAM, D. W., BRESTOFF, J. R., WICZER, B. M., ILKAYEVA, O. & CIANFLONE, K. 2010. Downregulation of adipose glutathione S-transferase A4 leads to increased protein carbonylation, oxidative stress, and mitochondrial dysfunction. *Diabetes,* 59**,** 1132-1142.

CURTIS, J. M., HAHN, W. S., LONG, E. K., BURRILL, J. S., ARRIAGA, E. A. & BERNLOHR, D. A. 2012. Protein carbonylation and metabolic control systems. *Trends in Endocrinology & Metabolism,* 23**,** 399-406.

CUYÀS, E., VERDURA, S., LLORACH-PARÉS, L., FERNÁNDEZ-ARROYO, S., JOVEN, J., MARTIN-CASTILLO, B., BOSCH-BARRERA, J., BRUNET, J., NONELL-CANALS, A. & SANCHEZ-MARTINEZ, M. 2018. Metformin is a direct SIRT1-activating compound: Computational modeling and experimental validation. *Frontiers in endocrinology,* 9**,** 657.

CZECH, M. P. & CORVERA, S. 1999. Signaling mechanisms that regulate glucose transport. *Journal of Biological Chemistry,* 274**,** 1865-1868.

DEBERARDINIS, R. J. & THOMPSON, C. B. 2012. Cellular metabolism and disease: what do metabolic outliers teach us? *Cell,* 148**,** 1132-1144.

DEFRONZO, R. A. 2004. Pathogenesis of type 2 diabetes mellitus. *Medical Clinics,* 88**,** 787-835.

DEWANGAN, H., TIWARI, R.K., SHARMA, V., SHUKLA, S.S., SATAPATHY, T. AND PANDEY, R., 2017. Past and Future of in-vitro and in-vivo Animal Models for Diabetes: A Review. *Indian Journal of Pharmaceutical Education and Research*, 51(4S), pp.s522-s530.

DEY, N., DAS, F., MARIAPPAN, M. M., MANDAL, C. C., GHOSH-CHOUDHURY, N., KASINATH, B. S. & CHOUDHURY, G. G. 2011. MicroRNA-21 orchestrates high glucose-induced signals to TOR complex 1, resulting in renal cell pathology in diabetes. *Journal of Biological Chemistry,* 286**,** 25586-25603.

DÍAZ-VILLANUEVA, J. F., DÍAZ-MOLINA, R. & GARCÍA-GONZÁLEZ, V. 2015. Protein folding and mechanisms of proteostasis. *International journal of molecular sciences,* 16**,** 17193-17230.

DIECI, G., FIORINO, G., CASTELNUOVO, M., TEICHMANN, M. & PAGANO, A. 2007. The expanding RNA polymerase III transcriptome. *TRENDS in Genetics,* 23**,** 614-622.

DI PINO, A. AND DEFRONZO, R.A., 2019. Insulin Resistance and Atherosclerosis: Implications for Insulin-Sensitizing Agents. *Endocrine Reviews*, 40(6), pp.1447-1467.

DIMEGLIO, L. A., EVANS-MOLINA, C. & ORAM, R. A. 2018. Type 1 diabetes. *The Lancet,* 391**,** 2449-2462.

DOS SANTOS MELLO, M., RIBAS, G. S., WAYHS, C. A. Y., HAMMERSCHMIDT, T., GUERREIRO, G. B. B., FAVENZANI, J. L., SITTA, Â., DE MOURA COELHO, D., WAJNER, M. & VARGAS, C. R. 2015. Increased oxidative stress in patients with 3-hydroxy-3-methylglutaric aciduria. *Molecular and cellular biochemistry,* 402**,** 149-155.

DRONAVALLI, S., DUKA, I. & BAKRIS, G. L. 2008. The pathogenesis of diabetic nephropathy. *Nature Reviews Endocrinology,* 4**,** 444.

E GONZÁLEZ-REYES, R., ALIEV, G., ÁVILA-RODRIGUES, M. & E BARRETO, G. 2016. Alterations in glucose metabolism on cognition: a possible link between diabetes and dementia. *Current pharmaceutical design,* 22**,** 812-818.

EISNER, V., PICARD, M. & HAJNÓCZKY, G. 2018. Mitochondrial dynamics in adaptive and maladaptive cellular stress responses. *Nature cell biology,* 20**,** 755.

EIZIRIK, D., MIANI, M. & CARDOZO, A. 2013. Signalling danger: endoplasmic reticulum stress and the unfolded protein response in pancreatic islet inflammation. *Diabetologia,* 56**,** 234-241.

EL-KOSSI, A. E. A., ABDELLAH, M. M., RASHAD, A. M. & HAMED, S. A. 2011. The effectiveness of evening primrose oil and alpha lipoic acid in recovery of nerve function in diabetic rats. *Journal of Clinical & Experimental Investigations/Klinik ve Deneysel Arastirmalar Dergisi*, 2.

ELAHI, M., HASAN, Z., MOTOI, Y., MATSUMOTO, S.-E., ISHIGURO, K. & HATTORI, N. 2016. Region-specific vulnerability to oxidative stress, neuroinflammation, and tau hyperphosphorylation in experimental diabetes mellitus mice. *Journal of Alzheimer's Disease,* 51**,** 1209-1224.

ELDOR, R., YEFFET, A., BAUM, K., DOVINER, V., AMAR, D., BEN-NERIAH, Y., CHRISTOFORI, G., PELED, A., CAREL, J. & BOITARD, C. 2006. Conditional and specific NF-κB blockade protects pancreatic beta cells from diabetogenic agents. *Proceedings of the National Academy of Sciences,* 103**,** 5072-5077.

FANG, J.-Y., LIN, C.-H., HUANG, T.-H. & CHUANG, S.-Y. 2019. In Vivo Rodent Models of Type 2 Diabetes and Their Usefulness for Evaluating Flavonoid Bioactivity. *Nutrients,* 11**,** 530.

FERNYHOUGH, P. 2015. Mitochondrial dysfunction in diabetic neuropathy: a series of unfortunate metabolic events. *Current diabetes reports,* 15**,** 89.

FORETZ, M., GUIGAS, B., BERTRAND, L., POLLAK, M. & VIOLLET, B. 2014. Metformin: from mechanisms of action to therapies. *Cell metabolism,* 20**,** 953-966.

FRAME, S. & COHEN, P. 2001. GSK3 takes centre stage more than 20 years after its discovery. *Biochemical Journal,* 359**,** 1-16.

FROHNERT, B. I. & BERNLOHR, D. A. 2013. Protein carbonylation, mitochondrial dysfunction, and insulin resistance. *Advances in nutrition,* 4**,** 157-163.

FU, M.-X., REQUENA, J. R., JENKINS, A. J., LYONS, T. J., BAYNES, J. W. & THORPE, S. R. 1996. The advanced glycation end product, N-(carboxymethyl) lysine, is a product of both lipid peroxidation and glycoxidation reactions. *Journal of Biological Chemistry,* 271**,** 9982-9986.

FULDA, S., GORMAN, A. M., HORI, O. & SAMALI, A. 2010. Cellular stress responses: cell survival and cell death. *International journal of cell biology,* 2010.

GANTOIS, I., POPIC, J., KHOUTORSKY, A. & SONENBERG, N. 2019. Metformin for treatment of fragile X syndrome and other neurological disorders. *Annual review of medicine,* 70**,** 167-181.

GARDNER, B. M. & WALTER, P. 2011. Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. *Science,* 333**,** 1891-1894.

GERICH, J. E. 2010. Role of the kidney in normal glucose homeostasis and in the hyperglycaemia of diabetes mellitus: therapeutic implications. *Diabetic medicine : a journal of the British Diabetic Association,* 27**,** 136-142.

GERICH, J. E., MEYER, C., WOERLE, H. J. & STUMVOLL, M. 2001. Renal gluconeogenesis: its importance in human glucose homeostasis. *Diabetes care,* 24**,** 382-391.

GHOSH, S. & KARIN, M. 2002. Missing pieces in the NF-κB puzzle. *Cell,* 109**,** S81-S96.

GNUDI, L., COWARD, R. J. M. & LONG, D. A. 2016. Diabetic Nephropathy: Perspective on Novel Molecular Mechanisms. *Trends in Endocrinology & Metabolism,* 27**,** 820-830.

GONG, C.-X., LIU, F., GRUNDKE-IQBAL, I. & IQBAL, K. 2005. Post-translational modifications of tau protein in Alzheimer's disease. *Journal of neural transmission,* 112**,** 813-838.

GONG, L., GOSWAMI, S., GIACOMINI, K. M., ALTMAN, R. B. & KLEIN, T. E. 2012. Metformin pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenetics and genomics,* 22**,** 820.

GOODPASTER, B. H., DELANY, J. P., OTTO, A. D., KULLER, L., VOCKLEY, J., SOUTH-PAUL, J. E., THOMAS, S. B., BROWN, J., MCTIGUE, K. & HAMES, K. C. 2010. Effects of diet and physical activity interventions on weight loss and cardiometabolic risk factors in severely obese adults: a randomized trial. *Jama,* 304**,** 1795-1802.

GRAHAM, G. G., PUNT, J., ARORA, M., DAY, R. O., DOOGUE, M. P., DUONG, J., FURLONG, T. J., GREENFIELD, J. R., GREENUP, L. C. & KIRKPATRICK, C. M. 2011. Clinical pharmacokinetics of metformin. *Clinical pharmacokinetics,* 50**,** 81-98.

GRATUZE, M., JULIEN, J., PETRY, F. R., MORIN, F. & PLANEL, E. 2017. Insulin deprivation induces PP2A inhibition and tau hyperphosphorylation in hTau mice, a model of Alzheimer's disease-like tau pathology. *Scientific Reports,* 7**,** 46359.

GREEN, K., BRAND, M. D. & MURPHY, M. P. 2004. Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. *Diabetes,* 53**,** S110-S118.

GREGOR, M.F. AND HOTAMISLIGIL, G.S., 2011. Inflammatory mechanisms in obesity. *Annual review of immunology*, 29, pp.415-445.

GRØNBÆK, H., VOGEL, I., ØSTERBY, R., LANCRANJAN, I., FLYVBJERG, A. & ØRSKOV, H. 1998. Effect of octreotide, captopril or insulin on renal changes and UAE in long-term experimental diabetes. *Kidney international,* 53**,** 173-180.

GUIMARÃES, J., BASTOS, M., MELO, M. & CARVALHEIRO, M. 2007. Diabetic nephropathy: glomerular filtration rate and estimated creatinine clearance. *Acta medica portuguesa,* 20**,** 145-50.

GUZZARDI, M. A., SANGUINETTI, E., BARTOLI, A., KEMENY, A., PANETTA, D., SALVADORI, P. A., BURCHIELLI, S. & IOZZO, P. 2018. Elevated glycaemia and brain glucose utilization predict BDNF lowering since early life. *Journal of Cerebral Blood Flow & Metabolism,* 38**,** 447-455.

HAAK, E. S., USADEL, K. H., KOHLEISEN, M., YILMAZ, A., KUSTERER, K. & HAAK, T. 1999. The effect of α-lipoic acid on the neurovascular reflex arc in patients with diabetic neuropathy assessed by capillary microscopy. *Microvascular research,* 58**,** 28-34.

HAIGIS, M. C. & YANKNER, B. A. 2010. The aging stress response. *Molecular cell,* 40**,** 333-344.

HAMPTON, R. Y. 2002. ER-associated degradation in protein quality control and cellular regulation. *Current opinion in cell biology,* 14**,** 476-482.

HAN, H.-S., KANG, G., KIM, J. S., CHOI, B. H. & KOO, S.-H. 2016. Regulation of glucose metabolism from a liver-centric perspective. *Experimental & molecular medicine,* 48**,** e218-e218.

HANGER, D. P., ANDERTON, B. H. & NOBLE, W. 2009. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends in molecular medicine,* 15**,** 112-119.

HARDIE, D. 2008. AMPK: a key regulator of energy balance in the single cell and the whole organism. *International journal of obesity,* 32**,** S7.

HARDIE, D. G. 2014. AMPK—sensing energy while talking to other signaling pathways. *Cell metabolism,* 20**,** 939-952.

HARDIE, D. G., ROSS, F. A. & HAWLEY, S. A. 2012. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nature reviews Molecular cell biology,* 13**,** 251.

HARDING, H. P., ZENG, H., ZHANG, Y., JUNGRIES, R., CHUNG, P., PLESKEN, H., SABATINI, D. D. & RON, D. 2001. Diabetes mellitus and exocrine pancreatic dysfunction in perk−/− mice reveals a role for translational control in secretory cell survival. *Molecular cell,* 7**,** 1153-1163.

HARDING, J. L., PAVKOV, M. E., MAGLIANO, D. J., SHAW, J. E. & GREGG, E. W. 2019. Global trends in diabetes complications: a review of current evidence. *Diabetologia,* 62**,** 3-16.

HARRIS, J. J., JOLIVET, R. & ATTWELL, D. 2012. Synaptic energy use and supply. *Neuron,* 75**,** 762-777.

HASNAIN, S., LOURIE, R., DAS, I., CHEN, A. & MCGUCKIN, M. 2012. The interplay between endoplasmic reticulum stress and inflammation. *Immunology and Cell Biology,* 90**,** 267-270.

HECKER, M. & WAGNER, A. H. 2018. Role of protein carbonylation in diabetes. *Journal of Inherited Metabolic Disease,* 41**,** 29-38.

HERAS-SANDOVAL, D., PÉREZ-ROJAS, J. M., HERNÁNDEZ-DAMIÁN, J. & PEDRAZA-CHAVERRI, J. 2014. The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. *Cellular signalling,* 26**,** 2694-2701.

HOCK, A. K. & VOUSDEN, K. H. 2014. The role of ubiquitin modification in the regulation of p53. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1843**,** 137-149.

HÖLSCHER, C., 2014. Drugs developed for treatment of diabetes show protective effects in Alzheimer's and Parkinson's diseases. *Sheng Li Xue Bao*, 66(5), pp.497-510.

HOTAMISLIGIL, G.S., 2010. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell,* 140(6), pp.900-917.

HOTTA, N. 2001. Clinical difference between thiazolidinediones and biguanides. *Nihon rinsho. Japanese journal of clinical medicine,* 59**,** 2219-2227.

HUMPTON, T. J. & VOUSDEN, K. H. 2016. Regulation of cellular metabolism and hypoxia by p53. *Cold Spring Harbor perspectives in medicine,* 6**,** a026146.

HUNDAL, R. S., KRSSAK, M., DUFOUR, S., LAURENT, D., LEBON, V., CHANDRAMOULI, V., INZUCCHI, S. E., SCHUMANN, W. C., PETERSEN, K. F. & LANDAU, B. R. 2000. Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes,* 49**,** 2063-2069.

ICHIKAWA, T., OEDA, T., OHMORI, H. & SCHILL, W. 1999. Reactive oxygen species influence the acrosome reaction but not acrosin activity in human spermatozoa. *International journal of andrology,* 22**,** 37-42.

INZUCCHI, S. E., BERGENSTAL, R. M., BUSE, J. B., DIAMANT, M., FERRANNINI, E., NAUCK, M., PETERS, A. L., TSAPAS, A., WENDER, R. & MATTHEWS, D. R. 2015. Management of hyperglycaemia in type 2 diabetes, 2015: a patient-centered approach: update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes care,* 38**,** 140-149.

ISHIBASHI, Y., YAMAGISHI, S.-I., MATSUI, T., OHTA, K., TANOUE, R., TAKEUCHI, M., UEDA, S., NAKAMURA, K.-I. & OKUDA, S. 2012. Pravastatin inhibits advanced glycation end products (AGEs)-induced proximal tubular cell apoptosis and injury by reducing receptor for AGEs (RAGE) level. *Metabolism,* 61**,** 1067-1072.

IWABU, M., YAMAUCHI, T., OKADA-IWABU, M., SATO, K., NAKAGAWA, T., FUNATA, M., YAMAGUCHI, M., NAMIKI, S., NAKAYAMA, R. & TABATA, M. 2010. Adiponectin and AdipoR1 regulate PGC-1α and mitochondria by Ca 2+ and AMPK/SIRT1. *Nature,* 464**,** 1313.

JACK, M. & WRIGHT, D. 2012. Role of advanced glycation endproducts and glyoxalase I in diabetic peripheral sensory neuropathy. *Translational Research,* 159**,** 355-365.

JÄGER, S., HANDSCHIN, C., PIERRE, J. S.-. & SPIEGELMAN, B. M. 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proceedings of the National Academy of Sciences,* 104**,** 12017-12022.

JIN, L., GALONEK, H., ISRAELIAN, K., CHOY, W., MORRISON, M., XIA, Y., WANG, X., XU, Y., YANG, Y. & SMITH, J. J. 2009. Biochemical characterization, localization, and tissue distribution of the longer form of mouse SIRT3. *Protein Science,* 18**,** 514-525.

JO, E.-K., KIM, J. K., SHIN, D.-M. & SASAKAWA, C. 2016. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cellular & molecular immunology,* 13**,** 148.

JONO, T., KIMURA, T., TAKAMATSU, J., NAGAI, R., MIYAZAKI, K., YUZURIHA, T., KITAMURA, T. & HORIUCHI, S. 2002. Accumulation of imidazolone, pentosidine and Nε-

(carboxymethyl) lysine in hippocampal CA4 pyramidal neurons of aged human brain. *Pathology international,* 52**,** 563-571.

JOSE, C., BELLANCE, N. & ROSSIGNOL, R. 2011. Choosing between glycolysis and oxidative phosphorylation: a tumor's dilemma? *Biochimica et Biophysica Acta (BBA)-Bioenergetics,* 1807**,** 552-561.

KADAMKODE, V. & BANERJEE, G. 2014. Micro RNA: an epigenetic regulator of type 2 diabetes. *Microrna,* 3**,** 86-97.

KAJIKAWA, M., NAKASHIMA, A., FUJIMURA, N., MARUHASHI, T., IWAMOTO, Y., IWAMOTO, A., MATSUMOTO, T., ODA, N., HIDAKA, T. & KIHARA, Y. 2015. Ratio of serum levels of AGEs to soluble form of RAGE is a predictor of endothelial function. *Diabetes Care,* 38**,** 119-125.

KAMALTA, N., KUMAR, A., VASHIST, H. & SHARMA, R. B. 2018. AN ANTHOLOGY OF DIABETES. *Innovat International Journal Of Medical & Pharmaceutical Sciences,* 3.

KANWAR, Y. S., SUN, L., XIE, P., LIU, F.-Y. & CHEN, S. 2011. A glimpse of various pathogenetic mechanisms of diabetic nephropathy. *Annual Review of Pathology: Mechanisms of Disease,* 6**,** 395-423.

KAO, C.-L., CHEN, L.-K., CHANG, Y.-L., YUNG, M.-C., HSU, C.-C., CHEN, Y.-C., LO, W.-L., CHEN, S.-J., KU, H.-H. & HWANG, S.-J. 2010. Resveratrol protects human endothelium from H2O2-induced oxidative stress and senescence via SirT1 activation. *Journal of atherosclerosis and thrombosis,* 17**,** 970-979.

KARTONO, A., MALIK, S., SYAFUTRA, H., WAHYUDI, S. & SUMARYADA, T. Pharmacokinetics simulation of Metformin in type 2 Diabetes Mellitus. *Journal of Physics: Conference Series*, 2019. IOP Publishing, 012044.

KAVANAGH, K., FLYNN, D. M., JENKINS, K. A., ZHANG, L. & WAGNER, J. D. 2011. Restoring HSP70 deficiencies improves glucose tolerance in diabetic monkeys. *American Journal of Physiology-Endocrinology and Metabolism,* 300**,** E894-E901.

KENSLER, T. W., WAKABAYASHI, N. & BISWAL, S. 2007. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.,* 47**,** 89-116.

KIM, B., BACKUS, C., OH, S., HAYES, J. M. & FELDMAN, E. L. 2009. Increased tau phosphorylation and cleavage in mouse models of type 1 and type 2 diabetes. *Endocrinology,* 150**,** 5294-5301.

KIM, Y. D., PARK, K.-G., LEE, Y.-S., PARK, Y.-Y., KIM, D.-K., NEDUMARAN, B., JANG, W. G., CHO, W.-J., HA, J. & LEE, I.-K. 2008. Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase–dependent regulation of the orphan nuclear receptor SHP. *Diabetes,* 57**,** 306-314.

KITADA, M., OGURA, Y., MONNO, I. & KOYA, D. 2019. Sirtuins and Type 2 diabetes: role in inflammation, oxidative stress, and mitochondrial function. *Frontiers in Endocrinology,* 10.

KLEIN, J. P. & WAXMAN, S. G. 2003. The brain in diabetes: molecular changes in neurons and their implications for end-organ damage. *The Lancet Neurology,* 2**,** 548-554.

KOPP, E. & GHOSH, S. 1994. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science,* 265**,** 956-959.

KRABBE, K., NIELSEN, A., KROGH-MADSEN, R., PLOMGAARD, P., RASMUSSEN, P., ERIKSTRUP, C., FISCHER, C., LINDEGAARD, B., PETERSEN, A. & TAUDORF, S. 2007. Brain-derived neurotrophic factor (BDNF) and type 2 diabetes. *Diabetologia,* 50**,** 431-438.

KUMAR, M., LU, Z., TAKWI, A. A. L., CHEN, W., CALLANDER, N. S., RAMOS, K. S., YOUNG, K. H. & LI, Y. 2011. Negative regulation of the tumor suppressor p53 gene by microRNAs. *Oncogene,* 30**,** 843.

KUMAR, M., NATH, S., PRASAD, H. K., SHARMA, G. & LI, Y. 2012. MicroRNAs: a new ray of hope for diabetes mellitus. *Protein & cell,* 3**,** 726-738.

KUME, S., UZU, T., HORIIKE, K., CHIN-KANASAKI, M., ISSHIKI, K., ARAKI, S.-I., SUGIMOTO, T., HANEDA, M., KASHIWAGI, A. & KOYA, D. 2010. Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney. *The Journal of clinical investigation,* 120**,** 1043-1055.

LARGE, V. & BEYLOT, M. 1999. Modifications of citric acid cycle activity and gluconeogenesis in streptozotocin-induced diabetes and effects of metformin. *Diabetes,* 48**,** 1251-1257.

LEE, H.-M., KIM, J.-J., KIM, H. J., SHONG, M., KU, B. J. & JO, E.-K. 2013. Upregulated NLRP3 inflammasome activation in patients with type 2 diabetes. *Diabetes,* 62**,** 194-204.

LEE, J., MIYAZAKI, M., ROMEO, G. R. & SHOELSON, S. E. 2014. Insulin receptor activation with transmembrane domain ligands. *Journal of Biological Chemistry,* 289**,** 19769-19777.

LEMIEUX, G., ARANDA, M. R., FOURNEL, P. & LEMIEUX, C. 1984. Renal enzymes during experimental diabetes mellitus in the rat. Role of insulin, carbohydrate metabolism, and ketoacidosis. *Canadian journal of physiology and pharmacology,* 62**,** 70-75.

LI, G., MONGILLO, M., CHIN, K.-T., HARDING, H., RON, D., MARKS, A. R. & TABAS, I. 2009. Role of ERO1-α–mediated stimulation of inositol 1, 4, 5-triphosphate receptor activity in endoplasmic reticulum stress–induced apoptosis. *The Journal of cell biology,* 186**,** 783-792.

LI, X. 2013. SIRT1 and energy metabolism. *Acta Biochim Biophys Sin,* 45**,** 51-60.

LI, X., FANG, P., MAI, J., CHOI, E. T., WANG, H. & YANG, X.-F. 2013. Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. *Journal of Hematology & Oncology,* 6**,** 19.

LI, Y., LIU, L., BARGER, S. W. & GRIFFIN, W. S. T. 2003. Interleukin-1 mediates pathological effects of microglia on tau phosphorylation and on synaptophysin synthesis in cortical neurons through a p38-MAPK pathway. *Journal of Neuroscience,* 23**,** 1605-1611.

LI, X.Y., WANG, S.S., HAN, Z., HAN, F., CHANG, Y.P., YANG, Y., XUE, M., SUN, B. AND CHEN, L.M., 2017. Triptolide restores autophagy to alleviate diabetic renal fibrosis through the miR-141-3p/PTEN/Akt/mTOR pathway. *Molecular Therapy-Nucleic Acids*, 9, pp.48-56.

LIKE, A. A. & ROSSINI, A. A. 1976. Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science,* 193**,** 415-417.

LIN, J., WU, H., TARR, P. T., ZHANG, C.-Y., WU, Z., BOSS, O., MICHAEL, L. F., PUIGSERVER, P., ISOTANI, E. & OLSON, E. N. 2002. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. *Nature,* 418**,** 797.

LIU, X.-H., GENG, Z., YAN, J., LI, T., CHEN, Q., ZHANG, Q.-Y. & CHEN, Z.-Y. 2015. Blocking GSK3β-mediated dynamin1 phosphorylation enhances BDNF-dependent TrkB endocytosis and the protective effects of BDNF in neuronal and mouse models of Alzheimer's disease. *Neurobiology of disease,* 74**,** 377-391.

LOPEZ, Y.O.N., GARUFI, G. AND SEYHAN, A.A., 2017. Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes. *Molecular biosystems,* 13(1), pp.106-121.

LUKIW, W. J., ANDREEVA, T. V., GRIGORENKO, A. P. & ROGAEV, E. I. 2013. Studying micro RNA function and dysfunction in Alzheimer's disease. *Frontiers in genetics,* 3**,** 327.

LUO, X., LI, R. & YAN, L.-J. 2015. Roles of pyruvate, NADH, and mitochondrial complex I in redox balance and imbalance in β cell function and dysfunction. *Journal of diabetes research,* 2015.

LUO, X., WU, J., JING, S. & YAN, L.-J. 2016. Hyperglycaemic stress and carbon stress in diabetic glucotoxicity. *Aging and disease,* 7**,** 90.

LV, W.-S., WEN, J.-P., LI, L., SUN, R.-X., WANG, J., XIAN, Y.-X., CAO, C.-X., WANG, Y.-L. & GAO, Y.-Y. 2012. The effect of metformin on food intake and its potential role in hypothalamic regulation in obese diabetic rats. *Brain research,* 1444**,** 11-19.

MA, J., MENG, X., KANG, S. Y., ZHANG, J., JUNG, H. W. & PARK, Y.-K. 2019. Regulatory effects of the fruit extract of Lycium chinense and its active compound, betaine, on muscle differentiation and mitochondrial biogenesis in C2C12 cells. *Biomedicine & Pharmacotherapy,* 118**,** 109297.

MACISAAC, R. J., JERUMS, G. & EKINCI, E. I. 2017. Effects of glycaemic management on diabetic kidney disease. *World journal of diabetes,* 8**,** 172-186.

MAISONNEUVE, E., DUCRET, A., KHOUEIRY, P., LIGNON, S., LONGHI, S., TALLA, E. & DUKAN, S. 2009. Rules governing selective protein carbonylation. *PloS one,* 4**,** e7269.

MANCUSO, C., SCAPAGINI, G., CURRO, D., GIUFFRIDA STELLA, A. M., DE MARCO, C., BUTTERFIELD, D. A. & CALABRESE, V. 2007. Mitochondrial dysfunction, free radical generation and cellular stress response in neurodegenerative disorders. *Front Biosci,* 12**,** 1107-23.

MARIATHASAN, S., NEWTON, K., MONACK, D. M., VUCIC, D., FRENCH, D. M., LEE, W. P., ROOSE-GIRMA, M., ERICKSON, S. & DIXIT, V. M. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature,* 430**,** 213.

MASI, S., ORLANDI, M., PARKAR, M., BHOWRUTH, D., KINGSTON, I., O'ROURKE, C., VIRDIS, A., HINGORANI, A., HUREL, S. J. & DONOS, N. 2018. Mitochondrial oxidative

stress, endothelial function and metabolic control in patients with type II diabetes and periodontitis: a randomised controlled clinical trial. *International journal of cardiology,* 271**,** 263-268.

MATHER, A. & POLLOCK, C. 2011. Glucose handling by the kidney. *Kidney international,* 79**,** S1-S6.

MATTICK, J. S. & MAKUNIN, I. V. 2006. Non-coding RNA. *Human Molecular Genetics,* 15**,** R17-R29.

MCCREIGHT, L. J., BAILEY, C. J. & PEARSON, E. R. 2016. Metformin and the gastrointestinal tract. *Diabetologia,* 59**,** 426-435.

MESSIER, C. 2005. Impact of impaired glucose tolerance and type 2 diabetes on cognitive aging. *Neurobiology of aging,* 26**,** 26-30.

MEYER, C., DOSTOU, J., NADKARNI, V. & GERICH, J. 1998. Effects of physiological hyperinsulinaemia on systemic, renal, and hepatic substrate metabolism. *American Journal of Physiology-Renal Physiology,* 275**,** F915-F921.

MEYER, C. & GERICH, J. E. 2000. Role of the human kidney in glucose homeostasis. *Current Opinion in Endocrinology, Diabetes and Obesity,* 7**,** 19-24.

MICHAN, S. & SINCLAIR, D. 2007. Sirtuins in mammals: insights into their biological function. *The Biochemical journal,* 404**,** 1.

MIJNHOUT, G., SCHELTENS, P., DIAMANT, M., BIESSELS, G., WESSELS, A., SIMSEK, S., SNOEK, F. & HEINE, R. 2006. Diabetic encephalopathy: a concept in need of a definition. *Diabetologia,* 49**,** 1447.

MINAMISHIMA, Y., TORAMARU, T., SUHARA, T. & YAMAGUCHI, K. 2019. Drug For Preventing Or Treating Lactic Acidosis. *Google Patents*.

MISHRA, P. K., TYAGI, N., KUMAR, M. & TYAGI, S. C. 2009. MicroRNAs as a therapeutic target for cardiovascular diseases. *Journal of cellular and molecular medicine,* 13**,** 778-789.

MITHIEUX, G., VIDAL, H., ZITOUN, C., BRUNI, N., DANIELE, N. & MINASSIAN, C. 1996. Glucose-6-phosphatase mRNA and activity are increased to the same extent in kidney and liver of diabetic rats. *Diabetes,* 45**,** 891-896.

MITRAKOU, A. 2011. Kidney: its impact on glucose homeostasis and hormonal regulation. *Diabetes research and clinical practice,* 93**,** S66-S72.

MORRE, D. M., LENAZ, G. & MORRE, D. J. 2000. Surface oxidase and oxidative stress propagation in aging. *Journal of Experimental Biology,* 203**,** 1513-1521.

MUHONEN, P. & HOLTHOFER, H. 2009. Epigenetic and microRNA-mediated regulation in diabetes. *Oxford University Press.*

MULLARKEY, C. J., EDELSTEIN, D. & BROWNLEE, M. 1990. Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochemical and biophysical research communications,* 173**,** 932-939.

NAGY, C. & EINWALLNER, E. 2018. Study of in vivo glucose metabolism in high-fat diet-fed mice using oral glucose tolerance test (OGTT) and insulin tolerance test (ITT). *JoVE (Journal of Visualized Experiments)*, e56672.

NAJEM, D., BAMJI-MIRZA, M., CHANG, N., LIU, Q. Y. & ZHANG, W. 2014. Insulin resistance, neuroinflammation, and Alzheimer's disease. *Reviews in the Neurosciences,* 25**,** 509-525.

NAKAGAWA, T., TSUCHIDA, A., ITAKURA, Y., NONOMURA, T., ONO, M., HIROTA, F., INOUE, T., NAKAYAMA, C., TAIJI, M. & NOGUCHI, H. 2000. Brain-derived neurotrophic factor regulates glucose metabolism by modulating energy balance in diabetic mice. *Diabetes,* 49**,** 436-444.

NAKAMURA, T., FURUHASHI, M., LI, P., CAO, H., TUNCMAN, G., SONENBERG, N., GORGUN, C.Z. AND HOTAMISLIGIL, G.S., 2010. Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. *Cell*, 140(3), pp.338-348.

NAKATO, R., OHKUBO, Y., KONISHI, A., SHIBATA, M., KANEKO, Y., IWAWAKI, T., NAKAMURA, T., LIPTON, S. A. & UEHARA, T. 2015. Regulation of the unfolded protein response via S-nitrosylation of sensors of endoplasmic reticulum stress. *Scientific reports,* 5**,** 14812.

NELSON, P. T., SMITH, C. D., ABNER, E. A., SCHMITT, F. A., SCHEFF, S. W., DAVIS, G. J., KELLER, J. N., JICHA, G. A., DAVIS, D. & WANG-XIA, W. 2009. Human cerebral neuropathology of Type 2 diabetes mellitus. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease,* 1792**,** 454-469.

NESCA, V., GUAY, C., JACOVETTI, C., MENOUD, V., PEYOT, M.-L., LAYBUTT, D. R., PRENTKI, M. & REGAZZI, R. 2013. Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes. Diabetologia, 56, 2203-2212.

NGO, J. K. & DAVIES, K. J. 2009. Mitochondrial Lon protease is a human stress protein. *Free Radical Biology and Medicine,* 46**,** 1042-1048.

NISHIKAWA, T., BROWNLEE, M. & ARAKI, E. 2015. Mitochondrial reactive oxygen species in the pathogenesis of early diabetic nephropathy. *Journal of diabetes investigation,* 6**,** 137-139.

NISHIKAWA, T., EDELSTEIN, D., DU, X. L., YAMAGISHI, S.-I., MATSUMURA, T., KANEDA, Y., YOREK, M. A., BEEBE, D., OATES, P. J. & HAMMES, H.-P. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature,* 404**,** 787.

NISR, R. B., SHAH, D. S., GANLEY, I. G. & HUNDAL, H. S. 2019. Proinflammatory NFkB signalling promotes mitochondrial dysfunction in skeletal muscle in response to cellular fuel overloading. *Cellular and Molecular Life Sciences*.

NODA, M., MATSUO, T., NAGANO, H., OHTA, M., SEKIGUCHI, M., SHIBOUTA, Y., NAKA, T. & IMURA, Y. 2001. Involvement of angiotensin II in progression of renal injury in

rats with genetic non-insulin-dependent diabetes mellitus (Wistar fatty rats). *The Japanese Journal of Pharmacology,* 85**,** 416-422.

NORTH, B. J. & VERDIN, E. 2004. Sirtuins: Sir2-related NAD-dependent protein deacetylases. *Genome biology,* 5**,** 224.

OATES, P. J. & MYLARI, B. L. 1999. Aldose reductase inhibitors: therapeutic implications for diabetic complications. *Expert opinion on investigational drugs,* 8**,** 2095-2119.

ONO, M., ICHIHARA, J., NONOMURA, T., ITAKURA, Y., TAIJI, M., NAKAYAMA, C. & NOGUCHI, H. 1997. Brain-derived neurotrophic factor reduces blood glucose level in obese diabetic mice but not in normal mice. *Biochemical and biophysical research communications,* 238**,** 633-637.

ORRENIUS, S., GOGVADZE, V. & ZHIVOTOVSKY, B. 2015. Calcium and mitochondria in the regulation of cell death. *Biochemical and biophysical research communications,* 460**,** 72-81.

OYA, T., HATTORI, N., MIZUNO, Y., MIYATA, S., MAEDA, S., OSAWA, T. & UCHIDA, K. 1999. Methylglyoxal Modification of Protein chemical and immunochemical characterization of methylglyoxal-arginine adducts. *Journal of Biological Chemistry,* 274**,** 18492-18502.

PACHER, P., LIAUDET, L., SORIANO, F. G., MABLEY, J. G., SZABÓ, É. & SZABO, C. 2002. The role of poly (ADP-ribose) polymerase activation in the development of myocardial and endothelial dysfunction in diabetes. *Diabetes,* 51**,** 514-521.

