EVALUATION OF THE THERAPEUTIC PROPERTIES OF A RUTHENIUM(II) URACIL-DERIVED DIIMINE COMPLEX ON SELECTED COMPLICATIONS ASSOCIATED WITH DIET-INDUCED PRE-DIABETES.

by

Lindokuhle Patience Mabuza

211509843

2019

UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

EVALUATION OF THE THERAPEUTIC PROPERTIES OF A RUTHENIUM(II) URACIL-DERIVED DIIMINE COMPLEX ON SELECTED COMPLICATIONS ASSOCIATED WITH DIET-INDUCED PRE-DIABETES.

by

Lindokuhle Patience Mabuza

211509843

Submitted as the dissertation component in fulfilment for the degree of Doctor of Philosophy in Health Sciences in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal

Supervisor: Dr A Khathi

Co-supervisor: Dr P.S Ngubane

Discipline of Human Physiology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

Date of submission: December 2019

UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

PREFACE

Westernised diets rich in high fats and high carbohydrates have been shown to lead to the development of prediabetes which precedes the onset of type 2 diabetes mellitus (T2DM). Once a patient is diagnosed with pre-diabetes, a preventative strategy including a combination of pharmacotherapy and dietary intervention is employed. The primary aim of this strategy is to prevent or delay the development of T2DM and its complications. However, there is reported poor patient compliance regarding dietary intervention as patients tend to heavily rely on the pharmacological treatments and neglect dietary intervention. This then reduces the efficacy of the drug and predisposes the patient to developing T2DM and complications such as non-alcoholic fatty liver disease (NAFLD), kidney disease and innate immune system dysregulation. Therefore, the search for therapeutic agents that can remain effective in both the presence and absence of dietary intervention continues. In our laboratory, we have created a diet-induced prediabetic rat model that accurately mimics the human condition. We have additionally synthesised a novel ruthenium(II) uracil-derived diamine complex that has shown promise as it was able to restore glucose homeostasis in this animal model. In this study, we sought to investigate the effects of this compound on pre-diabetes-associated complications such hepatic, renal and immune dysregulation in both the presence and absence of dietary intervention in a diet-induced pre-diabetic rat model.

DECLARATION

I, Lindokuhle Patience Mabuza hereby declare that the dissertation entitled:

"Evaluation of the therapeutic properties of a ruthenium(ii) uracil-derived diimine complex on selected complications associated with diet-induced pre-diabetes" is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

Student: L.P Mabuza   **Signature** _____ **Date:_____**

Supervisor: Dr A Khathi   **Signature**_____ **Date:_____**

Co-Supervisor: Dr P.S Ngubane   **Signature**_____ **Date:_____**

Last but not least, I would like to express my deepest gratitude to my entire family for their endless continuous support, encouragement and prayer. To my aunt, Lydia Mabuza I thank her from the bottom of my heart for the financial support she has given me. To my granny, Mathukulula Mabuza I thank her for her endless daily prayer and making me the person that I am today. "Your all sorts of support have never gone unnoticed, I deeply appreciate."

I DEDICATE ALL THIS RESEARCH WORK TO MY FAMILY AS A TOKEN OF APPRECIATION

TABLE OF CONTENTS

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

α	alpha
ADA	American Diabetes Association
ADP	adenosine diphosphate
ACR	albumin creatinine ratio
AER	albumin excretion rate
ALT	alanine transaminase
ANOVA	Analysis of variance
AGE's	advanced glycation end products
AST	aspartate transaminase
β	beta
BRU	biomedical research unit
BHT	butylated hydroxytoluene
Ca^{2+}	calcium ion
CC	creatinine clearance
CD40L	CD40 ligand
cDNA	complementary deoxyribonucleic acid
CETP	cholesteryl ester transfer protein
CHS	College of Health Sciences
CRP	C-reactive protein
CV	central vein
CVD	cardiovascular disease
DN	diabetic nephropathy
DNA	deoxyribonucleic acid
DMSO	dimethyl sulphoxide
DPP-IV	dipeptidyl peptidase 4
eNOS	endothelial cell nitric oxide synthase
ELISA	enzyme-linked immunosorbent assay
FBG	fasting blood glucose

FEK$^+$	fractional excretion of potassium ion
FENa$^+$	fractional excretion of sodium ion
FFA	free fatty acid
G	gram
GBM	glomerular basement membrane
GFB	glomerular filtration barrier
GFR	glomerular filtration rate
GLUT4	glucose transporter 4
h	hour
H	hepatocyte
H$_2$O$_2$	hydrogen peroxide
HCD	high carbohydrate diet
HCl	hydrochloric acid
HDL-C	high-density lipoprotein cholesterol
HFD	high fat diet
hIAPP	human islet amyloid polypeptide
HOMA-IR2	homeostasis model assessment of insulin resistance
HRP	horseradish peroxidase
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL-1β	interleukin-1β
IL-6	interleukin 6
Inos	Inducible nitric oxide synthase
IRS	insulin receptor substrate

JNK	c-Jun-NH2 terminal kinase
K^+	potassium ion
kg	kilogram
KIM-1	kidney injury molecular-1
Λ	lambda
L	litre
LD	lipid droplets
LDL-C	low-density lipoprotein cholesterol
LPS	lipopolysaccharides
MDA	malondialdehyde
mmol	millimole
MPV	mean platelet volume
MTF	metformin
μ	micro
μg	microgram
μl	microlitre
mg	milligram
ml	millilitre
mRNA	messenger ribonucleic acid
Na^+	sodium ion
Ng	nanogram
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NPD	non-prediabetic
NO_2	nitrogen dioxide
NO	nitric oxide
NF-κβ	nuclear factor kappa-beta
OGTT	oral glucose tolerance test
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PD	pre-diabetic
PDK1	phosphatidylinositol-dependent protein kinase 1
Pg	petagram
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
RAAS	renin angiotensin aldosterone system
RAGE	receptor for advanced glycation product
RNA	ribonucleic acid
ROS	reactive oxygen species
RU	ruthenium(II) complex
SEM	standard error of means
SGLT2	sodium-dependent glucose cotransporter-2
SOD	superoxide dismutase
SREBP-1c	steroid regulatory elementary binding Protein 1c
T2DM	type 2 diabetes mellitus
TBA	thiobarbituric acid
TBARs	thiobarbituric acid reactive substances
TGs	triglycerides
TNF-α	tumour necrosis factor α
TOF	time-of-flight
TZDs	thiazolidinediones
USA	United States of America
UKZN	University of KwaZulu-Natal
VLDL	very low-density lipoproteins
WBC	white blood cell
WHO	World Health Organization

STUDY OUTLINE

The current dissertation is presented in manuscript format, consisting of 7 sections viz. abstract, chapter 1: abstract, introduction/literature review, chapter 2: manuscript 1, chapter 3: manuscript 2, chapter 4: manuscript 3, chapter 5: synopsis and appendices. The abstract briefly summarizes the purpose and innovation of the present study in research. Chapter 1 entails a brief background and relevant literature review in problem solving and current gaps, and how the aims of the current research fill the gaps in the literature. Chapter 2 comprises of the first novel research study in manuscript format that seeks to investigate the hepatoprotective effects of a ruthenium(II) complex on diet-induced pre-diabetic rats and the management of pre-diabetes through pharmacotherapy combined with dietary intervention. This work was authored by L.P Mabuza, M.W Gamede, S Maikoo, IN Booysen, P.S Ngubane and A Khathi. This manuscript has been accepted for publication in the journal of **Current Therapeutic Research**. Chapter 3 comprises the second research study in manuscript formal that seeks to investigate amelioration of risk factors associated with diabetic nephropathy in diet-induced pre-diabetic rats by an uracil-derived diimine ruthenium(II) compound and the management of pre-diabetes through pharmacotherapy combined with dietary intervention. This work was authored by L.P Mabuza, M.W Gamede, S Maikoo, IN Booysen, P.S Ngubane and A Khathi and has been submitted for publication in the journal **Nutrients** and has been formatted according to journal's guidelines to authors. Chapter 4 comprises of the third research study in manuscript format that seeks to investigate the effects of a ruthenium(II) Schiff base complex administration on inflammatory-induced immune response biomarkers in a diet-induced pre-diabetic rat model. This work was authored by L.P Mabuza, M.W Gamede, S Maikoo, IN Booysen, P.S Ngubane and A Khathi and has been submitted for publication in the journal **Immune Network** and has been formatted according to journal's guidelines to authors. Chapter 5 is the discussion that links the findings of the three studies with the aims of the project. Appendices contain the ethical clearance letter, symposium presentation certificates, publications and journal's guideline to authors.

