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

By

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Master of Medical Science

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Supervisor

Professor Anil. A. Chuturgoon

2019
PREFACE AND DECLARATION

I, Taskeen Fathima Docrat, declare that:

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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 do 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

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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
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**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**

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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

PREFACE AND DECLARATION ...........................................................................................................i
DEDICATION.....................................................................................................................................ii
ACKNOWLEDGEMENTS .................................................................................................................. iii
PUBLICATIONS ............................................................................................................................ viii
PRESENTATIONS ........................................................................................................................... ix
LIST OF FIGURES ........................................................................................................................... x
LIST OF TABLES ............................................................................................................................... xiii
LIST OF ABBREVIATIONS ................................................................................................................ xiv
ABSTRACT ........................................................................................................................................ xix
CHAPTER 1 ........................................................................................................................................ 1
  1. Introduction .................................................................................................................................. 1
     1.1 Background .............................................................................................................................. 1
     1.2 Problem statement ................................................................................................................... 4
     1.3 Hypothesis ............................................................................................................................... 4
     1.4 Aim and Research questions ................................................................................................. 5
     1.5 Objectives ............................................................................................................................... 5
     1.6 Experimental approach .......................................................................................................... 5

CHAPTER 2 ........................................................................................................................................ 7
  2. Literature review .......................................................................................................................... 7
     2.1 Diabetes ................................................................................................................................... 7
        2.1.1 Definition and prevalence .............................................................................................. 7
        2.1.2 Types of diabetes mellitus ............................................................................................. 8
           2.1.2.1 Type 1 ....................................................................................................................... 8
           2.1.2.2 Type 2 ....................................................................................................................... 9
     2.2 Glucose metabolism ................................................................................................................. 10
     2.3 Insulin signalling ...................................................................................................................... 11
     2.4 Diabetes associated complications ......................................................................................... 13
        2.4.1 Organ damage ................................................................................................................ 13

v
# Metformin——The Physiological Beneﬁts of a Potential Therapeutic Agent for Type 2 Diabetes Mellitus

## Chapter 1: Introduction

1.1 Metformin: A Brief History

## Chapter 2: Metformin Pharmacology

### 2.1 Metformin and Diabetes Mellitus

2.1.1 Type 2 Diabetes Mellitus

### 2.2 Metformin Pharmacokinetics

2.2.1 Absorption

### 2.3 Metformin Pharmacodynamics

2.3.1 Glucose Transport

### 2.4 Metformin Interactions

2.4.1 Diabetic Neuropathy

- 2.4.1.1 Diabetic Neuropathy

2.4.2 Diabetic Nephropathy

- 2.4.2.1 Diabetic Nephropathy

### 2.5 Metformin

2.5.1 Pharmacokinetics of MF

- 2.5.1.1 Pharmacokinetics of MF

2.5.2 Canonical mechanisms of action

- 2.5.2.1 Canonical mechanisms of action

### 2.6 Molecular mechanisms underlying diabetic pathophysiology

2.6.1 Oxidative stress (OS)

- 2.6.1.1 Mitochondrial dysfunction

2.6.1.2 Other sources of ROS

- 2.6.1.2.1 Other sources of ROS

2.6.2 Advanced glycation end products

- 2.6.2.1 Advanced glycation end products

2.6.3 Sirtuins (Sirts)

- 2.6.3.1 Sirtuins (Sirts)

2.6.4 Hypoxia and pseudohypoxia

- 2.6.4.1 Hypoxia and pseudohypoxia

2.6.5 Endoplasmic reticulum (ER) stress

- 2.6.5.1 Endoplasmic reticulum (ER) stress

2.6.6 Inflammatory response

- 2.6.6.1 Inflammatory response

2.6.7 Neuroplasticity

- 2.6.7.1 Neuroplasticity

2.6.8 Metabolic dysfunction and cell death

- 2.6.8.1 Metabolic dysfunction and cell death

### 2.7 Cell defence mechanisms

2.7.1 Antioxidant defences

- 2.7.1.1 Antioxidant defences

2.7.2 Mitochondrial maintenance and chaperone proteins

- 2.7.2.1 Mitochondrial maintenance and chaperone proteins

2.7.3 Epigenetics — role of microRNAs

- 2.7.3.1 Epigenetics — role of microRNAs

2.7.4 Streptozotocin model of T2DM

- 2.7.4.1 Streptozotocin model of T2DM

### 2.8 References

- 2.8.1 References

---

# Chapter 3

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

---

# Chapter 4

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

---

# Chapter 5

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

---

vi
CHAPTER 6.................................................................................................................... 134