PALLEPATI, P. & AVERILL-BATES, D. 2012. Reactive oxygen species, cell death signaling and apoptosis. *Princ Free Rad Biomed,* 2**,** 513-546.

PALM, F., ORTSÄTER, H., HANSELL, P., LISS, P. & CARLSSON, P. O. 2004. Differentiating between effects of streptozotocin per se and subsequent hyperglycaemia on renal function and metabolism in the streptozotocin-diabetic rat model. *Diabetes/metabolism research and reviews,* 20**,** 452-459.

PANDEY, A. K., AGARWAL, P., KAUR, K. & DATTA, M. 2009. MicroRNAs in diabetes: tiny players in big disease. *Cellular Physiology and Biochemistry,* 23**,** 221-232.

PARAMITHIOTIS, E., PRENTKI, M., RABASA-LHORET, R., CROTEAU, P., LANOIX, J., MADIRAJU, S. M. & JOLY, È. 2019. Type 2 diabetes biomarkers and uses thereof. *Google Patents.*

PASQUINELLI, A. E. 2012. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nature Reviews Genetics,* 13**,** 271.

PEARSON-LEARY, J. & MCNAY, E. C. 2016. Novel Roles for the Insulin-Regulated Glucose Transporter-4 in Hippocampally Dependent Memory. *The Journal of Neuroscience,* 36**,** 11851.

PENTIKÄINEN, P., NEUVONEN, P. & PENTTILÄ, A. 1979. Pharmacokinetics of metformin after intravenous and oral administration to man. *European journal of clinical pharmacology,* 16**,** 195-202.

PETERSEN, M.C. AND SHULMAN, G.I., 2018. Mechanisms of insulin action and insulin resistance. *Physiological reviews,* 98(4), pp.2133-2223.

PIETROPAOLO, M., TOWNS, R. & EISENBARTH, G. S. 2012. Humoral autoimmunity in type 1 diabetes: prediction, significance, and detection of distinct disease subtypes. *Cold Spring Harbor perspectives in medicine,* 2**,** a012831.

PINTI, M., GIBELLINI, L., NASI, M., DE BIASI, S., BORTOLOTTI, C. A., IANNONE, A. & COSSARIZZA, A. 2016. Emerging role of Lon protease as a master regulator of mitochondrial functions. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1857**,** 1300-1306.

PLUM, L., BELGARDT, B. F. & BRÜNING, J. C. 2006. Central insulin action in energy and glucose homeostasis. *The Journal of clinical investigation,* 116**,** 1761-1766.

POLLA, B., KANTENGWA, S., FRANCOIS, D., SALVIOLI, S., FRANCESCHI, C., MARSAC, C. & COSSARIZZA, A. 1996. Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proceedings of the national academy of sciences,* 93**,** 6458-6463.

POZZILLI, P., DAVID LESLIE, R., CHAN, J., DE FRONZO, R., MONNIER, L., RAZ, I. & DEL PRATO, S. 2010. The A1C and ABCD of glycaemia management in type 2 diabetes: a physician's personalized approach. *Diabetes/metabolism research and reviews,* 26**,** 239-244.

QU, Z., JIAO, Z., SUN, X., ZHAO, Y., REN, J. & XU, G. 2011. Effects of streptozotocin-induced diabetes on tau phosphorylation in the rat brain. *Brain research,* 1383**,** 300-306.

RABINOVITCH, R. C., SAMBORSKA, B., FAUBERT, B., MA, E. H., GRAVEL, S.-P., ANDRZEJEWSKI, S., RAISSI, T. C., PAUSE, A., PIERRE, J. S.-. & JONES, R. G. 2017. AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species. *Cell reports,* 21**,** 1-9.

RAINA, M. & IBBA, M. 2014. tRNAs as regulators of biological processes. *Frontiers in genetics,* 5**,** 171.

RAINS, J. L. & JAIN, S. K. 2011. Oxidative stress, insulin signaling, and diabetes. *Free radical biology & medicine,* 50**,** 567-575.

REILLY, P., WINSTON, C. N., BARON, K. R., TREJO, M., ROCKENSTEIN, E. M., AKERS, J. C., KFOURY, N., DIAMOND, M., MASLIAH, E. & RISSMAN, R. A. 2017. Novel human neuronal tau model exhibiting neurofibrillary tangles and transcellular propagation. *Neurobiology of disease,* 106**,** 222-234.

REZNICK, R. M. & SHULMAN, G. I. 2006. The role of AMP-activated protein kinase in mitochondrial biogenesis. *The Journal of physiology,* 574**,** 33-39.

ROBBINS, G. R., WEN, H. & TING, J. P.-Y. 2014. Inflammasomes and metabolic disorders: old genes in modern diseases. *Molecular cell,* 54**,** 297-308.

RODGERS, J. T., LERIN, C., HAAS, W., GYGI, S. P., SPIEGELMAN, B. M. & PUIGSERVER, P. 2005. Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature,* 434**,** 113.

ROUSSEL, R., TRAVERT, F., PASQUET, B., WILSON, P. W., SMITH, S. C., GOTO, S., RAVAUD, P., MARRE, M., PORATH, A. & BHATT, D. L. 2010. Metformin use and mortality

among patients with diabetes and atherothrombosis. *Archives of internal medicine,* 170**,** 1892-1899.

ROVIRA-LLOPIS, S., APOSTOLOVA, N., BANULS, C., MUNTANE, J., ROCHA, M. & VICTOR, V. M. 2018. Mitochondria, the NLRP3 inflammasome, and sirtuins in type 2 diabetes: new therapeutic targets. *Antioxidants & redox signaling,* 29**,** 749-791.

ROWE, I., CHIARAVALLI, M., MANNELLA, V., ULISSE, V., QUILICI, G., PEMA, M., SONG, X. W., XU, H., MARI, S. & QIAN, F. 2013. Defective glucose metabolism in polycystic kidney disease identifies a new therapeutic strategy. *Nature medicine,* 19**,** 488.

RUFINI, A., TUCCI, P., CELARDO, I. & MELINO, G. 2013. Senescence and aging: the critical roles of p53. *Oncogene,* 32**,** 5129.

SAKAI, N., WADA, T., FURUICHI, K., IWATA, Y., YOSHIMOTO, K., KITAGAWA, K., KOKUBO, S., KOBAYASHI, M., HARA, A. & YAMAHANA, J. 2005. Involvement of extracellular signal-regulated kinase and p38 in human diabetic nephropathy. *American journal of kidney diseases,* 45**,** 54-65.

SALMINEN, A., KAUPPINEN, A., SUURONEN, T. & KAARNIRANTA, K. 2008. SIRT1 longevity factor suppresses NF-κB-driven immune responses: regulation of aging via NF-κB acetylation? *Bioessays,* 30**,** 939-942.

SANDIREDDY, R., YERRA, V. G., KOMIRISHETTI, P., ARETI, A. & KUMAR, A. 2016. Fisetin imparts neuroprotection in experimental diabetic neuropathy by modulating Nrf2 and NF-κB pathways. *Cellular and molecular neurobiology,* 36**,** 883-892.

SANDLER, S. & SWENNE, I. 1983. Streptozotocin, but not alloxan, induces DNA repair synthesis in mouse pancreatic islets in vitro. *Diabetologia,* 25**,** 444-447.

SANGODKAR, J., FARRINGTON, C. C., MCCLINCH, K., GALSKY, M. D., KASTRINSKY, D. B. & NARLA, G. 2016. All roads lead to PP2A: exploiting the therapeutic potential of this phosphatase. *The FEBS journal,* 283**,** 1004-1024.

SATO, K., TATSUNAMI, R., YAMA, K. & TAMPO, Y. 2013. Glycolaldehyde induces cytotoxicity and increases glutathione and multidrug-resistance-associated protein levels in Schwann cells. *Biological and Pharmaceutical Bulletin,* 36**,** 1111-1117.

SCHÄFER, G. 1983. Biguanides. a review of history, pharmacodynamics and therapy. *Diabete & metabolisme,* 9**,** 148-163.

SCHEEN, A. J. 1996. Clinical pharmacokinetics of metformin. *Clinical pharmacokinetics,* 30**,** 359-371.

SCHRAUWEN, P. & HESSELINK, M. K. 2004. Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. *Diabetes,* 53**,** 1412-1417.

SELVARAJU, V., JOSHI, M., SURESH, S., SANCHEZ, J. A., MAULIK, N. & MAULIK, G. 2012. Diabetes, oxidative stress, molecular mechanism, and cardiovascular disease – an overview. *Toxicology Mechanisms and Methods,* 22**,** 330-335.

SEMPERE, L. F., FREEMANTLE, S., PITHA-ROWE, I., MOSS, E., DMITROVSKY, E. & AMBROS, V. 2004. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome biology,* 5**,** R13.

SENFTLEBEN, U., CAO, Y., XIAO, G., GRETEN, F. R., KRÄHN, G., BONIZZI, G., CHEN, Y., HU, Y., FONG, A. & SUN, S.-C. 2001. Activation by IKKα of a second, evolutionary conserved, NF-κB signaling pathway. *Science,* 293**,** 1495-1499.

SERGEANT, N., BRETTEVILLE, A., HAMDANE, M., CAILLET-BOUDIN, M.-L., GROGNET, P., BOMBOIS, S., BLUM, D., DELACOURTE, A., PASQUIER, F. & VANMECHELEN, E. 2008. Biochemistry of Tau in Alzheimer's disease and related neurological disorders. *Expert review of proteomics,* 5**,** 207-224.

SHAH, S., IQBAL, M., KARAM, J., SALIFU, M. & MCFARLANE, S. I. 2007. Oxidative stress, glucose metabolism, and the prevention of type 2 diabetes: pathophysiological insights. *Antioxidants & redox signaling,* 9**,** 911-929.

SHAKHER, J. & STEVENS, M. J. 2011. Update on the management of diabetic polyneuropathies. *Diabetes, metabolic syndrome and obesity: targets and therapy,* 4**,** 289.

SHAO, B.-Z., XU, Z.-Q., HAN, B.-Z., SU, D.-F. & LIU, C. 2015. NLRP3 inflammasome and its inhibitors: a review. *Frontiers in pharmacology,* 6**,** 262.

SHARMA, M., GUPTA, S., SINGH, K., MEHNDIRATTA, M., GAUTAM, A., KALRA, O. P., SHUKLA, R. & GAMBHIR, J. K. 2016. Association of glutathione-S-transferase with patients of type 2 diabetes mellitus with and without nephropathy. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews,* 10**,** 194-197.

SHAW, R. J., LAMIA, K. A., VASQUEZ, D., KOO, S.-H., BARDEESY, N., DEPINHO, R. A., MONTMINY, M. & CANTLEY, L. C. 2005. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science,* 310**,** 1642-1646.

SHIMADA, K., CROTHER, T. R., KARLIN, J., DAGVADORJ, J., CHIBA, N., CHEN, S., RAMANUJAN, V. K., WOLF, A. J., VERGNES, L. & OJCIUS, D. M. 2012. Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity,* 36**,** 401-414.

SIMMONS, K. M. & MICHELS, A. W. 2015. Type 1 diabetes: A predictable disease. *World journal of diabetes,* 6**,** 380.

SIMPSON, I. A., CARRUTHERS, A. & VANNUCCI, S. J. 2007. Supply and Demand in Cerebral Energy Metabolism: The Role of Nutrient Transporters. *Journal of Cerebral Blood Flow & Metabolism,* 27**,** 1766-1791.

SINK, K. M., DIVERS, J., WHITLOW, C. T., PALMER, N. D., SMITH, S. C., XU, J., HUGENSCHMIDT, C. E., WAGNER, B. C., WILLIAMSON, J. D. & BOWDEN, D. W. 2015. Cerebral structural changes in diabetic kidney disease: African American–Diabetes Heart Study MIND. *Diabetes care,* 38**,** 206-212.

SIVITZ, W. I. & YOREK, M. A. 2010. Mitochondrial dysfunction in diabetes: from molecular mechanisms to functional significance and therapeutic opportunities. *Antioxidants & redox signaling,* 12**,** 537-577.

SKOVSO, S. 2014. Modeling type 2 diabetes in rats using high fat diet and streptozotocin. *J Diabet Investig* 5 (4): 349–358.

SONG, B., SCHEUNER, D., RON, D., PENNATHUR, S. & KAUFMAN, R. J. 2008. Chop deletion reduces oxidative stress, improves β cell function, and promotes cell survival in multiple mouse models of diabetes. *The Journal of clinical investigation,* 118**,** 3378-3389.

SONG, J., YANG, X. & YAN, L.-J. 2019. Role of pseudohypoxia in the pathogenesis of type 2 diabetes. *Hypoxia,* 7**,** 33.

SONG, Y., DING, W., BEI, Y., XIAO, Y., TONG, H.-D., WANG, L.-B. & AI, L.-Y. 2018. Insulin is a potential antioxidant for diabetes-associated cognitive decline via regulating Nrf2 dependent antioxidant enzymes. *Biomedicine & Pharmacotherapy,* 104**,** 474-484.

SRINIVASAN, K. & RAMARAO, P. 2007. Animal model in type 2 diabetes research: An overview. *Indian Journal of Medical Research,* 125**,** 451.

SRINIVASAN, S., OHSUGI, M., LIU, Z., FATRAI, S., BERNAL-MIZRACHI, E. & PERMUTT, M. A. 2005. Endoplasmic reticulum stress–induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3β in mouse insulinoma cells. *Diabetes,* 54**,** 968-975.

STEPHENNE, X., FORETZ, M., TALEUX, N., VAN DER ZON, G., SOKAL, E., HUE, L., VIOLLET, B. & GUIGAS, B. 2011. Metformin activates AMP-activated protein kinase in primary human hepatocytes by decreasing cellular energy status. *Diabetologia,* 54**,** 3101-3110.

STROWIG, T., HENAO-MEJIA, J., ELINAV, E. & FLAVELL, R. 2012. Inflammasomes in health and disease. *Nature,* 481**,** 278.

STRUM, J.C., JOHNSON, J.H., WARD, J., XIE, H., FEILD, J., HESTER, A., ALFORD, A. AND WATERS, K.M., 2009. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. *Molecular endocrinology,* 23(11), pp.1876-1884.

STUMVOLL, M., GOLDSTEIN, B. J. & VAN HAEFTEN, T. W. 2005. Type 2 diabetes: principles of pathogenesis and therapy. *The Lancet,* 365**,** 1333-1346.

SULAIMAN, M. K. 2019. Diabetic nephropathy: recent advances in pathophysiology and challenges in dietary management. *Diabetology & metabolic syndrome,* 11**,** 7-7.

SUMNER, C., SHETH, S., GRIFFIN, J., CORNBLATH, D. & POLYDEFKIS, M. 2003. The spectrum of neuropathy in diabetes and impaired glucose tolerance. *Neurology,* 60**,** 108-111.

SZKUDELSKI, T. 2001. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiological research,* 50**,** 537-546.

SZKUDELSKI, T. 2012. Streptozotocin–nicotinamide-induced diabetes in the rat. Characteristics of the experimental model. *Experimental biology and medicine,* 237**,** 481-490.

TAKASHIMA, M., OGAWA, W., HAYASHI, K., INOUE, H., KINOSHITA, S., OKAMOTO, Y., SAKAUE, H., WATAOKA, Y., EMI, A. & SENGA, Y. 2010. Role of KLF15 in regulation of hepatic gluconeogenesis and metformin action. *Diabetes,* 59**,** 1608-1615.

TAKIYAMA, Y. & HANEDA, M. 2014. Hypoxia in diabetic kidneys. *BioMed research international,* 2014.

THOMAS, N. J., JONES, S. E., WEEDON, M. N., SHIELDS, B. M., ORAM, R. A. & HATTERSLEY, A. T. 2018. Frequency and phenotype of type 1 diabetes in the first six decades of life: a cross-sectional, genetically stratified survival analysis from UK Biobank. *The lancet Diabetes & endocrinology,* 6**,** 122-129.

TODD, J. N. & FLOREZ, J. C. 2014. An update on the pharmacogenomics of metformin: progress, problems and potential. *Pharmacogenomics,* 15**,** 529-539.

TOĞAY, V. A., SEVIMLI, T. S., SEVIMLI, M., ÇELIK, D. A. & ÖZÇELIK, N. 2018. DNA damage in rats with streptozotocin-induced diabetes; protective effect of silibinin. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 825**,** 15-18.

TUCKER, G., CASEY, C., PHILLIPS, P., CONNOR, H., WARD, J. & WOODS, H. 1981. Metformin kinetics in healthy subjects and in patients with diabetes mellitus. *British journal of clinical pharmacology,* 12**,** 235-246.

VANDANMAGSAR, B., YOUM, Y.-H., RAVUSSIN, A., GALGANI, J. E., STADLER, K., MYNATT, R. L., RAVUSSIN, E., STEPHENS, J. M. & DIXIT, V. D. 2011. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nature medicine,* 17**,** 179.

VASCONCELOS-DOS-SANTOS, A., LOPONTE, H., MANTUANO, N., OLIVEIRA, I., DE PAULA, I., TEIXEIRA, L., DE-FREITAS-JUNIOR, J., GONDIM, K., HEISE, N. & MOHANA-BORGES, R. 2017. Hyperglycaemia exacerbates colon cancer malignancy through hexosamine biosynthetic pathway. *Oncogenesis,* 6**,** e306.

VIOLLET, B., GUIGAS, B., GARCIA, N. S., LECLERC, J., FORETZ, M. & ANDREELLI, F. 2011. Cellular and molecular mechanisms of metformin: an overview. *Clinical science,* 122**,** 253-270.

VOSSEL, K. A., ZHANG, K., BRODBECK, J., DAUB, A. C., SHARMA, P., FINKBEINER, S., CUI, B. & MUCKE, L. 2010. Tau reduction prevents Aβ-induced defects in axonal transport. *Science,* 330**,** 198-198.

WADHWA, R., TAKANO, S., KAUR, K., AIDA, S., YAGUCHI, T., KAUL, Z., HIRANO, T., TAIRA, K. & KAUL, S. C. 2005. Identification and characterization of molecular interactions between mortalin/mtHsp70 and HSP60. *Biochemical Journal,* 391**,** 185.

WAKINO, S., HASEGAWA, K. & ITOH, H. 2015. Sirtuin and metabolic kidney disease. *Kidney international,* 88**,** 691-698.

WALLACE, D. C. 1992. Diseases of the mitochondrial DNA. *Annual review of biochemistry,* 61**,** 1175-1212.

WANG, Z.-H., XIANG, J., LIU, X., YU, S. P., MANFREDSSON, F. P., SANDOVAL, I. M., WU, S., WANG, J.-Z. & YE, K. 2019. Deficiency in BDNF/TrkB Neurotrophic Activity Stimulates δ-Secretase by Upregulating C/EBPβ in Alzheimer's Disease. *Cell reports,* 28**,** 655-669. e5.

WANG, Z. & GLEICHMANN, H. 1998. GLUT2 in pancreatic islets: crucial target molecule in diabetes induced with multiple low doses of streptozotocin in mice. *Diabetes,* 47**,** 50-56.

WEBER, G., LEA, M. A., CONVERY, H. J. H. & STAMM, N. B. 1967. Regulation of gluconeogenesis and glycolysis: studies of mechanisms controlling enzyme activity. *Advances in enzyme regulation,* 5**,** 257-298.

WILCOCK, C. & BAILEY, C. 1994. Accumulation of metformin by tissues of the normal and diabetic mouse. *Xenobiotica,* 24**,** 49-57.

WILLIAMSON, J. R., CHANG, K., FRANGOS, M., HASAN, K. S., IDO, Y., KAWAMURA, T., NYENGAARD, J. R., VAN DEN ENDEN, M., KILO, C. & TILTON, R. G. 1993. Hyperglycaemic pseudohypoxia and diabetic complications. *Diabetes,* 42**,** 801-813.

WOLFF, S. P. & DEAN, R. 1987. Glucose autoxidation and protein modification. The potential role of'autoxidative glycosylation'in diabetes. *Biochemical journal,* 245**,** 243.

WRIGHT, E., HIRAYAMA, B. & LOO, D. 2007. Active sugar transport in health and disease. *Journal of internal medicine,* 261**,** 32-43.

WRIGHT, E. M. 2001. Renal Na+-glucose cotransporters. *American Journal of Physiology-Renal Physiology,* 280**,** F10-F18.

XOURGIA, E., PAPAZAFIROPOULOU, A. & MELIDONIS, A. 2019. Antidiabetic treatment on memory and spatial learning: From the pancreas to the neuron. *World journal of diabetes,* 10**,** 169.

YAMAGISHI, S.-I., MAEDA, S., MATSUI, T., UEDA, S., FUKAMI, K. & OKUDA, S. 2012. Role of advanced glycation end products (AGEs) and oxidative stress in vascular complications in diabetes. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1820**,** 663-671.

YAMAMOTO, Y., DOI, T., KATO, I., SHINOHARA, H., SAKURAI, S., YONEKURA, H., WATANABE, T., MYINT, K. M., HARASHIMA, A. & TAKEUCHI, M. 2005. Receptor for advanced glycation end products is a promising target of diabetic nephropathy. *Annals of the New York Academy of Sciences,* 1043**,** 562-566.

YAN, L.-J. 2014. Pathogenesis of chronic hyperglycaemia: from reductive stress to oxidative stress. *Journal of diabetes research,* 2014.

YAN, L. J. 2018. Redox imbalance stress in diabetes mellitus: Role of the polyol pathway. *Animal models and experimental medicine,* 1**,** 7-13.

YARIBEYGI, H., FARROKHI, F. R., BUTLER, A. E. & SAHEBKAR, A. 2019. Insulin resistance: Review of the underlying molecular mechanisms. *Journal of cel lular physiology,* 234**,** 8152-8161.

YIN, M.-J., YAMAMOTO, Y. & GAYNOR, R. B. 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature,* 396**,** 77.

YUAN, M., KONSTANTOPOULOS, N., LEE, J., HANSEN, L., LI, Z.-W., KARIN, M. & SHOELSON, S. E. 2001. Reversal of obesity-and diet-induced insulin resistance with salicylates or targeted disruption of Ikkβ. *Science,* 293**,** 1673-1677.

ZACCARDI, F., WEBB, D. R., YATES, T. & DAVIES, M. J. 2016. Pathophysiology of type 1 and type 2 diabetes mellitus: a 90-year perspective. *Postgraduate medical journal,* 92**,** 63-69.

ZAKHARY, S. M., AYUBCHA, D., DILEO, J. N., JOSE, R., LEHESTE, J. R., HOROWITZ, J. M. & TORRES, G. 2010. Distribution analysis of deacetylase SIRT1 in rodent and human nervous systems. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology,* 293**,** 1024-1032.

ZAMPETAKI, A., KIECHL, S., DROZDOV, I., WILLEIT, P., MAYR, U., PROKOPI, M., MAYR, A., WEGER, S., OBERHOLLENZER, F. & BONORA, E. 2010. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circulation research,* 107**,** 810-817.

ZHANG, X., DAMACHARLA, D., MA, D., QI, Y., TAGETT, R., DRAGHICI, S., KOWLURU, A. & YI, Z. 2016. Quantitative proteomics reveals novel protein interaction partners of PP2A catalytic subunit in pancreatic β-cells. *Molecular and cellular endocrinology,* 424**,** 1-11.

ZHONG, F., XIE, J., ZHANG, D., HAN, Y. & WANG, C. 2015. Polypeptide from Chlamys farreri suppresses ultraviolet-B irradiation-induced apoptosis through restoring ER redox homeostasis, scavenging ROS generation, and suppressing the PERK-eIF2a-CHOP pathway in HaCaT cells. *Journal of Photochemistry and Photobiology B: Biology,* 151**,** 10-16.

ZHOU, G., MYERS, R., LI, Y., CHEN, Y., SHEN, X., FENYK-MELODY, J., WU, M., VENTRE, J., DOEBBER, T. & FUJII, N. 2001. Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of clinical investigation,* 108**,** 1167-1174.

ZHOU, W. AND YE, S., 2018. Rapamycin improves insulin resistance and hepatic steatosis in type 2 diabetes rats through activation of autophagy. *Cell biology international,* 42(10), pp.1282-1291.

CHAPTER 3

The protective effect of Metformin on mitochondrial dysfunction and endoplasmic reticulum stress in diabetic mice brain

The pleiotropic effects of MF extend further than glycaemic control. A vast number of studies focus on glucose metabolism and related insulin signalling pathways in organs like the liver and pancreas. However, the mechanism of MF in the brain under hyperglycaemia is a neglected area. To fill the gaps, we assessed the interrelated mechanisms across metabolic pathways of MF's potential protective effects in diabetic mice brain. This paper integrates MFs effect on oxidative stress and mt biogenesis markers through epigenetic regulation in brain tissue from STZ-injected mice.

This manuscript has been accepted in *European Journal of Pharmacology* (Manuscript reference: EJP-52205) and is presented in this thesis as per journal formatting requirements.

The protective effect of Metformin on mitochondrial dysfunction and endoplasmic reticulum stress in diabetic mice brain

Taskeen Fathima Docrat, Savania Nagiah, Nikita Naicker, Sooraj Baijnath, Sanil Singh, Anil A. Chuturgoon

Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, South Africa

*Address correspondence to: Prof Anil Chuturgoon, Discipline of Medical Biochemistry, Faculty of Health Sciences, George Campbell Building, Howard College, University of KwaZulu-Natal, Durban, 4041, South Africa. Telephone: (031) 260 4404. Email: chutur@ukzn.ac.za

Abstract

Background and objectives: Diabetes is characterised by decreased insulin production (Type 1) and insulin resistance (Type 2) and is associated with mitochondrial (mt) dysfunction and oxidative stress. Insulin targets the brain, thus understanding diabetic complications such as neurological disorders is of prime interest. MicroRNAs control complex gene regulatory networks and have nascent roles in mt maintenance through transcriptional repression within the brain. Therefore, we investigated the potential protective effects of metformin (MF) on miR-132 and miR-148a, and their targets in STZ-induced diabetic mice brain.

Materials and Methods: Streptozotocin (STZ)-induced diabetic mice were treated with MF (20 mg/kg BW), and whole brain tissue was harvested for further analysis. Protein carbonylation was measured as a marker of neuronal oxidative stress. Protein expression of mt chaperones, maintenance proteins, and regulators of the unfolded protein response (UPR) were measured by western blot. Transcript levels of antioxidant enzyme *GSTA4*; mt biogenesis markers, ER stress regulators, and miR-132 and miR-148a were analysed using qPCR.

Results: The results showed that MF efficiently reduced protein carbonylation and oxidation through up-regulation of mt chaperone proteins (HSP60, HSP70 and LonP1). MF elicits the UPR to attenuate ER stress through a miR-132 repression mechanism. Additionally, MF was found to elevate deacetylases- Sirt1, Sirt3; and mt biogenesis marker PGC-1α through miR-148a repression.

Conclusions: This is the first study to demonstrate the epigenetic regulation of mt maintenance by MF in diabetic C57BL/6 mouse whole brain tissue. We thus conclude that MF, beyond its anti-hyperglycaemic role, can improve epigenomic neurodegenerative alterations in diabetes.

KEYWORDS: Diabetes, Neurodegeneration, Reactive oxygen species (ROS), Mitochondrial dysfunction, Unfolded protein response (UPR), Metformin

1. Introduction

Metformin (MF) is used for the treatment of type 2 diabetes mellitus (T2DM) and unlike other guanidine derivates has thrived therapeutically due to its superior safety profile. Its use has extended further to treat disorders including diabetic nephropathy, polycystic ovary disease, various cancers, gestational diabetes, as well as cardiovascular disease (Viollet et al., 2012). The pleiotropic effects of MF are attributed to transient mt respiratory chain inhibition of Complex I, and activation of AMP-activated protein kinase (AMPK) (Viollet et al., 2012).

Insulin action promotes optimal brain metabolism and is apposite to mt function. The brain is high in lipid content making it susceptible to oxidative damage. Several researchers have investigated the association of impaired antioxidant responses as well as loss of mt maintenance in the diabetic brain, contributing to the accumulation of damaged proteins and neuronal toxicity (Abdul et al., 2006, McCall, 1992, Roriz-Filho et al., 2009).

Mitochondria are energy generators that facilitate brain metabolism. In a diabetic state, redox imbalances exacerbate reactive oxygen species (ROS) production (Wu et al., 2016). Several stress response mechanisms including transcriptional activation of co-transcription factor, peroxisome-proliferator-activated receptor gamma coactivator-1α (PGC-1α) and related antioxidant enzymes are at the crossroads of diabetes and loss of mitochondrial dysfunction. PGC-1α together with sirtuin 1 (Sirt1), and sirtuin 3 (Sirt3) form an energy sensing network to maintain glucose homeostasis and mitochondrial density (Rodgers et al., 2005, Przemyslaw et al., 2009). Molecular chaperones such as Lon Protease (LonP1) through interaction with the heat shock proteins (HSPs): HSP60-mtHSP70 complex (Bota and Davies, 2016), is involved in cellular protein quality control. Their neuroprotective role has recently been outlined (Kim et al., 2017, Leak, 2014, Ngo et al., 2013).

STZ is a common inducer of experimental diabetes in mice through GLUT-2 transportation and is toxic to the beta cells of the pancreas (Kamat, 2015). STZ-induced diabetes leads to endoplasmic reticulum (ER) stress (Lind et al., 2013), triggering the UPR sensor. This response stimulates PKR (double-stranded RNA-dependent protein kinase)-like ER kinase (PERK) activation of eukaryotic initiation factor-1α (eIF2α), shutting down global protein synthesis. Numerous studies provide a link between chronic ER stress and neurodegeneration (De Felice and Ferreira, 2017, Wang and Kaufman, 2016, Zou et al., 2017).

MicroRNAs (miRNAs) belong to a class of single-stranded non-coding RNA that modulates gene expression through post-transcriptional binding of mature miRNAs to the 3′-UTR of target mRNAs. Initially, miR-132 was known to regulate neuronal function and survival (Miyazaki et al., 2014), however its upregulation has been shown in various diabetic animal models (Nesca et al., 2013, Tattikota et al., 2014). Additionally, MiR-148a was shown to be upregulated in serum of T1DM patients (Assmann et al., 2017) and possesses the potential to be a circulatory biomarker. However, the nascent roles of these miRNAs in STZ-induced neurodegeneration is unknown. Here, we investigated how STZ-induced diabetic model, and MF administration affect oxidative status, mt maintenance, and the UPR through expression of miR-132 and -148a in the mouse brain.

2. Methods and Materials

2.1 Materials

Streptozotocin (STZ) (S0130) and Metformin hydrochloride (PHR1084) were purchased from Sigma Aldrich (St Louis, MO, USA). All other consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

2.2 Animals and induction of diabetes

Animal Experiments were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (Reference AREC/057/016). Mice used in these experiments were acquired from the Biomedical Resource Unit from the University of KwaZulu-Natal (Westville Campus), Durban, South Africa. Male mice of the C57BL/6 strain at 6-weeks-old [(n=15, mean body weight (BW) 20 ± 2.99g)]. Mouse feed included a standard laboratory diet, normal drinking water *ad libitum* throughout the experimental period.

A preliminary investigation including a range of STZ concentrations (50 mg/kg, 100 mg/kg and 150 mg/kg BW) was performed to establish the optimal dosage. For this study, following an overnight fast (12 hr), STZ was administered three times intraperitoneally [(50 mg/kg BW, dissolved in 0.1M citrate buffer (pH 4.4)] on day 0, 3, and 10 to induce T2DM. The control group received citrate buffer solution without STZ (vehicle control). Blood glucose levels were monitored using a glucometer (Accu-Chek®). Mice with blood sugar levels of 7-16mmol/L were considered T2 diabetic, and the treatment period was inducted thereafter.

2.3 Treatment preparations

The dose of metformin administered to mice in this study was calculated according to clinically relevant human dose based on body surface area. MF was made up in 0.1M phosphate-buffered saline (PBS) and filter sterilized (0.45-μm filter), to make a final concentration of 20 mg/kg BW based on previous animal studies (Cho et al., 2015, Zou et al., 2004b). Mice were randomly divided into 3 groups of 5 mice per group (n=5): Group 1 are control (C) normal mice. Group 2 are Streptozotocin (STZ) induced T2 diabetic mice (HG control) and were fed PBS (vehicle

control) during the treatment period. However, the mice in group 3 were diabetic and treated with MF (20 mg/kg BW) via oral gavage once daily for the 15-day treatment period. Mice were housed in polycarbonated cages (40-60% humidity, 23 ± 1°C) with a 12 hr light dark cycle. On the last day mice were sacrificed, and whole brain tissue was used for ex vivo studies.

2.4 Oral glucose tolerance test (OGTT)

The OGGT was performed on the last day (day 25) of the experimental period. Following an overnight fast (12 hr), mice in control and STZ groups were orally dosed with a D-glucose solution (2.0g/kg BW). Thereafter, blood glucose concentrations, and changes in body weight of each group were subsequently measured.

2.5 Tissue collection

At the end of the treatment period, the mice were sacrificed using isoflurane. Fasting plasma samples were measured from mice tail-veins. The blood glucose levels were measured at an accredited pathology laboratory (AMPATH, Amanzimtoti, Durban, South Africa). Whole brain tissue was harvested, rinsed twice in saline, dissected and then stored in Cytobuster (Novagen, Darmstadt, Germany) or Qiazol (Qiagen; Hildenburg, Germany) at -80°C for downstream protein and mRNA analysis respectively.

2.6 Protein Carbonyl Assay

The protein carbonyl levels were determined by reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) according to a protocol described by Levine *et al* (Levine et al., 1994). All samples consisted of a blank prepared by treatment with 2.5M HCl. Each mouse sample was plated in triplicate (100µl/well) and the carbonyl content was calculated from the maximum absorbance (370nm) (Augustyniak et al., 2015).

2.7 Mitochondrial maintenance and chaperone protein expression

Western blotting was performed using an in house protocol (Raghubeer et al., 2015). Crude protein samples obtained post homogenisation were quantified by the Bicinchoninic acid (BCA) assay and standardized to 1 mg/ml. Protein expression was determined by incubating membranes with the following primary antibodies (1:1000, 5% BSA): Heat Shock Protein 60 (HSP60) (BD 611563), HSP70 (BD610618), LonP1 (ab76487), PGC-1α (3G6), SIRT1 (#2314L), SIRT3 (ab86671), TFAM (D5C8), p-PERK (THr980) (16F8), p-eIF2α (D9G8), and eIF2α (D7D3). This was followed with incubation of horseradish peroxidase (HRP) conjugated secondary antibody [Anti rabbit IgG #7074, Anti-mouse IgG #7076, 1:10,000 in 5% BSA] (RT, 1h). Protein bands were obtained through chemiluminescent detection [Clarity western ECL substrate (Bio-Rad)] and images were captured on the Chemidoc™ imaging system (Bio-Rad). Protein expression was determined using the Image Lab Software version 5.0 (Bio-Rad). Densitometric protein measurements were normalised against house-keeping protein, HRP-conjugated anti-β-actin

(CS1615, Sigma) for 1h at RT. Results are reflected as a ratio of relative fold change (RFC) of proteins of interest over β-actin.

2.8 Messenger RNA quantification

Following homogenisation of brain tissue in Qiazol reagent (232 Qiagen, Germany), total RNA was quantified (Nano-Drop 2000) and standardised (900 ng/µl). Subsequent complementary DNA (cDNA) synthesis was performed using the iScript™ cDNA synthesis kit (Bio-Rad, SA, cat. no. 1708891). Gene expression was determined using the iScript SYBR Green PCR kit (Bio-Rad), according to the manufacturer's instructions.