ABSTRACT

Pre-diabetes is a chronic metabolic condition where blood glucose levels are above the upper threshold considered normal but below the threshold for a diagnosis of diabetes. Pre-diabetes predisposes individuals to a high probability of future progression to overt T2DM. Pre-diabetic patients are at increased risk of developing other pathologies such as NAFLD, diabetic nephropathy (DN) and immune dysregulation complications. As a chronic disease, the long-term implications of diabetes contribute to poor quality of life and significantly increase costs associated with healthcare. Pre-diabetes may however be reversible, through the implementation of lifestyle modification programmes based around dietary modification and increased physical activity. Where lifestyle modifications are ineffective, both pharmacotherapy and lifestyle modification are recommended. However, there is reported poor patient compliance in terms of dietary intervention as patients tend to heavily rely on the pharmacological treatment, thus reducing the efficacy of the drug and increasing the possibility to develop T2DM. Hence, there is a need for novel drugs that will remain therapeutic even in the absence of dietary modification. In our laboratory, we have synthesized a novel ruthenium(II) uracil-derived diimine complex that has been shown to improve insulin sensitivity and restore glucose homeostasis in diet-induced prediabetes. In this study, we further sought to evaluate the effects of this compound on selected complications associated with diet-induced pre-diabetes. Estimating whether ruthenium(II) uracil-derived diimine complex in both the presence and/or absence of dietary intervention will show to be effective in the management of prediabetic-related complications. A high fat high carbohydrate (HFHC) diet was used to induce pre-diabetes for 20 weeks. After the induction, pre-diabetic rats were randomly allocated to the following treatment groups: non-prediabetes (NPD); pre-diabetic (PD); metformin plus HFHC; metformin plus normal diet (ND); Ruthenium plus HFHC and ruthenium plus ND. The animals were treated with subcutaneous injection of ruthenium complex (15 mg/ kg) and oral dose of metformin (500 mg/ kg). The rats were treated once a day every third day at 09:00 am for 12 weeks. Every 4 weeks, parameters such as body weight, food intake, fasting blood glucose, fluid intake and urinary output were monitored for 12 weeks treatment period. In study 1, the administration of ruthenium(II) complex resulted in the restoration of liver and body weights in the pre-diabetic treated rats when compared to the PD group. This treatment also reduced liver damage enzyme biomarkers and plasma total bilirubin

levels in the pre-diabetic treated rats when compared to the PD group   whilst administration of ruthenium(II) with dietary intervention reduced plasma sterol regulatory element binding protein 1c (SREBP-1c) concentration in the pre-diabetic treated rats when compared to the PD group. These findings were further supported by the histological analysis of the liver, showing reduced hepatic lipid droplet accumulation, hepatocyte ballooning and locular disarray in the ruthenium(II)-treated rats when compared to the PD group as seen in chapter 2 of the study. In study 2, the administration of ruthenium(II) complex resulted in reduced blood glucose, aldosterone, fluid intake and urinary output in the pre-diabetic treated rats when compared to the PD group which positively correlated with a restoration in plasma and urinary electrolytes along with plasma antioxidants concentrations in the ruthenium(II)-treated rats. Furthermore, there was a decrease in kidney injury molecular-1 (KIM-1) concentration, albumin excretion rate (AER) albumin creatinine ratio (ACR) and mRNA expression of podocin in urine in the ruthenium(II)-treaded rats. These observetions were further demonstrated by the histological analysis of the kidney, displaying improved histology of renal glomerulus in ruthenium-treated rats when compared to the PD group as seen in chapter 3 of the study. In study 3, treatment with ruthenium(II) complex resulted in reduction of platelet activation markers mean platelet volume (MPV) and CD40 Ligand (CD40 L) concentrations, which positively correlated with decreased plasma triglycerides (TG) and very low-density lipoproteins (VLDL) levels in the pre-diabetic treated rats when compared to the PD group. Whilst administration of ruthenium(II) with dietary intervention reduced plasma fibrinogen concentration in the pre-diabetic treated rats when compared to the PD group. These was further evidenced by normalization of immune cell counts in the ruthenium(II)-treated rats. Furthermore, there was a decrease in pro-inflammatory cytokines, tumor necrosis factor α (TNF-α) concentration in the ruthenium(II)-treated rats and decreased interleukin-1β (IL-1β) concentration in the ruthenium(II) with dietary interventiontreated rats when compared to the PD group as seen in chapter 4 of the study. Taken together, the results observed suggest that ruthenium(II) complex exhibited hepato and renoprotective effects while ameliorating immune dysregulation underlying pre-diabetes in diet-induced pre-diabetic rats. However, further studies are still required to find out the exact mechanism behind potential effect of this metal-based compound.

CHAPTER 1: INTRODUCTION/LITERATURE REVIEW

1.1 Background

According to the American Diabetes Association (ADA), the criteria to define pre-diabetes consists of impaired fasting glucose (IFG) with fasting plasma glucose levels of 5.6-6.9 mmol/L, impaired glucose tolerance (IGT) with 2-hour postprandial glucose levels of 7.8-11.0 mmol/L or glycated haemoglobin (HbA1c) of 5.7-6.4% (Jesudason *et al.*, 2003; Syed, 2011; ADA, 2019). HbA1c is considered as a more reliable test of impaired glucose homeostasis as it is reflective of steady-state blood glucose levels over a period of several months (Syed, 2011; ADA, 2019). In pre-diabetes, an HbA1c level higher than 5.7 % indicates poor blood glucose control (Hostalek, 2019). Pre-diabetic dysglycaemia as a result of alterations in insulin sensitivity, pancreatic β-cell function, inflammatory cytokines and hepatic glucose production, has been shown to lead to the development of microvascular and macrovascular complications in pre-diabetic patients (Bugianesi *et al.*, 2005; Brannick *et al.*, 2016). Insulin resistance is associated with NAFLD which encompasses a histological spectrum ranging from simple steatosis, non-alcoholic steatohepatitis (NASH) and cirrhosis (Kumashiro *et al.*, 2011; Byrne and Targher, 2014). Non-alcoholic fatty liver disease carries a higher risk of cardio-metabolic and liver-related complications, the latter being confined to NASH and demanding specific treatment (Mavrogiannaki and Migdalis, 2013). The liver plays an important role in the maintenance of systemic lipid and glucose homeostasis (Hamelet *et al.*, 2007). However, pre-diabetic liver damage leads to derangements in the regulation of both lipid and glucose metabolism and become a vicious cycle (Hamelet *et al.*, 2007). Recently, researchers have shown that hyperglycaemia promotes overproduction of reactive oxygen species (ROS) causing oxidative stress (Brown and Griendling, 2015). The body has its own anti-oxidative defence enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) to transform ROS into less reactive products (Brown and Griendling, 2015). Diabetic dyslipidaemia is also characterised by elevated triglycerides (TG), low high-density lipoprotein (HDL) cholesterol and higher concentrations of apoB-containing particles which lead to acceleration of atherosclerosis and liver-related complications in pre-diabetes (Boren *et al.*, 2013; Parhofer, 2015). Studies indicate that up to one-third of adults with newly diagnosed diabetes mellitus are already showing signs of kidney damage (Okada *et al.*, 2014). Hyperglycaemia has been shown to evoke the onset and progression of DN because of its role in causing hemodynamic dysregulation along with abnormal morphological and functional nephron changes, ranging from increased glomerular basement membrane (GBM) thickness, mesangial expansion, extracellular matrix deposition, glomerulosclerosis, overt proteinuria and decreased glomerular function and filtration rate to

eventually, end-stage renal damage (Tabák *et al.*, 2012; Hostalek, 2019). Furthermore, the progression of pre-diabetes to overt T2DM involves a complex interaction between metabolism and inflammatory-induced immune response (Hameed *et al.*, 2015). Increased lipid accumulation in adipocytes have been shown to activate c-Jun N-terminal kinase (JNK) and nuclear factor kappa B (NF-κB) signalling pathways and subsequently increase the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). These pro-inflammatory cytokines have been shown to activate both the innate and adaptive immune response in diabetic patients, which further contribute to inflammatory-induced immune dysregulation (Lontchi-Yimagou *et al.*, 2013; Hameed *et al.*, 2015).