6.1 Synthesis and conclusion ..................................................................................... 134

ADDENDUM A ............................................................................................................. 139

ADDENDUM B.............................................................................................................. 140

ADDENDUM C.............................................................................................................. 145
PUBLICATIONS


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


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

LIST OF FIGURES

Chapter 1

Figure 1: Metformin—a Galegine derivative

Chapter 2

Figure 2.1: Global prevalence of diabetes

Figure 2.2: Interaction between the pancreatic β-cell and innate and adaptive immune systems. Type 1 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

Figure 2.3: Pathophysiology associated with Type 2 DM

Figure 2.4: Fates of glucose. Glycolysis occurs in the cytoplasm whilst ATP is produced in the mitochondrion

Figure 2.5: Insulin signalling and related pathways

Figure 2.6: Neurological responses to hyperglycaemia can be active or passive

Figure 2.7: Glucose reabsorption in the kidney

Figure 2.8: Pharmacokinetics of metformin transport

Figure 2.9: Metformin’s mechanism of action

Figure 2.10: Redox cycling within the mitochondrion

Figure 2.11: Diabetes increases flux through the polyol pathway

Figure 2.12: Biochemistry of protein carbonylation

Figure 2.13: Balance of the redox status is crucial in cellular homeostasis

Figure 2.14: Schematic diagram of hyperglycaemic stress

Figure 2.15: Sirtuin/HIF-1 axis and their effect on glycolysis-OxPhos switch

Figure 2.16: The adaptive UPR response to ER stress

Figure 2.17: Mechanism of diabetic ER stress-mediated cell death

Figure 2.18: Inflammasome activation in Diabetes Mellitus

Figure 2.19: Mechanism of tau phosphorylation and inflammation linked to high glucose levels

x
Figure 2.20: Intrinsic apoptotic pathway

Figure 2.21: HSPs stabilise misfolded and denatured proteins

Figure 2.22: Mechanism of miRNA biogenesis and gene regulation

Figure 2.23: The pros and cons of different models used in diabetes research

Figure 2.24: Mechanism of streptozotocin action.

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.

Chapter 3

Figure 1: Glucose tolerance following experimental period

Figure 2: MF reduced protein carbonylation in diabetic mice brain

Figure 3: MiR-132 repression by MF in STZ brain tissue. Seed sequence between miR-132 and predicted target sequence of HSP70-12B

Figure 4: STZ promotes ER stress through PERK phosphorylation

Figure 5: MF regulates mt biogenic markers

Figure 6: MiR-148a repression by MF in STZ brain tissue

Chapter 4

Figure 1: MF increases PP2A gene expression in a miR-141 regulatory manner

Figure 2: MF dephosphorylates tau in diabetic mice brain

Figure 3: Effects of MF on BDNF and TrkB expressions

Figure 4: MF inhibited NF-κB signalling in an IκB-α-dependent manner

Figure 5: MF regulates NLRP3 inflammatory response in diabetic mice brain

Chapter 5
**Figure 1:** MF mitigates oxidative stress in the kidney of diabetic mice

**Figure 2:** MF activates AMPK signalling

**Figure 3:** Increased Sirt1 protein expression promotes decreased PARP-1 and HIF-1α

**Figure 4:** STZ upregulates gene expression of ER stress signals

**Figure 5:** STZ significantly increased stress signals in mice kidney tissue

**Figure 6:** Diabetic stress promotes dysfunctional Mdm2-p53 axis

**Figure 7:** Damage response protein p21 and pro apoptotic Bax are diminished by MF in mice kidney tissue

**Figure 8:** Metformin mediates intrinsic mt caspase inhibition by decreasing cyt-c, intrinsic caspase-9, and caspase-3 protein expression in treated mice kidney