Table 1: Primer sequences used to determine gene expression profiles

Gene	**Primer sequence:** (5'-3')	**Annealing temperature (^{o} C)**
GSTA4	**F:** TACCTCGCTGCCAAGTACAAC	59
	R: GAGCCACGGCAATCATCATCA	
SIRT1	**F:** CAGCCGTCTCTGTGTCACAAA	61
	R: GCACCGAGGAACTACCTGAT	
SIRT3	**F:** TACAGGCCCAATGTCACTCA	58.5
	R: ACAGACCGTGCATGTAGCTG	
PGC-1α	**F:** GCAACATGCTCAAGCCAAAC	56.2
	R: TGCAGTTCCAGAGAGTTCCA	
PERK	**F:** GCACTTTAGATGGACGAATCGC	59
	R: TGCTGAGGCTAGATGAAACCA	
eIF2α	**F**: AAACTGGAGCATGTTTGAAATCG	59
	R: GGGCACCTTTACTTCCTGGG	
GAPDH	**F:** ATGTGTCCGTCGTGGATCTGAC	Variable
	R: AGACAACCTGGTCCTCAGTGTAG	

2.9 MicroRNA quantification and target prediction

The cDNA for miR-132 (MS00024143), -148a (MS00011193) was prepared by using a 10µl reaction mix of the miScript II RT kit [cat. No. 218161 (5 × miScript HiSpec Buffer, miScript Reverse Transcriptase Mix 10 × miScript Nucleics Mix; Qiagen)] as per manufacturer's instructions. Data normalisation was performed against human RNA U6 small nuclear 2 (RNU6-2) and was analysed using the method described by (Livak and Schmittgen, 2001). The targets of miR -132, and miR-148a were determined by pathway analysis using Target Scan V7.2. This database was utilised as no experimental data for these miRNAs in mouse brain exists. The results were filtered for targets with highly predicted confidence for an interaction with miR-132 and miR-148a. Among the multiple targets for both miRNAs, we determined the 3' UTR of *HSP70-12B* was complimentary to the seed sequence of miR-132; and the 3' UTR of *PGC-1α* to miR-148a.

2.10 Statistical Analysis

The GraphPad prism V5.0 software (GraphPad Software Inc., La Jolla, USA) was utilised for statistical analysis. Results were statistically compared using a one-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple group comparison (data is presented as mean ± SD). Statistical significance was considered at $p \leq 0.05$ ($n = 5$).

3. Results

3.1 MF modulates glucose tolerance

There was no significant difference in body weights between MF-treated and STZ groups, however body weights were significantly increased after injection of STZ (*** $p < 0.0001$). Animal blood glucose levels were significantly elevated in the STZ-treated group, and MF treatment significantly lowered blood glucose in diabetic mice compared with the STZ group (Fig 1: *** $p<0.0001$, $^{###}p<0.0001$).

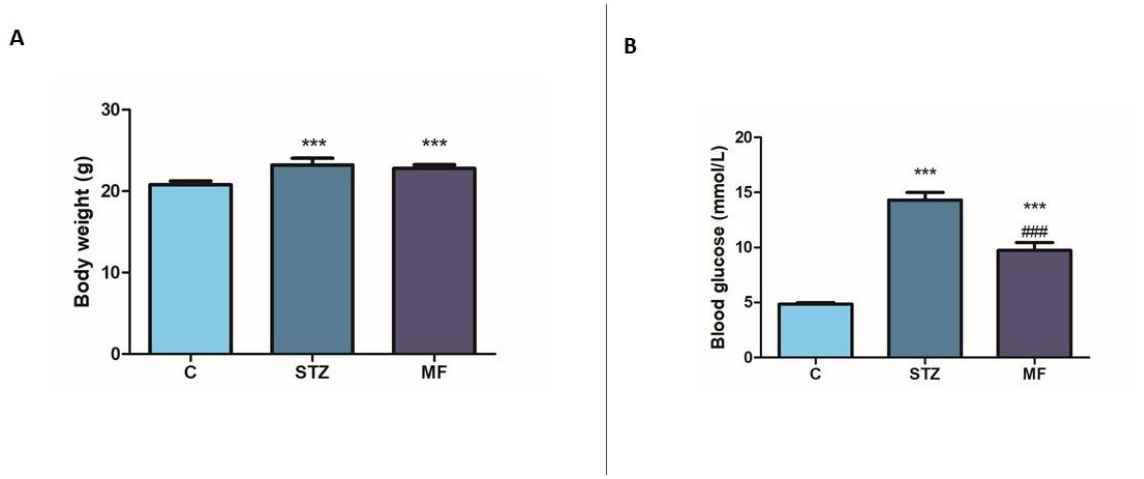


Figure 1: Glucose tolerance following experimental period (n=5). STZ-treatment significantly elevated blood glucose levels. MF did not influence body weight (A: $^{***}p < 0.0001$ *vs* control) and was able to counteract the hyperglycaemic effect by efficiently lowering mice blood glucose levels (B: $^{***}p < 0.0001$ *vs* control, $^{###}p < 0.001$ *vs* STZ).

3.2 MF improves protein oxidation and antioxidant enzyme GSTA4 expression

To estimate MFs ability to combat oxidative stress, protein carbonylation and antioxidant expression was measured. MF significantly reduced protein carbonylation in STZ brain tissue (Fig 2A: ***, $^{###}$: p<0.0001). *GSTA4* expression was decreased in diabetic mice, whilst MF enhanced its expression (Fig 2B ***, $^{###}$: p<0.0001). Additionally, we assessed LonP1 expression, to determine if MF induced maintenance and stress response protein to clear protein aggregates and degrade oxidised proteins. STZ treated mice revealed a significant reduction in LonP1 protein expression in comparison to the control, whereas MF was able to enhance LonP1 expression in diabetic mice brain (Fig 2C ***, $^{###}$: p<0.0001).

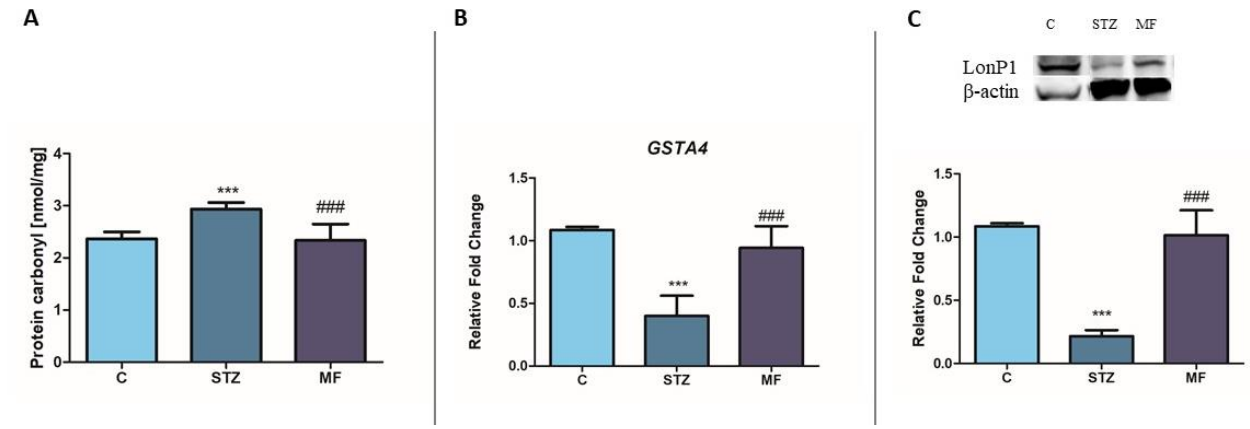


Figure 2: MF reduced protein carbonylation in diabetic mice brain (n=5), (A: $^{***}p < 0.0001$ *vs* control, $^{###}p < 0.0001$ *vs* STZ), upregulated *GSTA4* gene expression in the diabetic group (B: $^{***}p < 0.0001$ *vs* control, $^{###}p < 0.0001$ *vs* STZ), and modulates LonP1 expression (C: $^{***}p < 0.0001$ *vs* control, $^{###}p < 0.0001$ *vs* STZ) in diabetic mice.

3.3 MiR-132 regulation and induction of Heat shock proteins (HSPs): HSP60 and HSP70

The 3′-UTR complementary prediction with Target Scan 7.2 revealed that miR-132 is complementary to HSP70-12B. STZ significantly increased expression of miR-132 in brain tissue, whilst its expression was significantly decreased following MF treatment (Fig 3A: ***, $^{###}$ p<0.0001). Western blotting revealed that MF effectively increased protein expression of both HSP60 (Fig 3CA: ***, $^{###}$: p<0.0001, *: p<0.05) and HSP70 (Fig 3B: **p<0.005, $^{###}$p=0.0001) in the MF treated group compared to the control.

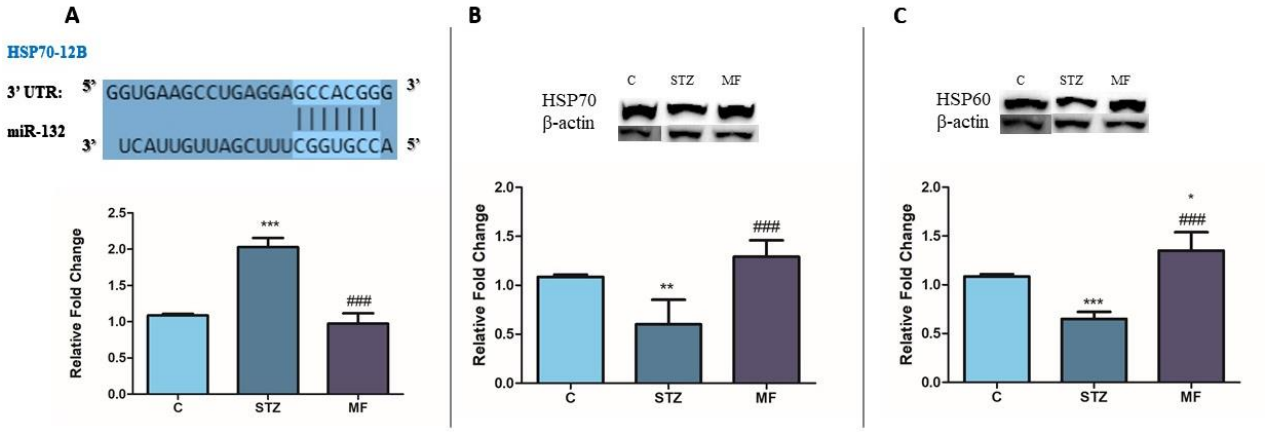


Figure 3: MiR-132 repression by MF in STZ brain tissue. Seed sequence between miR-132 and predicted target sequence of HSP70-12B. STZ reduces activity in diabetic groups and MF improves the HSP response in diabetic mice brain tissue (A: $^{***}p < 0.0001$ *vs* control, $^{###}p < 0.0001$ *vs* STZ, B: $^{**}p < 0.005$ *vs* control, $^{###}$: p = 0.0001 *vs* STZ; C: $^{*}p < 0.05$, $^{***}p < 0.0001$ *vs* control, $^{###}p < 0.0001$ *vs* STZ), (n=5).

3.4 MF improves protein translation through inhibition of stress sensor PERK and eIF2α phosphorylation

To determine whether MF represses ER stress in mice brain tissue, we assessed the phosphorylated form of PERK. Diabetic mice brain exhibited increased protein levels of PERK phosphorylation (active), as well as elevated gene expression. Whereas MF significantly reduced both protein expression (Fig 4A: ***, ### p<0.0001) and transcript levels (Fig 4B: *p<0.05, **p<0.005, ###p<0.0001) as compared to the control and diabetic groups respectively. The activity of UPR signalling protein, eif2α, is increased upon phosphorylation (p-eif2α). MF

decreased p-eif2α expression (Fig 4C: **: p<0.005, ***, ### p=0.0001) in diabetic brain tissue as compared to control and STZ animals. Moreover, expression of eif2α was reduced following MF treatment in the diabetic group (Fig 4D: **p<0.005, ***, ###: p<0.0001).

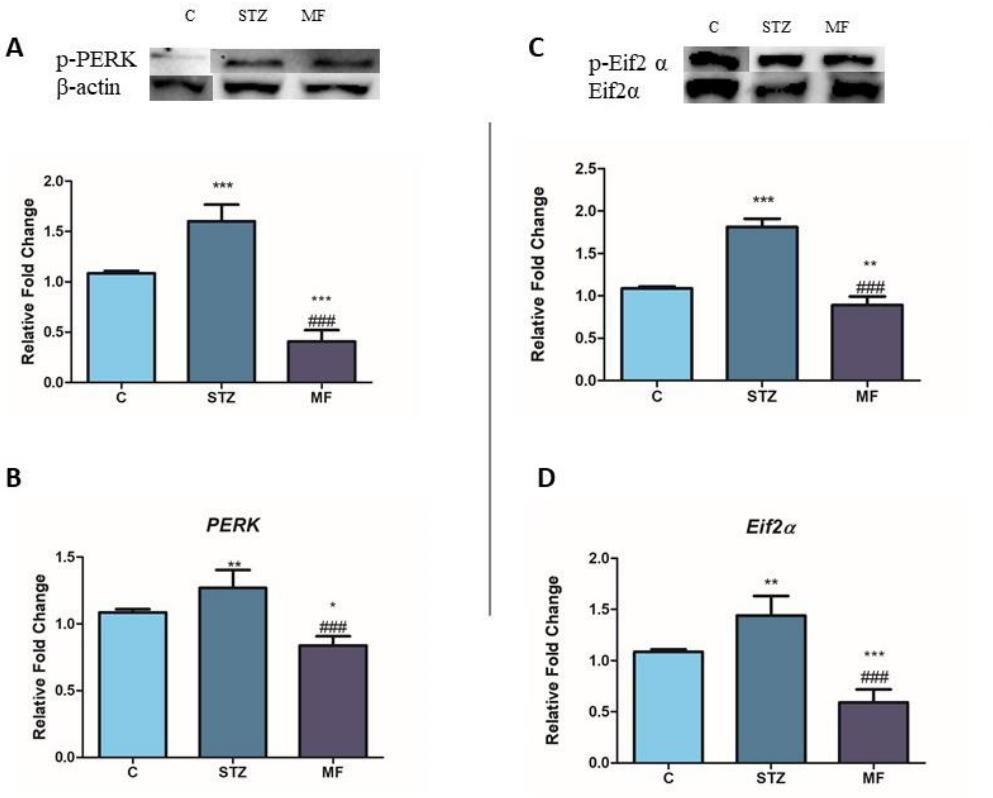


Figure 4: STZ promotes ER stress through PERK phosphorylation, and MF concomitantly reduces p-PERK protein (A) and gene (B) expression in the STZ group (A: ***p < 0.0001 *vs* control, ### p < 0.0001 *vs* STZ; B: *p< 0.05, **p < 0.005 *vs* control, ###p < 0.0001 *vs* STZ). MF decreased both p-eIF2α (C), and gene expression of *eIF2α* (D), with a subsequent increase in total eIF2α protein expression (E) as compared to the control and STZ mice (C: ***p < 0.0001, *p < 0.05 *vs* control, ### p = 0.0001 *vs* STZ; D: **p < 0.005, ***p < 0.0001 *vs* control, ##p < 0.0001 *vs* STZ; E: *p < 0.05 *vs* control, ###p < 0.0001 *vs* STZ;), (n=5).

3.5 Mt regulators: Sirt1, Sirt3 and TFAM are modulated by MF

The lysine deacetylases Sirt1 and Sirt3 protect mt function and prevent neuronal degeneration and dysfunction. Sirt1 activity (Fig 5A: ***, ### p<0.0001) and mRNA expression (Fig 5B: **p<0.005, ***p<0.0001, ###p<0.0001) was increased by MF in STZ treated brain tissue. Sirt3 protein and its gene expression (Fig -5C, -5D: ***, ###p<0.0001) showed a similar trend post MF treatment.

Downstream mitochondriogenesis protein TFAM (Fig 5E: ***, $^{\#\#\#}$p<0.0001) was induced following MF treatment in diabetic brain tissue.

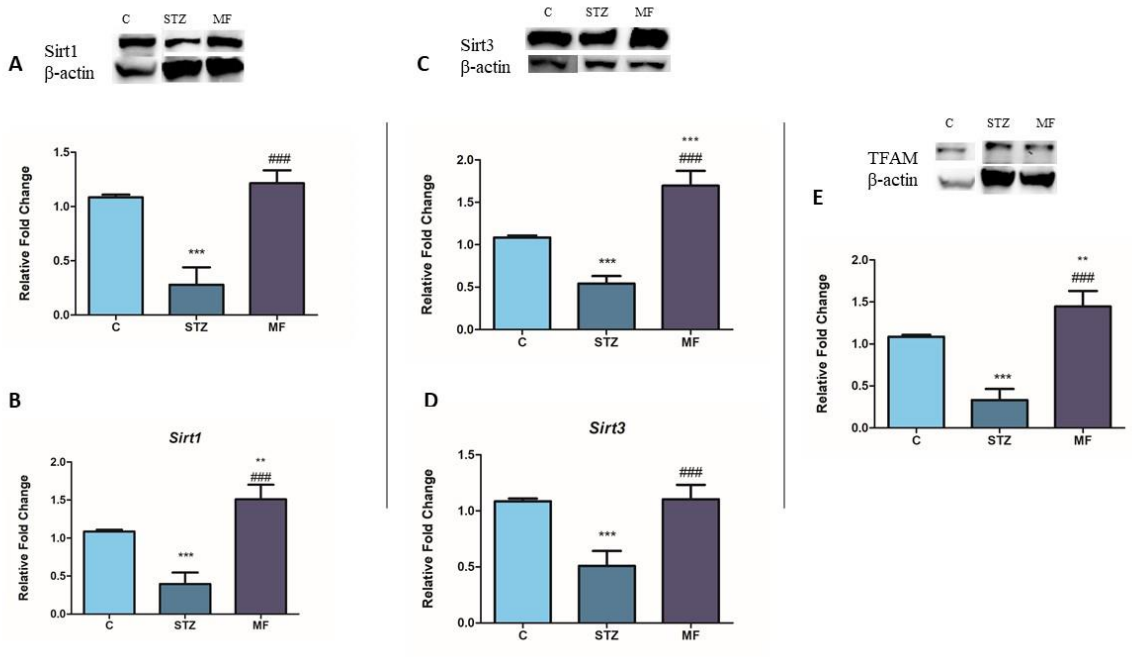


Figure 5: MF regulates mt biogenic markers. Sirt1 (A: ***p < 0.0001 *vs* control, $^{\#\#\#}$ p < 0.005 *vs* STZ), *Sirt1* (B: **p < 0.005, ***p < 0.0001 *vs* control, $^{\#\#\#}$ p < 0.005 *vs* STZ). Sirt3 (C: ***p < 0.0001 *vs* control, $^{\#\#\#}$ p < 0.005 *vs* STZ), *Sirt3* (D: ***p < 0.0001 *vs* control, $^{\#\#\#}$ p < 0.005 *vs* STZ). TFAM (E: ***p < 0.0001 *vs* control, $^{\#\#\#}$ p < 0.005 *vs* STZ), (n=5).

3.6 MF modulates PGC-1α through miR-148a repression

Based on the 3′-UTR complementary prediction with Target Scan 7.2, PGC-1α (Fig 6A) was chosen as it is an important regulator of multiple metabolic processes in diabetes. STZ treatment increased miR-148a expression, with associated decreases in PGC-1α gene and protein expression. MF decreased miR-148a expression post-STZ treatment of mice (Fig 6A: ** p<0.005 ***, $^{\#\#\#}$ p<0.0001). This was accompanied by elevated PGC-1α gene expression (Fig 6B: **p<0.005, $^{\#\#}$p=0.0019), and protein (Fig 6C: ***, $^{\#\#\#}$p<0.0001) as compared to control mice brain tissue.

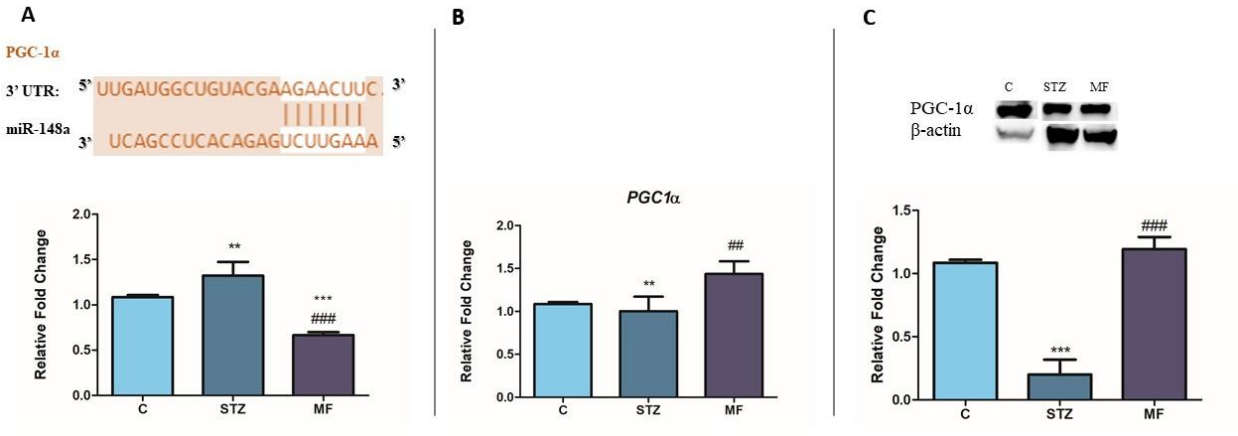


Figure 6: MiR-148a repression by MF in STZ brain tissue. Seed sequence between miR-148a and predicted target sequence of PGC-1α, (**, *** $p < 0.005$ *vs* control, $^{###}$ $p < 0.0001$ *vs* STZ). MF upregulates PGC-1α gene (B: $^{**}p < 0.005$ *vs* control, $^{##}$ $p = 0.0019$ *vs* STZ) expression, and associated protein activity (C: $^{***}p < 0.0001$ *vs* control, $^{###}p < 0.0001$ *vs* STZ) in STZ treated mice brain tissue (n=5).

4. Discussion

MF counteracts oxidative stress induced by diabetes through direct action on the mt and alters cellular bioenergetics (Andrzejewski et al., 2014, Lee et al., 2012). Insulin resistance and elevated ROS levels in the brain impairs synaptic and metabolic functions (De Felice and Ferreira, 2014). Hypothalamic insulin action is a master regulator of the mt stress response, by controlling mt proteostasis and regulating function and metabolism (Wardelmann et al., 2019).

In this study a STZ-induced diabetic mouse model (10 days) was used to establish an insulin deficient system through partial knock out of pancreatic β-cell function (Busineni and Goud, 2015). The minimal increase in body weight in STZ and MF groups (Fig 1A) may be due to their development and degree of hyperphagia. Fasting blood glucose levels increased in the STZ group, confirming that the mice developed diabetes, this was efficiently reduced by MF (Fig 1B). Horakova *et.al.*, have recently shown that glucose uptake in the brain is significantly increased through oral MF treatment, validating our finding (Horakova et al., 2019). However, MF-treated mice exhibited blood glucose significantly higher than the control group (Fig 1B), this may be due to the low dose of MF treatment used in our study, as higher MF doses reduce blood glucose more efficiently (Horakova et al., 2019). Here, we evaluated the effect of MF on protein oxidation and related mt protective circuitry in the brain of diabetic mice.

Oxidative stress is a tissue damaging feature of diabetic complications including neurodegeneration. In neurons, the oxidation of mt proteins, lipids and DNA exacerbates the

progression of neuropathologies (Islam, 2017). Mice exhibited protein carbonylation following STZ-induced diabetes, whilst MF efficiently protected proteins from carbonylation (Fig 2A). Reactive dicarbonyls are directly neutralised by MF through binding of its guanidine group to the α-dicarbonyl group produced in high oxidative states (Dziubak et al., 2018). Depletion of the antioxidant molecule glutathione (GSH) persists in oxidative states. A study by Curtis *et al.* demonstrated that a loss of GSTA4 is correlated with mt dysfunction and increased protein carbonylation (Curtis et al., 2010). We showed diminished transcript levels of the antioxidant enzyme, *GSTA4*, following STZ administration, and a concomitant increase in the MF group (Fig 2B). This suggests that MF has a protective effect by counteracting ROS production and stabilising the antioxidant status in mice brain. LonP1 is an ATP dependent protease that prevents the accumulation of protein aggregates in the mitochondria by degrading them into short peptides, thereby, optimizing bioenergetics and preserving cellular viability (Bota and Davies, 2016, Bota et al., 2005, Fukuda et al., 2007). We showed decreased LonP1 protein expression in STZ treated animals as compared to MF treated mice where it significantly upregulated LonP1 expression. LonP1 upregulation enables mt protein quality maintenance in diabetic brain tissue (Fig 2C). MF's regulation against protein oxidation and removal of damaged proteins in diabetic mice brain corresponds with the observed reduced protein carbonyl content (Fig 2B). Silencing studies of LonP1 have confirmed its role in protein stress responses by protecting against oxidative protein damage and diminished mitochondrial function (Ngo and Davies, 2009b).

Previous studies revealed that LonP1 is required for the proper assembly of the ETC subunits (Mottis et al., 2014). Furthermore, proteomic studies showed that LonP1 partners with HSP60 and HSP70 to assist in protein re-folding during stress conditions (Gregersen et al., 2001). Research demonstrates the neuroprotective roles of HSP60 and HSP70, with loss of HSPs being implicated in protein oxidation and neurodegeneration (Bruening et al., 1999, Kleinridders et al., 2013, Winklhofer et al., 2003, Zhang et al., 2017d). In keeping with this, the pharmacological induction of HSPs in transgenic mice have been shown to lower disease progression (Kieran et al., 2004). The HSP response is elicited in the brain to combat its vulnerability to oxidative stress (Abdul et al., 2006). These molecular chaperones detect misfolded proteins and refold them into their native or non-toxic shapes. Here we showed that MF significantly increased HSP60 and HSP70 expression in diabetic brain tissue (Fig 3B and 3C), potentially through LonP1 stabilisation of HSP60-mtHSP70 chaperone complex formed during stressful states (Kao et al., 2016). Increased LonP1 expression (Fig 2C) parallels with the upregulated HSP response (Fig 3B and 3C), further confirming MFs ability to decrease the levels of oxidatively modified protein in the diabetic brain. By activating chaperone effects and catalytically removing oxidised proteins, MF prevents the accumulation of aggregated proteins in the cell promoting efficient mt function and establishes homeostasis.

Further, the heat-shock response, Sirt1, Sirt3, and the unfolded protein response (UPR) act to counteract mt oxidative stress and prevent accumulation of misfolded proteins (Min et al., 2013). A primary adaptive mechanism of ER stress during diabetes involves eIF2α phosphorylation via PERK activation, thus attenuating misfolded protein translation (Piperi et al., 2012). A study by Quentin *et. al.*, demonstrated the protective role of MF through late activation of PERK, keeping the stress response below a threshold level that would induce apoptosis (Quentin et al., 2012). Our results agree with this study as MF lowered phosphorylation and activation of PERK protein (Fig 4A) as well as mRNA expression (Fig 4B) in STZ mice. This PERK-specific induction of UPR signalling by MF is consistent with the idea that PERK is not directly activated by MF and may result through MFs effect on the energy status of the cell. Subsequent depletion of phosphorylated/active eif2α (p- eif2α) (Fig 4C), and lowered transcript levels of eif2α (Fig 4D) was exhibited by MF in diabetic mice brain. This observation parallels the abovementioned PERK result, further indicating MF's ability to counteract STZ-induced ER stress in mice brain. These results are consistent with a study by Simon-Szabó *et. al.*, where MF abolished ER stress in a diabetic rat model (Simon-Szabó et al., 2014). Additionally, Diaz-Morales *et al*., *demonstrated* the protective role of MF against ER stress through UPR signalling in DM (Diaz-Morales et al., 2017, Diaz-Morales et al., 2018). The reduced protein oxidation (Fig 2A) is consistent with these results as the protein folding process is disrupted by STZ-induced ROS that yields misfolded proteins in the UPR (Lee et al., 2009, Piperi et al., 2012). Furthermore, MF-induced LonP1 (Fig 2C) allows the mt to counteract oxidative stress experienced in diabetic brain tissue. Our data also agrees with a study by Wardelmann *et. al.*, that demonstrated the role of brain insulin in adaptive mt stress responses that control neuronal health and metabolic regulation (Wardelmann et al., 2019). Our data indicates a plausible mechanism of MFs ability to promote mt and ER function, preventing STZ-induced diabetic stress in mice brain.

Defective mt biogenesis is a common feature of diabetic neuropathies. The co-transcription factor, PGC-1α is regarded as the master regulator of mt biogenesis and plays a pivotal role in attenuating mt and oxidative stress. A synergistic energy sensing role exists between Sirt1 and PGC-1α, thus modulating metabolic control through regulation of mt biogenesis (Rodgers et al., 2005). Research suggests MF-induced AMPK activation enhances blood-brain barrier functions (Takata et al., 2013). Additionally, MF promotes mt biogenesis through elevated PGC-1α levels (Onyango et al., 2010, Suwa et al., 2006). MF inhibits complex 1 in the mt ETC, increasing the AMP/ATP ratio (Hardie et al., 2006). Increased PGC-1α protein, and mRNA levels (Fig 8) restores energy levels mediated by AMPK in diabetic mice brain.

Sirt1 exerts partial neuroprotection by preventing mitochondrial impairment through PGC-1α activation (Min et al., 2013), whereas Sirt3 resides in the mitochondrial matrix and regulates the acetylation of several key metabolic enzymes in the mitochondria. In our study, MF increased both Sirt1 protein and gene expression in diabetic mice brain as compared to the control mice

brain (Fig 5A and B). A plausible explanation may be through MFs regulation of AMPK and restoration of NAD^+ levels (Bonkowski and Sinclair, 2016). To corroborate this observation with decreased protein carbonyl levels (Fig 2A), it was reported that reduced Sirt1 activity was due to concomitant carbonyl modifications on its cysteine residues (Caito et al., 2010). Considering that Sirt1 induces PGC-1α, a known transcription factor that regulates Sirt3 activity, we measured its expression and transcript levels. MF significantly increased Sirt3 protein and transcript levels (Fig 5C and D) in diabetic mice. A study by Aatsiniki *et. al.*, demonstrated that AMPK and Sirt1 siRNAs attenuated the increase in PGC-1α induced by metformin, providing evidence for their direct roles as metabolic sensors (Aatsinki et al., 2014). PGC-1α, Sirt1, and Sirt3 act synergistically to regulate mt biogenesis and increase mt content through increased TFAM expression (Jornayvaz and Shulman, 2010). The increased protein level of TFAM (Fig 5E) in diabetic mice correlates with the restoration/increase in both Sirt1 and Sirt3 levels by MF (Fig 5A-D). Collectively, we provide novel action of MF in the diabetic brain by alleviating neuronal stress through Sirt1 activation of PGC-1α and corresponding increases in Sirt3 and TFAM expression, hence, mediating mt biogenesis.

MicroRNAs, non-coding single stranded RNA, pair with bases of complementary target mRNA, resulting in their degradation or translational inhibition (He and Hannon, 2004). Alterations in the miRNA regulatory pathways may predispose to neurodegenerative pathogenesis (Basavaraju and de Lencastre, 2016). Mir-132 is enriched in neuronal cells and its dysregulation leads to brain related disorders (Wanet et al., 2012). Furthermore, limited information exists on neuronal miR-148a dysregulation in diabetes, yet, its regulation in neurodegenerative diseases has been reported (Cogswell et al., 2008). We assessed both miR-132, and -148a and their modulation of mt function and protein translation. The seed sequence of miR-132 is complementary to gene product HSP70-12B (Fig 3A). STZ increased miR-132 expression, as shown in situations under diabetic stress (Kim et al., 2014), correlating to the reduced protein expression of HSP70 in mice brain (Fig 3A and B). A concomitant decrease in miR-132 expression and increased HSP70 protein levels was exerted by MF in diabetic mice brain (Fig 3A and B). MiR-132 overexpression negatively regulates Sirt1 and its downstream targets (Miyazaki et al., 2014). In agreement with this, the increased Sirt1 levels (Fig 5A and B) corroborate the effect of MF on miR-132 in STZ-treated mice.

The PGC-1α gene (*PPARGC1A*) is located on chromosome 4p15.1-2 (Esterbauer et al., 1999), a region that is associated with basal insulin levels (Pratley et al., 1998). A compelling relationship exists between diabetes-related phenotypes and single nucleotide polymorphisms on *PPARGC1A* (Andrulionyte et al., 2004, Bhat et al., 2007, Ek et al., 2001). The seed sequence of miR-148a was found to be complementary to the PGC-1α transcript as shown by a study indicating decreased PGC-1α levels by miR-148a (Chen et al., 2017). Tryggestad *et. al.* specify lower AMPK activity with miR-148a mimics (Tryggestad et al., 2016). The suppression of miR-148a may occur through

enhanced activity of AMPK by MF in diabetic mice brain (Fig 6A). Additionally, the increased PGC-1α mRNA and protein levels (Fig 6) in MF-treated mice brain, correlates with enhanced mt biogenesis factors, Sirt1, Sirt3 and TFAM (Fig 5), suggesting that MF prevents silencing of PGC-1α by miR-148a. This is the first study to demonstrate the epigenetic regulation of HSP70 and PGC-1α by MF through miR-132 and -148a suppression respectively. In summary, we indicate the stress induced by STZ in mice brain contributes to elevated levels of miR-132 and -148a resulting in mt dysregulation. The molecular mechanism of MF in alleviating this stress involves suppression of these miRs and modulation of metabolic networks within the mt, promoting neuroprotection.

5. Conclusion

In conclusion, our results strongly suggest that MF exhibits neuronal protection against oxidative damage and mt dysfunction caused by STZ in mouse whole brain homogenates. MFs regulation of miRs-132 and -148a and their integration with mt stress responses attributes to the novelty of this study. The multi-targeted actions of MF illustrate that the neuroprotective effects in diabetic whole mouse brains found were due to synergistic activity. The primary mechanism of MF is established and may now prove efficient in counteracting diabetes associated neurodegeneration. Our study proved MF's efficacy over an acute treatment period, however, future translational research should consider chronic MF treatment periods in the diabetic brain. Additionally, downstream ER pathway analysis should be considered for future work. This will extend pharmacologic knowledge on MF and its neuroprotective role in diabetics.

6. Conflict of interest

The authors declare that there are no conflicts of interest

7. Acknowledgements

Authors would like to thank the national research foundation (NRF) grant, South Africa (UID98379) and College of Health Sciences – University of KwaZulu-Natal for funding this study.

8. References

AATSINKI, S. M., BULER, M., SALOMÄKI, H., KOULU, M., PAVEK, P. & HAKKOLA, J. 2014. Metformin induces PGC-1α expression and selectively affects hepatic PGC-1α functions. *British journal of pharmacology,* 171**,** 2351-2363.

ABDUL, H. M., CALABRESE, V., CALVANI, M. & BUTTERFIELD, D. A. 2006. Acetyl-L-carnitine-induced up-regulation of heat shock proteins protects cortical neurons against

amyloid-beta peptide 1–42-mediated oxidative stress and neurotoxicity: Implications for Alzheimer's disease. *Journal of neuroscience research,* 84**,** 398-408.

ANDRULIONYTE, L., ZACHAROVA, J., CHIASSON, J.-L., LAAKSO, M. & GROUP, S.-N. S. 2004. Common polymorphisms of the PPAR-γ2 (Pro12Ala) and PGC-1α (Gly482Ser) genes are associated with the conversion from impaired glucose tolerance to type 2 diabetes in the STOP-NIDDM trial. *Diabetologia,* 47**,** 2176-2184.

ANDRZEJEWSKI, S., GRAVEL, S.-P., POLLAK, M. & ST-PIERRE, J. 2014. Metformin directly acts on mitochondria to alter cellular bioenergetics. *Cancer & metabolism,* 2**,** 12-12.