The management of hyperglycaemia and pre-diabetes associated complications relies on a combination of oral hypoglycaemic drugs and lifestyle modification such as dietary intervention (Giacco *et al.*, 2013). Lifestyle and dietary interventions are recognised as one of the cornerstones in preventing diabetes, managing existing diabetes and delaying the development of diabetic complications through improved glycaemic control and insulin sensitivity (Giacco *et al.*, 2013). Upon diagnosis, patients are recommended to adhere to both pharmacotherapy and lifestyle modification (Spoto *et al.*, 2016). Lifestyle modification includes, but is not limited to, dietary intervention and the incorporation of moderate exercise (Spoto *et al.*, 2016). However, there have been reports on low patient compliance as patients take the hypoglycaemic drugs and neglect lifestyle intervention thus compromising the efficacy of the drugs (Lindström *et al.*, 2013; Parajuli *et al.*, 2014; Tseng *et al.*, 2017). Therefore, the development of novel drugs that can remain therapeutic even in the absence of dietary modification is required. Research has shown significant progress in the utilization of transition metal complexes as drugs to treat several human diseases (Allardyce and Dyson, 2006; Rafique *et al.*, 2010). Transition metals exhibit different oxidation states and can interact with many negatively charged molecules. These activities of transition metals have started the development of metal-based drugs with promising pharmacological application and may offer alternative therapeutic opportunities (Allardyce and Dyson, 2006). Organoruthenium compounds emerge as the most promising with biological features including their low toxicity, mechanism of action and a different preference for protein rather than DNA binding in comparison to platinum compounds (Komers and Anderson, 2003; Antonyan *et al.*, 2014; Booysen *et al.*, 2014). In our laboratory, we have previously reported on a diet-induced pre-diabetic rat model that accurately depicts the human condition (Luvuno *et al.*, 2018). Additionally, we have used this rat model to show that administration of the ruthenium(II) complex improves glycaemic control, insulin sensitivity as well

as decreases the risk of developing diabetes-related cardiovascular diseases (Mabuza *et al.*, 2018; Mabuza *et al.*, 2019). However, the hepatoprotective, renoprotective and immune response effects of the ruthenium(II) uracil-derived diimine complex in diet-induced pre-diabetic state remain unclear. Therefore, the present study sought to investigate the effects of a ruthenium(II) uracil-derived diimine complex on liver and kidney function as well as the inflammatory-induced immunity response in diet-induced pre-diabetes rats in both the presence and absence of dietary intervention.

1.2 Pre-diabetes

Pre-diabetes is a chronic metabolic condition where blood glucose levels are above the upper threshold for normal but below the threshold for a diagnosis of diabetes mellitus (Tabák *et al.*, 2012; Hostalek, 2019). Pre-diabetes is increasingly recognised as an important metabolic state that predisposes individuals to a high probability of future progression to overt T2DM (Tabák *et al.*, 2012). The diagnostic criteria and terminology associated with pre-diabetes vary considerably between organisations, and care must be taken when interpreting and describing prevalence and incidence data (Tabák *et al.*, 2012; Hostalek, 2019). Type 2 diabetes mellitus progression is however, not only connected with changes in metabolic biomarkers such as blood glucose levels, HbA1c and cholesterol, but also pro-inflammatory cytokines, antioxidants, endothelial dysfunction, oxidative stress, lipid peroxidation products and immune system dysregulation markers (Maschirow *et al.*, 2015). The risks of diabetic-related complications are significantly increased in pre-diabetic patients, where lifestyle modifications have been recommended as a method to manage pre-diabetes (Giacco *et al.*, 2013). Pre-diabetes may, however, be reversible, through the implementation of lifestyle modification programmes based on the intervention of healthier diet and increased levels of physical activity (ADA, 2019). The benefits of this have been confirmed in many prospective, randomized studies where lifestyle modification and pharmacological intervention have been shown to significantly improve clinical markers related to the risk of pre-diabetes (ADA, 2019). Medical guidelines currently considers pre-diabetes as a risk factor for T2DM and related complications (Laaksonen *et al.*, 2005; Giacco *et al.*, 2013; Evert *et al.*, 2014). As the disease progresses, tissues or vascular damage ensues, leading to severe diabetic complications (Laaksonen *et al.*, 2005; Giacco *et al.*, 2013; Evert *et al.*, 2014). Thus, pre-diabetes covers a wide range of heterogeneous diseases. The pre-diabetic state has been reported to lead to the development of T2DM and related complications such as diabetic retinopathy, neuropathy, nephropathy and macrovascular complications (Ahmed *et al.*, 2010). Therefore, the key to prevention of diabetes development and its vascular complications are early detection and treatment of pre-diabetes. A major part of understanding disease progression is to unravel pathological processes

associated with asymptomatic or preclinical stages of disease (Ahmed *et al.*, 2010). In this study, we investigated the evaluation of the therapeutic properties of a ruthenium(II) uracil-derived diimine complex on selected complications associated with diet-induced pre-diabetes.

1.3 Diagnosis of pre-diabetes

The diagnostic criteria and terminology associated with pre-diabetes vary considerably between organisations (Hostalek, 2019; ADA, 2019). Both the World Health Organization (WHO) and the ADA provide guidance on screening for pre-diabetes based on the assessment of IGT and IFG levels (Hostalek, 2019; ADA, 2019). Impaired fasting glucose, reflective of hepatic insulin resistance is considered the more important predictor of diabetes risk than skeletal muscle insulin resistance described by IGT (Yip *et al.*, 2017). Impaired fasting glucose (WHO: 6.1-6.9 mmol/L; ADA: 5.6-6.9 mmol/L) is assessed based on the fasting plasma glucose (FPG) level and IGT (WHO: 7.8-11.0 mmol/L; ADA: 7.8-11.0 mmol/L) using the 2-h plasma glucose during a 75 g oral glucose tolerance test (OGTT) (Tabák *et al.*, 2012; Hostalek, 2019). While the defined thresholds for IGT are common to both guidelines, the ADA recommends a lower threshold for IFG compared to the WHO guidelines (Tabák *et al.*, 2012; Hostalek, 2019). This strategy was an attempt to improve the concordance of prevalence estimates between IFG and IGT which when defined using WHO thresholds can differ considerably from each other. The criteria used by the WHO were derived not on overall prevalence, but to reflect the relative likelihood of progression to overt T2DM (ADA, 2019).

The ADA have also recommended the assessment of HbA1c (5.7-6.4 %) to screen for pre-diabetes (Sequeira and Poppitt, 2017). However, this view is not recommended by the WHO (Sequeira and Poppitt, 2017). These criteria have evolved over the years and discussions on the topic continue. Recently, HbA1c has been widely used in the diagnosis of pre-diabetes and is considered as a more reliable test for monitoring the glycaemic control in individuals with diabetes (Syed, 2011; ADA, 2019). In pre-diabetes, higher amounts of HbA1c indicate more inadequate control of blood glucose levels (Jesudason *et al.*, 2003; Tran *et al.*, 2004; Syed, 2011). Once a haemoglobin molecule is glycated, glucose remains in the red blood cell for the rest of its lifespan (120 days). As such, HbA1c provides information about the degree of long-term blood glucose control (Jesudason *et al.*, 2003; Tran *et al.*, 2004; Syed, 2011). Furthermore, HbA1c is also a more convenient screening test than FBG or OGTT, as fasting is not required (Sequeira and Poppitt, 2017). In some cases, the diagnosis of pre-diabetes relies on one criterion (such as IGT), while others evaluate results from more than one test,

therefore relying on one test may underestimate prevalence. Therefore, with a range of criteria available for pre-diabetes, population with pre-diabetes diagnosed by each method varies (Hostalek, 2019). Therefore, according to the ADA guideline, an abnormal finding of any of the three criteria, the IFG, IGT and HbA1c is sufficient to confirm the diagnosis of pre-diabetes (ADA, 2019). Moreover, the ADA recommendations are regularly updated with the most recent scientific data and may be considered more globally accepted than the other guidelines (Hostalek, 2019). Thus, in this study the ADA pre-diabetes criteria were used to diagnose for pre-diabetes.