**ADDENDUM C**

**Figure 1:** DNA electrophoresis from control, STZ, and MF-treated DNA isolates. No significant differences were established within acute treatment period (15 day)
LIST OF TABLES

Chapter 3

Table 1: Primer sequences used to determine gene expression profiles

Chapter 4

Table 1: List of antibodies used

Table 2: Primer sequences used to determine gene expression profiles

Chapter 5

Table 1: List of antibodies used

Table 2: Primer sequences used to determine gene expression profiles

ADDENDUM B

Table S1: Blood glucose measurements (mmol/L) at day 3, 10, and 13 for the duration of the Pilot study (n=3)

Table S2: Blood glucose measurements (mmol/L) at day 0, 3, and 10 for the duration of the diabetic induction period

Table S3: Blood glucose measurements (mmol/L) at day 0, 5, 10, and 15 for the duration of the MF-treatment period

Table S4: OGTT test readings on the last day of experimental treatment period (Day 25)

Table S5: Mice body weight measurements (g) at days 0, 5, 10, and 15 for the duration of the MF-treatment period
<table>
<thead>
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<td>Enhanced Chemiluminescence</td>
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<td>eukaryotic translation initiation factor 2</td>
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<td>GFR</td>
<td>Glomerular filtration rate</td>
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<td>OCTs</td>
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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 7-16 mmol/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 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 Ser396. 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 turnover 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 (Stephene 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)](image)

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 do 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.

![Diagram of Neurological responses to hyperglycaemia](image_url)

**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).

![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.
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•), and superoxide anion radicals (O₂•⁻) 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).

![Diagram of the polyol pathway](image)

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 (Maisonuneuve 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$_2$O 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 (Ishibushi 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.
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).
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-1\(\alpha\) and NF-\(\kappa\)B 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.

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

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)](image)

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)
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.
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; Ca^{2+} 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)](image)

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).
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)
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.
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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.

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

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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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence: (5’-3’)</th>
<th>Annealing temperature (° C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTA4</td>
<td>F: TACCTCGCTGCCAAGTACAAC</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>R: GAGCCACGGCAATCATCATCA</td>
<td></td>
</tr>
<tr>
<td>SIRT1</td>
<td>F: CAGCCGTCTCTGTGTCACAAA</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R: GCACCGAGGAACTACCTGAT</td>
<td></td>
</tr>
<tr>
<td>SIRT3</td>
<td>F: TACAGGCCCAATGTCACTCA</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td>R: ACAGACCCTGCTGATAGCTG</td>
<td></td>
</tr>
<tr>
<td>PGC-1α</td>
<td>F: GCAACATGCTCAAGCCAAAC</td>
<td>56.2</td>
</tr>
<tr>
<td></td>
<td>R: TGCAGTTCAGAGAGTTCCA</td>
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</tr>
<tr>
<td>PERK</td>
<td>F: GCACCTTAGATGGACGAATCG</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>R: TGCTGAGGCTAGATGAACCA</td>
<td></td>
</tr>
<tr>
<td>eIF2α</td>
<td>F: AAACCTGGAGCATGTTGAAATCG</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>R: GGGCACCCTTTACTTCTGGG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: ATGTGTCGGTCGTGGATCTGAC</td>
<td>Variable</td>
</tr>
<tr>
<td></td>
<td>R: AGACAACCTGGTCCTCAGTAG</td>
<td></td>
</tr>
</tbody>
</table>
**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 ≤ 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) 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 \( \alpha \)-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 re-fold 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 mouse 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

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8. References


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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.

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

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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 Ser396 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\textsuperscript{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, antidiabetic 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 (Labuzek 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.