ASSMANN, T. S., RECAMONDE-MENDOZA, M., DE SOUZA, B. M. & CRISPIM, D. 2017. MicroRNA expression profiles and type 1 diabetes mellitus: systematic review and bioinformatic analysis. *Endocrine connections,* 6**,** 773-790.

AUGUSTYNIAK, E., ADAM, A., WOJDYLA, K., ROGOWSKA-WRZESINSKA, A., WILLETTS, R., KORKMAZ, A., ATALAY, M., WEBER, D., GRUNE, T. & BORSA, C. 2015. Validation of protein carbonyl measurement: a multi-centre study. *Redox biology,* 4**,** 149-157.

BASAVARAJU, M. & DE LENCASTRE, A. 2016. Alzheimer's disease: presence and role of microRNAs. *Biomolecular concepts,* 7**,** 241-252.

BHAT, A., KOUL, A., RAI, E., SHARMA, S., DHAR, M. & BAMEZAI, R. 2007. PGC-1α Thr394Thr and Gly482Ser variants are significantly associated with T2DM in two North Indian populations: a replicate case-control study. *Human Genetics,* 121**,** 609-614.

BONKOWSKI, M. S. & SINCLAIR, D. A. 2016. Slowing ageing by design: the rise of NAD+ and sirtuin-activating compounds. *Nature Reviews Molecular Cell Biology,* 17**,** 679.

BOTA, D. A. & DAVIES, K. J. 2016. Mitochondrial Lon protease in human disease and aging: Including an etiologic classification of Lon-related diseases and disorders. *Free Radical Biology and Medicine,* 100**,** 188-198.

BOTA, D. A., NGO, J. K. & DAVIES, K. J. 2005. Downregulation of the human Lon protease impairs mitochondrial structure and function and causes cell death. *Free Radical Biology and Medicine,* 38**,** 665-677.

BRUENING, W., ROY, J., GIASSON, B., FIGLEWICZ, D. A., MUSHYNSKI, W. E. & DURHAM, H. D. 1999. Up-regulation of protein chaperones preserves viability of cells expressing toxic Cu/Zn-superoxide dismutase mutants associated with amyotrophic lateral sclerosis. *Journal of neurochemistry,* 72**,** 693-699.

BUSINENI, A. & GOUD, J. 2015. Streptozotocin-A Diabetogenic Agent in Animal Models.

CAITO, S., RAJENDRASOZHAN, S., COOK, S., CHUNG, S., YAO, H., FRIEDMAN, A. E., BROOKES, P. S. & RAHMAN, I. 2010. SIRT1 is a redox-sensitive deacetylase that is post-translationally modified by oxidants and carbonyl stress. *The FASEB Journal,* 24**,** 3145-3159.

CHEN, Z., LUO, J., SUN, S., CAO, D., SHI, H. & LOOR, J. J. 2017. miR-148a and miR-17–5p synergistically regulate milk TAG synthesis via PPARGC1A and PPARA in goat mammary epithelial cells. *RNA biology,* 14**,** 326-338.

CHO, K., CHUNG, J. Y., CHO, S. K., SHIN, H.-W., JANG, I.-J., PARK, J.-W., YU, K.-S. & CHO, J.-Y. 2015. Antihyperglycemic mechanism of metformin occurs via the AMPK/LXRα/POMC pathway. *Scientific reports,* 5**,** 8145.

COGSWELL, J. P., WARD, J., TAYLOR, I. A., WATERS, M., SHI, Y., CANNON, B., KELNAR, K., KEMPPAINEN, J., BROWN, D. & CHEN, C. 2008. Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. *Journal of Alzheimer's disease,* 14**,** 27-41.

CURTIS, J. M., GRIMSRUD, P. A., WRIGHT, W. S., XU, X., FONCEA, R. E., GRAHAM, D. W., BRESTOFF, J. R., WICZER, B. M., ILKAYEVA, O. & CIANFLONE, K. 2010. Downregulation of adipose glutathione S-transferase A4 leads to increased protein carbonylation, oxidative stress, and mitochondrial dysfunction. *diabetes,* 59**,** 1132-1142.

DE FELICE, F. G. & FERREIRA, S. T. 2014. Inflammation, defective insulin signaling, and mitochondrial dysfunction as common molecular denominators connecting type 2 diabetes to Alzheimer disease. *Diabetes,* DB_131954.

DE FELICE, F. G. & FERREIRA, S. T. 2017. Getting a "GRiP" on Hypothalamic Endoplasmic Reticulum Stress to Combat Obesity. *Diabetes,* 66**,** 17-19.

DIAZ-MORALES, N., IANNANTUONI, F., ESCRIBANO-LOPEZ, I., BANULS, C., ROVIRA-LLOPIS, S., SOLA, E., ROCHA, M., HERNANDEZ-MIJARES, A. & VICTOR, V. M. 2018. Does Metformin Modulate Endoplasmic Reticulum Stress and Autophagy in Type 2 Diabetic Peripheral Blood Mononuclear Cells? : Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA.

DIAZ-MORALES, N., ROVIRA-LLOPIS, S., BANULS, C., LOPEZ-DOMENECH, S., ESCRIBANO-LOPEZ, I., VESES, S., JOVER, A., ROCHA, M., HERNANDEZ-MIJARES, A. & VICTOR, V. M. 2017. Does metformin protect diabetic patients from oxidative stress and leukocyte-endothelium interactions? : Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA.

DZIUBAK, A., WÓJCICKA, G., WOJTAK, A. & BEŁTOWSKI, J. 2018. Metabolic Effects of Metformin in the Failing Heart. *International journal of molecular sciences,* 19**,** 2869.

EK, J., ANDERSEN, G., URHAMMER, S., GAEDE, P., DRIVSHOLM, T., BORCH-JOHNSEN, K., HANSEN, T. & PEDERSEN, O. 2001. Mutation analysis of peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) and relationships of identified amino acid polymorphisms to Type II diabetes mellitus. *Diabetologia,* 44**,** 2220-2226.

ESTERBAUER, H., OBERKOFLER, H., KREMPLER, F. & PATSCH, W. 1999. Human peroxisome proliferator activated receptor gamma coactivator 1 (PPARGC1) gene: cDNA

sequence, genomic organization, chromosomal localization, and tissue expression. *Genomics,* 62**,** 98-102.

FUKUDA, R., ZHANG, H., KIM, J.-W., SHIMODA, L., DANG, C. V. & SEMENZA, G. L. 2007. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell,* 129**,** 111-122.

GREGERSEN, N., BROSS, P., ANDRESEN, B. S., PEDERSEN, C. B., CORYDON, T. J. & BOLUND, L. 2001. The role of chaperone-assisted folding and quality control in inborn errors of metabolism: protein folding disorders. *Journal of inherited metabolic disease,* 24**,** 189-212.

HARDIE, D. G., HAWLEY, S. A. & SCOTT, J. W. 2006. AMP-activated protein kinase–development of the energy sensor concept. *The Journal of physiology,* 574**,** 7-15.

HE, L. & HANNON, G. J. 2004. MicroRNAs: small RNAs with a big role in gene regulation. *Nature Reviews Genetics,* 5**,** 522.

HORAKOVA, O., KROUPOVA, P., BARDOVA, K., BURESOVA, J., JANOVSKA, P., KOPECKY, J. & ROSSMEISL, M. 2019. Metformin acutely lowers blood glucose levels by inhibition of intestinal glucose transport. *Scientific Reports,* 9**,** 6156.

ISLAM, M. T. 2017. Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. *Neurological research,* 39**,** 73-82.

JORNAYVAZ, F. R. & SHULMAN, G. I. 2010. Regulation of mitochondrial biogenesis. *Essays in biochemistry,* 47**,** 69-84.

KAMAT, P. K. 2015. Streptozotocin induced Alzheimer's disease like changes and the underlying neural degeneration and regeneration mechanism. *Neural regeneration research,* 10**,** 1050.

KAO, T., CHIU, Y., FANG, W., CHENG, C., KUO, C., JUAN, H., WU, S. & LEE, A. Y. 2016. Mitochondrial Lon regulates apoptosis through the association with Hsp60–mtHsp70 complex. *Cell death & disease,* 6**,** e1642.

KIERAN, D., KALMAR, B., DICK, J. R., RIDDOCH-CONTRERAS, J., BURNSTOCK, G. & GREENSMITH, L. 2004. Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. *Nature medicine,* 10**,** 402.

KIM, J. Y., LEE, J. E. & YENARI, M. A. 2017. Neuroprotection of Heat Shock Proteins (HSPs) in Brain Ischemia. *Translational Research in Stroke.* Springer.

KIM, Y. S., KANG, H. J., HONG, M. H., KANG, W. S., CHOE, N., KOOK, H., JEONG, M. H. & AHN, Y. 2014. Angiopoietin-like 4 is involved in the poor angiogenic potential of high glucose-insulted bone marrow stem cells. *Korean circulation journal,* 44**,** 177-183.

KLEINRIDDERS, A., LAURITZEN, H. P., USSAR, S., CHRISTENSEN, J. H., MORI, M. A., BROSS, P. & KAHN, C. R. 2013. Leptin regulation of Hsp60 impacts hypothalamic insulin signaling. *The Journal of clinical investigation,* 123**,** 4667-4680.

LEAK, R. K. 2014. Heat shock proteins in neurodegenerative disorders and aging. *Journal of cell communication and signaling,* 8**,** 293-310.

LEE, H., LEE, Y. J., CHOI, H., KO, E. H. & KIM, J.-W. 2009. Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. *Journal of Biological Chemistry,* 284**,** 10601-10609.

LEE, J. O., LEE, S. K., KIM, J. H., KIM, N., YOU, G. Y., MOON, J. W., KIM, S. J., PARK, S. H. & KIM, H. S. 2012. Metformin regulates glucose transporter 4 (GLUT4) translocation through AMP-activated protein kinase (AMPK)-mediated Cbl/CAP signaling in 3T3-L1 preadipocyte cells. *Journal of Biological Chemistry***,** jbc. M112. 361386.

LEVINE, R. L., WILLIAMS, J. A., STADTMAN, E. P. & SHACTER, E. 1994. [37] Carbonyl assays for determination of oxidatively modified proteins. *Methods in enzymology*. Elsevier.

LIND, K. R., BALL, K. K., CRUZ, N. F. & DIENEL, G. A. 2013. The unfolded protein response to endoplasmic reticulum stress in cultured astrocytes and rat brain during experimental diabetes. *Neurochemistry international,* 62**,** 784-795.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods,* 25**,** 402-408.

MCCALL, A. L. 1992. The impact of diabetes on the CNS. *Diabetes,* 41**,** 557-570.

MIN, S.-W., SOHN, P. D., CHO, S.-H., SWANSON, R. A. & GAN, L. 2013. Sirtuins in neurodegenerative diseases: an update on potential mechanisms. *Frontiers in aging neuroscience,* 5**,** 53.

MIYAZAKI, Y., LI, R., REZK, A., MISIRLIYAN, H., MOORE, C., FAROOQI, N., SOLIS, M., GOIRY, L. G., DE FARIA JUNIOR, O. & COLMAN, D. 2014. A novel microRNA-132-surtuin-1 axis underlies aberrant B-cell cytokine regulation in patients with relapsing-remitting multiple sclerosis. *Plos one,* 9**,** e105421.

MOTTIS, A., JOVAISAITE, V. & AUWERX, J. 2014. The mitochondrial unfolded protein response in mammalian physiology. *Mammalian genome,* 25**,** 424-433.

NESCA, V., GUAY, C., JACOVETTI, C., MENOUD, V., PEYOT, M.-L., LAYBUTT, D. R., PRENTKI, M. & REGAZZI, R. 2013. Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes. *Diabetologia,* 56**,** 2203-2212.

NGO, J. K. & DAVIES, K. J. A. 2009. Mitochondrial Lon protease is a human stress protein. *Free Radical Biology and Medicine,* 46**,** 1042-1048.

NGO, J. K., POMATTO, L. C. & DAVIES, K. J. 2013. Upregulation of the mitochondrial Lon Protease allows adaptation to acute oxidative stress but dysregulation is associated with chronic stress, disease, and aging. *Redox biology,* 1**,** 258-264.

ONYANGO, I. G., LU, J., RODOVA, M., LEZI, E., CRAFTER, A. B. & SWERDLOW, R. H. 2010. Regulation of neuron mitochondrial biogenesis and relevance to brain health. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1802**,** 228-234.

PIPERI, C., ADAMOPOULOS, C., DALAGIORGOU, G., DIAMANTI-KANDARAKIS, E. & PAPAVASSILIOU, A. G. 2012. Crosstalk between advanced glycation and endoplasmic reticulum stress: emerging therapeutic targeting for metabolic diseases. *The Journal of Clinical Endocrinology & Metabolism,* 97**,** 2231-2242.

PRATLEY, R. E., THOMPSON, D. B., PROCHAZKA, M., BAIER, L., MOTT, D., RAVUSSIN, E., SAKUL, H., EHM, M. G., BURNS, D. K. & FOROUD, T. 1998. An autosomal genomic scan for loci linked to prediabetic phenotypes in Pima Indians. *The Journal of clinical investigation,* 101**,** 1757-1764.

PRZEMYSLAW, W., VAARMANN, A., CHOUBEY, V., SAFIULINA, D., LIIV, J., KUUM, M. & KAASIK, A. 2009. PGC-1 [alpha] AND PGC-1 [beta] regulate mitochondrial density in neurons. *Journal of biological chemistry***,** jbc. M109. 018911.

QUENTIN, T., STEINMETZ, M., POPPE, A. & THOMS, S. 2012. Metformin differentially activates ER stress signaling pathways without inducing apoptosis. *Disease models & mechanisms,* 5**,** 259-269.

RAGHUBEER, S., NAGIAH, S., PHULUKDAREE, A. & CHUTURGOON, A. 2015. The phytoalexin resveratrol ameliorates ochratoxin A toxicity in human embryonic kidney (HEK293) cells. *Journal of cellular biochemistry,* 116**,** 2947-2955.

RODGERS, J. T., LERIN, C., HAAS, W., GYGI, S. P., SPIEGELMAN, B. M. & PUIGSERVER, P. 2005. Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature,* 434**,** 113.

RORIZ-FILHO, J. S., SÁ-RORIZ, T. M., ROSSET, I., CAMOZZATO, A. L., SANTOS, A. C., CHAVES, M. L., MORIGUTI, J. C. & RORIZ-CRUZ, M. 2009. (Pre) diabetes, brain aging, and cognition. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease,* 1792**,** 432-443.

SIMON-SZABÓ, L., KOKAS, M., MANDL, J., KÉRI, G. & CSALA, M. 2014. Metformin attenuates palmitate-induced endoplasmic reticulum stress, serine phosphorylation of IRS-1 and apoptosis in rat insulinoma cells. *PLoS One,* 9**,** e97868.

SUWA, M., EGASHIRA, T., NAKANO, H., SASAKI, H. & KUMAGAI, S. 2006. Metformin increases the PGC-1α protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. *Journal of Applied Physiology,* 101**,** 1685-1692.

TAKATA, F., DOHGU, S., MATSUMOTO, J., MACHIDA, T., KANESHIMA, S., MATSUO, M., SAKAGUCHI, S., TAKESHIGE, Y., YAMAUCHI, A. & KATAOKA, Y. 2013. Metformin induces up-regulation of blood–brain barrier functions by activating AMP-

activated protein kinase in rat brain microvascular endothelial cells. *Biochemical and Biophysical Research Communications,* 433**,** 586-590.

TATTIKOTA, S. G., RATHJEN, T., MCANULTY, S. J., WESSELS, H.-H., AKERMAN, I., VAN DE BUNT, M., HAUSSER, J., ESGUERRA, J. L., MUSAHL, A. & PANDEY, A. K. 2014. Argonaute2 mediates compensatory expansion of the pancreatic β cell. *Cell metabolism,* 19**,** 122-134.

TRYGGESTAD, J. B., VISHWANATH, A., JIANG, S., MALLAPPA, A., TEAGUE, A. M., TAKAHASHI, Y., THOMPSON, D. M. & CHERNAUSEK, S. D. 2016. Influence of gestational diabetes mellitus on human umbilical vein endothelial cell microRNA. *Clinical Science***,** CS20160305.

VIOLLET, B., GUIGAS, B., GARCIA, N. S., LECLERC, J., FORETZ, M. & ANDREELLI, F. 2012. Cellular and molecular mechanisms of metformin: an overview. *Clinical science,* 122**,** 253-270.

WANET, A., TACHENY, A., ARNOULD, T. & RENARD, P. 2012. miR-212/132 expression and functions: within and beyond the neuronal compartment. *Nucleic acids research,* 40**,** 4742-4753.

WANG, M. & KAUFMAN, R. J. 2016. Protein misfolding in the endoplasmic reticulum as a conduit to human disease. *Nature,* 529**,** 326.

WARDELMANN, K., BLÜMEL, S., RATH, M., ALFINE, E., CHUDOBA, C., SCHELL, M., CAI, W., HAUFFE, R., WARNKE, K. & FLORE, T. 2019. Insulin action in the brain regulates mitochondrial stress responses and reduces diet-induced weight gain. *Molecular Metabolism*.

WINKLHOFER, K. F., HENN, I. H., KAY-JACKSON, P. C., HELLER, U. & TATZELT, J. 2003. Inactivation of Parkin by oxidative stress and C-terminal truncations a protective role of molecular chaperones. *Journal of Biological Chemistry,* 278**,** 47199-47208.

WU, J., JIN, Z., ZHENG, H. & YAN, L.-J. 2016. Sources and implications of NADH/NAD+ redox imbalance in diabetes and its complications. *Diabetes, metabolic syndrome and obesity: targets and therapy,* 9**,** 145.

ZHANG, X., LI, C., FOWLER, S. C., ZHANG, Z., BLAGG, B. S. & DOBROWSKY, R. T. 2017. Targeting Heat Shock Protein 70 to Ameliorate c-Jun Expression and Improve Demyelinating Neuropathy. *ACS chemical neuroscience,* 9**,** 381-390.

ZOU, M.-H., KIRKPATRICK, S. S., DAVIS, B. J., NELSON, J. S., WILES, W. G., SCHLETTNER, U., NEUMANN, D., BROWNLEE, M., FREEMAN, M. B. & GOLDMAN, M. H. 2004. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo: role of mitochondrial reactive nitrogen species. *Journal of Biological Chemistry*.

ZOU, W., YUAN, J., TANG, Z.-J., WEI, H.-J., ZHU, W.-W., ZHANG, P., GU, H.-F., WANG, C.-Y. & TANG, X.-Q. 2017. Hydrogen sulfide ameliorates cognitive dysfunction in

streptozotocin-induced diabetic rats: involving suppression in hippocampal endoplasmic reticulum stress. *Oncotarget*, 8**,** 64203.

CHAPTER 4

Metformin mediates neuroprotection by regulating miR-141 and dampening the NF-κB-mediated inflammasome pathway in a diabetic mouse model

In chapter 3, the antioxidant effects of MF and its neuroprotective role on mitochondrial dysfunction and ER stress were established in diabetic mice. This chapter further explores MFs role as an anti-inflammatory agent with focus on NF- κB and inflammasome signalling. MiR-141 targets are regulated by MF and controls neuroplasticity pathways.

This manuscript has been submitted to *Biomedicine and Pharmacotherapy* (Manuscript reference: BIOPHA-D-19-00128) and is presented in this thesis as per journal formatting requirements.

Metformin mediates neuroprotection by regulating miR-141 and dampening the NF-κB-mediated inflammasome pathway in a diabetic mouse model

Taskeen Fathima Docrat, Savania Nagiah, Anil A. Chuturgoon

Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, South Africa

*Address correspondence to:

Prof Anil Chuturgoon, Discipline of Medical Biochemistry, Faculty of Health Sciences, George Campbell Building, Howard College, University of KwaZulu-Natal, Durban, 4041, South Africa. Telephone: (031) 260 4404. Email: chutur@ukzn.ac.za

Abstract

Acute inflammation is a feature of diabetes that affects several organs including the brain. This leads to compromised function and eventual neurodegeneration. Tau protein is hyperphosphorylated under diabetic conditions exacerbating degenerative processes. The antidiabetic drug metformin (MF) plays a role in neuroprotection by various pathways including activation of protein phosphatase 2A (PP2A). The microRNA (miR)-200 family, specifically miR-141, is differentially expressed in diseased states including cognitive decline, thereby triggering changes in downstream genes. We hypothesised that miR-141 regulates PP2A and associated NF-κB-mediated inflammasome expression in diabetic mice brain. Diabetes was induced by intraperitoneal injection of Streptozotocin (STZ), thereafter mice were treated with MF (20 mg/kg BW). Whole brain tissue was harvested for further analysis. Protein and gene expressions were established through western blotting and qPCR respectively. Diabetic mice brain tissue revealed increased *miR-141* and decreased *PP2A* expressions. Following MF treatment, *miR-141*, *PP2A*, and p-tau at Ser^{396} protein expressions were regulated in the brain of diabetic mice. Additionally, down-regulation of NF-κB and increased IκB-α protein expression by MF treatment. Further experimentation revealed that MF induces BDNF overexpression, suggesting that the NF-κB signalling pathway may contribute to alleviating neuroinflammation. Metformin also repressed *IL-1β* and *IL-18* expression, proposing its inhibitory action on the inflammasome system, as well as regulated expression of related cytokines and the upstream controller *NLRP3*. Collectively, we demonstrate that MF promotes neuroplasticity in diabetic mice by constraining inflammatory responses through its inhibitory effects on various signalling pathways. This suggests that MF may be effective in the treatment of inflammation-mediated pathologies in diabetic mice.

1. Introduction

Insulin signalling in the brain stimulates various molecular processes (Kleinridders et al., 2014). The disruption of which, under hyperglycaemic conditions, promotes early manifestations of Alzheimer's disease (AD) including neurofibrillary tangles (NFT) and hyperphosphorylation of tau protein (Liu et al., 2016, Whittington et al., 2013, Ye et al., 2017, Zhang et al., 2016a). Tau protein is found within the cytoplasmic and axonal region of neurons where it stabilises microtubules and confers proper brain function (Morris et al., 2011). Its regulation is dependent on phosphorylation and dephosphorylation. The phosphorylation of tau occurs at 85 potential serine (Ser), threonine (Thr), and tyrosine (Tyr) phosphorylation sites, of which Ser^{396} phosphorylation is primarily responsible for the functional loss of tau (Noble et al., 2013). The balance between tau kinases and phosphatase activity regulates its phosphorylation. When this equilibrium is disturbed, hyperphosphorylation and aggregation of tau occurs. Thus, understanding the modes of tau phosphorylation is important in determining protection strategies to cope with the associated neurodegeneration. The Ser/Thr kinase, glycogen synthase kinase-3β (GSK3β), has essential roles in protein synthesis, cell differentiation, proliferation, and death (Frame and Cohen, 2001b); and has additional functions in tau phosphorylation, which is correlated with destabilised microtubules, tauopathies, and neuronal degeneration (Noble et al., 2005). Protein phosphatase 2A (PP2A) is a phosphoesterase that is intracerebrally located and functions in tau dephosphorylation. Its function is further outlined through PP2A-mediated regulation of GSK3β activity by dephosphorylation (Mitra et al., 2012), hence PP2A and GSK3β are the most important enzyme regulators of tau phosphorylation in the brain. Studies demonstrate that reduced PP2A activity leads to tau hyperphosphorylation in Alzheimer's disease (AD) mouse models (Xiong et al., 2013, Zhao et al., 2013, Zhou et al., 2008), and STZ mouse models (Planel et al., 2007). Additionally, tau-induced neurotoxicity is also associated with its ability to decrease trophic support for affected neurons.

Brain-derived neurotrophic factor (BDNF) has a high affinity to the receptor, Tyrosine kinase receptor B (TrkB), which is important for neuronal growth, development and survival through its

ability to defend against free radicals and prevent inflammation in the brain (Huang and Reichardt, 2001, Sampaio et al., 2017). Tau has been shown to downregulate BDNF expression in animal and cellular models of AD (Rosa et al., 2016). Studies have also linked GSK3β inhibition to the protective effects of BDNF/TrkB in neuronal mouse models (Liu et al., 2015). This sheds light on the important co-regulation of tau phosphorylation kinase, GSK3β, and PP2A function in proper brain function. Considering the signalling pathways affected by hyperglycaemia in the brain, anti-diabetic drugs such as Metformin (MF) may positively regulate brain metabolism. Numerous studies depict the neuroprotective effects of MF additional to its glucose lowering activity. The beneficial outcomes of MF in the brain are demonstrated through increased PP2A activation (Demir et al., 2014, Kickstein et al., 2010), which reduced the tau phosphorylation both *in vitro* and *in vivo* (Kickstein et al., 2010). Additionally, MF improved cell proliferation in the hippocampus by inducing BDNF levels (Yoo et al., 2011), with similar effects in a metabolic syndrome patient study (Hristova, 2011).

Neurodegenerative disorders result from increased inflammatory responses and cytokine release in diabetes mellitus (DM) including NF-κB signalling and its activation of the NLRP3 inflammasome (Yang et al., 2014). The inflammasome facilitates caspase-1 activation and maturation of interleukin 1 β (IL-1β) cytokine (Grebe et al., 2018, de Zoete et al., 2014). The concept that the NLRP3 inflammasome is activated by pathways that culminate in metabolic stress is further supported by the crucial role of NLRP3 production in patients with type 2 DM (Masters et al., 2010, Lee et al., 2013). *In vivo* research shows that MF crosses the blood-brain barrier and accumulates in the brain (Łabuzek et al., 2010), where it exerts neuroprotective effects through regulating oxidative stress, inflammatory responses, and neuronal apoptosis (Ge et al., 2017, Liu et al., 2014, Zhang et al., 2017a). For instance, MF was found to inhibit proinflammatory IL-1β and NF-κB expression (Isoda et al., 2006), and provide neuroprotection through suppression of inflammation and apoptosis (Wang et al., 2016). The anti-inflammatory effects of MF have also been documented in diabetics (Lee et al., 2013). Sirtuin 1 (Sirt1) is known to maintain mitochondrial homeostasis and promote its function through its NAD^+-dependent protein deacetylase activity (Araki et al., 2004, Braidy et al., 2012). The physical interaction of Sirt1 and NF-κB inhibits its transcriptional activity through deacetylation of the RelA/p65 subunit at lysine 310 (Yeung et al., 2004). *In silico* analysis reveal the putative interactions between MF and Sirt1 through molecular docking (Cuyàs et al., 2018) thus, making it an important target for disease mechanisms. In addition, targeting the expression of NLRP3 inflammasome along with enhancing neuroprotective mechanisms may provide a tailored solution to attenuate diabetes induced neurodegeneration.

Epigenetics is a rapidly growing field in medical research. Metformin may influence the activity of numerous epigenetic modifying enzymes, mostly by modulating the activation of AMP-activated protein kinase (AMPK). Increased Sirt1 activity *in vitro* has been shown to prevent Aβ

peptides formation by increasing α-secretase activity (Qin et al., 2006) indicating its protective role in the brain. Additionally, the regulatory role of MF to increase Sirt1 in an AMPK manner has been demonstrated (Caton et al., 2011). The expression levels of numerous microRNAs are also reportedly influenced by MF treatment. Although they do not interact directly with DNA, microRNAs (miR) have epigenetic-like effects as they alter protein expression through the suppression of mRNA translation. Altered expression of numerous miRNAs has been shown to be extensively involved in the pathogenesis of various diseases including diabetes and its associated pathologies (Wang et al., 2014). There are five members that comprise the miR-200 family, of which are grouped into two independent transcriptional clusters: miR-200c and -141, located on 12p13, and miR-200a, -200b, and -429, located on chromosome 1p36 (Bracken et al., 2015). MiR-200c/141 is the predominant member of the miR-200 family, with numerous studies indicating their aberrant expressions in various cancers (Antolín et al., 2015, Dimri et al., 2016). Additionally, MF has been shown to inhibit tumourigenesis by increasing MiR-200c/141 expression (Zhang et al., 2017b). A clinical study has indicated that miR-141 is significantly upregulated in diabetic nephropathy (Li et al., 2019), and has been identified to play inflammatory roles in rodent brain tissue (Verma et al., 2018). Increased miR-141 levels have been correlated to decreased Sirt1 levels in a patient study, indicating its role in the development of insulin resistance (Nourbakhsh et al., 2018). These reports suggest that miR-141 may be involved in the development and progression of neurological diseases related to diabetes. Ours is the first study to demonstrate the effects of MF on miR-141 expression in diabetic brain tissue *in vivo,* attributing to its novelty.

Administration of streptozotocin (STZ), a glucosamine-nitrosourea compound has been shown to mimic diabetic conditions including oxidative stress, biochemical alterations, and neuroinflammation in rodent brain. This method of experimental hyperglycaemia provides a means to assess the eventual neurodegenerative states (Kamat, 2015). A study by Gao *et. al*. showed manifestation of sporadic AD through STZ-induced tau phosphorylation in mice brain (Gao et al., 2014). In this study, we explored the neuroprotective mechanism of MF through miR-141 modulation and its downstream implications on tau phosphorylation status and inflammation in an STZ mouse model.

1.2 Methods and materials

1.2.1 Animal care and treatment

C57BL/6 male mice and their littermates (6 weeks of age, (n=15, mean body weight (BW) 20 ± 2.99g) were acclimated to the animal research facilities (Biomedical Resource Unit from the University of KwaZulu-Natal (Westville Campus), Durban, South Africa) for 5 days. The

following investigations were conducted in accordance to Animal Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (Reference AREC/057/016) guidelines. were used to house the mice. C57BL/6 rodents (5 per cage) were housed in polycarbonated cages (40-60% humidity, 23 ± 1°C) with a 12 hr light dark cycle, a normal laboratory diet, and drinking water *ad libitum*.

All consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated. To determine optimal dosage, C57BL/6 mice were given STZ (Sigma Aldrich (S0130), St Louis, MO, USA) through a preliminary investigation including a range of concentrations (50 mg/kg, 100 mg/kg and 150 mg/kg BW) as per our previous protocol. DM was induced following an overnight fast (12 hr). Briefly, the compound was dissolved in a citrate buffer (pH 4.4) and injected intraperitoneally (50 mg/kg/d) within 15 min of dissolution. The control group received the vehicle: citrate buffer solution without STZ correspondingly.

Metformin hydrochloride (PHR1084) was made up to a final concentration of 20 mg/kg BW (Cho et al., 2015, Zou et al., 2004a) in 0.1M phosphate-buffered saline (PBS). Post diabetic stimulation, animals with random blood glucose values of 7-16mmol/L were defined as STZ-induced diabetic mice. The mice were then divided into three groups: 1. Control/ normal mice (C); 2. Streptozotocin (STZ) induced diabetic mice (HG control) which were fed PBS (vehicle control) during the treatment period; and 3. STZ diabetic mice treated with MF (20 mg/kg BW) via oral gavage once daily for the 15-day treatment period. Following treatment, mice were euthanised (isoflurane) and whole brain tissue was harvested. Samples were stored in Cytobuster (Novagen, Darmstadt, Germany) or Qiazol (Qiagen; Hildenburg, Germany) at -80°C for further analysis. (Qiagen; Hildenburg, Germany) at -80°C for downstream analysis.

1.2.2 Western blot analysis

Brain tissue samples were homogenized with ice-cold cell lysis buffer. The tissue homogenate was centrifuged (to remove debris) at 4°C for 5 min at 12,000 rpm, and the supernatant was transferred to a fresh tube. Protein concentration was determined using BCA protein assay kit (Thermo-Fisher Scientific). Equivalent amount of protein sample was loaded, and the western blotting procedure was carried out using an in-house protocol (Abdul et al., 2019). Protein expression was established by incubating membranes with primary antibodies (Table 1) at a 1:1000, 5% BSA dilution. Thereafter membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody [Anti rabbit IgG #7074, Anti-mouse IgG #7076, 1:10,000 in 5% BSA] for 1hr (RT). Densitometric protein measurements were normalised against house-keeping protein, HRP-conjugated anti-β-actin (CS1615, Sigma) for 1h at RT. Finally, the membrane was incubated with enhanced chemiluminescence reagents [Clarity western ECL substrate (Bio-Rad)] and exposed in a luminescence image analyser (Chemidoc™ imaging

system, Image Lab Software version 5.0, Bio-Rad) to detect the immunoreactive complex. Density of each blot was assessed and expressed as a relative fold change (RFC) ratio over β-actin.

Table 1: List of antibodies used

Primary antibody	**Cat. No.**
BDNF	AB1534
p-tau	5396 (PHF13)
NF-κB (p65)	D14E12
IκB-α	L35A5

1.2.3 RNA analysis

To evaluate the abundance of mRNA of interest in cell lysate, total RNA was extracted from homogenised brain tissue using Qiazol reagent (232 Qiagen, Germany) as per manufacturer's instructions and standardised (900 ng/µl). Total RNA was reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad, SA, cat. no. 1708891) following manufacturer's instructions. cDNA was quantified using iScript SYBR Green PCR kit (Bio-Rad) as per instructions. The following primers were used for qPCR amplification:

Table 2: Primer sequences used to determine gene expression profiles

Gene	**Primer sequence:** (5'-3')	**Annealing temperature (°C)**
PP2A	**F:** GTTCAAGAGGTTCGATGTCCAG	58
	R: AGCTACAAGCAGTGTAACTGTTT	
tau	**F:** TGGGGAACATTCCGTATGAGG	61.9
	R: CAGAAGCCATAACCCTTGGG	
TrkB	**F:** CGGCACATAATTTCACACG	58
	R: TTACCCGTCAGGATCAGGTC	
BDNF	**F:** AAACATCCGAGGACAAGGTG	58
	R: AGAAGAGGAGGCTCCAAAGG	
NF-κB	**F:** GAAATTCCTGATCCAGACAAAAA	57.5
	R: ATCACTTCAATGGCCTCTGTGTAG	
NLRP3	**F:** TGCTCTTCACTGCTATCAAGCCCT	61.9
	R: ACAAGCCTTTGCTCCAGACCCTAT	
IL-1β	**F:** ATGGCAACTGTTCCTGAACTCAACT	58.7

	R: CAGGACAGGTATAGATTCTTTCCTTT	
TNF-α	**F:** CCAACATGCTGATTGATGACACC	64.4
	R: GAGAATGCCAATTTTGATTGCCA	
Caspase-1	**F:** AATACAACCACTGGTACACGTC	58
	R: AGCTCCAACCCTCGGAGAAA	
GAPDH	**F:** AATGGATTTGGACGCATTGGT	variable
	R: TTTGCACTGGTACGTGTTGAT	

1.2.4 MicroRNA quantification and target prediction

Total RNA was reverse transcribed into cDNA using the miR-141 miScript primer assay kit (MS00001610, Qiagen). A 10µl reaction mix (5 × miScript HiSpec Buffer, miScript Reverse Transcriptase Mix 10 × miScript Nucleics Mix; Qiagen)] was prepared as per manufacturer's instructions. Human RNA U6 small nuclear 2 (RNU6-2) was used as the internal control. Amplification specificity was assessed by melting curve analysis and the relative miRNA levels in each sample were calculated using the $2^{-\Delta\Delta CT}$ method. The target of miR-141 was determined by pathway analysis using Target Scan V7.2. This database was utilised as no experimental data for this miR in mouse brain exists. The results were filtered for targets with highly predicted confidence for an interaction with miR-141. Among the multiple targets of miR-141, we determined that the 3' untranslated region (UTR) of *Sirt1* and *PP2A* were complimentary to its seed sequence.

1.2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Inc., La Jolla, USA). Five independent replicates were included in each data set. All data were presented as mean ± SD for comparison with control group when applicable. Statistical significance was determined with one-way analysis of variance (ANOVA) and the Bonferroni multiple comparison test. A difference was considered statistically significant when $p \leq 0.05$.