1.4 Epidemiology of pre-diabetes

Glycaemic concentrations are rapidly rising in people living in developed and developing countries (Danaei *et al.*, 2011; Katikireddi *et al.*, 2011). The estimates of pre-diabetes vary due to the diagnostic criteria used and the choice of test (Tabák *et al.*, 2012; Hostalek, 2019; ADA, 2019). The global prevalence of IGT was estimated at 7.3% of the adult population in 2017, equivalent to 352.1 million individuals (ADA, 2019). The prevalence is anticipated to increase to 8.3% of the global adult population, equivalent to an estimated 587 million individuals by 2045, with the Africa region expected to experience the greatest increase (ADA, 2019). Several epidemiological studies have demonstrated a strong relationship between ethnicity and likelihood of pre-diabetes in African American, Native American, South Asian and Hispanic people (Yip *et al.*, 2017). This data suggest that individuals progress from normoglyaemia to IGT and that the likelihood for further progression to T2DM is the same across ethnic groups (Yip *et al.*, 2017). Factors that influence the prevalence rates of pre-diabetes involve a complex interaction of further factors like life-expectancy, socioeconomic status, wealth, access to healthcare services, levels of education, public health awareness initiatives, and regional levels of obesity (Anjana *et al.*, 2017). Increases in prevalence are expected to be more pronounced in developing rather than in developed countries as lifestyles become more westernised (Anjana *et al.*, 2017).

1.5 Pathophysiology of pre-diabetes

The progression of prediabetes to overt diabetes often occurs over many years, although its onset can be rapid (Gastaldelli and Ferrannini, 2014). The first stage of the multistage model of pre-diabetes development is defined by a long period of insulin resistance accompanied by a compensatory increased rate of insulin secretion and increased β-cell mass (Chen *et al.*, 2015). The second stage is the stable adaptation period when β cells are no longer compensating the increase in insulin resistance with the fasting, and postprandial glucose values are not entirely maintained. This period is said to start when

fasting and postprandial glucose levels are still within the normal range and is usually accompanied by a decrease in acute insulin secretion at FBG concentrations of around 5.6 mmol/L (Chen *et al.*, 2015). The first and second stages occur before the pre-diabetic phase. During the unstable early decompensation period, the third stage of diabetes development where β cells become unable to compensate for insulin resistance and consequently glucose concentrations start to increase rapidly, leading to glucolipotoxicity and extends from pre-diabetes to manifest T2DM (Chen *et al.*, 2015).

1.5.1 Glucolipotoxicity

Glucotoxicity and lipotoxicity have been positively correlated with insulin resistance and β-cell toxicity, a phenomenon referred to as glucolipotoxicity (Tabák *et al.*, 2012). The relation between glucose and lipid toxicity with insulin resistance and β-cell toxicity have been shown and the mechanism on how they affect pancreas is said to be distinct (Kim and Yoon, 2011; Schwartz and Reaven, 2012). Isolated hyperglycaemia or elevated circulating free fatty acids (FFAs) may not be detrimental to the β-cell (Kim and Yoon, 2011; Schwartz and Reaven, 2012). Elevated glucose levels alone results in oxidation and isolated elevation in FFAs are oxidized instead of glucose (Kim and Yoon, 2011; Schwartz and Reaven, 2012). However, when both glucose and FFA levels are elevated, progressive tissue toxicity may ensue. Free fatty acids impair β-cell function, particularly in the setting of elevated glucose levels (Kim and Yoon, 2011; Schwartz and Reaven, 2012). Free fatty acids act via multiple, ultimately converging, pathways that include suppression of cellular proliferation, impairments in β-cell gene transcription, alterations in glycerolipid, and elevations in reactive oxidative species production (Kim and Yoon, 2011; Schwartz and Reaven, 2012). Lipid accumulation in the liver appears to be a principal mechanism associated with obesity-related insulin resistance and T2DM(Haus *et al.*, 2010). Altered metabolism of triglyceride-rich lipoproteins is an integral part of the atherogenic dyslipidaemia in insulin resistant pre-diabetic individuals and in T2DM (Huynh *et al.*, 2013). It is characterized by elevated serum TG levels and decreased high-density lipoprotein cholesterol (HDL-c) (Haus *et al.*, 2010). Increased hepatic secretion and decreased clearance of VLDL and intestinally derived chylomicrons result in prolonged plasma retention of these particles and accumulation of highly atherogenic partially lipolyzed cholesterol-enriched intermediate density lipoprotein (IDL) remnants and small dense LDL particles (Avramoglu *et al.*, 2006; Lorenzo *et al.*, 2013). Excess triacylglycerol's beyond the oxidative needs of lean tissue leads to impairment in the liver, skeletal muscle, cardiac muscle and endocrine pancreas, resulting in tissue dysfunction or lipotoxicity (Schwartz and Reaven, 2012). This eventually leads to lipoapoptosis or lipid-induced cell death (Avramoglu *et al.*, 2006; Lorenzo *et al.*, 2013). This toxic tissue effect has been attributed to the generation of

specific proapoptotic lipid species or signalling molecules such as reactive oxygen species generation, *de novo* ceramide synthesis, nitric oxide generation, decreases in phosphatidylinositol-3-kinase and primary effects on mitochondrial structure or function (Bergman, 2013). Free fatty acids resulting from hydrolysis of stored triacylglycerols result in decreased glucose transport via inhibition of key glucose transporters in insulin responsive tissues such as skeletal muscle, resulting in insulin resistance (Schwartz and Reaven, 2012). Free fatty acid oxidation stimulates the activity of key gluconeogenic enzymes (pyruvate carboxylase, phosphoenolpyruvate carboxykinase, glucose-6-phodphastase) (Bergman, 2013). Thus, lipotoxicity results in pancreatic and peripheral defects eventually resulting in sustained hyperglycaemia and the development of insulin resistance (Bergman, 2013).

1.5.2   Effect of insulin resistance on prediabetic-related complications

Insulin resistance is a complicated condition in which three primary metabolic tissues that are sensitive to insulin, namely, skeletal muscle, liver and white adipose tissue become less sensitive to insulin and its downstream metabolic actions under normal serum glucose concentrations (Laakso and Kuusisto, 2014). The stimulation of glucose uptake by insulin is mediated by phosphatidylinositol (PI) 3-kinase–dependent and independent pathways (Saltiel and Pessin, 2003; Watson *et al.*, 2004). Upon tyrosine phosphorylation, insulin receptor substrate (IRS) proteins interact with the p85 regulatory subunit of PI 3-kinase, leading to the activation of the enzyme and its targeting to the plasma membrane. The classic insulin-like signalling cascades involve the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) by the phosphatidylinositol 3-kinase (PI3K). Phosphatidylinositol 3,4,5-trisphosphate recruits the Serine/Threonine kinases phosphatidylinositol-dependent protein kinase 1 (PDK1) and AKT to the plasma membrane, where AKT is activated by PDK1-mediated phosphorylation at the threonine 308 residue (Shepherd, 2005). This signalling event primes AKT for phosphorylation at serine 473 by mTORC2 (the complex rictor/mTOR), which activates the AKT serine/threonine kinase activity (Shepherd, 2005). Activated AKT phosphorylates many proteins, including glycogen synthase kinase 3b (GSK3B) in the liver, AS160 (GLUT4 translocation), the BADBCL2 heterodimer (anti-apoptotic), and forkhead box O transcription factors (regulation of gene expression) (Taguchi and White, 2008). Processes such as glucose uptake, lipid synthesis, protein synthesis, and glycogen deposition are controlled by insulin (White, 2003; Stolar, 2010). Protein phosphatases and phospholipid phosphatases modulate the strength and duration of insulin signals (White, 2003). In pre-diabetes, insulin resistance in the liver has been shown to be the predominant causative factor (Bugianesi *et al.*, 2005; Larter and Farrell, 2006; Leclercq *et al.*, 2007). In the liver, insulin increases the activity of glucose utilizing pathways

(glycolysis, glycogenesis) and decreases glucose producing pathways (gluconeogenesis, glycogenolysis), whereas glucagon and epinephrine have contrary effects (Nuttall *et al.*, 2008). Insulin receptor substrate 1and 2 are complementary key players in the regulation of hepatic insulin signalling and expression of genes involved in gluconeogenesis, glycogen synthesis and lipid metabolism (Fritsche *et al.*, 2008). The dysfunction of IRS has been reported to impair glycogenesis, enhance gluconeogenesis and reduce suppression of lipolysis in adipose tissue. These have been considered as a major pathophysiological mechanism for the development of insulin resistance and pre-diabetes related complications (Taniguchi *et al.*, 2005; Simmgen *et al.*, 2006; Dong *et al.*, 2006). Ruthenium(II) uracil-derived diimine complex improves insulin sensitivity as evidenced by reduced HOMA-IR2 and HbAc1 levels in a diet-induced pre-diabetic rat model (Mabuza *et al.*, 2018). Therefore, the study aims to evaluate the therapeutic properties of the ruthenium(II) uracil-derived diimine complex on selected complications associated with pre-diabetes in a diet-induced prediabetic rat model.