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 housekeeping 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

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>AB1534</td>
</tr>
<tr>
<td>p-tau</td>
<td>5396 (PHF13)</td>
</tr>
<tr>
<td>NF-κB (p65)</td>
<td>D14E12</td>
</tr>
<tr>
<td>IκB-α</td>
<td>L35A5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence: (5’-3’)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PP2A</strong></td>
<td>F: GTTCAAGAGGTTTGTATGTCCAG</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R: AGCTACAAGCAGTGTAACTGTIT</td>
<td></td>
</tr>
<tr>
<td>tau</td>
<td>F: TGGGGAACATTCCGTATGAGG</td>
<td>61.9</td>
</tr>
<tr>
<td></td>
<td>R: CAGAAGCCATAACCCTTGGG</td>
<td></td>
</tr>
<tr>
<td><strong>TrkB</strong></td>
<td>F: CGGCACAATAATTTCACCG</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R: TTACCCGTCAGGATCAGGTC</td>
<td></td>
</tr>
<tr>
<td><strong>BDNF</strong></td>
<td>F: AAACATCCGAGGACAAGGTG</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R: AGAAGGAGGGCTCCAAAGG</td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>F: GAAATTCCTGATCCAGACAAAA</td>
<td>57.5</td>
</tr>
<tr>
<td></td>
<td>R: ATCACTCCAATGGCCTGTGTAG</td>
<td></td>
</tr>
<tr>
<td>NLRP3</td>
<td>F: TGCTCTTCACTGCTATCAAGCCCT</td>
<td>61.9</td>
</tr>
<tr>
<td></td>
<td>R: ACAAAGCTTTGTCAGCAGCCTAT</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: ATGGCAACTGTTCCTGAACTCAACT</td>
<td>58.7</td>
</tr>
</tbody>
</table>
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^−ΔΔCT 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 its 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 (Cuff 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 (Verna 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 Ser396, 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 (Ghademzehad 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 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
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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.

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

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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/PGC1α 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-$\kappa$B (NF-$\kappa$B), HIF-1$\alpha$ and peroxisome proliferator-activated receptor gamma coactivator-1$\alpha$ (PGC)-1$\alpha$ (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 glycero- phosphate 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°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°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.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-AMPK</td>
<td>T172</td>
</tr>
<tr>
<td>AMPK</td>
<td>2532</td>
</tr>
<tr>
<td>Sirt1</td>
<td>ab32441</td>
</tr>
<tr>
<td>PARP1</td>
<td>9542P</td>
</tr>
<tr>
<td>CHOP</td>
<td>L63F7</td>
</tr>
<tr>
<td>p38</td>
<td>ab4822</td>
</tr>
<tr>
<td>NF-κB</td>
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<tr>
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</tr>
<tr>
<td>p21</td>
<td>p1484</td>
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<tr>
<td>Bax</td>
<td>BD610982</td>
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<td>9502P</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>9662P</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence: (5’-3’)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>F: CTTTAGTCAGCGACAGAAGGAC</td>
<td>58</td>
</tr>
</tbody>
</table>
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 \( \text{O}_2^- \) 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-1\(\alpha\) expression (Fig. 2C). PGC-1\(\alpha\) is a nuclear-encoded transcriptional coactivator, that plays an important role in mt biogenesis and the anti-oxidant defence response. Sirt1 deacetylates PGC-1\(\alpha\) to enhance its activity to promote and maintain mt function (Cantó and Auwerx, 2009). Under diabetic conditions, the downregulation of AMPK/Sirt-1/PGC-1\(\alpha\) 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 (Malinská 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 normaliz
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
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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 Sirt's 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.

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Hyperglycaemia induces apoptosis in pre-implantation embryos through cell death effector