1.3 Results

1.3.1 MF influences *miR-141* and its targets, *Sirt1* and *PP2A*

To study the effects of MF on *miR-141* in diabetic brain tissue, we assessed mRNA expressions. We determined the 3′-UTR of Sirt1 and PP2A were complementary to miR-141 with Target Scan v7.2 (Fig 1A). Diabetic mice revealed increased *miR-141* (Fig 1A) expression, with associated decreases in *PP2A* (Fig 1B) expression in comparison to normal mice. However, *miR-141*

expression was significantly reduced by MF treatment in comparison to hyperglycaemic mice. This was accompanied by elevated PP2A gene expression as compared to control mice brain tissue. This suggests that *miR-141* decreases PP2A expression in diabetic mice brain tissue, which is reversed by treatment with MF.

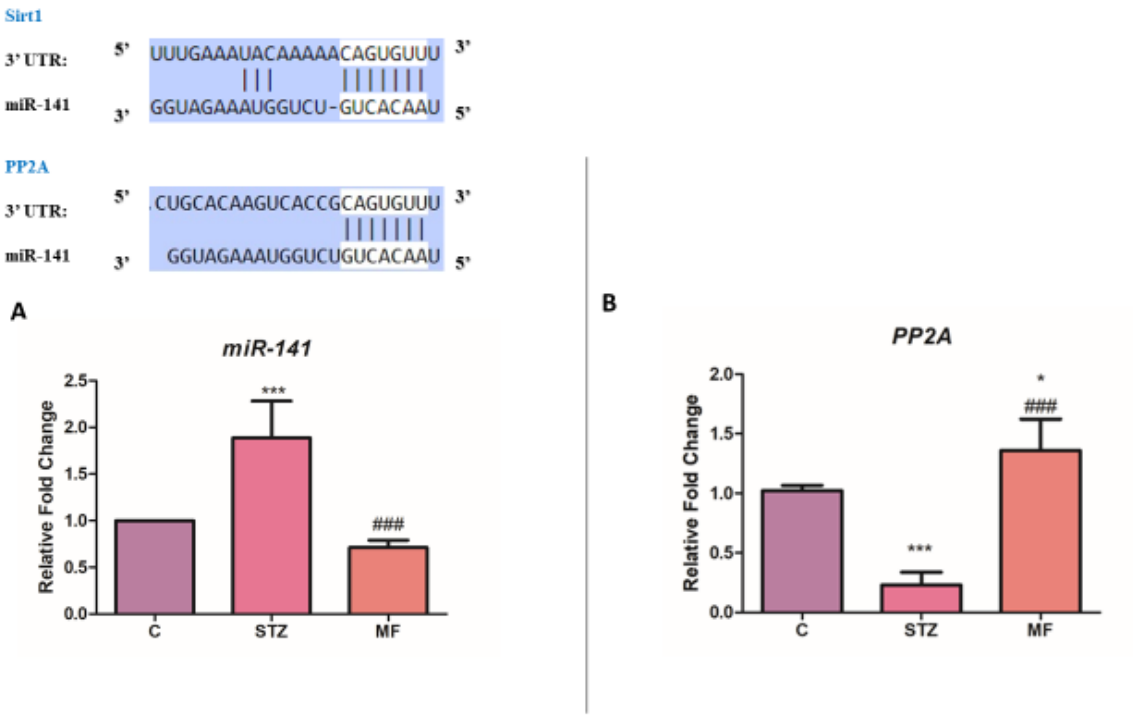


Figure 1: MF increases PP2A gene expression in a miR-141 regulatory manner. MF-treated mice exhibited reduced miR-141 expression (A: *** $p < 0.0001$ vs control, ### $p < 0.0001$ vs STZ) with concomitant increases in PP2A transcript levels (B: * $p < 0.05$, *** $p < 0.0001$ vs control; ### $p < 0.0001$ vs STZ) in diabetic mice (n=5).

1.3.2 **MF Induces Dephosphorylation of tau in Vivo**

Metformin has been demonstrated to be active in the brain after oral administration (Koenig et al., 2017). To determine if MF could mediate the dephosphorylation of tau, we assessed both protein and gene expressions in diabetic mice brain. Interestingly, tau protein was significantly dephosphorylated in the MF-treated mice brain tissue (Fig 2A). In agreement with this, expression of *tau* was also reduced in brain tissue of hyperglycaemic mice confirming the inhibitory effect of MF (Fig 2B).

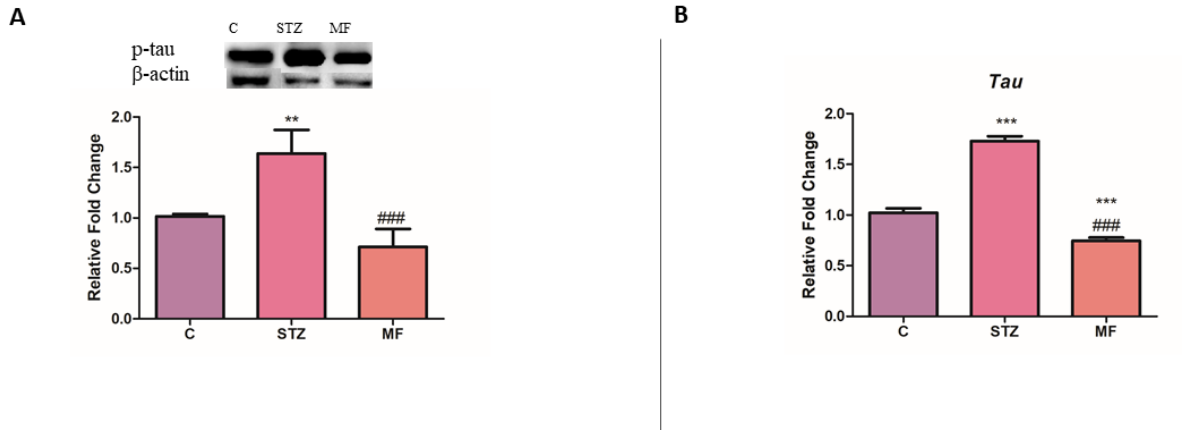


Figure 2: MF dephosphorylates tau in diabetic mice brain. Mice treated with STZ experienced tau hyperphosphorylation depicted by increased protein (A: ** $p < 0.005$ vs control, ### $p < 0.0001$ vs STZ) as well as gene (B: *** $p < 0.0001$ vs control, ### $p < 0.0001$ vs STZ) expression. MF effectively reduced both p-tau protein and its transcript levels following treatment in mice tissue (n=5).

1.3.3 BDNF and its receptor TrkB are modulated by MF

In order to explore effect of MF on level of the neurotrophic factors, we investigated BDNF expression. STZ-treatment decreased both protein and gene expressions in rodent brain tissue (Fig 3A and B), along with potent inhibition of *TrkB* receptor level (Fig 3C). Conversely, MF treatment increased BDNF protein, gene, and associated *TrkB* expressions in extracted diabetic mice brain tissue. This result indicates that the neuroprotection of MF was at least in part mediated by BDNF/TrkB upregulation.

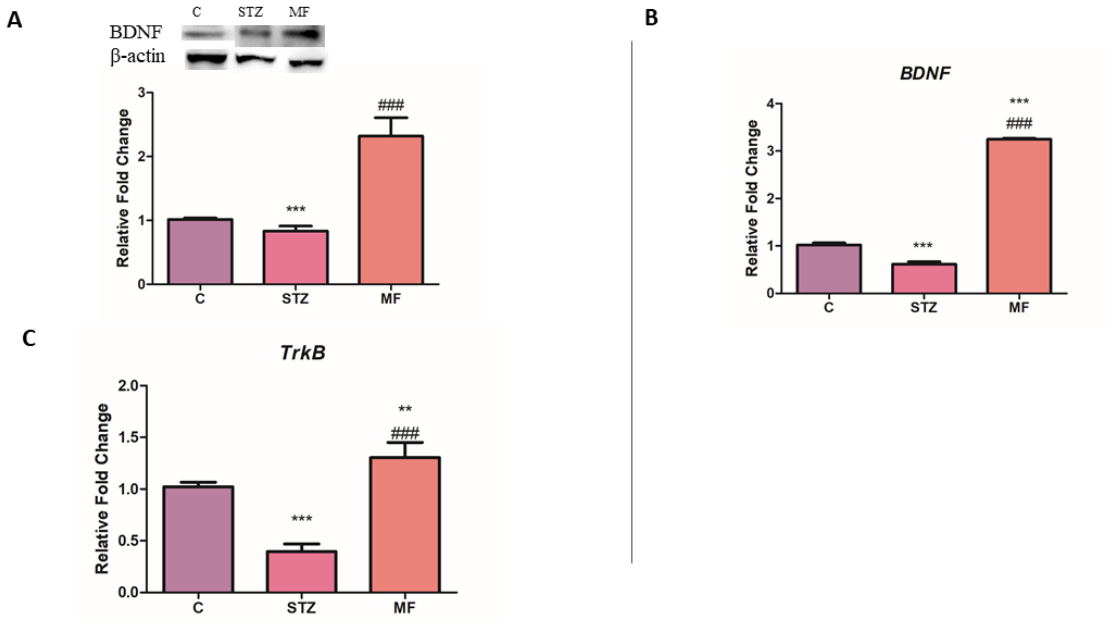


Figure 3: Effects of MF on BDNF and TrkB expressions. Protein expression (A: *** p< 0.0001 vs control, ### p< 0.0001 vs STZ) and transcript level (B: *** p< 0.0001 vs control, ### p< 0.0001 vs STZ) of BDNF was increased in diabetic mice treated with MF. The depleted levels of *TrkB* expression following diabetic induction was reversed in MF-treated mice (C: ** p < 0.005, *** p< 0.0001 vs control; ### p< 0.0001 vs STZ) (n=5).

1.3.4 NF-κB signalling and inflammasome-related transcript regulation

Previous research has demonstrated that PP2A reduces NF-κB activation in macrophages (Qadri et al., 2018), and Sirt1 inhibits its activity through direct inhibition of the p65 subunit (Yeung et al., 2004). We therefore investigated whether MF exerts it anti-inflammatory effects through NF-κB related inflammasome gene expressions in diabetic mice brain tissue as it is linked to NLRP3 activation (Luo et al., 2014). Our data showed that NF-κB protein and gene expression was increased in diabetic mice, whilst inhibitor of kappa B-α (IκB- α) kinase gene expression was depleted (Fig 4A-C). The downregulation of NF-κB protein (Fig 1A) and gene (Fig 1C) expression accompanied by upregulated IκB-α protein expression was mediated by MF treatment (Fig. 4A-C).

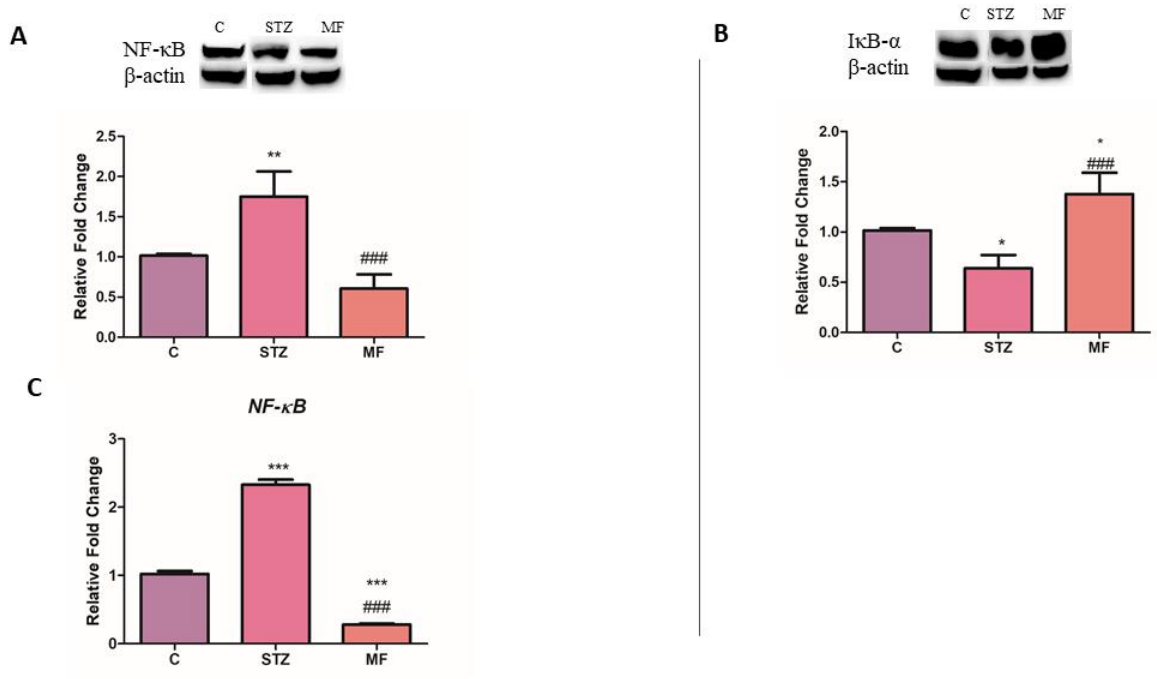


Figure 4: MF inhibited NF-κB signalling in an IκB- α -dependent manner. Diabetic mice exhibited decreased NF-κB protein (A: ** p< 0.005 vs control, ### p < 0.0001 vs STZ) and mRNA (C: *** p< 0.0001 vs control, ### p < 0.0001 vs STZ) expressions. Concomitant analysis of IκB-α (B: * p< 0.05 vs control, ### p < 0.0001 vs STZ) showed upregulated protein expression in diabetic mice brain treated with MF (n=5).

The function of PP2A as a potent inhibitor of NLRP3 inflammasome through NF-κB regulation has been elucidated (Haneklaus et al., 2017). Hence, we determined the expressions of related inflammasome components. MF significantly downregulated gene expressions of NLRP3 (Fig 5A), Caspase-1 (Fig 5B), IL-1β (Fig 5C), IL-18 (Fig 5D), and TNF-α (Fig 5E) in comparison to hyperglycaemic mice. Taken together, MF exerts potent anti-inflammatory action in diabetic mice brain tissue through inhibition of the inflammasome-related genes.

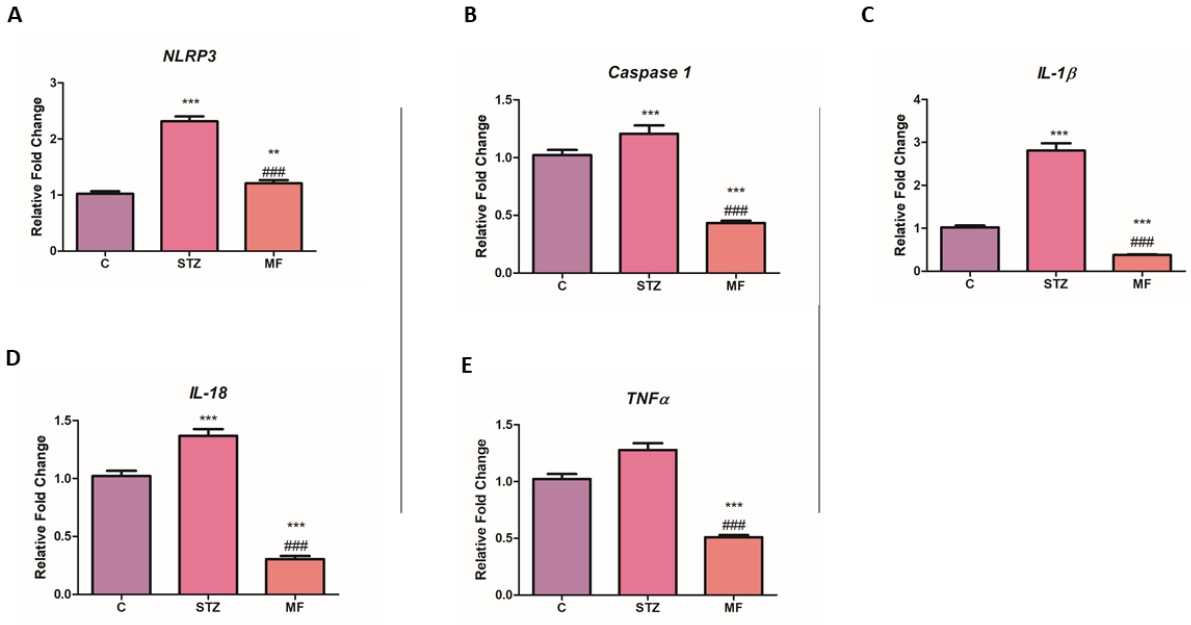


Figure 5: MF regulates NLRP3 inflammatory response in diabetic mice brain. The mRNA levels of *NLRP3* (A: ** p < 0.005, *** p< 0.0001 vs control; ### p < 0.0001 vs STZ) and associated *caspase-1* (B: *** p< 0.0001 vs control, ### p < 0.0001 vs STZ) were significantly decreased. Furthermore, cytokine gene expressions were downregulated in MF-treated mice (C-E: *** p< 0.0001 vs control, ### p < 0.0001 vs STZ) (n=5).

1.4 Discussion

To understand the mechanisms involved in diabetes-associated neurodegeneration, we established an animal model to mimic the disease. Experimentally, administration of STZ is a validated model for inducing a hyperglycaemic state with subsequent inflammation and degeneration in mice brain (Gao et al., 2014, Kamat, 2015). In line with earlier MF studies (Nikolakopoulou et al., 2018, Tahara et al., 2008), we show favourable outcomes on glucose tolerance following MF administration in STZ injected mice (Supp Fig 1). Therefore, we examined the potential of MF to

repress the expression of neurodegenerative markers and attenuate neuroinflammation in mice by modulating miR-141 expression.

MiRNAs regulate target genes directly through interactions with the conserved and target recognition elements, which leads to decreased transcript levels. The miR-200 family has been shown to regulate cellular proliferation and apoptosis, with majority of the findings involving cancer research (Takahashi et al., 2014, Wang et al., 2018). Direct regulation of miR-200 expression is facilitated by MF in stress-induced senescence (Cufí et al., 2012). Subdivision of the miR-200 family includes two clusters, miR-200a/b/429 and miR-200c/141 (Humphries and Yang, 2015), of which our study focused on the latter. Elevated expression of *miR-141* is correlated to insulin resistance in transgenic mouse models suggests a prominent role for inducing a diabetic phenotype (Belgardt et al., 2015) as well as neuroinflammation (Verma et al., 2018). Further, significantly increased levels of miR-141 was observed in patients with diabetic nephropathy (Li et al., 2019), a clear indication that this miR is involved in diabetic pathologies. Researchers have shown that MF has regulatory roles at the epigenetic level, particularly through miR modulation (Zhou et al., 2015). The seed sequence of miR-141 was found to be complementary to 3'UTR gene product of Sirt1 and PP2A (Fig 1A). Our experiments on miR-141 showed a persistent increase in its expression along with concomitant decreased *PP2A* in mice administered with STZ alone (Fig 1A) confirming the prediction target. Protein phosphorylation is an ATP-dependent process involving the covalent addition of phosphate groups to specific residues, allowing for protein modifications. Tau hyperphosphorylation specifically at Ser^{396}, promotes intraneuronal neurofibrillary tangles, a hallmark of neurodegenerative disease (Grundke-Iqbal et al., 1986), and further depletes ATP levels in diabetes. PP2A is an enzyme that promotes catalytic removal of phosphate groups from protein serine and threonine residues (Clark and Ohlmeyer, 2019). The phosphorylation of tau is maintained by equilibrium between kinases and phosphatases. The kinase GSK-3β functions to promote tau phosphorylation (Zhao et al., 2013) whilst PP2A inhibits this process through GSK-3β and subsequent tau dephosphorylation (Mitra et al., 2012). Insulin modulates tau phosphorylation by mediating GSK-3β activity (El Khoury et al., 2014), this explains the co-morbidity between DM and neuronal cognitive decline. The administration of STZ over an acute 10-day period (Planel et al., 2007) is reported to inhibit PP2A by insulin dysfunction, thus enhancing hyperphosphorylated tau levels (Clodfelder-Miller et al., 2006, Qu et al., 2011). In harmony with this, we demonstrated increased *tau* expression and subsequent phosphorylation at the protein level (Fig 2) following STZ injection, which is concomitant to PP2A inhibition (Fig 1B). Diabetic mice treated with MF displayed enhanced *PP2A* expression (Fig 1B) potentially through its ability to activate AMPK and restore ATP levels, hence dephosphorylating tau (Fig 2) in the brain. Metformin plays an essential role in alleviating diabetes associated oxidative stress through GSK-3β inhibition (Markowicz-Piasecka et al., 2017). Additionally, the formation of an active PP2A complex which then targets GSK-3β has been

demonstrated by MF treatment (Elgendy et al., 2019). Thus, forming a consensus to the mechanism of MF and reduced tau phosphorylation at Ser^{396} through kinase and phosphatase regulation in neuronal tissue of diabetic mice. In addition, the mechanism of Ca^{2+} ions inducing tau hyperphosphorylation has been reviewed (Wang, 2019). Metformin protects neuronal cells by modulating intracellular Ca^{2+} levels (Jang and Park, 2018), substantiating our decreased p-tau finding in diabetic mice brain tissue. Overall, we found that the neuroprotective effects of MF can be attributed to its influence on miR-141 regulation of *PP2A*, and subsequent dephosphorylation of tau which is a unique finding in terms of neuroprotection for this glucose-lowering agent.

The neurotrophin BDNF is negatively expressed in a diabetic state, rendering neurons of the brain vulnerable to diabetic insult. The ability of BDNF to regenerate neuronal circuits through regulating glucose metabolism is described (Fargali et al., 2012). In our study, STZ-treated mice displayed reduced BDNF protein and associated TrkB receptor gene expressions in brain tissue (Fig 3A-C), keeping with the findings of a clinical study that determined the number of cells expressing BDNF and TrkB mRNA was reduced in brain samples of diabetic patients (Bochukova et al., 2018, Zhen et al., 2013). Reduced BDNF expression is associated with tau hyperphosphorylation in neurodegenerative disorders. Following treatment with MF, diabetic mice revealed increased *TrkB* receptor, along with elevated protein and gene expression of BDNF (Fig 3A-C), potentially through an AMPK-mediated mechanism (Cho et al., 2015, Huang et al., 2015). Pre-treatment with MF has been shown to increase BDNF levels in hippocampal neurons (Ghadernezhad et al., 2016), supporting our finding. A study showed that exogenously introduced BDNF does not affect tau hyperphosphorylation (Jiao et al., 2016). Contrarily, *in vivo* treatment with BDNF provides a balance between tau phosphorylation through the co-regulation of GSK-3β and PP2A (Götz et al., 2010), correlating with our findings. These results suggest that MF promotes neuronal connectivity and neuroplasticity, which contributes to synaptic efficacy through BDNF upregulation and tau dephosphorylation.

Diabetic conditions are associated with increased microglial numbers and inflammation in the brain (Wanrooy et al., 2018, Wong et al., 2018). MF has proved efficient in alleviating chronic inflammation by direct action preclinically and clinically (Saisho, 2015). The most prevalent form of pro-inflammatory mediator, NF-κB, exists as a heterodimer composed of p50 and the active RelA/p65 polypeptides. Han *et. al*. observed BDNF and its receptor, TrkB, to prevent microglial activation and inhibit TNF-α and IL-6 inflammatory factors in STZ-treated mice thus mitigating the synaptic impairments experienced in hyperglycaemia through RelA/p65 NF-κB signalling (Han et al., 2019). Similarly, we show that MF prevents overexpression of the active subunit p65 NF-κB protein and gene expression (Fig 4), as well as *TNF-α* expression (Fig 5E) in STZ-treated mice and control mice; collaborating with the increased BDNF levels (Fig 3). The deacetylase activity of Sirt1 inhibits NF-κB activity at the RelA/p65 location (Yeung et al., 2004). We previously showed that MF upregulates Sirt1 expression in diabetic mice brain tissue (Supp Fig

2). In this study, we show that miR-141 also targets Sirt1 (Fig 1A), suggesting that MF regulates exerts its anti-inflammatory effect on NF-κB through an epigenetic mechanism. The IκB family sequesters the p65 NF-κB subunit in the cytoplasm and inhibits it in a normal setting (Vadapalli et al., 2018). Diabetes associated inflammation prevents this inhibition by activating the IκB kinase (IKK) which phosphorylates and blocks IκB protein activity (Ghosh and Karin, 2002). We show that MF treatment significantly increased IκB-α (Fig 4B) expression in diabetic mice brain tissue. Mechanistically, PP2A prevents sustained activation of the IKK complex, and readily dephosphorylates IκB-α, thereby mediating control of NF-κB transcription (Tsuchiya et al., 2017). We demonstrate that MF can regulate acute inflammation in diabetic brain homogenates through miR-141 inhibition, PP2A-mediated dephosphorylation and subsequent NF-κB regulation.

Furthermore, diabetes induced ROS formation promotes inflammatory processes through NF-κB activation of the NLRP3 inflammasome (An et al., 2019). This mediates pro-inflammatory cytokines IL-1β and IL-18 which are implicated in metabolic disorders (Fullerton et al., 2013, Masters et al., 2010, Nakamura et al., 2005). Thus, we assessed mRNA expression of inflammasome related genes *in vivo* to fully clarify the anti-inflammatory effects of MF at the transcriptional level in diabetic mice brain tissue. Our data suggested that MF alleviates the pro-inflammatory response to STZ injection by inhibiting *NLRP3* inflammasome expression (Fig 5A). The NLRP3 inflammasome activates caspase-1 which induces a proinflammatory state by cleaving of pro- IL-1β and IL-18 into mature IL-1β and IL-18 (Hong et al., 2019). Downregulation of *caspase-1*, *IL-1β*, *IL-18*, and *TNF-α* (Fig 5B-E) mRNA expression by MF treatment in diabetic mice brain tissue was mediated by its effect on the PP2A/NF-κB cascade. This finding is corroborated by a study showing the inhibitory effects of MF on NLRP3 inflammasome through AMPK regulation in a diabetic mouse cardiomyopathy model (Yang et al., 2019).

In conclusion, this study strongly demonstrates MF's neuroprotective effects by mediating PP2A dephosphorylation of tau protein through miR-141 inhibition over an acute time period. Additionally, the anti-inflammatory effects of MF are demonstrated by subsequent BDNF activation through its receptor TrkB, and inhibition of NF-κB mediated NLRP3 inflammasome-related transcripts. These findings provide novel insight on the potential of MF to alleviate neuroinflammation and associated neurodegeneration *in vivo* in a diabetic ambiance. Further research is warranted over longer time periods *in vivo* to clarify the mechanism of the anti-inflammatory effect of MF in diabetic-related neurodegeneration.

1.5 Conflict of interest

The authors declare that there are no conflicts of interest

1.6 Acknowledgements

Authors would like to thank the national research foundation (NRF) grant, South Africa (UID98379) and College of Health Sciences – University of KwaZulu-Natal for funding this study. We would also like to thank Dr N. Naicker, Dr. S. Baijnath, and Dr S. Singh for their assistance with obtaining mice tissue.

1.7 References

ABDUL, N. S., NAGIAH, S. & CHUTURGOON, A. A. 2019. Fusaric acid induces NRF2 as a cytoprotective response to prevent NLRP3 activation in the liver derived HepG2 cell line. *Toxicology in Vitro,* 55**,** 151-159.

AN, Y., ZHANG, H., WANG, C., JIAO, F., XU, H., WANG, X., LUAN, W., MA, F., NI, L. & TANG, X. 2019. Activation of ROS/MAPKs/NF-κB/NLRP3 and inhibition of efferocytosis in osteoclast-mediated diabetic osteoporosis. *The FASEB Journal***,** fj. 201802805RR.

ANTOLÍN, S., CALVO, L., BLANCO-CALVO, M., SANTIAGO, M. P., LORENZO-PATIÑO, M. J., HAZ-CONDE, M., SANTAMARINA, I., FIGUEROA, A., ANTÓN-APARICIO, L. M. & VALLADARES-AYERBES, M. 2015. Circulating miR-200c and miR-141 and outcomes in patients with breast cancer. *BMC cancer,* 15**,** 297-297.

ARAKI, T., SASAKI, Y. & MILBRANDT, J. 2004. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science,* 305**,** 1010-1013.

BELGARDT, B.-F., AHMED, K., SPRANGER, M., LATREILLE, M., DENZLER, R., KONDRATIUK, N., VON MEYENN, F., VILLENA, F. N., HERRMANNS, K. & BOSCO, D. 2015. The microRNA-200 family regulates pancreatic beta cell survival in type 2 diabetes. *Nature medicine,* 21**,** 619.

BOCHUKOVA, E. G., LAWLER, K., CROIZIER, S., KEOGH, J. M., PATEL, N., STROHBEHN, G., LO, K. K., HUMPHREY, J., HOKKEN-KOELEGA, A. & DAMEN, L. 2018. A transcriptomic signature of the hypothalamic response to fasting and BDNF deficiency in Prader-Willi syndrome. *Cell reports,* 22**,** 3401-3408.

BRACKEN, C. P., KHEW-GOODALL, Y. & GOODALL, G. J. 2015. Network-based approaches to understand the roles of miR-200 and other microRNAs in cancer. *Cancer research,* 75**,** 2594-2599.

BRAIDY, N., JAYASENA, T., POLJAK, A. & SACHDEV, P. S. 2012. Sirtuins in cognitive ageing and Alzheimer's disease. *Current opinion in psychiatry,* 25**,** 226-230.

CATON, P., KIESWICH, J., YAQOOB, M., HOLNESS, M. & SUGDEN, M. 2011. Metformin opposes impaired AMPK and SIRT1 function and deleterious changes in core clock protein expression in white adipose tissue of genetically-obese db/db mice. *Diabetes, Obesity and Metabolism,* 13**,** 1097-1104.

CHO, K., CHUNG, J. Y., CHO, S. K., SHIN, H.-W., JANG, I.-J., PARK, J.-W., YU, K.-S. & CHO, J.-Y. 2015. Antihyperglycemic mechanism of metformin occurs via the AMPK/LXRα/POMC pathway. *Scientific reports,* 5**,** 8145.

CLARK, A. R. & OHLMEYER, M. 2019. Protein phosphatase 2A as a therapeutic target in inflammation and neurodegeneration. *Pharmacology & therapeutics.*

CLODFELDER-MILLER, B. J., ZMIJEWSKA, A. A., JOHNSON, G. V. & JOPE, R. S. 2006. Tau is hyperphosphorylated at multiple sites in mouse brain in vivo after streptozotocin-induced insulin deficiency. *Diabetes,* 55**,** 3320-3325.

CUFÍ, S., VAZQUEZ-MARTIN, A., OLIVERAS-FERRAROS, C., QUIRANTES, R., SEGURA-CARRETERO, A., MICOL, V., JOVEN, J., BOSCH-BARRERA, J., DEL BARCO, S. & MARTIN-CASTILLO, B. 2012. Metformin lowers the threshold for stress-induced senescence: a role for the microRNA-200 family and miR-205. *Cell Cycle,* 11**,** 1235-1246.

CUYÀS, E., VERDURA, S., LLORACH-PARÉS, L., FERNÁNDEZ-ARROYO, S., JOVEN, J., MARTIN-CASTILLO, B., BOSCH-BARRERA, J., BRUNET, J., NONELL-CANALS, A. & SANCHEZ-MARTINEZ, M. 2018. Metformin is a direct SIRT1-activating compound: Computational modeling and experimental validation. *Frontiers in endocrinology,* 9**,** 657.

DE ZOETE, M. R., PALM, N. W., ZHU, S. & FLAVELL, R. A. 2014. Inflammasomes. *Cold Spring Harbor perspectives in biology,* 6**,** a016287.

DIMRI, M., KANG, M. & DIMRI, G. P. 2016. A miR-200c/141-BMI1 autoregulatory loop regulates oncogenic activity of BMI1 in cancer cells. *Oncotarget,* 7**,** 36220-36234.

EL KHOURY, N., GRATUZE, M., PAPON, M.-A., BRETTEVILLE, A. & PLANEL, E. 2014. Insulin dysfunction and Tau pathology. *Frontiers in cellular neuroscience,* 8**,** 22.

ELGENDY, M., CIRÒ, M., HOSSEINI, A., WEISZMANN, J., MAZZARELLA, L., FERRARI, E., CAZZOLI, R., CURIGLIANO, G., DECENSI, A. & BONANNI, B. 2019. Combination of Hypoglycemia and Metformin Impairs Tumor Metabolic Plasticity and Growth by Modulating the PP2A-GSK3β-MCL-1 Axis. *Cancer cell,* 35**,** 798-815. e5.

FARGALI, S., SADAHIRO, M., JIANG, C., FRICK, A. L., INDALL, T., COGLIANI, V., WELAGEN, J., LIN, W.-J. & SALTON, S. R. 2012. Role of neurotrophins in the development and function of neural circuits that regulate energy homeostasis. *Journal of Molecular Neuroscience,* 48**,** 654-659.

FRAME, S. & COHEN, P. 2001. The renaissance of GSK3. *Nat Rev Mol Cell Biol,* 2**,** 769-776.

FULLERTON, M. D., STEINBERG, G. R. & SCHERTZER, J. D. 2013. Immunometabolism of AMPK in insulin resistance and atherosclerosis. *Molecular and cellular endocrinology,* 366**,** 224-234.

GAO, C., LIU, Y., JIANG, Y., DING, J. & LI, L. 2014. Geniposide Ameliorates Learning Memory Deficits, Reduces Tau Phosphorylation and Decreases Apoptosis via GSK 3β Pathway in Streptozotocin-Induced A lzheimer Rat Model. *Brain pathology,* 24**,** 261-269.

GE, X.-H., ZHU, G.-J., GENG, D.-Q., ZHANG, H.-Z., HE, J.-M., GUO, A.-Z., MA, L.-L. & YU, D.-H. 2017. Metformin protects the brain against ischemia/reperfusion injury through PI3K/Akt1/JNK3 signaling pathways in rats. *Physiology & Behavior,* 170**,** 115-123.

GHADERNEZHAD, N., KHALAJ, L., PAZOKI-TOROUDI, H., MIRMASOUMI, M. & ASHABI, G. 2016. Metformin pretreatment enhanced learning and memory in cerebral forebrain ischaemia: the role of the AMPK/BDNF/P70SK signalling pathway. *Pharmaceutical biology,* 54**,** 2211-2219.

GHOSH, S. & KARIN, M. 2002. Missing pieces in the NF-κB puzzle. *cell,* 109**,** S81-S96.

GÖTZ, J., GLADBACH, A., PENNANEN, L., VAN EERSEL, J., SCHILD, A., DAVID, D. & ITTNER, L. M. 2010. Animal models reveal role for tau phosphorylation in human disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease,* 1802**,** 860-871.

GREBE, A., HOSS, F. & LATZ, E. 2018. NLRP3 Inflammasome and the IL-1 Pathway in Atherosclerosis. *Circulation research,* 122**,** 1722-1740.

GRUNDKE-IQBAL, I., IQBAL, K., TUNG, Y.-C., QUINLAN, M., WISNIEWSKI, H. M. & BINDER, L. I. 1986. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences,* 83**,** 4913-4917.

HAN, R., LIU, Z., SUN, N., LIU, S., LI, L., SHEN, Y., XIU, J. & XU, Q. 2019. BDNF Alleviates Neuroinflammation in the Hippocampus of Type 1 Diabetic Mice via Blocking the Aberrant HMGB1/RAGE/NF-κB Pathway. *Aging and disease,* 10**,** 611.