1.5.3   Low-grade inflammation and oxidative stress

The development of insulin resistance and pathogenesis of pre-diabetes are directly interlinked with each other (Eringa *et al.*, 2013). Pro-inflammatory and oxidative stress mediators play a key role causative factors in the pathogenesis of pre-diabetes and development of insulin resistance (Akash *et al.*, 2013; Rehman and Akash, 2016). The most essential pro-inflammatory mediators that induce inflammation include, IL-1β, IL-6 TNF-α and chemokines (Akash *et al.*, 2013; Rehman and Akash, 2016). Experimental, clinical and epidemiology studies have reported that the levels of these pro-inflammatory cytokines in insulin resistant and T2DM experimental animal models and/or patients are found to be increased (Donath, 2014; Chen *et al.*, 2015). This reveals the critical role of pro-inflammatory mediators in the development of insulin resistance and pathogenesis of T2DM (Donath, 2014; Chen *et al.*, 2015). Pro-inflammatory mediators play a crucial role in the induction of insulin resistance and T2DM via involvement of oxidative stress and activation of various transcriptional mediated molecular and/or metabolic pathways (Akash *et al.*, 2018). Glucolipotoxicity induces the generation of ROS and oxidative stress that are responsible for generating various pro-inflammatory mediators (Kim and Yoon, 2011). ROS and reactive nitrogen species (RNS) are primarily produced through activity of the electron transport chain in mitochondria and by other pathways including xanthine oxidase, lipoxygenase, myeloperoxidase and NO synthase (Brown and Griendling, 2015). Alterations in electron transport chain activity result in increased electrochemical gradients and free radical leakage thus generating oxidative stress (Pitocco *et al.*, 2013). Inactivation and degradation of ROS/RNS are regulated by complex networks of proteins and signalling pathways

including superoxide dismutase, catalase, glutathione peroxidase, peroxiredoxins and thioredoxins (Pitocco *et al.*, 2013). Furthermore, experimental studies have found that pro-inflammatory mediators are interdependently involved in inducing tissue-specific inflammation which may lead to the development of insulin resistance and pathogenesis of T2DM (Akash *et al.*, 2013; Rehman and Akash, 2016). The association between oxidative stress and cardiovascular disease in diabetes via endothelial dysfunction and the resulting pathological changes in inflammation, coagulation status and cell-proliferation has been extensively investigated (Al-Aubaidy and Jelinek, 2011; Fiorentino *et al.*, 2013; Markiewicz *et al.*, 2013). In the case of pre-diabetes, the situation is less understood, but changes in redox-status and the onset of hyperglycaemia-associated complications are already identifiable before diabetes diagnosis (Broedbaek *et al.*, 2011). The central mechanism that is responsible for the increased cardiovascular risk in prediabetes is endothelial dysfunction due to the elevated formation of ROS and advanced glycation end products (AGEs) as well as increased lipid peroxidation under hyperglycaemic conditions (Maschirow *et al.*, 2015). The consequences of endothelial dysfunction include impaired balance between coagulation and fibrinolysis, platelet activation, vascular smooth muscle cell proliferation and stimulation of inflammatory processes, which all together create a pro-thrombotic environment and, if hyperglycaemia remains untreated, cause cardiovascular disease, nephropathy, retinopathy, neuropathy and other diabetic-related complications in the long-term (Maschirow *et al.*, 2015). Of interest, this study will be investigating the hepatic, renal and immune dysregulation complications in diet-induced prediabetic rats. Furthermore, the antioxidant effects of ruthenium are not known hence the study will also seek to evaluate whether the ameliorative effects of the metallo-drug in glycaemic control and cardiovascular disease (CVD) complications will further ameliorate these pre-diabetic complications in the presence and absence of dietary intervention.

1.6.1 Pre-diabetic-related hepatic complications

Pre-diabetes related NAFLD is characterised with lipid abnormalities affecting 60% to 70% of pre-diabetic patients and hyperglycaemia accelerates atheroma formation in the setting of diabetic dyslipidaemia (Guven *et al.*, 2006; Mavrogiannaki and Migdalis, 2013). Metabolism of very-low-density lipoprotein (VLDL), the main transporter for fasting TG, is insulin-regulated at multiple levels (Taskinen, 2003; Erion *et al.*, 2016). Insulin suppresses lipolysis and regulates circulating FFA which are substrates for VLDL cholesterol assembly and secretion (Adiels *et al.*, 2008) . In the liver, insulin mediates transfer of TG to apoB and regulates lipoprotein lipase activity to delipidate VLDL cholesterol (Michael *et al.*, 2000; Laakso, 2010). Lipoprotein lipase activity can be disrupted by increased circulating FFA and

inhibited by apoCIII, whereas apoCIII hinders hepatic uptake of TG-rich lipoproteins and is inhibited by insulin (Michael *et al.*, 2000; Laakso, 2010). Thus, in the insulin-resistant state, hypertriglyceridemia may be a consequence of elevated FFA level and decreased degradation of apoB leading to overproduction of VLDL cholesterol, impaired lipoprotein lipase activity and decreased hepatic uptake of VLDL with reduced VLDL cholesterol clearance (Adiels *et al.*, 2008). Other lipid abnormalities observed in pre-diabetes can be attributed, in part, to elevated TG (Taskinen, 2003; Erion *et al.*, 2016). The transfer of TG from TG-rich lipoproteins to HDL and LDL cholesterol is facilitated by cholesteryl ester transfer protein (CETP) (Taskinen, 2003; Erion *et al.*, 2016). Hypertriglyceridemia stimulates CETP activity resulting in HDL and LDL cholesterol with high TG content (Girona *et al.*, 2016). Enrichment with TG makes HDL particles subject to increased catabolism, lowering plasma HDL cholesterol concentration, whereas TG-enriched LDL particles undergo hydrolysis, decreasing particle size (Girona *et al.*, 2016). Elevated FFA's impair insulin signalling and cause subclinical inflammation with subsequent pancreatic β-cell dysfunction (Rachek, 2013; Parhofer, 2015). In addition, insulin resistance increases fatty acid flux from adipose tissue to the liver through increased adipose tissue lipolysis by the action of hormone sensitive lipase and increased *de novo* lipogenesis by upregulation of steroid regulatory element-binding protein 1-c (SREBP1-c) (Oliver, 2015). Recent investigations have shown that torcetrapib, a CETP inhibitor, raises not only HDL cholesterol concentration but also improves hyperglycaemia (Barter *et al.*, 2011). In addition, recombinant HDL cholesterol infusions improve glucose dysregulation in patients with T2DM (Barter *et al.*, 2011). These observations obtained suggest that HDL cholesterol may play a role in glucose metabolism. The proposed mechanisms included anti-inflammatory properties of HDL cholesterol and the central role of HDL cholesterol in mediating reverse cholesterol transport, or cholesterol efflux, which may subsequently improve insulin sensitivity or secretion (Drew *et al.*, 2009; Drew *et al.*, 2012; Parhofer, 2015). Excessive accumulation of fatty acids also disturbs the β-oxidation system in the hepatic mitochondria and leads to further infiltration of fats in the liver (Moscatiello *et al.*, 2007; Regnell and Lernmark, 2011; Mavrogiannaki and Migdalis, 2013). Furthermore, studies have revealed the prominent histopathological changes in the livers of T2DM patients that is characterised by the accumulation of fat in the vesicles, excess lipid distortion in the nucleus and decreased glycogen content (Moscatiello *et al.*, 2007; Hamden *et al.*, 2008; Lucchesi *et al.*, 2015). Moreover, several cytokines related with insulin resistance are involved with the development of pre-diabetes related NAFLD and activation of inflammatory pathways (Pradhan *et al.*, 2001; Makki *et al.*, 2013).