NEUMANN, B., BAROR, R., ZHAO, C., SEGEL, M., DIETMANN, S., RAWJI, K. S.,


ADDENDUM A

Ethics letter (year mice tissue was harvested)
ADDENDUM B

Table S1: Blood glucose measurements (mmol/L) at day 3, 10, and 13 for the duration of the Pilot study (n=3)
<table>
<thead>
<tr>
<th>Mice DAY 3</th>
<th>Weight (g)</th>
<th>Blood glucose (mmol/L)</th>
<th>Blood glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td></td>
<td></td>
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<tr>
<td>STZ 50mg/kg BW</td>
<td>22</td>
<td>6.8</td>
<td>122.4</td>
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<tr>
<td></td>
<td></td>
<td>22</td>
<td>5.6</td>
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<tr>
<td></td>
<td></td>
<td>22</td>
<td>5.9</td>
</tr>
<tr>
<td>STZ 100mg/kg BW</td>
<td>22</td>
<td>7.4</td>
<td>133.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>5.7</td>
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<tr>
<td></td>
<td></td>
<td>25</td>
<td>6.6</td>
</tr>
<tr>
<td>STZ 150mg/kg BW</td>
<td>23</td>
<td>-</td>
<td>-</td>
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<td>24</td>
<td>-</td>
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<td></td>
<td></td>
<td>25</td>
<td>15.7</td>
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<tr>
<td>Mice DAY 10</td>
<td>Weight (g)</td>
<td>Blood glucose (mmol/L)</td>
<td>Blood glucose (dL)</td>
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<td></td>
<td></td>
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<tr>
<td>STZ 50mg/kg BW</td>
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<td>9.3</td>
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<td></td>
<td></td>
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<tr>
<td>STZ 100mg/kg BW</td>
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<td>144</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>19</td>
<td>563.4</td>
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<tr>
<td>Mice DAY 13</td>
<td>Weight (g)</td>
<td>Blood glucose (mmol/L)</td>
<td>Blood glucose (dL)</td>
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<td>STZ 100mg/kg BW</td>
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<td>10</td>
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</table>
The administration of 50mg/kg BW yielded similar blood glucose levels as the 100mg/kg BW group over the 2 week 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.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day 0 (glucose in mmol/L)</th>
<th>Day 3 (glucose in mmol/L)</th>
<th>Day 10 (glucose in mmol/L)</th>
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<td>7.8</td>
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<td>2</td>
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<tr>
<td></td>
<td>5</td>
<td>5.5</td>
<td>7.7</td>
</tr>
<tr>
<td>STZ+MF</td>
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<td>4.3</td>
<td>7.9</td>
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<tr>
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<td>7</td>
<td>5.0</td>
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<tr>
<td></td>
<td>10</td>
<td>4.9</td>
<td>8.5</td>
</tr>
</tbody>
</table>

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.

<table>
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<th>Mice</th>
<th>Day 0 (glucose in mmol/L)</th>
<th>Day 5 (glucose in mmol/L)</th>
<th>Day 10 (glucose in mmol/L)</th>
<th>Day 15 (glucose in mmol/L)</th>
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<td><strong>Control</strong></td>
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<td>15</td>
<td>15.2</td>
<td>12.9</td>
<td>11.5</td>
<td>10.4</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day 0 (g)</th>
<th>Day 5 (g)</th>
<th>Day 10 (g)</th>
<th>Day 15 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>19</td>
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<td>21</td>
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<tr>
<td>2</td>
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<td>19</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
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<td>20</td>
<td>21</td>
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<tr>
<td>4</td>
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<td>20</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>21</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>STZ</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>6</td>
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<td>21</td>
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<tr>
<td>7</td>
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<tr>
<td>8</td>
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<td>23</td>
<td>23</td>
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<tr>
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<td>23</td>
<td>24</td>
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</tr>
<tr>
<td>10</td>
<td>22</td>
<td>23</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Diabetic + MF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>20</td>
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<td>22</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>21</td>
<td>22</td>
<td>23</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ</th>
<th>Diabetic + MF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>4.80</td>
<td>11.00</td>
<td>10.00</td>
</tr>
<tr>
<td>30 min</td>
<td>4.90</td>
<td>11.20</td>
<td>9.80</td>
</tr>
<tr>
<td>60 min</td>
<td>5.00</td>
<td>11.50</td>
<td>9.60</td>
</tr>
<tr>
<td>90 min</td>
<td>4.90</td>
<td>11.60</td>
<td>9.70</td>
</tr>
<tr>
<td>120 min</td>
<td>4.95</td>
<td>11.40</td>
<td>9.65</td>
</tr>
</tbody>
</table>

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

![Agarose gel electrophoresis](image)

**Figure 1**: DNA electrophoresis from control, STZ, and MF-treated DNA isolates. No significant differences were established within acute treatment period (15 day)