HANEKLAUS, M., O'NEIL, J. D., CLARK, A. R., MASTERS, S. L. & O'NEILL, L. A. 2017. The RNA-binding protein Tristetraprolin (TTP) is a critical negative regulator of the NLRP3 inflammasome. *Journal of Biological Chemistry,* 292**,** 6869-6881.

HONG, P., GU, R.-N., LI, F.-X., XIONG, X.-X., LIANG, W.-B., YOU, Z.-J. & ZHANG, H.-F. 2019. NLRP3 inflammasome as a potential treatment in ischemic stroke concomitant with diabetes. *Journal of neuroinflammation,* 16**,** 121.

HUANG, E. J. & REICHARDT, L. F. 2001. Neurotrophins: roles in neuronal development and function. *Annual review of neuroscience,* 24**,** 677-736.

HUANG, W., CAO, J., LIU, X., MENG, F., LI, M., CHEN, B. & ZHANG, J. 2015. AMPK plays a dual role in regulation of CREB/BDNF pathway in mouse primary hippocampal cells. *Journal of Molecular Neuroscience,* 56**,** 782-788.

HUMPHRIES, B. & YANG, C. 2015. The microRNA-200 family: small molecules with novel roles in cancer development, progression and therapy. *Oncotarget,* 6**,** 6472.

ISODA, K., YOUNG, J. L., ZIRLIK, A., MACFARLANE, L. A., TSUBOI, N., GERDES, N., SCHONBECK, U. & LIBBY, P. 2006. Metformin inhibits proinflammatory responses and nuclear factor-κB in human vascular wall cells. *Arteriosclerosis, thrombosis, and vascular biology,* 26**,** 611-617.

JANG, S. & PARK, S.-H. 2018. Antidiabetic drug metformin protects neuronal cells against quinolinic acid-induced excitotoxicity by decreasing intracellular calcium. *Chonnam medical journal,* 54**,** 24-30.

JIAO, S. S., SHEN, L. L., ZHU, C., BU, X. L., LIU, Y. H., LIU, C. H., YAO, X. Q., ZHANG, L. L., ZHOU, H. D., WALKER, D. G., TAN, J., GÖTZ, J., ZHOU, X. F. & WANG, Y. J. 2016. Brain-derived neurotrophic factor protects against tau-related neurodegeneration of Alzheimer's disease. *Translational psychiatry,* 6**,** e907-e907.

KAMAT, P. K. 2015. Streptozotocin induced Alzheimer's disease like changes and the underlying neural degeneration and regeneration mechanism. *Neural regeneration research,* 10**,** 1050.

KLEINRIDDERS, A., FERRIS, H. A., CAI, W. & KAHN, C. R. 2014. Insulin action in brain regulates systemic metabolism and brain function. *Diabetes,* 63**,** 2232-2243.

KOENIG, A. M., MECHANIC-HAMILTON, D., XIE, S. X., COMBS, M. F., CAPPOLA, A. R., XIE, L., DETRE, J. A., WOLK, D. A. & ARNOLD, S. E. 2017. Effects of the insulin sensitizer metformin in Alzheimer's disease: Pilot data from a randomized placebo-controlled crossover study. *Alzheimer disease and associated disorders,* 31**,** 107.

ŁABUZEK, K., LIBER, S., GABRYEL, B., ADAMCZYK, J. & OKOPIEŃ, B. 2010. Metformin increases phagocytosis and acidifies lysosomal/endosomal compartments in AMPK-dependent manner in rat primary microglia. *Naunyn-Schmiedeberg's archives of pharmacology,* 381**,** 171-186.

LEE, H.-M., KIM, J.-J., KIM, H. J., SHONG, M., KU, B. J. & JO, E.-K. 2013. Upregulated NLRP3 inflammasome activation in patients with type 2 diabetes. *Diabetes,* 62**,** 194-204.

LI, Y., HUANG, D., ZHENG, L., CAO, H. & FAN, Z. 2019. Effect of microRNA-141 on the development of diabetic nephropathy through regulating AKT/AMPK signaling pathway by targeting insulin receptor substrate 2. *Journal of cellular biochemistry,* 120**,** 8008-8015.

LIU, H., WANG, P., BIAN, Y., WANG, J. & WEI, Y. 2016. Effects of eye acupuncture therapy on neurological function and brain-derived neurotrophic factor expression in a rat model of cerebral ischemia/reperfusion injury. *Chinese Journal of Tissue Engineering Research,* 20**,** 2634-2641.

LIU, X.-H., GENG, Z., YAN, J., LI, T., CHEN, Q., ZHANG, Q.-Y. & CHEN, Z.-Y. 2015. Blocking GSK3β-mediated dynamin1 phosphorylation enhances BDNF-dependent TrkB endocytosis and the protective effects of BDNF in neuronal and mouse models of Alzheimer's disease. *Neurobiology of disease,* 74**,** 377-391.

LIU, Y., TANG, G., LI, Y., WANG, Y., CHEN, X., GU, X., ZHANG, Z., WANG, Y. & YANG, G.-Y. 2014. Metformin attenuates blood-brain barrier disruption in mice following middle cerebral artery occlusion. *Journal of Neuroinflammation,* 11**,** 177.

LUO, B., LI, B., WANG, W., LIU, X., XIA, Y., ZHANG, C., ZHANG, M., ZHANG, Y. & AN, F. 2014. NLRP3 gene silencing ameliorates diabetic cardiomyopathy in a type 2 diabetes rat model. *PloS one,* 9**,** e104771.

MARKOWICZ-PIASECKA, M., SIKORA, J., SZYDŁOWSKA, A., SKUPIEŃ, A., MIKICIUK-OLASIK, E. & HUTTUNEN, K. M. 2017. Metformin – a Future Therapy for Neurodegenerative Diseases. *Pharmaceutical Research,* 34**,** 2614-2627.

MASTERS, S. L., DUNNE, A., SUBRAMANIAN, S. L., HULL, R. L., TANNAHILL, G. M., SHARP, F. A., BECKER, C., FRANCHI, L., YOSHIHARA, E. & CHEN, Z. 2010. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β in type 2 diabetes. *Nature immunology,* 11**,** 897.

MITRA, A., MENEZES, M., PANNELL, L., MULEKAR, M., HONKANEN, R., SHEVDE, L. & SAMANT, R. 2012. DNAJB6 chaperones PP2A mediated dephosphorylation of GSK3β to downregulate β-catenin transcription target, osteopontin. *Oncogene,* 31**,** 4472.

MORRIS, M., MAEDA, S., VOSSEL, K. & MUCKE, L. 2011. The many faces of tau. *Neuron,* 70**,** 410-426.

NAKAMURA, A., SHIKATA, K., HIRAMATSU, M., NAKATOU, T., KITAMURA, T., WADA, J., ITOSHIMA, T. & MAKINO, H. 2005. Serum interleukin-18 levels are associated with nephropathy and atherosclerosis in Japanese patients with type 2 diabetes. *Diabetes care,* 28**,** 2890-2895.

NIKOLAKOPOULOU, P., CHATZIGEORGIOU, A., KOURTZELIS, I., TOUTOUNA, L., MASJKUR, J., ARPS-FORKER, C., POSER, S. W., ROZMAN, J., RATHKOLB, B. & AGUILAR-PIMENTEL, J. A. 2018. Streptozotocin-induced β-cell damage, high fat diet, and metformin administration regulate Hes3 expression in the adult mouse brain. *Scientific reports,* 8**,** 11335.

NOBLE, W., HANGER, D. P., MILLER, C. C. J. & LOVESTONE, S. 2013. The importance of tau phosphorylation for neurodegenerative diseases. *Frontiers in neurology,* 4**,** 83-83.

NOBLE, W., PLANEL, E., ZEHR, C., OLM, V., MEYERSON, J., SULEMAN, F., GAYNOR, K., WANG, L., LAFRANCOIS, J. & FEINSTEIN, B. 2005. Inhibition of glycogen synthase kinase-3 by lithium correlates with reduced tauopathy and degeneration in vivo. *Proceedings of the National Academy of Sciences,* 102**,** 6990-6995.

NOURBAKHSH, M., YOUSEFI, Z., NIKROO, N. D., MALEK, M., PAZOUKI, A. & MOKHBER, S. MicroRNA-141 directly targets and inhibits sirtuins 1 gene expression and its elevation in obese subjects is responsible for reduced levels of sirtuin1 and the subsequent hepatic steatosis and insulin resistance. HORMONE RESEARCH IN

PAEDIATRICS, 2018. KARGER ALLSCHWILERSTRASSE 10, CH-4009 BASEL, SWITZERLAND, 46-47.

PLANEL, E., TATEBAYASHI, Y., MIYASAKA, T., LIU, L., WANG, L., HERMAN, M., YU, W. H., LUCHSINGER, J. A., WADZINSKI, B. & DUFF, K. E. 2007. Insulin dysfunction induces in vivo tau hyperphosphorylation through distinct mechanisms. *Journal of Neuroscience,* 27**,** 13635-13648.

QADRI, M., ALMADANI, S., JAY, G. D. & ELSAID, K. A. 2018. Role of CD44 in regulating TLR2 activation of human macrophages and downstream expression of proinflammatory cytokines. *The Journal of Immunology,* 200**,** 758-767.

QIN, W., YANG, T., HO, L., ZHAO, Z., WANG, J., CHEN, L., ZHAO, W., THIYAGARAJAN, M., MACGROGAN, D. & RODGERS, J. T. 2006. Neuronal SIRT1 activation as a novel mechanism underlying the prevention of Alzheimer disease amyloid neuropathology by calorie restriction. *Journal of Biological Chemistry,* 281**,** 21745-21754.

QU, Z., JIAO, Z., SUN, X., ZHAO, Y., REN, J. & XU, G. 2011. Effects of streptozotocin-induced diabetes on tau phosphorylation in the rat brain. *Brain research,* 1383**,** 300-306.

ROSA, E., MAHENDRAM, S., KE, Y. D., ITTNER, L. M., GINSBERG, S. D. & FAHNESTOCK, M. 2016. Tau downregulates BDNF expression in animal and cellular models of Alzheimer's disease. *Neurobiology of aging,* 48**,** 135-142.

SAISHO, Y. 2015. Metformin and inflammation: its potential beyond glucose-lowering effect. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders),* 15**,** 196-205.

SAMPAIO, T. B., SAVALL, A. S., GUTIERREZ, M. E. Z. & PINTON, S. 2017. Neurotrophic factors in Alzheimer's and Parkinson's diseases: implications for pathogenesis and therapy. *Neural regeneration research,* 12**,** 549.

TAHARA, A., MATSUYAMA-YOKONO, A., NAKANO, R., SOMEYA, Y. & SHIBASAKI, M. 2008. Hypoglycaemic effects of antidiabetic drugs in streptozotocin-nicotinamide-induced mildly diabetic and streptozotocin-induced severely diabetic rats. *Basic & clinical pharmacology & toxicology,* 103**,** 560-568.

TAKAHASHI, R.-U., MIYAZAKI, H. & OCHIYA, T. 2014. The role of microRNAs in the regulation of cancer stem cells. *Frontiers in genetics,* 4**,** 295.

TSUCHIYA, Y., OSAKI, K., KANAMOTO, M., NAKAO, Y., TAKAHASHI, E., HIGUCHI, T. & KAMATA, H. 2017. Distinct B subunits of PP 2A regulate the NF-κB signalling pathway through dephosphorylation of IKK β, IκBα and RelA. *FEBS letters,* 591**,** 4083-4094.

VADAPALLI, J., VANAM, A., MOTOHASHI, N. & GOLLAPUDI, R. 2018. Chronic Inflammation: Prospective Prevention and/or Control by the Regulation of Nuclear Factor Kappa B with Natural Products as Dietary Supplements. *Journal of Community and Preventive Medicine• Vol,* 1**,** 1.

VERMA, R., RITZEL, R. M., HARRIS, N. M., LEE, J., KIM, T., PANDI, G., VEMUGANTI, R. & MCCULLOUGH, L. D. 2018. Inhibition of miR-141-3p Ameliorates the Negative Effects of Poststroke Social Isolation in Aged Mice. *Stroke,* 49**,** 1701-1707.

WANG, C., JI, B., CHENG, B., CHEN, J. & BAI, B. 2014. Neuroprotection of microRNA in neurological disorders. *Biomedical reports,* 2**,** 611-619.

WANG, C., LIU, C., GAO, K., ZHAO, H., ZHOU, Z., SHEN, Z., GUO, Y., LI, Z., YAO, T. & MEI, X. 2016. Metformin preconditioning provide neuroprotection through enhancement of autophagy and suppression of inflammation and apoptosis after spinal cord injury. *Biochemical and biophysical research communications,* 477**,** 534-540.

WANG, P. 2019. Calcium Ions Stimulate the Hyperphosphorylation of Tau by Activating Microsomal Prostaglandin E Synthase 1. *Frontiers in aging neuroscience,* 11**,** 108.

WANG, Y., WU, Z. & HU, L. 2018. The regulatory effects of metformin on the [SNAIL/miR-34]:[ZEB/miR-200] system in the epithelial-mesenchymal transition(EMT) for colorectal cancer(CRC). *European Journal of Pharmacology,* 834**,** 45-53.

WANROOY, B. J., KUMAR, K. P., WEN, S. W., QIN, C. X., RITCHIE, R. H. & WONG, C. H. 2018. Distinct contributions of hyperglycemia and high-fat feeding in metabolic syndrome-induced neuroinflammation. *Journal of neuroinflammation,* 15**,** 293.

WHITTINGTON, R. A., BRETTEVILLE, A., DICKLER, M. F. & PLANEL, E. 2013. Anesthesia and tau pathology. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 47**,** 147-155.

WONG, C. H., WANROOY, B. J. & BRUCE, D. G. 2018. Neuroinflammation, Type 2 Diabetes, and Dementia. *Type 2 Diabetes and Dementia*. Elsevier.

XIONG, Y., JING, X.-P., ZHOU, X.-W., WANG, X.-L., YANG, Y., SUN, X.-Y., QIU, M., CAO, F.-Y., LU, Y.-M. & LIU, R. 2013. Zinc induces protein phosphatase 2A inactivation and tau hyperphosphorylation through Src dependent PP2A (tyrosine 307) phosphorylation. *Neurobiology of aging,* 34**,** 745-756.

YANG, F., QIN, Y., WANG, Y., MENG, S., XIAN, H., CHE, H., LV, J., LI, Y., YU, Y. & BAI, Y. 2019. Metformin Inhibits the NLRP3 Inflammasome via AMPK/mTOR-dependent Effects in Diabetic Cardiomyopathy. *International Journal of Biological Sciences,* 15**,** 1010.

YANG, F., WANG, Z., WEI, X., HAN, H., MENG, X., ZHANG, Y., SHI, W., LI, F., XIN, T. & PANG, Q. 2014. NLRP3 deficiency ameliorates neurovascular damage in experimental ischemic stroke. *Journal of Cerebral Blood Flow & Metabolism,* 34**,** 660-667.

YE, S., WANG, T.-T., CAI, B., WANG, Y., LI, J., ZHAN, J.-X. & SHEN, G.-M. 2017. Genistein protects hippocampal neurons against injury by regulating calcium/calmodulin dependent protein kinase IV protein levels in Alzheimer's disease model rats. *Neural regeneration research,* 12**,** 1479.

YEUNG, F., HOBERG, J. E., RAMSEY, C. S., KELLER, M. D., JONES, D. R., FRYE, R. A. & MAYO, M. W. 2004. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *The EMBO journal,* 23**,** 2369-2380.

ZHANG, D., XUAN, J., ZHENG, B.-B., ZHOU, Y.-L., LIN, Y., WU, Y.-S., ZHOU, Y.-F., HUANG, Y.-X., WANG, Q. & SHEN, L.-Y. 2017a. Metformin improves functional recovery after spinal cord injury via autophagy flux stimulation. *Molecular neurobiology,* 54**,** 3327-3341.

ZHANG, J., LI, G., CHEN, Y., FANG, L., GUAN, C., BAI, F., MA, M., LYU, J. & MENG, Q. H. 2017b. Metformin inhibits tumorigenesis and tumor growth of breast cancer cells by upregulating miR-200c but downregulating AKT2 expression. *Journal of Cancer,* 8**,** 1849.

ZHANG, S.-G., WANG, X.-S., ZHANG, Y.-D., DI, Q., SHI, J.-P., QIAN, M., XU, L.-G., LIN, X.-J. & LU, J. 2016. Indirubin-3′-monoxime suppresses amyloid-beta-induced apoptosis by inhibiting tau hyperphosphorylation. *Neural regeneration research,* 11**,** 988.

ZHAO, H.-H., DI, J., LIU, W.-S., LIU, H.-L., LAI, H. & LÜ, Y.-L. 2013. Involvement of GSK3 and PP2A in ginsenoside Rb1's attenuation of aluminum-induced tau hyperphosphorylation. *Behavioural brain research,* 241**,** 228-234.

ZHEN, Y. F., ZHANG, J., LIU, X. Y., FANG, H., TIAN, L. B., ZHOU, D. H., KOSTEN, T. R. & ZHANG, X. Y. 2013. Low BDNF is associated with cognitive deficits in patients with type 2 diabetes. *Psychopharmacology,* 227**,** 93-100.

ZHOU, J. Y., XU, B. & LI, L. 2015. A New Role for an Old Drug: Metformin Targets Micro RNA s in Treating Diabetes and Cancer. *Drug development research,* 76**,** 263-269.

ZHOU, X.-W., GUSTAFSSON, J.-Å., TANILA, H., BJORKDAHL, C., LIU, R., WINBLAD, B. & PEI, J.-J. 2008. Tau hyperphosphorylation correlates with reduced methylation of protein phosphatase 2A. *Neurobiology of disease,* 31**,** 386-394.

ZOU, M.-H., KIRKPATRICK, S. S., DAVIS, B. J., NELSON, J. S., WILES, W. G., SCHLATTNER, U., NEUMANN, D., BROWNLEE, M., FREEMAN, M. B. & GOLDMAN, M. H. 2004. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo role of mitochondrial reactive nitrogen species. *Journal of Biological Chemistry,* 279**,** 43940-43951.

CHAPTER 5

Metformin regulates renal stress by Sirtuin 1 signalling axes and prevents intrinsic cell death in diabetic mice

In addition to the brain, another major organ that is affected by high blood glucose is the kidney. These two organs are involved in energy metabolism and are linked through the vascular system. Diabetic injury to the brain negatively affects kidney function by inducing inflammatory processes. The renal system has gluconeogenetic roles making it prone to hyperglycaemic injury. In this chapter, we outline the protective role of MF in diabetic mice through Sirt1 regulation of mt metabolism linked to ER stress and nephropathy.

This manuscript was submitted to *Journal of Cellular Physiology* (Manuscript reference: JCP-19-5700) and is presented in this thesis as per journal formatting requirements.

Metformin regulates renal stress by Sirtuin 1 signalling axes and prevents intrinsic cell death in diabetic mice

Taskeen Fathima Docrat, Savania Nagiah, Anil A. Chuturgoon

Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, South Africa

*Address correspondence to:

Prof Anil Chuturgoon, Discipline of Medical Biochemistry, Faculty of Health Sciences, George Campbell Building, Howard College, University of KwaZulu-Natal, Durban, 4041, South Africa. Telephone: (031) 260 4404. Email: chutur@ukzn.ac.za

Abstract

Diabetic nephropathy is considered as the leading cause of end-stage renal disease. Hyperglycaemia contributes to oxidative stress and pseudohypoxia which play prominent roles in the progression of this condition. The NAD^+ dependent deacetylase, Sirtuin 1 (Sirt1) has been established as an energy sensor and key regulator of cell defence mechanisms that may prevent renal cell damage and apoptosis. Therefore, we investigated the potential protective effects of metformin (MF) through modulation of Sirt1 mediated pathways and apoptosis in Streptozotocin (STZ)-induced diabetic mice kidney. Diabetic mice were treated with MF (20 mg/kg BW), and whole kidney tissue was harvested for further analysis. Protein carbonylation was assessed as a marker of oxidative stress in whole kidney lysates. Western blot and qPCR experiments were conducted to determine the effect of MF on Sirt1 and modulation of its downstream targets. We examined the ability of MF to disrupt the intrinsic apoptotic pathway as an end stage contributor to DN. Metformin inhibited oxidative damage to proteins by up-regulation of an anti-oxidant response. We further established that MF positively regulates the SIRT1/AMPK/PGC1a activation loop suggesting enhanced mitochondrial activity and improved metabolic homeostasis. Additionally, MF dampened ER stress, demonstrated by decreased CHOP protein expression along with concomitant decrease to *eIF2α* and *PERK*. Metformin was also shown to suppress intrinsic apoptosis by inhibiting the expression of Bax and *cyt-c*. Herein we show that the protective effect of MF is closely tied to the enhanced expression of Sirt1 in the diabetic kidney. Mechanistically, Sirt1 is at the forefront of modulating cell defence to metabolic stress and associated pathologic outcomes, particularly in DN and represents a therapeutic target for MF.

Keywords:

Metformin, Diabetes, Kidney injury, Sirt1, ER stress, Apoptosis

1. Introduction

Diabetes mellitus (DM) is characterised by hyperglycaemia which results in glycation of macromolecules and associated organ damage (Fowler, 2008). The kidney requires high levels of ATP to function and is rich in mitochondria. Endogenous glucose promotes ATP production through mitochondrial (mt) oxidative phosphorylation. In a diabetic ambiance, kidney cells are incapable of adequate regulation of intracellular glucose and are subjected to extreme oxidative distress mediated by reactive oxygen species (ROS) (Sifuentes-Franco et al., 2018). Diabetic nephropathy (DN) and associated metabolic dysregulation often triggers oxidative, mt, and endoplasmic reticulum (ER) stress-related responses (Sifuentes-Franco et al., 2018). This contributes to changes in intracellular signalling cascades promoting apoptotic cell death and organ damage (Khanra et al., 2015).

The mitochondrion is integral to mediating signal transduction cascades and altered gene expression profiles associated with chronic over-production of ROS as a secondary signalling messenger (Hensley et al., 2000). With overwhelming increases in the production of ROS, antioxidant defence systems are easily exhausted, promoting mt dysfunction and exacerbating ROS production (Niedowicz and Daleke, 2005). The increased cytosolic ratio of free NAD^+ to NADH in cells, caused by hyperglycaemia has been termed pseudohypoxia and maybe seen as a critical driver of DM pathology (Williamson et al., 1993). The presence of a hypoxia inducible component in the transcriptome in response to hyperglycaemia has been observed and an overlap with ROS and aberrant metabolism proven.

Pseudohypoxia and ROS regulate the expression and activity of Sirt1 (Gomes et al., 2013, Zheng et al., 2012). This deacetylase enzyme is regarded as a metabolic sensor (dependence on NAD^+) tasked with maintaining genome stability and anti-oxidant responses. Previous studies have reported that Sirt1 mediates a wide range of cellular responses through its deacetylation activity targeting numerous transcription factors such as p53, nuclear factor-κB (NF-κB), HIF-1α and peroxisome proliferator-activated receptor gamma coactivator-1α (PGC)-1α (Ryu et al., 2019). Sirtuin 1 bridges the transcriptome to cell defence responses and is recognised as a survival factor.

Sirt1 activation has been shown to inhibit hyperglycaemia-induced apoptosis by reducing oxidative stress and mt dysfunction (Wang et al., 2017) Thus, a pseudohypoxic state that disrupts

Sirt1 function and expression contributes to the decline in mt function and cell damage. Further mechanistic studies revealed elevated apoptosis and caspase activation following hypoxic injury accompanied by mt mediated intrinsic apoptosis through Bax accumulation and cytochrome-c (cyt-c) release (Allison, 2014). The molecular mechanisms involved in cell defence have gained interest due to the societal impact of diabetes.

Metformin acts to suppress hyperglycaemia and hepatic gluconeogenesis by activation of AMPK signalling. Additionally, this biguanide inhibits complex 1 of the mitochondria with consequent compromised ATP and AMP homeostasis as well as inhibition of mt glycerophosphate dehydrogenase, thereby attenuating transfer of reducing equivalents from the cytoplasm to mitochondria. As a result, raised lactate/pyruvate ratio and redox-dependent inhibition of gluconeogenesis occurs from reduced but not oxidized substrates. Taken together these mechanisms suggest a profound effect on the redox potential of the cell (NAD:NADH ratio) which can directly affect Sirt1 expression and modulate associated cell survival mechanisms. Given that both MF and Sirt1 play important roles in the cellular response to hyperglycaemic stress and redox potential, we propose that MF can prevent renal damage in a diabetic state by alleviating mt stress and the intrinsic apoptotic pathway.

2. Methods and Materials

3.1 Materials

Treatments including MF hydrochloride (PHR1084) and STZ (S0130) were purchased from Sigma Aldrich (St Louis, MO, USA). All other consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

3.2 Animals and induction of diabetes

This study used male mice of the C57BL/6 strain at 6-weeks-old [(n=15, mean body weight (BW) 20 ± 2.99g)] from the Biomedical Resource Unit from the University of KwaZulu-Natal (Westville Campus), Durban, South Africa. Mouse feed included a standard laboratory diet, normal drinking water *ad libitum* throughout the experimental period. All experimental procedures were performed in accordance to the Animal Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (Reference AREC/057/016).

The optimal dosage for STZ treatment was conducted through a preliminary investigation including a range of concentrations (50 mg/kg, 100 mg/kg and 150 mg/kg BW). DM was induced following an overnight fast (12 hr) by a single intraperitoneal administration of 50 mg/kg of STZ which was dissolved in sodium citrate buffer at pH 4.4. Control animals were injected with the vehicle (sodium citrate buffer, pH 4.2-4.5). Blood glucose levels were monitored using a

glucometer (Accu-Chek®), where levels of 7-16mmol/L were considered T2 diabetic. The treatment period was then inducted.

3.3 Treatments

MF was prepared in 0.1M phosphate-buffered saline (PBS) and filter sterilized (0.45-µm filter), to a final concentration of 20 mg/kg BW based on previous animal studies (Cho et al., 2015, Zou et al., 2004b). Random division of mice was performed into 3 groups of 5 mice per group (n=5). Group 1: control (C) normal mice, Group 2: Streptozotocin-induced T2 diabetic mice (HG control) and were fed PBS (vehicle control) during the treatment period. Group 3: STZ diabetic mice treated with MF (20 mg/kg BW) via oral gavage once daily for the 15-day treatment period. Polycarbonated cages (40-60% humidity, 23 ± 1^{o}C) with a 12 hr light dark cycle were used to house the mice.

Post treatment, mice were euthanised (isoflurane) and whole kidney tissue was harvested. Samples were stored in Cytobuster (Novagen, Darmstadt, Germany) or Qiazol (Qiagen; Hildenburg, Germany) at -80^{o}C for downstream analysis.

3.4 Protein Carbonyl content

Carbonylation of protein causing oxidative damage was measured in whole kidney tissue of diabetic mice through the reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) as per protocol described by Levine *et. al*., (1994) (Levine et al., 1994). A blank was prepared by treatment with 2.5M HCl for all treatments. Mice samples were plated in triplicate (100µl/well) and the maximum absorbance (370nm) was used to determine the carbonyl content (Augustyniak et al., 2015).

3.5 Western blot analysis

Kidney protein was extracted in cell lysis buffer. The extract was centrifuged (12,000 rpm, 5min, 4°C) to remove debris. Total protein concentration was determined using the bicinchoninic acid (BCA) protein assay. Western blotting was performed using an in house protocol (Nagiah et al., 2016). To determine protein expression, membranes were incubated with primary antibodies (Table 1) at a 1:1000, 5% BSA dilution. Thereafter membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody [Anti rabbit IgG #7074, Anti-mouse IgG #7076, 1:10,000 in 5% BSA] for 1hr (RT). Densitometric protein measurements were normalised against house-keeping protein, HRP-conjugated anti-β-actin (CS1615, Sigma) for 1h at RT. Chemiluminescence [Clarity western ECL substrate (Bio-Rad)] was used to detect protein bands followed by image detection (Chemidoc™ imaging system, Bio-Rad). The expression of protein

was established using Image Lab Software version 5.0 (Bio-Rad). Proteins of interest are expressed as a relative fold change (RFC) ratio over β-actin.

Table 1: List of antibodies used.

Primary antibody	**Cat. No.**
p-AMPK	T172
AMPK	2532
Sirt1	ab32441
PARP1	9542P
CHOP	L63F7
p38	ab4822
NF-κB	D14E12
Mdm2	m4328
p53	SC6243
p21	p1484
Bax	BD610982
Caspase-9	9502P
Caspase-3	9662P

3.6 RNA analysis

Total RNA was isolated from homogenised kidney tissue using Qiazol reagent (232 Qiagen, Germany) as per manufacturer's instructions and standardised (1000 ng/µl). Total RNA (1µl) was reverse transcribed in a 15µl reaction using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time quantitative PCR analyses were performed using iScript SYBR Green PCR kit (Bio-Rad) as per instructions. The following primers were used for qPCR amplification.

Table 2: Primer sequences used to determine gene expression profiles

Gene	**Primer sequence:** (5'-3')	**Annealing temperature (^{o} C)**
Nrf2	**F:** CTTTAGTCAGCGACAGAAGGAC	58

	R: AGGCATCTTGTTTGGGAATGTG	
GPx	**F:** GGGACTACACCGAGATGAACGA	57
	R: ACCATTCACTTGGCACTTCTCA	
PGC-1α	**F:** GCAACATGCTCAAGCCAAAC	58
	R: TGCAGTTCCAGAGAGAGTTCCA	
Sirt1	**F:** CAGCCGTCTCTGTGTCACAAA	57
	R: GCACCGAGGAACTACCTGAT	
HIF-1	**F:** GTCCCAGCTACGAAGTTACAGC	67
	R: CAGTGCAGGATACACAAGGTTT	
PERK	**F**: TGCTGAGGCTAGATGAAACCA	59
	R: GCACTTTAGATGGACGAATGC	
eIF2α	**F:** ATCTTGTCCTCAACCTCAGACT	58
	R: TTCTTTAGCCTGGCTTTCTTTCA	
Chop	**F:** AAGCCTGGTATGAGGATCTGC	59
	R: TTCCTGGGGATGAGATATAGGTG	
NF-κB	**F:** GAAATTCCTGATCCAGACAAAAA	54
	R: ATCACTTCAATGGCCTCTGTGTAG	
TNF- α	**F:** CCAACATGCTGATTGATGACACC	58
	R: GAGAATGCCAATTTTGATTGCCA	
P53	**F:** GGGCCCGTGTTGGTTCATCC	60
	R: CCGCGAGACTCCTGGCACAA	
Cyt-c	**F:** CCCATCTTTGAGCATCTTGGT	57
	R: GCCCAGCCTGAGTAGTGAAG	
GAPDH	**F:** AATGGATTTGGACGCATTGGT	variable
	R: TTTGCACTGGTACGTGTTGAT	

3.7 Statistical Analysis

Data are expressed as means ± SDs. Statistical significance was determined with one-way analysis of variance (ANOVA) and the Bonferroni multiple comparison test. All data were analysed with GraphPad software (Inc., La Jolla, USA), and $p \leq 0.05$ was considered as significant ($n = 5$).

4. Results

4.1 Effects of MF on STZ-induced oxidative stress related parameters

In the present study, the kidney of diabetic mice showed a significant increase in protein carbonyl content with paralleled decreases in antioxidant *Nrf2* and glutathione peroxidase 1 (*GPx)* expression (Fig. 1A) as compared to the control group. However, treatment with MF for 15 days significantly decreased protein carbonyl levels in the kidney of diabetic mice, with significant upregulation of antioxidant genes after treatment with MF (Fig. 1B and C). Reversal of these oxidative distress related parameters suggests that MF is a good antioxidant agent that protects mice kidney from diabetes-induced oxidative damage.

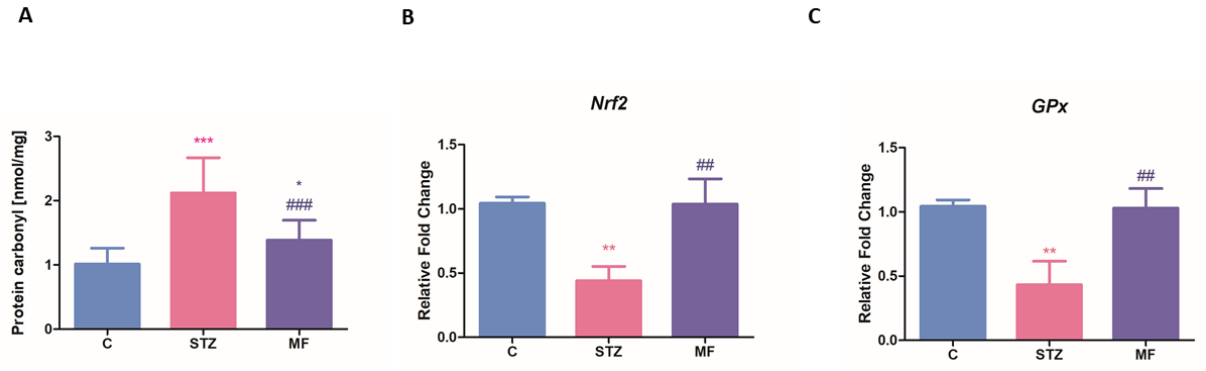


Figure 1: MF mitigates oxidative stress in the kidney of diabetic mice. MF reduced protein carbonylation (n=5), (A: *p < 0.05, ***p < 0.0001 vs control, ### p < 0.0001 vs STZ), with concomitant increases in antioxidant Nrf2 (B: **p < 0.005 vs control, ### p < 0.0001 vs STZ), and GPx (C: **p < 0.001 vs control, ### p < 0.0001 vs STZ) gene expression in diabetic mice.

4.2 MF increases PGC-1α gene expression, in parallel with AMPK phosphorylation

We examined changes in AMPK phosphorylation and *PGC1α* expression in diabetic conditions. AMPK is an important regulator of cellular metabolism, and direct activation of PGC1α by AMPK increases its transcriptional activity (Cantó and Auwerx, 2009). Fig. 2 shows that increased AMPK activity (Fig. 2A) restores *PGC-1α* (Fig. 2C) expression (Fig 2) indicating restored mt functionality in mice kidney tissue exposed to MF treatment.

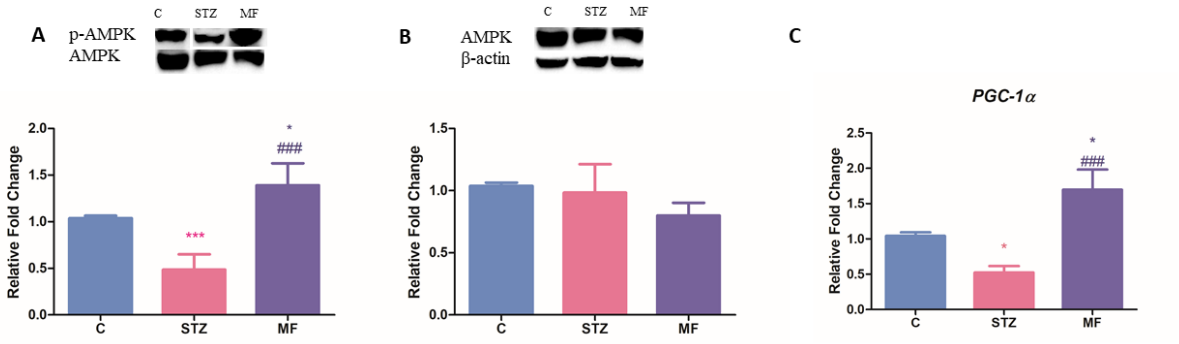


Figure 2: MF activates AMPK signalling (A: * $p < 0.05$ ***, $p < 0.0001$ vs control; ### $p < 0.0001$ vs STZ) and induces the expression of PGC-1α mRNA in mouse renal tissue. MF upregulated PGC-1α (A: *$p < 0.05$ vs control, ### $p < 0.0001$ vs STZ) (n=5).