Hyperglycaemia has been reported to cause increased production of ROS in the liver via formation of Amadori products (Brownlee, 2005). These are oxidized to form AGEs which in turn activate RAGE to stimulate NADPH oxidase-1 with

intracellular ROS production (Brownlee, 2005). The secretion of abnormal liver enzyme levels of plasma alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGT) indicate liver injury (Nannipieri *et al.*, 2005; Mavrogiannaki and Migdalis, 2013). High caloric diets increase plasma levels of these enzymes through the induction of oxidative stress in the liver and these enzymes are found to be increased in pre-diabetic patients (Moscatiello *et al.*, 2007; Mavrogiannaki and Migdalis, 2013). Increased oxidative stress further triggers the inflammatory cascade which leads to liver injury (Manna *et al.*, 2010; Palsamy *et al.*, 2010; Stefan *et al.*, 2014; Iroz *et al.*, 2015). Furthermore, low-grade inflammation has been shown to lead to hepatic insulin resistance and the development of hepatic steatosis (Lumeng and Saltiel, 2011). Pre-diabetes and obesity substantially increase the number of hepatic macrophages and increase their migration into the liver (Obstfeld *et al.*, 2010). Hyperlipidaemia within the atherosclerotic plaque results in the recruitment and migration of monocytes and other immune and inflammatory cells into the vascular sub-endothelial layer (Nishizawa and Bornfeldt, 2012). The recruitment of immune and inflammatory cells contributes to liver injury through the production of proinflammatory cytokines in the liver (Nishizawa and Bornfeldt, 2012).

Currently, there is no effective treatment for NAFLD and related complications. Dietary and lifestyle interventions like those recommended for obesity remain the best therapeutic option for the management of NAFLD. These, when combined with pharmacological therapy are aimed at improving hepatic, pancreatic and peripheral insulin sensitivity (Bugianesi *et al.*, 2005; Hamad *et al.*, 2011; DeFronzo *et al.*, 2013a). The clinical value of MTF, pioglitazone and thiazolidinediones (TZDs) together with other treatments such as betaine, atorvastatin, losartan and orlistat is very subjective. Patients taking these drugs should be closely monitored due to possible contra-indications with T2DMmedications and the vulnerable condition of the liver during the drug detoxification process (Hamad *et al.*, 2011; Mohamed *et al.*, 2016). Additionally, the success of these drugs is often dependent on lifestyle intervention to reduce the risk of developing overt T2DM (Sullivan and Ratner, 2011). Therefore, drugs and lifestyle changes to manage pre-diabetes and the associated complications can only be effective through adherence to the overall prescribed regimen (Sullivan and Ratner, 2011). We have previously shown that the administration of our novel ruthenium(II) complex in diet-induced pre-diabetes ameliorates both peripheral insulin resistance and weight gain (Mabuza *et al.*, 2018), and may perhaps ameliorate hepatic dysfunction . Hence, in chapter 2 of this study, we investigated the effects of a ruthenium(II) uracil-derived diimine complex on markers associated with diabetic liver dysfunction in a diet-induced pre-diabetic rat model in both the presence and absence of dietary intervention.

1.6.2 Pre-diabetes-related renal complications

Several studies have reported that up to one-third of adults with newly diagnosed T2DMalready have kidney damage (Tabák *et al.*, 2012; Hostalek, 2019). Hyperglycaemia is a crucial factor in the development of DN because of its effects on glomerular and mesangial cells, suggesting that DN may occur in the early stages of the disease (Tabák *et al.*, 2012). The effect of hyperglycaemia on the occurrence of DN may start before glucose levels reach the diabetic range (Weil *et al.*, 2012). Persistent hyperglycaemia increases proximal tubular reabsorption of glucose and sodium secondary to the induction of tubular growth and upregulated expression of sodium-dependent glucose cotransporter-2 (SGLT2) which contribute to renal glucose reabsorption (Norton *et al.*, 2017). In the kidneys, all filtered glucose in the glomerulus has to be reabsorbed along the nephron (DeFronzo *et al.*, 2013b). During hyperglycaemia, glucose is excreted in urine causing glucosuria because SGLT2 are saturated with the filtered glucose (Norton *et al.*, 2017). The Na^+/K^+ ATPase pump on the basolateral membrane of the proximal tubule cell uses ATP molecules in exchange of 2 potassium ions (K^+) and 3 sodium ions (Na^+) (Wilding, 2014). Sodium-dependent glucose cotransporter-2 utilizes the electrochemical potential of Na^+ as an energy source to transport glucose across the apical membrane against the glucose gradient (Wilding, 2014). Glucose accumulates within the epithelium in the brush-border membrane and is then transported out of the cell across the basolateral membrane and into peritubular capillaries by facilitated members of the glucose transporter family of glucose uniporters (Kahn, 2002). The increase in proximal reabsorption leads to a decrease in sodium delivery to the macula densa with deactivation of tubuloglomerular feedback and a consequent increase in glomerular pressure and filtration (De Nicola *et al.*, 2016). In turn, these hemodynamic changes are associated with the histopathology of the kidney characterised by mesangial expansion, thickening of the basement membrane and nodular glomerulosclerosis (De Nicola *et al.*, 2016). Studies have shown that pre-diabetes is associated with the subsequent development of glomerular hyperfiltration and albuminuria (Ritz, 2008; DeFronzo *et al.*, 2013b). Glomerular hyperfiltration is a key mediator of progressive kidney damage caused by a variety of initiating injuries, including pre-diabetes (Ritz, 2008; Kanasaki *et al.*, 2013). In the kidney, insulin resistance in pre-diabetes is a common and very early alteration risk factor for a decline in GFR as well as for the onset and progression of DN (Ritz, 2008; Kanasaki *et al.*, 2013). A significant event that precedes renal injury is an increase in permeability of plasma proteins such as albumin through damage to the glomerular filtration barrier (Weil *et al.*, 2012). This results in excessive urinary albumin excretion (UAE) through the nephron (DeFronzo *et al.*, 2013b). Excess albumin excretion into the urine is used as a prominent marker for diabetic kidney disease (De Nicola *et al.*, 2016; Tagawa *et al.*, 2016). Increased albumin leakage results from the impaired integrity of the glomerular

filtration barrier (GFB) which further results in early podocyte loss at the onset of diabetes and initiates increased protein excretion in urine (Suh and Miner, 2013; Anil Kumar *et al.*, 2014). Kidney injury molecule-1 (KIM-1) is another biomarker for renal proximal tubule injury and has been shown to be increased in diabetic patients (Carlsson *et al.*, 2014; El-Ashmawy *et al.*, 2015).