4.3 MF restores Sirt1 expression and improves diabetic hypoxic state

Sirt1 expression is decreased in diabetic conditions of chronic metabolic stress, oxidative stress, or hypoxia (Yacoub et al., 2014). MF treatment positively regulates Sirt1 protein and gene expression (Fig. 3A and B), with associated decline in *HIF-1α* expression (Fig. 3D). PARP-1 protein expression increased markedly in the kidneys from diabetic mice compared to normal, however reduced levels of protein were noted in MF treatment (Fig. 3C).

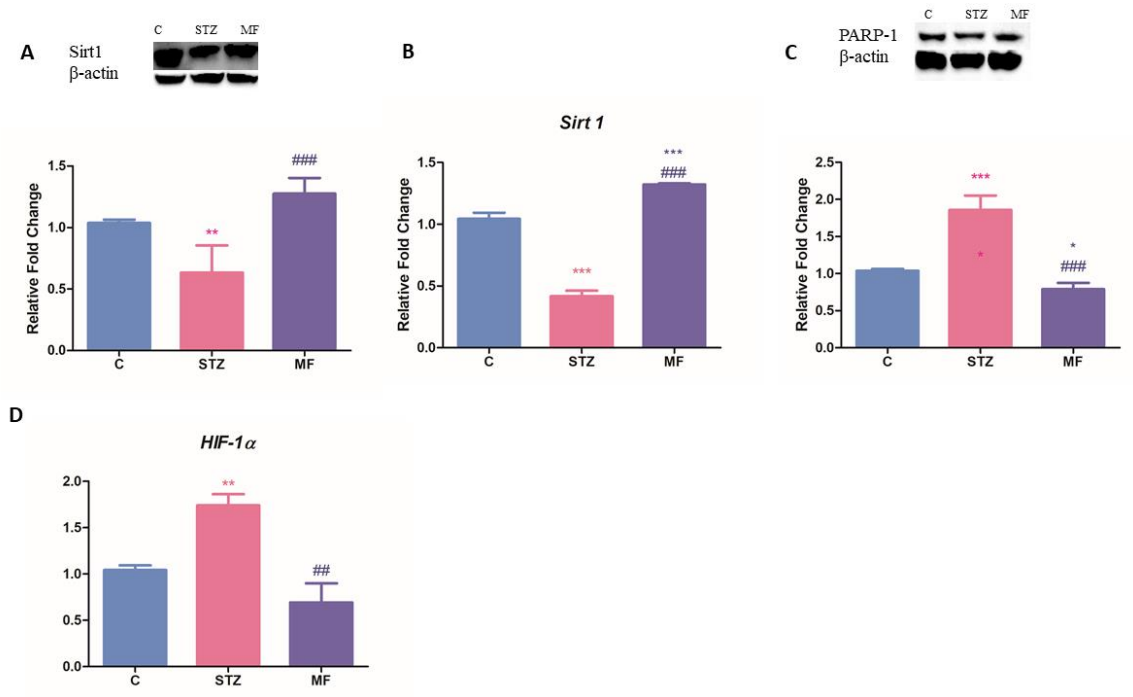


Figure 3: Increased Sirt1 protein (A: **$p < 0.005$ vs control, ### $p < 0.0001$ vs STZ), and gene (B: ***$p < 0.0001$, ***$p < 0.0001$ vs control, ### $p < 0.0001$ vs STZ) expression promotes decreased PARP-1 (C: ***$p < 0.0001$, *$p < 0.05$ vs control, ### $p < 0.0001$ vs STZ), and HIF-1α gene expression (D: **$p < 0.005$ vs control, ## $p < 0.005$ vs STZ) in diabetic mice kidney.

4.4 MF improves ER stress through inhibition of CHOP activation

Aberrant metabolic conditions such as hyperglycaemic induction of ROS can differentially affect ER trafficking (Ron and Walter, 2007). In this study, the increased expression of *PERK* and *eIF-2α* by STZ was significantly decreased following MF treatment (Fig. 4A and B). CHOP, a classic

marker of ER stress, displayed reduced expression profiles in the presence of MF (Fig. C and D). Collectively, we show that MF efficiently prevents ER stress signals in diabetic kidney tissue.

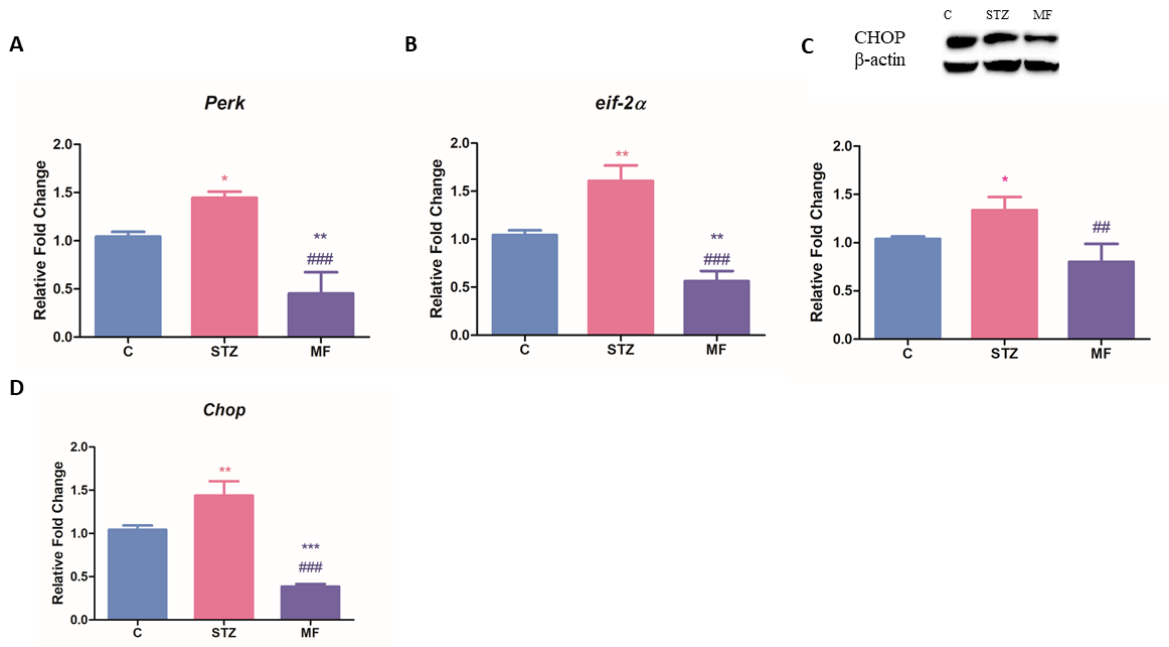


Figure 4: STZ upregulates gene expression of ER stress signals. MF significantly reduces gene expression of PERK (A: *p < 0.05, **p < 0.005 vs control, ## p < 0.005 vs STZ), and eIF2α (B: **p < 0.005 vs control, ## p < 0.005 vs STZ). Protein expression of both CHOP protein (C: *p < 0.05 vs control, ## p < 0.005 vs STZ), and gene (D: **p < 0.005, ***p < 0.0001 vs control, ## p < 0.005 vs STZ) expressions are reduced in MF-treated mice (n=5)

4.5 MF prevents cell death by inhibition of apoptotic signals

Diabetic conditions also stimulate the generation of intracellular ROS through mt pathways and NADPH oxidase, leading to activation of the pro-apoptotic p38 mitogen-activated protein kinase (p38 MAPK) and caspases (Susztak et al., 2006). On the other hand, MF treatment, post to diabetic induction, significantly reversed the activation of p38 (Fig. 5A). Next, we examined whether of NF-κB has a role in STZ-induced diabetic nephropathy and whether MF can inhibit this occurrence. We showed that, in STZ-induced diabetic kidney tissue, protein and gene expression of NF-κB increased (Fig. 5B and C). MF treatment however, effectively decreased NF-κB expression. The level of *TNF-α* was significantly augmented in STZ-induced diabetic mice whilst MF effectively inhibited the expression (Fig. 5D).

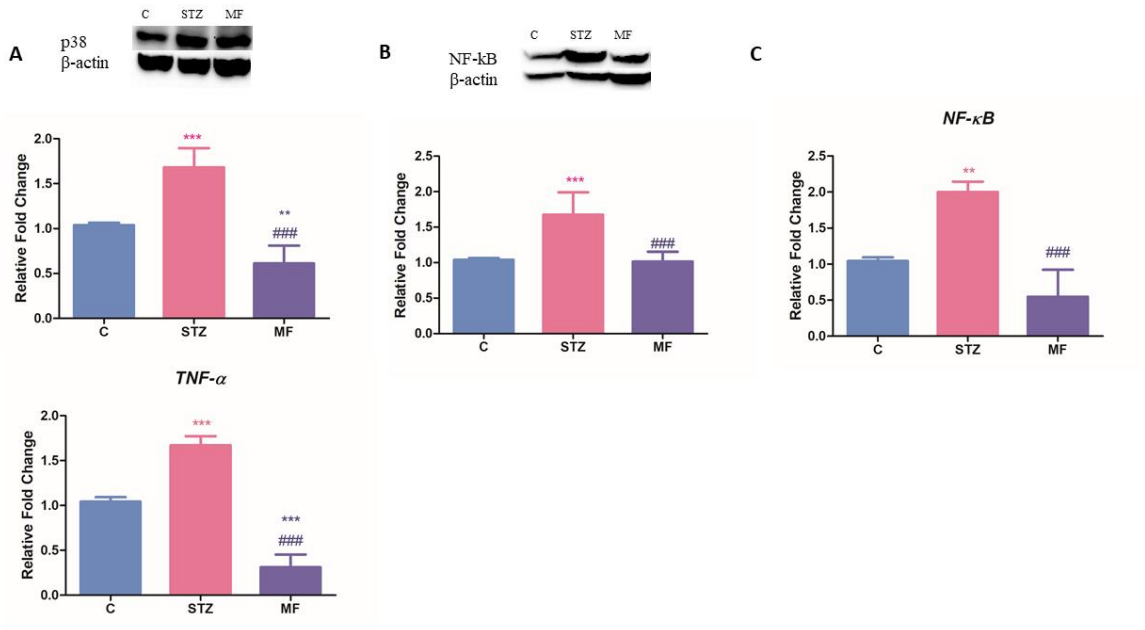


Figure 5: STZ significantly increased stress signals in mice kidney tissue. MF treatment lowered p38 protein expression (A: ***p < 0.0001, ** p < 0.005 vs control, ### p < 0.0001 vs STZ), NF-κB transcription factor protein (B: ***p < 0.0001, ### p < 0.0001 vs STZ) and gene expression (C: ** p < 0.005 vs control, ### p < 0.0001 vs STZ). Additionally, gene expression of TNF-α cytokine (D: ***p < 0.0001, vs control, ### p < 0.0001 vs STZ) is reduced following MF treatment (n=5).

Next, we explored the possible mechanisms involved in Mdm2-associated diabetes dysfunction. p53 is a classic downstream target of Mdm2 and STZ administration inhibits Mdm2-mediated p53 degradation, which has been reported to play a beneficial role in variety of kidney diseases (Allam et al., 2011, Mulay et al., 2013, Mulay et al., 2016). Thus, we treated the STZ-induced diabetic mice with MF to determine the p53-dependent role of Mdm2. By western blotting and qPCR, we found that protein and gene expressions of p53 was markedly decreased in the kidney of mice following MF treatment (Fig. 6B and C), indicating the efficient upregulation of Mdm2-p53 signalling (Fig. 6A). Collectively, these findings suggest a Mdm2-dependent effect on p53 expression in kidney tissue under diabetic status *in vivo*.

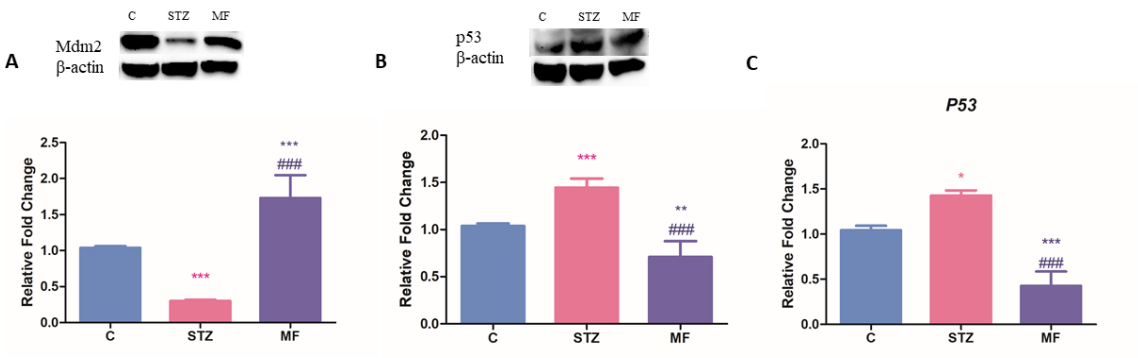


Figure 6: Diabetic stress promotes dysfunctional Mdm2-p53 axis. MF positively regulates Mdm2 protein expression (A: ***p < 0.0001 vs control, ### p < 0.0001 vs STZ), and lowers p53 (B: ***p < 0.0001, ** p < 0.005 vs control, ### p < 0.0001 vs STZ) protein, and gene (C: *p < 0.05, *** p < 0.0001 vs control, ### p < 0.0001 vs STZ) expression following treatment (n=5).

Furthermore, western blot showed that the expression of p21, Bax, caspase-9 and -3 proteins were both significantly enhanced in the STZ-treated mice compared with that of the control, while treatment with MF markedly decreased the expression (Fig. 7 and 8). In addition, the apoptogenic protein cyt-c was upregulated in mice kidneys exposed to STZ (Fig. 8A). However, treatment with MF effectively inhibited these parameters suggesting its potential anti-apoptotic effect in diabetes-mediated mt dependent apoptotic pathways in kidney tissue.

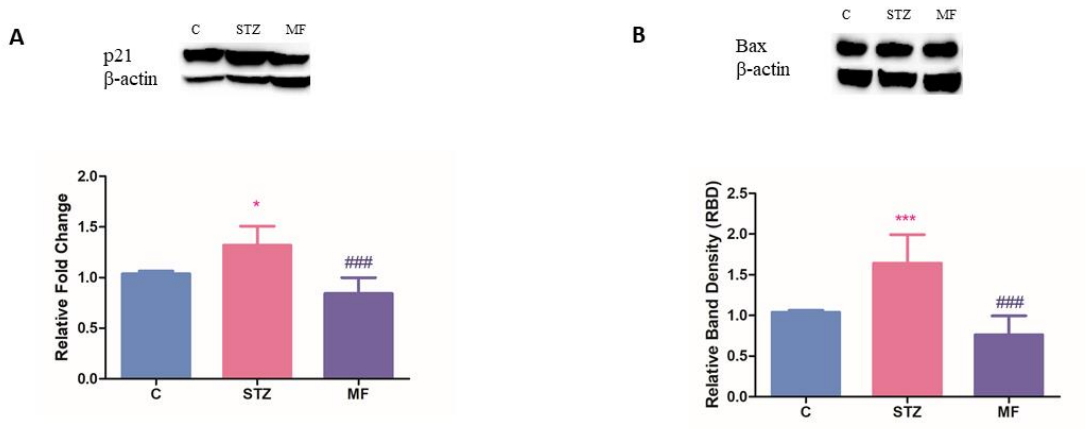


Figure 7: Damage response protein p21 (A: *p < 0.05 vs control, ### p < 0.0001 vs STZ), and pro apoptotic Bax (B: ***p < 0.0001 vs control, ### p < 0.0001 vs STZ) are diminished by MF in mice kidney tissue (n=5).

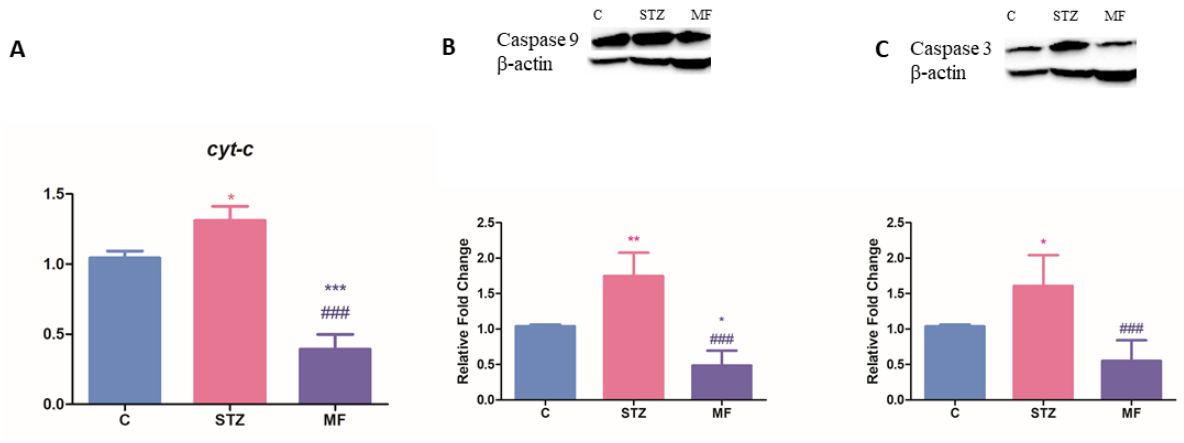


Figure 8: Metformin mediates intrinsic mt caspase inhibition by decreasing cyt-c (A: * $p < 0.05$, ***$p < 0.0001$, vs control, ### $p < 0.0001$ vs STZ) gene expression, intrinsic caspase-9 (B: **$p < 0.005$, * $p < 0.05$ vs control, ### $p < 0.0001$ vs STZ), and caspase-3 (C: * $p < 0.05$ vs control, ### $p < 0.0001$ vs STZ) protein expression in treated mice kidney.

5. Discussion

The kidneys maintain glucose homeostasis through glucose release into circulation via gluconeogenesis, uptake of glucose from circulation to maintain their energy requirements, and glucose reabsorption at the proximal tubule (Triplitt, 2012). Renal glucose release contributes to the pathophysiology of diabetes (Marsenic, 2009). The production of ROS in diabetic milieu is said to be among the important factors that alter cellular metabolism and function in DN (Sifuentes-Franco et al., 2018). STZ induced hyperglycaemia promotes free radical formation which overwhelms antioxidant defences (Sadi et al., 2019), leading to imbalances between pro and antioxidants and modifies protein activity making them susceptible to degradation. Amino acids are modified in the presence of excessive ROS by formation of cysteine disulphide bonds or addition of carbonyl groups (aldehydes and ketones) (Therond, 2006). In this study, we found that multiple injections of diabetogenic STZ in C57BL/6 mice produced significant increases in blood glucose with little change in body weight (Fig S1). We selected an acute STZ exposure time based on a recent study depicting severe insulitis and diabetic manifestations over a short period (Han et al., 2017). Following oral administration, MF is not metabolised, with 90% is absorbed and excreted through the renal route (Gong et al., 2012). Metformin exerts its glucose-lowering effect by inhibition of gluconeogenesis in the liver and kidney (Foretz et al., 2014). It also displays renoprotective roles by enhancing glucose uptake and preventing podocyte loss, this has been shown in a recent study (Polianskyte-Prause et al., 2018). Similarly, we demonstrate MFs efficacy to decrease blood glucose levels in STZ-treated mice potentially through gluconeogenesis inhibition (Fig S1). Protein carbonyl levels were increased after STZ administration (Fig. 1A), indicating oxidative protein damage and rendering the model efficient. Studies have reported the

antioxidant benefits of MF in diabetic patients. Metformin lowered protein carbonylation under hyperglycaemic conditions (Fig. 1A) potentially through its radical scavenging ability. It has been shown that hydroxyl free radicals but not $O_2^{\cdot-}$ react with MF, suggesting that this drug is not a very good scavenger of ROS at the molecular level (Khouri et al., 2004). Consequently, we further investigated the capacity of MF to up-regulate intracellular defence systems for targeted ROS alleviation. Nrf2 is an important mediator of anti-oxidant signalling by promoting cytoprotective genes, and significantly improved metabolic indices associated with DM (Zheng et al., 2011). Metformin significantly upregulated *Nrf2* expression in STZ mice (Fig 1B). Evidence that MF maintains and improves the anti-oxidant capacity of the cell was further supported by enhanced transcription of *GPx* (Fig. 1C), a crucial enzyme known to protect tissue from oxidative damage by detoxifying hydrogen peroxide and GSH recycling. The mechanism of improving the GSH redox state has been shown to prevent the induction of complications by oxidative stress in diabetic models and patients (Diaz-Flores et al., 2012, Sadi and Güray, 2009, Hamanishi et al., 2004). Taken together these data lend support to an altered gene expression profile associated with MF and subsequently reduce macromolecular modifications and oxidative damage in DN.

Additional to MFs anti-oxidant effects, it is established as a direct modulator of metabolic homeostasis through activation of AMPK (Rada et al., 2019) and Sirt1 (Cuyàs et al., 2018). The requirement for NAD^+ as a co-factor for Sirt1 activity makes this deacetylase an energy sensor that couples its function and expression to the NAD^+/NADH ratio of the cell (Gambini et al., 2011). Extensive research elucidates the primary effect of MF is inhibition of mt respiratory chain complex I, AMPK activation, and subsequent manipulation of cellular NAD^+ levels. Consequently, Sirt1 is expressed (Cantó et al., 2009, Song et al., 2015). The current study demonstrates that the activation of AMPK (Fig. 2A) and enhanced expression of Sirt1 (Fig. 3A and B) by MF by promoting mt function through NAD^+ restoration and enhanced PGC-1α expression (Fig. 2C). PGC-1α is a nuclear-encoded transcriptional coactivator, that plays an important role in mt biogenesis and the anti-oxidant defence response. Sirt1 deacetylates PGC-1α to enhance its activity to promote and maintain mt function (Cantó and Auwerx, 2009). Under diabetic conditions, the downregulation of AMPK/Sirt-1/PGC-1α axis induces hypertrophy, OS, and mt dysfunction which contributes to the development of DN (Cantó and Auwerx, 2009). It is therefore plausible that the cytoprotective effects of MF in DN is at least partially due to an increase in Sirt1 expression and activity.

Pseudohypoxia is a cellular state that creates transcriptional, translational and post-translational regulatory networks which are sensitive to environmental cues such as metabolic and genotoxic stress in DM patients (Gomes et al., 2013, Berthiaume et al., 2018). Given that hyperfiltration and/or metabolic changes in diabetic kidneys cause excessive oxygen consumption (Wang et al., 2019a), pseudohypoxia can be considered as hyperglycaemia-induced metabolic hypoxia. Sirt1 and PARP-1 affect two key post-translational modifications: acetylation and ADP-ribosylation

(PAR), respectively. Under stress conditions PARP-1 is acetylated enhancing its enzymatic activity. Sirt1 can regulate PARP-1 activity through deacetylation at both the transcriptional and posttranslational level (Rajamohan et al., 2009). Thus, by repression of the PARP-1 gene promoter leads to reduced synthesis of the PARP-1 protein (Rajamohan et al., 2009). Overactivation of PARP-1 during diabetes increases PAR synthesis and induces mt membrane leakiness, allowing mt apoptosis-inducing factor (AIF) to translocate to cytosolic and nuclear compartments (Puthanveetil et al., 2012). Additionally, Sirt1 activation blocks the release of AIF from mitochondria suggesting that it may counterbalance PARP-1 activity, and control cellular fate (Kolthur-Seetharam et al., 2006). In 2016, MF was shown to reduce PARP-1 activity and expression via the AMPK/PARP-1 cascade in a diabetic model (Shang et al., 2016). Three years later we show that MF suppresses PARP-1 expression in the kidneys of STZ treated mice (Fig 3C), further supporting the protective role of MF by mitigating PARP-1 through elevation in Sirt1 expression (Fig 3A and B) and establishing its role in cell survival.

Under normal glucose conditions, HIF-1α is expressed at basal levels and is degraded by the proteasome. However, under hyperglycaemic induced hypoxic conditions, hydroxylation is inhibited and HIF-1α accumulates in the nucleus, promoting a pro-oxidant ambiance by upregulating NADPH oxidase expression, and exacerbating OS (Nanduri et al., 2015). The increased protein oxidation (Fig. 1A) wires the upregulated HIF-1α expression exhibited in kidney of STZ injected mice (Fig 3D). Downregulated Sirt1 leads to greater acetylation and activation of HIF-1α (Ryu et al., 2019, Lim et al., 2010). Compelling evidence of known Sirt1 activator, resveratrol, promotes deacetylation and subsequent inactivation of HIF-1α to prevent HG-induced kidney damage (Shao et al., 2016). Similarly, our study suggests that MF promotes kidney health by preventing the expression of *HIF1* (Fig 3D) and promoting degradation in a Sirt1-dependent manner. Thus, MFs ability to enhance energy metabolism alleviates both protein oxidation and diabetic renal hypoxia.

Inflammatory responses in DN further impairs kidney function. The increased expression and activity of NF-κB is observed in experimental models and patients of DN (Mezzano et al., 2004, Starkey et al., 2006). The NF-κB inhibitor BAY 11-7082 reduced renal injury, inflammation, and oxidative stress in experimental models of DN (Kolati et al., 2015). Similar anti-inflammatory effects have been depicted in hypertensive rats (Malínská et al., 2016), and rat glomerular mesangial cells (Gu et al., 2014), where MF induced AMPK blocks NF-κB and related cytokine activation. Our study fits in with these findings as we show that MF decreased the expression of the transcriptionally active p65 NF-κB subunit at gene (Fig 5 C) and protein (Fig 5 B) levels and is congruent with decreased pro-inflammatory cytokine *TNF-α* transcription (Fig 5D) in hyperglycaemic mice. Analogous activation of Sirt1 by resveratrol in diabetic cardiomyocytes led to inactivation of NF-κB p65 as a result of deacetylation at lysine 310. Sirt1 activation leads to decreased binding of NF-κB-p65 to DNA (Bagul et al., 2015), suggesting that MF can normalize

several regulatory mechanisms through Sirt1 activity to prevent aberrant inflammatory processes of DN.

Diabetic nephropathy primes cellular responses to restore intracellular homeostasis by enhancing the unfolded protein response - an ER stress defence mechanism (Inagi, 2010). This enables cells to inhibit protein aggregation and translation and induce the proteasome machinery system for degradation of mis- and un-folded proteins. Interestingly, MF reduces ER stress via activation of AMPK-PI3k signalling (Jung et al., 2012) and reduced ER stress related markers in diabetic patients (Diaz-Morales et al., 2018). In the present study is we showed MF diminished ER stress in kidneys of STZ treated mice as evidenced with decreased levels of *PERK* (Fig 4A), *eIF2α* (Fig 4B), and CHOP (Fig 4C), demonstrating MFs ability to restore redox balance. Kidney homeostatic function can be achieved through inhibition of the UPR (Thériault et al., 2011), which is in accordance with our finding. Concurrent evidence *in vitro* and *in vivo* highlights the role of Sirt1 in alleviating ER stress (Guo et al., 2015, Li et al., 2011).

Uncontrolled ER stress in the diabetic kidney may result in cell death through other adaptive mechanisms including the p38 MAPK network (Adhikary et al., 2004) which is well documented as an upstream mediator of apoptotic cell death and oxidative stress (Igarashi et al., 1999). Interestingly, the expression of p38 is increased in the diabetic mouse kidney but was significantly decreased by MF treatment (Fig 5A). Inactivated p38 under diabetic condition suggests decrease in the progression of DN. We therefore investigated the effects of MF on renal apoptotic cell death. Hyperglycaemia sensitizes renal cells to hypoxic injury, accompanied by a heightened mt accumulation of Bax and release of cytochrome c and initiates p53- and mitochondria mediated apoptosis (Peng et al., 2015). In response to injury, diabetic kidney tissues of STZ treated mice showed marked p53 induction (Fig 6B and C). Mdm2 is a known suppressor of p53-dependent cell cycle arrest (p21) (Lei et al., 2017). The upregulation of Mdm2 protein expression (Fig. 6A) following MF-treatment in diabetic mice kidney correlates to reduced p53 protein and gene expression (Fig. 6B and C), as well as p21 protein levels (Fig. 7A), suggesting degradation through ubiquitination. Auxiliary apoptogenic machinery involve Bax and cyt-c release into the cytosol with subsequent apoptosome formation and activation of effector caspase-3. In turn, active caspase-3 degrades cell stabilizing proteins and other DNA repair enzymes, resulting in apoptotic cell death (Fuchs and Steller, 2015). For over a decade translocation of Bax protein into the mt membrane has been shown to increase in caspase-3 activity following high glucose treatment (Nakagami et al., 2001). STZ injection ensued upregulation of Bax (Fig. 7B) and cyt-c (Fig. 8A) in mice kidney tissue, with successive intrinsic cell death by increased caspase-9 and -3 protein expression (Fig. 8B and C). These results are indicative of mt induced dysfunction, OS, and kidney cell death. The cell death mechanisms mediated by Bax targeting mitochondria could be inhibited by MF-treatment, in agreement with previous studies (Zhang et al., 2017c). Intriguingly, the induction of CHOP (Fig. 4C and D), induces apoptosis (Oyadomari and Mori, 2004) by increased

cyt-c, and caspase-9 and -3/7 expression (Fig 8). Metformin's able to reverse ER stress in a CHOP-dependent manner by impeding cytoplasmic calcium release (Timmins et al., 2009), this is documented by Liu et. al., (2008) in STZ-treated rat kidney (Liu et al., 2008).

Collectively, our results demonstrate a plausible mechanism of MFs nephroprotective effects in diabetic mice connecting Sirt1, mt, and ER signalling and hypoxia in the regulation of apoptosis. We suggest that mt damage and associated integrated stress responses in diabetic mice can be pharmacologically repaired by MF to improve kidney function.

6. Conflict of interest

The authors declare that there are no conflicts of interest

7. Acknowledgements

Authors would like to thank the national research foundation (NRF) grant, South Africa (UID98379) and College of Health Sciences – University of KwaZulu-Natal for funding this study. We would also like to thank Dr N. Naicker, Dr. S. Baijnath, and Dr S. Singh for their assistance with obtaining mice tissue.

8. References

ADHIKARY, L., CHOW, F., NIKOLIC-PATERSON, D. J., STAMBE, C., DOWLING, J., ATKINS, R. C. & TESCH, G. H. 2004. Abnormal p38 mitogen-activated protein kinase signalling in human and experimental diabetic nephropathy. *Diabetologia,* 47**,** 1210-1222.

ALLAM, R., SAYYED, S. G., KULKARNI, O. P., LICHTNEKERT, J. & ANDERS, H.-J. 2011. Mdm2 promotes systemic lupus erythematosus and lupus nephritis. *Journal of the American Society of Nephrology,* 22**,** 2016-2027.

ALLISON, S. J. 2014. Acute kidney injury: mechanism of AKI sensitivity in diabetic nephropathy. *Nature Reviews Nephrology,* 10**,** 484.

AUGUSTYNIAK, E., ADAM, A., WOJDYLA, K., ROGOWSKA-WRZESINSKA, A., WILLETTS, R., KORKMAZ, A., ATALAY, M., WEBER, D., GRUNE, T. & BORSA, C. 2015. Validation of protein carbonyl measurement: a multi-centre study. *Redox biology,* 4**,** 149-157.

BAGUL, P. K., DEEPTHI, N., SULTANA, R. & BANERJEE, S. K. 2015. Resveratrol ameliorates cardiac oxidative stress in diabetes through deacetylation of NFkB-p65 and histone 3. *The Journal of nutritional biochemistry,* 26**,** 1298-1307.

BERTHIAUME, J. M., KURDYS, J. G., MUNTEAN, D. M. & ROSCA, M. G. 2018. Mitochondrial NAD+/NADH redox state and diabetic cardiomyopathy. *Antioxidants & redox signaling,* 30**,** 375-398.

CANTÓ, C. & AUWERX, J. 2009. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Current opinion in lipidology,* 20**,** 98.

CANTÓ, C., GERHART-HINES, Z., FEIGE, J. N., LAGOUGE, M., NORIEGA, L., MILNE, J. C., ELLIOTT, P. J., PUIGSERVER, P. & AUWERX, J. 2009. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature,* 458**,** 1056.

CHO, K., CHUNG, J. Y., CHO, S. K., SHIN, H.-W., JANG, I.-J., PARK, J.-W., YU, K.-S. & CHO, J.-Y. 2015. Antihyperglycaemic mechanism of metformin occurs via the AMPK/LXRα/POMC pathway. *Scientific reports,* 5**,** 8145.

CUYÀS, E., VERDURA, S., LLORACH-PARÉS, L., FERNÁNDEZ-ARROYO, S., JOVEN, J., MARTIN-CASTILLO, B., BOSCH-BARRERA, J., BRUNET, J., NONELL-CANALS, A. & SANCHEZ-MARTINEZ, M. 2018. Metformin is a direct SIRT1-activating compound: Computational modeling and experimental validation. *Frontiers in endocrinology,* 9**,** 657.

DIAZ-FLORES, M., ANGELES-MEJIA, S., BAIZA-GUTMAN, L., MEDINA-NAVARRO, R., HERNANDEZ-SAAVEDRA, D., ORTEGA-CAMARILLO, C., ROMAN-RAMOS, R., CRUZ, M. & ALARCON-AGUILAR, F. 2012. Effect of an aqueous extract of Cucurbita ficifolia Bouché on the glutathione redox cycle in mice with STZ-induced diabetes. *Journal of ethnopharmacology,* 144**,** 101-108.

DIAZ-MORALES, N., IANNANTUONI, F., ESCRIBANO-LOPEZ, I., BANULS, C., ROVIRA-LLOPIS, S., SOLA, E., ROCHA, M., HERNANDEZ-MIJARES, A. & VICTOR, V. M. 2018. Does metformin modulate endoplasmic reticulum stress and autophagy in type 2 diabetic peripheral blood mononuclear cells? : Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA.

DEWANGAN, H., TIWARI, R., SHARMA, V., SHUKLA, S. S., SATAPATHY, T. & PANDEY, R. 2017. Past and Future of in-vitro and in-vivo Animal Models for Diabetes: A Review. *IJPER,* 51.

FORETZ, M., GUIGAS, B., BERTRAND, L., POLLAK, M. & VIOLLET, B. 2014. Metformin: from mechanisms of action to therapies. *Cell metabolism,* 20**,** 953-966.

FOWLER, M. J. 2008. Microvascular and macrovascular complications of diabetes. *Clinical diabetes,* 26**,** 77-82.

FUCHS, Y. & STELLER, H. 2015. Live to die another way: modes of programmed cell death and the signals emanating from dying cells. *Nature reviews Molecular cell biology,* 16**,** 329.

GAMBINI, J., GOMEZ-CABRERA, M. C., BORRAS, C., VALLES, S. L., LOPEZ-GRUESO, R., MARTINEZ-BELLO, V. E., HERRANZ, D., PALLARDO, F. V., TRESGUERRES, J. A. & SERRANO, M. 2011. Free [NADH]/[NAD+] regulates sirtuin expression. *Archives of biochemistry and biophysics,* 512**,** 24-29.

GOMES, A. P., PRICE, N. L., LING, A. J., MOSLEHI, J. J., MONTGOMERY, M. K., RAJMAN, L., WHITE, J. P., TEODORO, J. S., WRANN, C. D. & HUBBARD, B. P. 2013.

Declining NAD+ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. *Cell,* 155**,** 1624-1638.

GONG, L., GOSWAMI, S., GIACOMINI, K. M., ALTMAN, R. B. & KLEIN, T. E. 2012. Metformin pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenetics and genomics,* 22**,** 820.

GU, J., YE, S., WANG, S., SUN, W. & HU, Y. 2014. Metformin inhibits nuclear factor-κB activation and inflammatory cytokines expression induced by high glucose via adenosine monophosphate-activated protein kinase activation in rat glomerular mesangial cellsin vitro. *Chinese medical journal,* 127**,** 1755-1760.

GUO, R., LIU, W., LIU, B., ZHANG, B., LI, W. & XU, Y. 2015. SIRT1 suppresses cardiomyocyte apoptosis in diabetic cardiomyopathy: an insight into endoplasmic reticulum stress response mechanism. *International Journal of Cardiology,* 191**,** 36-45.

HAMANISHI, T., FURUTA, H., KATO, H., DOI, A., TAMAI, M., SHIMOMURA, H., SAKAGASHIRA, S., NISHI, M., SASAKI, H. & SANKE, T. 2004. Functional variants in the glutathione peroxidase-1 (GPx-1) gene are associated with increased intima-media thickness of carotid arteries and risk of macrovascular diseases in Japanese type 2 diabetic patients. *Diabetes,* 53**,** 2455-2460.

HAN, X., TAO, Y.-L., DENG, Y.-P., YU, J.-W., CAI, J., REN, G.-F., SUN, Y.-N. & JIANG, G.-J. 2017. Metformin ameliorates insulitis in STZ-induced diabetic mice. *PeerJ,* 5**,** e3155.

HENSLEY, K., ROBINSON, K. A., GABBITA, S. P., SALSMAN, S. & FLOYD, R. A. 2000. Reactive oxygen species, cell signaling, and cell injury. *Free Radical Biology and Medicine,* 28**,** 1456-1462.

IGARASHI, M., WAKASAKI, H., TAKAHARA, N., ISHII, H., JIANG, Z.-Y., YAMAUCHI, T., KUBOKI, K., MEIER, M., RHODES, C. J. & KING, G. L. 1999. Glucose or diabetes activates p38 mitogen-activated protein kinase via different pathways. *The Journal of clinical investigation,* 103**,** 185-195.

INAGI, R. 2010. Endoplasmic reticulum stress as a progression factor for kidney injury. *Current opinion in pharmacology,* 10**,** 156-165.

JUNG, T. W., LEE, M. W., LEE, Y. J. & KIM, S. M. 2012. Metformin prevents endoplasmic reticulum stress-induced apoptosis through AMPK-PI3K-c-Jun NH2 pathway. *Biochemical and biophysical research communications,* 417**,** 147-152.

KHANRA, R., DEWANJEE, S., DUA, T. K., SAHU, R., GANGOPADHYAY, M., DE FEO, V. & ZIA-UL-HAQ, M. 2015. Abroma augusta L.(Malvaceae) leaf extract attenuates diabetes induced nephropathy and cardiomyopathy via inhibition of oxidative stress and inflammatory response. *Journal of translational medicine,* 13**,** 6.

KHOURI, H., COLLIN, F., BONNEFONT-ROUSSELOT, D., LEGRAND, A., JORE, D. & GARDÈS-ALBERT, M. 2004. Radical-induced oxidation of metformin. *European journal of biochemistry,* 271**,** 4745-4752.

KOLATI, S. R., KASALA, E. R., BODDULURU, L. N., MAHAREDDY, J. R., UPPULAPU, S. K., GOGOI, R., BARUA, C. C. & LAHKAR, M. 2015. BAY 11-7082 ameliorates diabetic nephropathy by attenuating hyperglycaemia-mediated oxidative stress and renal inflammation via NF-κB pathway. *Environmental toxicology and pharmacology,* 39**,** 690-699.

KOLTHUR-SEETHARAM, U., DANTZER, F., MCBURNEY, M. W., MURCIA1, G. D. & SASSONE-CORSI, P. 2006. Control of AIF-mediated cell death by the functional interplay of SIRT1 and PARP-1 in response to DNA damage. *Cell cycle,* 5**,** 873-877.

LEI, C.-T., TANG, H., YE, C., YOU, C.-Q., ZHANG, J., ZHANG, C.-Y., XIONG, W., SU, H. & ZHANG, C. 2017. MDM2 contributes to high glucose-induced glomerular mesangial cell proliferation and extracellular matrix accumulation via Notch1. *Scientific reports,* 7**,** 10393.

LEVINE, R. L., WILLIAMS, J. A., STADTMAN, E. P. & SHACTER, E. 1994. [37] Carbonyl assays for determination of oxidatively modified proteins. *Methods in enzymology*. Elsevier.

LI, Y., XU, S., GILES, A., NAKAMURA, K., LEE, J. W., HOU, X., DONMEZ, G., LI, J., LUO, Z. & WALSH, K. 2011. Hepatic overexpression of SIRT1 in mice attenuates endoplasmic reticulum stress and insulin resistance in the liver. *The FASEB Journal,* 25**,** 1664-1679.

LIM, J.-H., LEE, Y.-M., CHUN, Y.-S., CHEN, J., KIM, J.-E. & PARK, J.-W. 2010. Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1α. *Molecular cell,* 38**,** 864-878.

LIU, G., SUN, Y., LI, Z., SONG, T., WANG, H., ZHANG, Y. & GE, Z. 2008. Apoptosis induced by endoplasmic reticulum stress involved in diabetic kidney disease. *Biochemical and biophysical research communications,* 370**,** 651-656.

MALÍNSKÁ, H., OLIYARNYK, O., ŠKOP, V., ŠILHAVÝ, J., LANDA, V., ZÍDEK, V., MLEJNEK, P., ŠIMÁKOVÁ, M., STRNAD, H. & KAZDOVÁ, L. 2016. Effects of metformin on tissue oxidative and dicarbonyl stress in transgenic spontaneously hypertensive rats expressing human C-reactive protein. *PloS one,* 11**,** e0150924.

MARSENIC, O. 2009. Glucose control by the kidney: an emerging target in diabetes. *American Journal of Kidney Diseases,* 53**,** 875-883.

MEZZANO, S., AROS, C., DROGUETT, A., BURGOS, M. E., ARDILES, L., FLORES, C., SCHNEIDER, H., RUIZ-ORTEGA, M. & EGIDO, J. 2004. NF-κB activation and overexpression of regulated genes in human diabetic nephropathy. *Nephrology Dialysis Transplantation,* 19**,** 2505-2512.

MULAY, S. R., ROMOLI, S., DESAI, J., HONARPISHEH, M. M., KUMAR, S. V., ANDERS, H.-J. & THOMASOVA, D. 2016. Murine double minute-2 inhibition ameliorates established crescentic glomerulonephritis. *The American journal of pathology,* 186**,** 1442-1453.

MULAY, S. R., THOMASOVA, D., RYU, M., KULKARNI, O. P., MIGLIORINI, A., BRUNS, H., GRÖBMAYR, R., LAZZERI, E., LASAGNI, L. & LIAPIS, H. 2013. Podocyte loss involves MDM2-driven mitotic catastrophe. *The Journal of pathology,* 230**,** 322-335.

NAGIAH, S., PHULUKDAREE, A. & CHUTURGOON, A. A. 2016. Lon protease and eif2α are involved in acute, but not prolonged, antiretroviral induced stress response in HepG2 cells. *Chemico-biological interactions,* 252**,** 82-86.

NAKAGAMI, H., MORISHITA, R., YAMAMOTO, K., YOSHIMURA, S.-I., TANIYAMA, Y., AOKI, M., MATSUBARA, H., KIM, S., KANEDA, Y. & OGIHARA, T. 2001. Phosphorylation of p38 mitogen-activated protein kinase downstream of bax-caspase-3 pathway leads to cell death induced by high D-glucose in human endothelial cells. *Diabetes,* 50**,** 1472-1481.

NANDURI, J., VADDI, D. R., KHAN, S. A., WANG, N., MAKARENKO, V., SEMENZA, G. L. & PRABHAKAR, N. R. 2015. HIF-1α activation by intermittent hypoxia requires NADPH oxidase stimulation by xanthine oxidase. *PloS one,* 10**,** e0119762.

NIEDOWICZ, D. M. & DALEKE, D. L. 2005. The role of oxidative stress in diabetic complications. *Cell biochemistry and biophysics,* 43**,** 289-330.

OYADOMARI, S. & MORI, M. 2004. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell death and differentiation,* 11**,** 381.

PENG, J., LI, X., ZHANG, D., CHEN, J.-K., SU, Y., SMITH, S. B. & DONG, Z. 2015. Hyperglycaemia, p53, and mitochondrial pathway of apoptosis are involved in the susceptibility of diabetic models to ischemic acute kidney injury. *Kidney international,* 87**,** 137-150.

POLIANSKYTE-PRAUSE, Z., TOLVANEN, T. A., LINDFORS, S., DUMONT, V., VAN, M., WANG, H., DASH, S. N., BERG, M., NAAMS, J.-B. & HAUTALA, L. C. 2018. Metformin increases glucose uptake and acts renoprotectively by reducing SHIP2 activity. *The FASEB Journal,* 33**,** 2858-2869.

PUTHANVEETIL, P., ZHANG, D., WANG, Y., WANG, F., WAN, A., ABRAHANI, A. & RODRIGUES, B. 2012. Diabetes triggers a PARP1 mediated death pathway in the heart through participation of FoxO1. *Journal of molecular and cellular cardiology,* 53**,** 677-686.

RADA, P., MOSQUERA, A., MUNTANÉ, J., FERRANDIZ, F., RODRIGUEZ-MAÑAS, L., DE PABLO, F., GONZÁLEZ-CANUDAS, J. & VALVERDE, Á. M. 2019. Differential effects of metformin glycinate and hydrochloride in glucose production, AMPK phosphorylation and insulin sensitivity in hepatocytes from non-diabetic and diabetic mice. *Food and chemical toxicology,* 123**,** 470-480.

RAJAMOHAN, S. B., PILLAI, V. B., GUPTA, M., SUNDARESAN, N. R., BIRUKOV, K. G., SAMANT, S., HOTTIGER, M. O. & GUPTA, M. P. 2009. SIRT1 promotes cell survival

under stress by deacetylation-dependent deactivation of poly (ADP-ribose) polymerase 1. *Molecular and cellular biology,* 29**,** 4116-4129.

RON, D. & WALTER, P. 2007. Signal integration in the endoplasmic reticulum unfolded protein response. *Nature reviews Molecular cell biology,* 8**,** 519.

RYU, D. R., YU, M. R., KONG, K. H., KIM, H., KWON, S. H., JEON, J. S., HAN, D. C. & NOH, H. 2019. Sirt1–hypoxia-inducible factor-1α interaction is a key mediator of tubulointerstitial damage in the aged kidney. *Aging cell,* 18**,** e12904.

SADI, G. & GÜRAY, T. 2009. Gene expressions of Mn-SOD and GPx-1 in streptozotocin-induced diabetes: effect of antioxidants. *Molecular and cellular biochemistry,* 327**,** 127-134.

SADI, G., ŞAHIN, G. & BOSTANCI, A. 2019. Modulation of renal insulin signaling pathway and antioxidant enzymes with streptozotocin-induced diabetes: effects of resveratrol. *Medicina,* 55**,** 3.

SHANG, F., ZHANG, J., LI, Z., ZHANG, J., YIN, Y., WANG, Y., MARIN, T. L., GONGOL, B., XIAO, H. & ZHANG, Y.-Y. 2016. Cardiovascular protective effect of metformin and telmisartan: reduction of PARP1 activity via the AMPK-PARP1 cascade. *PloS one,* 11**,** e0151845.

SHAO, Y., LV, C., WU, C., ZHOU, Y. & WANG, Q. 2016. Mir-217 promotes inflammation and fibrosis in high glucose cultured rat glomerular mesangial cells via Sirt1/HIF-1α signaling pathway. *Diabetes/metabolism research and reviews,* 32**,** 534-543.

SIFUENTES-FRANCO, S., PADILLA-TEJEDA, D. E., CARRILLO-IBARRA, S. & MIRANDA-DÍAZ, A. G. 2018. Oxidative Stress, Apoptosis, and Mitochondrial Function in Diabetic Nephropathy. *International journal of endocrinology,* 2018**,** 1875870-1875870.

SONG, Y. M., LEE, Y.-H., KIM, J.-W., HAM, D.-S., KANG, E.-S., CHA, B. S., LEE, H. C. & LEE, B.-W. 2015. Metformin alleviates hepatosteatosis by restoring SIRT1-mediated autophagy induction via an AMP-activated protein kinase-independent pathway. *Autophagy,* 11**,** 46-59.

STARKEY, J. M., HAIDACHER, S. J., LEJEUNE, W. S., ZHANG, X., TIEU, B. C., CHOUDHARY, S., BRASIER, A. R., DENNER, L. A. & TILTON, R. G. 2006. Diabetes-induced activation of canonical and noncanonical nuclear factor-κB pathways in renal cortex. *Diabetes,* 55**,** 1252-1259.

SUSZTAK, K., RAFF, A. C., SCHIFFER, M. & BÖTTINGER, E. P. 2006. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes,* 55**,** 225-233.

THÉRIAULT, J. R., PALMER, H. J. & PITTMAN, D. D. 2011. Inhibition of the Unfolded Protein Response by metformin in renal proximal tubular epithelial cells. *Biochemical and biophysical research communications,* 409**,** 500-505.

THEROND, P. Oxidative stress and damages to biomolecules (lipids, proteins, DNA). Annales pharmaceutiques francaises, 2006. 383-389.

TIMMINS, J. M., OZCAN, L., SEIMON, T. A., LI, G., MALAGELADA, C., BACKS, J., BACKS, T., BASSEL-DUBY, R., OLSON, E. N. & ANDERSON, M. E. 2009. Calcium/calmodulin-dependent protein kinase II links ER stress with Fas and mitochondrial apoptosis pathways. *The Journal of clinical investigation,* 119**,** 2925-2941.

TRIPLITT, C. L. 2012. Understanding the kidneys' role in blood glucose regulation. *The American journal of managed care,* 18**,** S11-6.

WANG, S., WANG, J., ZHAO, A. & LI, J. 2017. SIRT1 activation inhibits hyperglycaemia-induced apoptosis by reducing oxidative stress and mitochondrial dysfunction in human endothelial cells. *Molecular medicine reports,* 16**,** 3331-3338.

WANG, W., SUN, W., CHENG, Y., XU, Z. & CAI, L. 2019. Role of sirtuin-1 in diabetic nephropathy. *Journal of Molecular Medicine,* 97**,** 291-309.

WILLIAMSON, J. R., CHANG, K., FRANGOS, M., HASAN, K. S., IDO, Y., KAWAMURA, T., NYENGAARD, J. R., VAN DEN ENDEN, M., KILO, C. & TILTON, R. G. 1993. Hyperglycaemic pseudohypoxia and diabetic complications. *Diabetes,* 42**,** 801-813.

YACOUB, R., LEE, K. & HE, J. C. 2014. The role of SIRT1 in diabetic kidney disease. *Frontiers in endocrinology,* 5**,** 166.

ZHANG, S., XU, H., YU, X., WU, Y. & SUI, D. 2017. Metformin ameliorates diabetic nephropathy in a rat model of low-dose streptozotocin-induced diabetes. *Experimental and therapeutic medicine,* 14**,** 383-390.

ZHENG, H., WHITMAN, S. A., WU, W., WONDRAK, G. T., WONG, P. K., FANG, D. & ZHANG, D. D. 2011. Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy. *Diabetes,* 60**,** 3055-3066.

ZHENG, Z., CHEN, H., LI, J., LI, T., ZHENG, B., ZHENG, Y., JIN, H., HE, Y., GU, Q. & XU, X. 2012. Sirtuin 1–mediated cellular metabolic memory of high glucose via the LKB1/AMPK/ROS pathway and therapeutic effects of metformin. *Diabetes,* 61**,** 217-228.

ZOU, M.-H., KIRKPATRICK, S. S., DAVIS, B. J., NELSON, J. S., WILES, W. G., SCHLETTNER, U., NEUMANN, D., BROWNLEE, M., FREEMAN, M. B. & GOLDMAN, M. H. 2004. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo: role of mitochondrial reactive nitrogen species. *Journal of Biological Chemistry*.

CHAPTER 6

6.1 Synthesis and conclusion

Metformin is the most commonly prescribed oral anti-diabetic treatment with minimal adverse effects. Current research demonstrates its beneficial role in other pathologies including anti-aging (Kumar and Baquer, 2018), anti-cancer (Vancura et al., 2018), as well as brain cell regeneration (Neumann et al., 2019). Metformin's suppression of hepatic gluconeogenesis in hyperglycaemia has been extensively studied, however, the mechanisms involved in diabetic brain and kidney organ damage remain elusive.

The brain relies on glucose for optimal function, whilst the kidney plays a role in glucose homeostasis through filtration and excretion, indicating a complementary link between these two organs. Therefore, these organs are prone to the harmful effects of uncontrolled hyperglycaemia. Diabetes leads to neurodegenerative states including dementia and AD which has high mortality and morbidity rates. Consistent evidence has been provided by research displaying that patients with DM have twice the risk of developing dementia (Ott et al., 1999), highlighting its detrimental effect on the brain. Additionally, diabetes-induced renal dysfunction is a common cause of chronic kidney disease (Dabla, 2010). Whilst most research provides vascular insight on the association; no studies display the nonvascular mechanisms.

The present study demonstrates that oral administration of MF (20 mg/kg BW) over an acute time period (15 days) was able to reduce blood glucose levels in STZ (50 mg/kg BW) induced diabetic mice. The oxidatively challenged status caused by protein carbonylation was effectively decreased by MF in whole brain and kidney tissue. This finding was coupled with concomitant increases in *GSTA4* expression in the brain; as well as *Nrf2* and *GPx* mRNA levels in the kidney. Thus, MF can overcome the redox imbalance associated with hyperglycaemia by inducing antioxidant effects.

The ancestral role of AMPK being the "fuel gauge of the cell" and its targets that uphold mt health are well established (Herzig and Shaw, 2018). However, during diabetic conditions dysregulated ATP metabolism downregulates AMPK, which leads to severe hypoxic states portrayed by upregulated *HIF-1* activation in mice kidney tissue. Treatment with the antidiabetic drug MF upregulated AMPK phosphorylation, and Sirt1 expressions with concomitant decreased PARP-1 protein expression, indicating its ability to restore depleted NAD^+ levels. Further, MF treatment led to a Sirt1-dependent decrease in *HIF-1* expression and overall improvement of hyperglycaemic-induced renal hypoxia.

Similar effects of MF on expression of Sirt1 and Sirt3 were noted in diabetic mice brain tissue. These two proteins have synergistic effects on PGC-1α, which positively regulates mt biogenesis by regulating TFAM and promoting mt replication. Metformin alleviated the

neuronal stress caused by hyperglycaemia through upregulated expressions of Sirt1, Sirt3, PGC-1α and TFAM. Additional to Sirts control over PGC-1α, is the post-transcriptional ability of miR-148a to bind to and inhibit its expression. Metformin induced an epigenetic change in neuronal tissue by altering the expression of miR-148a which modulates *PGC-1α* expression and enhances overall metabolic homeostasis. Furthermore, miR-132 was found to target a key chaperone protein HSP70. Mitochondrial surveillance is carried out by stress response chaperones like LonP1, HSP60 and HSP70 and are important for mt health (Yi et al., 2018). Inhibition of these chaperones in STZ-treated mice brain tissue exacerbated the level of oxidative stress and ablated their protective role in mt function. Metformin repressed miR-132 expression, and concomitantly upregulated HSP70 protein, associated HSP60, and protease LonP1; thus, maintaining proper mt metabolism and function in diabetic mice brain tissue.

The ability of cells to defend against hyperglycaemic insult and resultant misfolded and aggregated proteins involves the ER stress response system (Ramírez and Claret, 2015). This metabolic response is highlighted in both the brain (De Felice and Ferreira, 2017), and kidney (Cunard and Sharma, 2011). The UPR features as the primary signalling pathway in association with ER stress. The present study demonstrated upregulated UPR markers such as PERK and p-eIF2α after STZ-injection, indicating general inhibition of protein translation and synthesis in both mice brain and kidney tissue. Metformin was able to overcome the stress by reducing expression of these markers and restoring homeostasis. Furthermore, MF decreased the levels of CHOP protein and mRNA expressions in the hyperglycaemic kidney, thus counteracting ER-dependent degradative processes that are triggered by this protein.

The positive feedback loop between ER stress and mt oxidative stress reinforces the diseased state in diabetics. Their dual effects tend to overwhelm the cell's defence and mt apoptotic pathways are triggered. The elevated levels of the apoptotic marker p38 MAPK in diabetic mice kidney indicated that cell death signals were triggered. P53 activation is strongly related to kidney dysfunction (Zhou et al., 2010), and its inhibition has protective effects (Bhatt et al., 2010). The activation of inhibitor of p53-mediated apoptosis, Mdm2, and subsequent negative regulation of p53 and damage response protein, p21 levels by MF displays its protective effects against diabetic induced nephropathy. Additionally, high glucose prompts Bax-induced apoptosis highlighting one of the first findings in kidney cell apoptosis (Moley et al., 1998, Ortiz et al., 1997). Streptozotocin initiated apoptosis via the intrinsic pathway as demonstrated by increased Bax, cyt-c, caspase-9 and caspase-3 protein expressions in mice kidney tissue. Following MF treatment, the opposing effects provide evidence for its anti-apoptotic role in the diabetic kidney.

The suggested "alarm response" of the UPR and its ability to trigger apoptosis is accompanied by the activation of proinflammatory pathways in a diabetic ambiance (Cameron, 2013). The

upregulated Rel/A p65 NF-κB subunit exhibits further impairment in diabetic mice brain and kidney tissue. Potent anti-inflammatory effects were exerted by MF through decreased p65 NF-κB gene and protein expressions in both brain and kidney of diabetic mice. Additionally, MF treatment was associated with increased IκB-α levels in the diabetic brain indicating NF-κB sequestration in the cytoplasm preventing its translocation to the nucleus. From the two clusters of the miR-200c family, the miR-141 subunit has been associated with neuroinflammation (Verma et al., 2018). The inhibition of miR-141 by MF in diabetic mice brain tissue induced its target *Sirt1*, thus providing a mechanism for Rel/A p65 NF-κB inhibition through deacetylation. The NLRP3 inflammasome activation is primed by NF-κB signals (Bauernfeind et al., 2009), leading to the induction of NLRP3 related signals. Hyperglycaemic mice revealed increased mRNA expressions of *NLRP3*, *caspase-1*, *IL-1β*, *IL-18*, and *TNF-α* in the brain. These pro-inflammatory genes were decreased after acute MF treatment highlighting its suppressive role in neuronal inflammation *in vivo*. The catalytic activity of PP2A regulates IκB-α and tau protein by removal of phosphate groups. Thus, the PP2A/ NF-κB cascade mediates the regulation of the inflammasome at the transcriptional level. Diabetic mice treated with MF further revealed significantly downregulated miR-141 expressions and concomitantly increased *PP2A* mRNA. The associated decrease in tau hyperphosphorylation at Ser^{396} suggests MFs neuroprotective role by preventing protein aggregation and accumulation. Neuronal survival and function is further supported by BDNF (Rosa et al., 2016). Hyperphosphorylated tau protein was associated with reduced BDNF protein levels in diabetic mice, however, significant increases in both BDNF and its receptor TrkB demonstrated MFs function in neuroplasticity.

Both brain and kidney are the most metabolically active organs, however overcompensating their energy requirements in hyperglycaemic conditions leads to eventual organ-damage. This study fills the gap of mechanistic insight into MFs protection against mt oxidation, ER stress, inflammation, and apoptosis in renal and neuronal diabetic mice tissue. We provide novel epigenetic regulatory mechanisms in relation to these pathways, further highlighting its therapeutic effect.

In summary, MF's multi-directional properties, pharmacokinetic profile, and safety yields it a promising candidate in the prevention of diabetic- induced neuropathy and nephropathy. However, due to time constrains this study was conducted with a small number of mice. The effects of MF should be investigated over a chronic time period, where assays are conducted every 4 weeks to determine its efficacy. In addition, investigating other miRNA regulatory roles will uncover differential gene regulation during T2DM-organ damage.

Future work may include assessment of cytokines involved in low-grade systemic inflammation such as C-reactive protein (CRP), TNF-α, and IL-6. The effect of MF on inflammatory processes induced by STZ on co-secreting hormone, amylin, is unexplored in

the brain and should be addressed in future studies. Furthermore, the immunomodulatory effect of neuropeptides (e.g., Neuropeptide Y) and how these contribute to MF's neuroprotective actions requires attention.

References

BAUERNFEIND, F. G., HORVATH, G., STUTZ, A., ALNEMRI, E. S., MACDONALD, K., SPEERT, D., FERNANDES-ALNEMRI, T., WU, J., MONKS, B. G. & FITZGERALD, K. A. 2009. Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *The Journal of Immunology*, 183, 787-791.

BHATT, K., ZHOU, L., MI, Q.-S., HUANG, S., SHE, J.-X. & DONG, Z. 2010. MicroRNA-34a is induced via p53 during cisplatin nephrotoxicity and contributes to cell survival. *Molecular medicine*, 16, 409-416.

CAMERON, N. E. 2013. Role of endoplasmic reticulum stress in diabetic neuropathy. *Diabetes*, 62, 696-697.

CUNARD, R. & SHARMA, K. 2011. The endoplasmic reticulum stress response and diabetic kidney disease. *American Journal of Physiology-Renal Physiology*, 300, F1054-F1061.

DABLA, P. K. 2010. Renal function in diabetic nephropathy. *World journal of diabetes*, 1, 48.

DE FELICE, F. G. & FERREIRA, S. T. 2017. Getting a "GRiP" on hypothalamic endoplasmic reticulum stress to combat obesity. *Diabetes*, 66, 17-19.

HERZIG, S. & SHAW, R. J. 2018. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nature reviews Molecular cell biology*, 19, 121.

KUMAR, P. & BAQUER, N. 2018. Anti-diabetic and anti-aging effects of metformin on metabolic and molecular parameters in diabetic aging female rats. *Free Radical Biology and Medicine*, 120, S106.

MOLEY, K., CHI, M.-Y., KNUDSON, C., KORSMEYER, S. & MUECKLER, M. 1998. Hyperglycaemia induces apoptosis in pre-implantation embryos through cell death effector pathways. *Nature medicine*, 4, 1421.

NEUMANN, B., BAROR, R., ZHAO, C., SEGEL, M., DIETMANN, S., RAWJI, K. S., FOERSTER, S., MCCLAIN, C. R., CHALUT, K. & VAN WIJNGAARDEN, P. 2019. Metformin

Restores CNS Remyelination Capacity by Rejuvenating Aged Stem Cells. *Cell stem cell*, 25, 473-485. e8.

ORTIZ, A., ZIYADEH, F. N. & NEILSON, E. G. 1997. Expression of apoptosis-regulatory genes in renal proximal tubular epithelial cells exposed to high ambient glucose and in diabetic kidneys. *Journal of investigative medicine: the official publication of the American Federation for Clinical Research*, 45, 50-56.

OTT, A., STOLK, R. P., VAN HARSKAMP, F., POLS, H. A. P., HOFMAN, A. & BRETELER, M. M. B. 1999. Diabetes mellitus and the risk of dementia. *Neurology*, 53, 1937.

RAMÍREZ, S. & CLARET, M. 2015. Hypothalamic ER stress: a bridge between leptin resistance and obesity. *FEBS letters*, 589, 1678-1687.

ROSA, E., MAHENDRAM, S., KE, Y. D., ITTNER, L. M., GINSBERG, S. D. & FAHNESTOCK, M. 2016. Tau downregulates BDNF expression in animal and cellular models of Alzheimer's disease. *Neurobiology of Aging*, 48, 135-142.

VANCURA, A., BU, P., BHAGWAT, M., ZENG, J. & VANCUROVA, I. 2018. Metformin as an anticancer agent. *Trends in pharmacological sciences*, 39, 867-878.

VERMA, R., RITZEL, R. M., HARRIS, N. M., LEE, J., KIM, T., PANDI, G., VEMUGANTI, R. & MCCULLOUGH, L. D. 2018. Inhibition of miR-141-3p Ameliorates the Negative Effects of Poststroke Social Isolation in Aged Mice. *Stroke*, 49, 1701-1707.

YI, H.-S., CHANG, J. Y. & SHONG, M. 2018. The mitochondrial unfolded protein response and mitohormesis: a perspective on metabolic diseases. *Journal of molecular endocrinology*, 61, R91-R105.

ZHOU, L., FU, P., HUANG, X. R., LIU, F., LAI, K. N. & LAN, H. Y. 2010. Activation of p53 promotes renal injury in acute aristolochic acid nephropathy. *Journal of the American Society of Nephrology*, 21, 31-41.

ADDENDUM A

Ethics letter (year mice tissue was harvested)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

03 October 2016

Professor Anil Chuturgoon (34866)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Professor Chuturgoon,

Protocol reference number: AREC/057/016
Project title: Effect of fenugreek seed extract, 4-OH-Ile and metformin on insulin signalling, lipid metabolism and epigenetic regulation of type 2 diabetic C57B/6 black male mice

Full Approval – Research Application

With regards to your revised application received on 15 September 2016 and 31 August 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 03 October 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....
Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Acting Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc BRU – Dr Sanil Singh

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

1910 - 2010
100 YEARS OF ACADEMIC EXCELLENCE
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

ADDENDUM B

Table S1: Blood glucose measurements (mmol/L) at day 3, 10, and 13 for the duration of the Pilot study (n=3)

Mice DAY 3	Weight (g) Day 0	Blood glucose (mmol/L)	Blood glucose (mg/dL)
STZ 50mg/kg BW	22	6.8	122.4
	22	5.6	100.8
	22	5.9	106.2
STZ 100mg/kg BW	22	7.4	133.2
	21	5.7	102.6
	25	6.6	117.9
STZ 150mg/kg BW	23	-	-
	24	-	-
	25	15.7	282.6
Mice DAY 10	**Weight (g)**	**Blood glucose (mmol/L)**	**Blood glucose (dL)**
STZ 50mg/kg BW	22	9.3	164.7
	23	6.4	115.2
	22	7.1	127.8
STZ 100mg/kg BW	22	8	144
	21	5.2	93.6
	26	5.9	106.2
STZ 150mg/kg BW	-	-	-
	-	-	-
	19		563.4
Mice DAY 13	**Weight (g)**	**Blood glucose (mmol/L)**	**Blood glucose (dL)**
STZ 50mg/kg BW	21	11	198
	22	11.5	207
	21	12	216
STZ 100mg/kg BW	22	12.1	217.8
	21	11.9	214.2
	25	10	180
	-	-	-

STZ 150mg/kg BW	-	-	-
	19	32	576

The administration of 50mg/kg BW yielded similar blood glucose levels as the 100mg/kg BW group over the 2week experimental period. Hence, we selected the lower dose of STZ (50mg/kg BW) for this study. The dash (-) represents mice that did not tolerate the high doses of STZ (150mg/kg BW) and died by day 3 of administration.

Table S2: Blood glucose measurements (mmol/L) at day 0, 3, and 10 for the duration of the diabetic induction period.

		Day 0 (glucose in mmol/L)	**Day 3 (glucose in mmol/L)**	**Day 10 (glucose in mmol/L)**
Mice				
STZ	**1**	5.6	7.8	13.5
	2	4.3	6.5	14.4
	3	4.2	6.5	13.9
	4	4.1	6.3	14.0
	5	5.5	7.7	13.9
STZ+MF	**6**	4.3	7.9	14.3
	7	5.0	8.9	15.1
	8	4.7	7.9	14.6
	9	5.3	8.0	15.5
	10	4.9	8.5	15.2

All mice were labelled by an ear piecing, to ensure the same mice were treated within the same group.

Table S3: Blood glucose measurements (mmol/L) at day 0, 5, 10, and 15 for the duration of the MF-treatment period.

		Day 0 (glucose in mmol/L)	**Day 5 (glucose in mmol/L)**	**Day 10 (glucose in mmol/L)**	**Day 15 (glucose in mmol/L)**
Mice					
Control	**1**	5.1	5.0	5.1	5.0
	2	4.8	4.2	4.2	4.8
	3	5.0	4.9	4.9	4.7
	4	4.8	4.9	4.9	5.0
	5	4.3	4.2	4.7	4.8
STZ	**6**	13.5	13.8	14.2	14.3
	7	14.4	14.5	14.6	14.9
	8	13.9	13.3	13.8	13.2
	9	14.5	14.2	14.5	14.2
	10	13.9	13.9	13.8	14.9
Diabetic + MF	**11**	14.3	12.1	11.4	9.0
	12	15.1	11.0	10.6	8.9
	13	14.6	12.5	9.0	10.0
	14	15.5	12.5	12.0	10.3
	15	15.2	12.9	11.5	10.4

All mice were labelled by an ear piecing, to ensure the same mice were treated within the same group.

Table S4: OGTT test readings on the last day of experimental treatment period (Day 25).

		Day 0 (g)	Day 5 (g)	Day 10 (g)	Day 15 (g)
Mice					
Control	**1**	18	19	20	21
	2	26	20	19	20
	3	21	21	20	21
	4	21	22	20	21
	5	21	21	20	21
STZ	**6**	20	21	22	22
	7	22	22	22	23
	8	22	22	23	23
	9	22	23	24	24
	10	22	23	23	24
Diabetic + MF	**11**	20	21	22	22
	12	21	22	23	23
	13	22	22	23	23
	14	21	22	22	23
	15	20	21	22	23

All mice were labelled by an ear piecing, to ensure the same mice were treated within the same group.

Table S5: Mice body weight measurements (g) at days 0, 5, 10, and 15 for the duration of the MF-treatment period.

	Control	**STZ**	**Diabetic + MF**
0 min	4.80	11.00	10.00
30 min	4.90	11.20	9.80
60 min	5.00	11.50	9.60
90 min	4.90	11.60	9.70
120 min	4.95	11.40	9.65

All mice were labelled by an ear piecing, to ensure the same mice were treated within the same group.

ADDENDUM C

Agarose gel electrophoresis of DNA fragments in Control, STZ, and MF-treated mice

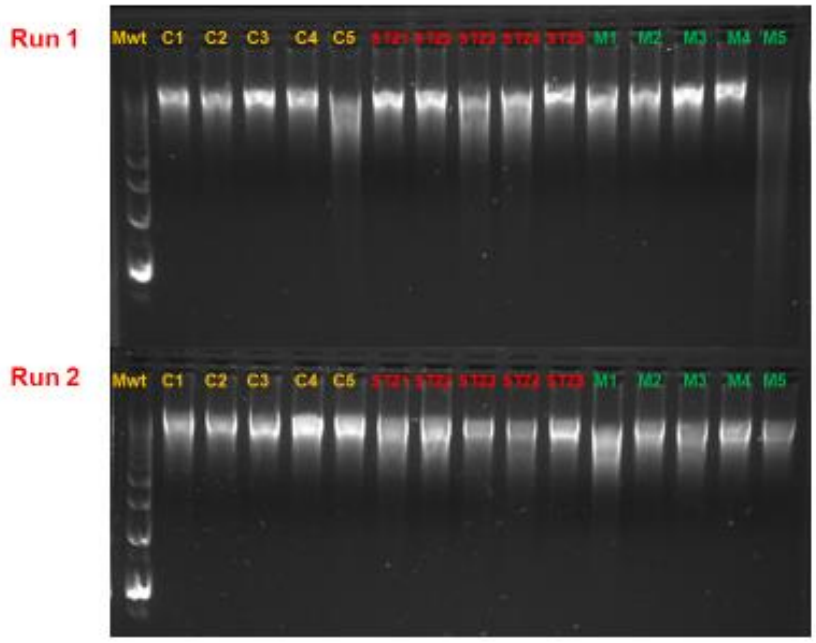


Figure 1: DNA electrophoresis from control, STZ, and MF-treated DNA isolates. No significant differences were established within acute treatment period (15 day)