However, as the onset and progression of DN may be independent of glycaemia, other factors such as humoral signals from metabolic tissues might have a role in the early stages of impaired renal function (Moriwaki *et al.*, 2003; Navarro *et al.*, 2006; Navarro-González *et al.*, 2011). Loss of renal function has been shown to be associated with elevated pro-inflammatory cytokine levels with creatinine clearance (CC) which has a positive correlation with cytokines, including IL-1, IL-6 and TNF-α (Moriwaki *et al.*, 2003; Navarro *et al.*, 2006; Navarro-González *et al.*, 2011). In addition, pre-diabetes is linked to the onset and progression of DN through inappropriate activation of the renin–angiotensin system (RAS) and oxidative stress in the kidney (Remuzzi *et al.*, 2005). The resulting pathology results in aldosterone excess which results in sodium and water retention leading to elevated blood pressure (Remuzzi *et al.*, 2005; Jansen *et al.*, 2009; Lim, 2014). In pre-diabetes, there is an increase in plasma osmolality, which causes an increase in ADH secretion and stimulation of thirst (Bouby and Fernandes, 2003; Krug and Ehrhart-Bornstein, 2008). Therefore, inhibition of the RAS could be effective in preventing glomerular hypertension and hyperfiltration as well as in reducing albuminuria and kidney damage (Lim, 2014). In addition, lifestyle and dietary intervention are recommended in the management of DN progression (Spoto *et al.*, 2016). Lifestyle intervention has been found to result in increased insulin sensitivity by enhancing glucose uptake and reduce renal mass (Spoto *et al.*, 2016). Various randomized controlled trials have shown that intensive lifestyle interventions and the use of pharmacological agents can significantly reduce the incidence of overt DM in individuals with pre-diabetes (Plantinga *et al.*, 2010; Roumen *et al.*, 2011; Ghody *et al.*, 2015). Several oral agents including MTF, TZDs, acarbose, orlistat and insulin have been evaluated for the prevention of T2DM and renal complications in patients with pre-diabetes (Ghody *et al.*, 2015). However, these studies have only had modest success. Despite the benefits of therapy, studies have indicated that recommended glycaemic goals are achieved by less than 50% of patients, which may be associated with decreased adherence to therapies (Ghody *et al.*, 2015). As a result, hyperglycaemia and long-term complications increase morbidity and premature mortality, and lead to increased costs to health services (Ghody *et al.*, 2015). Furthermore, recent studies have reported that a ruthenium(II) complex with a diimine uracil chelating ligand possesses anti-diabetic properties through the improvement of glycaemic control, insulin sensitivity and decreased risk of developing diabetes-related CVD in diet-induced pre-diabetic rats (Mabuza *et al.*, 2018;

Mabuza *et al.*, 2019). Ruthenium may therefore ameliorate renal dysfunction since. Hence, in chapter 3 of this study, we investigated the effects of a ruthenium(II) uracil-derived diimine complex on markers associated with DN in a diet-induced pre-diabetic rat model in both the presence and absence of dietary intervention.

1.6.3   Prediabetes-related immune dysregulation complications

The progression of pre-diabetes to overt T2DM involves a complex interaction between metabolism and immunity response (Hameed *et al.*, 2015). Metabolic inflammation is regulated by both the innate and adaptive immune cell interactions and has been shown to be a precipitating factor sustaining insulin resistance, preceding T2DM (Winer and Winer, 2012). Activation of innate immunity in T2DM is linked to the activation of toll-like receptors (TLRs). These receptors have been implicated in diabetes-induced inflammation and vascular complications (Schaeffler *et al.*, 2009; Jialal *et al.*, 2012). The initiation events of a pro-inflammatory response involve synergistic contributions of various mechanisms which include an increase in nuclear factor κB (NF-κB) and c-Jun NH2-terminal kinase (JNK) activity (Cinti *et al.*, 2005; Hotamisligil, 2010; Donath and Shoelson, 2011). Adipocytes are an important initiator of inflammatory response. Stressed adipocytes produce various cytokines and chemokines promoting immune cell activation and accumulation in adipose tissue (Cinti *et al.*, 2005). An increase in adiposity is associated with upregulation of genes encoding pro-inflammatory molecules and accumulation of immune cells (Cinti *et al.*, 2005; Hotamisligil, 2010; Donath and Shoelson, 2011). The sustained accumulation of lipids in adipose tissue results in the switching of macrophages from an anti-inflammatory to a pro-inflammatory phenotype (Prieur *et al.*, 2011). Elevated concentrations of biomarkers such as C-reactive protein (CRP), TNF- α, white blood cell (WBC) count, IL-1 β, IL-6 and fibrinogen point to a chronic, often subclinical degree of inflammation and are increased already before the onset of T2DM (Grossmann *et al.*, 2015). The progression of T2DM has been linked to various immune cells types but the primary sources of inflammatory effectors contributing to insulin resistance are macrophages (McArdle *et al.*, 2013). Besides macrophages, the representation of several other immune cell populations is also altered in diabetes. These include, eosinophils and neutrophils which serve as a negative regulator of adipose tissue inflammation (Talukdar *et al.*, 2012; Goh *et al.*, 2013). In addition, several factors like impaired fibrinolysis, increased coagulation, endothelial dysfunction and platelet hyperreactivity contribute to prothrombic condition that describes patients with T2DM (Ferreiro *et al.*, 2010; Sena *et al.*, 2013; Gaiz *et al.*, 2017). Furthermore, various metabolic abnormalities caused by diabetes leads to increased platelet activation and reactivity, evidenced by elevated levels of surface P-selectin and CD40 ligand markers as well as an increased count and mean

platelet volume (MPV) (Undas *et al.*, 2008; Vizioli *et al.*, 2009; Kodiatte *et al.*, 2012). Type 2 diabetes mellitus is preceded by an extensive period of disease development and the activation of inflammatory-induced immune response is activated before the onset of the disease (Makki *et al.*, 2013). Therefore, beginning treatment during the prediabetic state could be used as a therapeutic target in the management and prevention of pre-diabetes inflammatory-induced immune dysregulation complications. In addition, a study by Mzimela *et al.,* has shown the changes in immune cell concentration during the progression of pre-diabetes to T2DM in a high-fat high carbohydrate diet-induced prediabetic rat model (Mzimela *et al.*, 2019). A combination of pharmacological and dietary intervention is also recommended for the management of inflammatory-induced immune dysregulation to result in increased peripheral insulin sensitivity and improving glycaemic control (Giacco *et al.*, 2013; Evert *et al.*, 2014). Recent studies have reported that a ruthenium(II) Schiff base complex with a diimine uracil chelating ligand possesses anti-diabetic properties through improvement of glycaemic control, insulin sensitivity and decreased pro-inflammatory cytokines in diet-induced pre-diabetic rats (Mabuza *et al.*, 2018; Mabuza *et al.*, 2019). However, the effects of a ruthenium(II) uracil-derived diimine complex on immune response in diet-induced pre-diabetic state remain unclear. Hence, in chapter 4 of this study, we investigated the effects of a ruthenium(II) uracil-derived diimine complex on markers associated with immune dysregulation in a diet-induced pre-diabetic rat model in both the presence and absence of dietary intervention.

1.7   Conventional management of pre-diabetes

1.7.1   Lifestyle intervention

Lifestyle and dietary modification are critical in the management of pre-diabetes and its related complications (Evert *et al.*, 2014; ADA, 2019). The emphasis of nutrition therapy for pre-diabetic patients is on lifestyle strategies that will improve glycaemic control (Franz, 2003; Giacco *et al.*, 2013). Meta-analyses demonstrated that lifestyle interventions including diet and physical activity led to a 63% reduction in diabetes incidence in those at high risk and have demonstrated encouraging improvement (Stiegler *et al.*, 2009). The dietary management of pre-diabetes is a complement of lifestyle management with positive long-term health and quality of life (Frost *et al.*, 2014). Since many pre-diabetic patients are insulin resistant and overweight, nutrition therapy often begins with lifestyle strategies that reduce energy intake and increase energy expenditure through dietary intervention and physical activity (Lindström *et al.*, 2006). These strategies should be implemented as soon as the diagnosis of pre-diabetes or diabetes is made (Lindström *et al.*, 2006; Giacco *et al.*, 2013). The ADA recommends a hypocaloric diet for overweight or obese patients with pre-diabetes to

induce weight loss (ADA, 2019). Implied are the many pleiotropic effects of lifestyle intervention such as a reduction in lipids, blood pressure, and biomarkers that go beyond the straightforward glucose-lowering effect of oral hypoglycaemic drugs (Franz, 2003; Evert *et al.*, 2014). Therefore, dietary intervention may be important in pre-diabetic patients who are often characterized by the aggregation of multiple risk factors such as obesity, poor glycaemic control and insulin resistance (Franz, 2003; Evert *et al.*, 2014). There are various diets that are recommended such as banting, high fat and ketogenic diets in the management of pre-diabetes (McLellan *et al.*, 2014; Opie, 2014; Cooper *et al.*, 2018). The westernization of lifestyle characterized by decreasing physical activity and a dietary pattern with high intake of foods rich in hydrogenated fat, refined grains, and red meat is related to the increase in obesity, overt T2DM and associated complications (Viscogliosi *et al.*, 2013). In developing countries, the diets are based mainly on carbohydrates and high saturated fat which is associated with higher risk of IGT (Misra *et al.*, 2010; Rahati *et al.*, 2014). Furthermore, high caloric diets contribute to poor patient compliance (Misra *et al.*, 2010). Therefore, there is a necessity for patients to change diets once diagnosed with pre-diabetes (Misra *et al.*, 2010). Dietary changes include reduction of total saturated fat and increased dietary fiber intake, along with increased physical activity and weight loss (Lindström *et al.*, 2013). Lifestyle modification focuses on improving dietary quality, physical activity while pharmacological intervention improves glycaemic control and other risk factors of T2DM (Lindström *et al.*, 2013).

Despite the significance of lifestyle measures in pre-diabetes therapy, pharmacotherapy is used to maintain target glucose concentrations. Different oral hypoglycaemic drugs have been in use to aid in the maintenance of blood glucose level at the requisite threshold in people with diabetes through distinct mechanisms (Inzucchi, 2002; Curtis, 2007).

1.7.2 Pharmacological therapy

1.7.2.1 Biguanides

Metformin is an anti-hyperglycaemic drug that belongs to a class of insulin-sensitizing drugs known as biguanides (Ferrannini, 2014). Upon diagnosis with pre-diabetes, metformin form part of first-line treatment (Ferrannini, 2014). Biguanides improve insulin sensitivity via activation of the AMP-K pathway and hence decrease gluconeogenesis by approximately 20-30% when given orally but not intravenously (Musso *et al.*, 2010). Furthermore, biguanides decrease intestinal glucose absorption and increase insulin-mediated glucose uptake in skeletal muscle (Musso *et al.*, 2010). Metformin has also been shown to decrease serum triglycerides and fatty acid concentrations and slows the rate of lipid oxidation, actions that indirectly inhibit gluconeogenesis (Loomba *et al.*, 2009). Metformin does not cause insulin release

from the pancreas and does not cause hypoglycaemia, even in large doses (Loomba *et al.*, 2009). Furthermore, metformin treatment is associated with statistically and clinically significant reduction in body weight in obese patients with T2DM (Loomba *et al.*, 2009). Metformin has been reported to improve liver injury but could not prevent fibrosis in patients with steatosis (Loomba *et al.*, 2009). As metformin is not metabolized via the hepatic CYP450 system, its pharmacokinetic characteristics do not expose patients to drug–drug interactions (Holstein and Beil, 2009). However, there have only been a few reported cases of the hepatotoxic side effects of metformin, but there may be an increased risk of developing lactic acidosis in the setting of diabetes-induced impaired liver function (Kutoh, 2005; Holstein and Beil, 2009). Additionally, a recent meta-analysis concluded that 6-12 months treatment of metformin coupled with lifestyle intervention did not improve liver histology or aminotransferases compared with lifestyle intervention alone, independently of dose, treatment duration or diabetic state (Musso *et al.*, 2010). Moreover, metformin is also contraindicated for use in patients with renal insufficiency or with congestive heart failure because of the risk of lactic acidosis (Holstein and Beil, 2009). Renal dysfunction appears to be the most common risk factor implicated with lactic acidosis and many current guidelines suggest discontinuation of metformin at a GFR of <60 mL/min (Pongwecharak *et al.*, 2009). Furthermore, metformin has been shown to inhibit monocyte secretory function and reduce systemic inflammation in pre-diabetic patients (Krysiak and Okopien, 2013). However, lifestyle intervention was about twice as effective as metformin for prevention of diabetes and was the only intervention associated with regression to normal glucose regulation (Krysiak and Okopien, 2013). Therefore, there is a necessity to evaluate other alternative treatments in the management and prevention of NAFLD, DN and immune dysregulation complications during pre-diabetic state.

1.7.2.2 TZDs

Among insulin-sensitizing drugs,TZDs are a therapeutic class that are selective agonists for peroxisome proliferator-activated receptor-γ (PPAR-γ), a transcription factor that regulates gene expression in liver, adipose, vascular endothelium and muscle tissue (Esterson *et al.*, 2013). The currently available TZDs include rosiglitazone and pioglitazone and they are reported to improve insulin sensitivity at the level of adipose tissue, muscle and liver (Esterson *et al.*, 2013). Thiazolidinediones bind to PPAR-γ which in turn activate insulin-responsive genes that regulate carbohydrate and lipid metabolism (Esterson *et al.*, 2013). They require insulin to be present to exert their mechanism of action (Ceriello, 2008). There is strong evidence to indicate that these receptors may be important regulators of adipose differentiation, lipid homeostasis, insulin action, and vascular endothelial function (Yang *et al.*, 2012). They increase glucose transport into

muscle and adipose tissue by enhancing the synthesis and translocation of specific forms of glucose transporter proteins such as GLUT4 (Yang *et al.*, 2012). In addition, TZDs can also activate genes that regulate FFA metabolism in peripheral tissue thus lowering triglycerides and non-esterified fatty acid levels and inducing differentiation of adipocytes (Miyazaki *et al.*, 2001). Despite a concern for hepatotoxicity raised by an earlier drug of the same class, troglitazone, no cases of severe hepatitis or exacerbation of non-alcoholic steatohepatitis (NASH) have been noted in clinical studies of pioglitazone and rosiglitazone. However, there have been a few isolated case reports linking these drugs to cholestatic liver injury (May *et al.*, 2002; Mannucci *et al.*, 2010). Thiazolidinediones may have salutary effects on DN by decreasing both macro- and microalbuminuria, therefore use of them requires careful monitoring for volume retention and hepatitis (Yang *et al.*, 2012). In rodents, TZDs have direct protective effects on the β-cell against oxidative stress and apoptosis, which may contribute to preservation of β-cell mass (Esterson *et al.*, 2013). Thiazolidinediones have been shown to decrease inflammatory markers in visceral adipose tissue, liver, atherosclerotic plaques and circulating plasma (Esterson *et al.*, 2013). Pioglitazone treatment decreased invasion of adipose tissue by pro-inflammatory macrophages and increased hepatic and peripheral insulin sensitivity (Ceriello, 2008). Various clinical studies have examined the anti-inflammatory and antiatherogenic properties of TZDs (Zhao *et al.*, 2010; Schöndorf *et al.*, 2011; Erem *et al.*, 2014). A meta-analysis showed that pioglitazone and rosiglitazone significantly decreased serum CRP levels in both nondiabetic and diabetic patients, irrespective of effects on glycaemia (Zhao *et al.*, 2010). Treatment with TZDs also improved endothelial function, decreased hs-CRP and inflammatory markers, and increased adiponectin levels (Schöndorf *et al.*, 2011; Erem *et al.*, 2014). However, the most common side effect of TZDs such as weight gain are still present (Musso *et al.*, 2010). There is also increased risk of congestive heart failure which occurs in about 60-70% of patients (Musso *et al.*, 2010).

1.7.2.3 Sodium-glucose co-transport 2 (SGLT-2) inhibitors

Sodium-glucose co-transport 2 inhibitors are the latest approved class of glucose-lowering agents (Raskin, 2013; Mudaliar *et al.*, 2015). Sodium-glucose co-transport 2 inhibitors work by blocking sodium/glucose uptake in the proximal tubules of the nephron thereby inducing glucose excretion in the urine (Raskin, 2013; Mudaliar *et al.*, 2015). As excess glucose is excreted, there is a decrease in blood glucose level, decreased hepatic storage of glucose, decreased insulin secretion and, subsequently, decreased carbohydrate conversion to fat and, ultimately, reduced accumulated fat (Whaley *et al.*, 2012). Selective inhibition of SGLT-2 is expected to normalize plasma glucose by enhancing glucose excretion (Whaley *et al.*, 2012). Thus, SGLT-2 inhibitors work independent of insulin and β-cell function, focusing on the kidney (Whaley

et al., 2012). Renal SGLT-2 expression is increased in hyperglycaemic mice and in humans with pre-diabetes (Bailey and Day, 2010). Recently, SGLT-2 inhibitors have been found to be effective in treating pre-diabetes and T2DM (Raskin, 2013; Mudaliar *et al.*, 2015). Sodium-glucose co-transport 2 inhibitors are effective in lowering plasma glucose when used as monotherapy or in combination with other oral antidiabetic agents (Vallianou *et al.*, 2017). Although metformin is the first choice for treating T2DM, however if intolerable gastrointestinal side effects results SGLT-2 inhibitors are used as monotherapy (Vallianou *et al.*, 2017). The SGLT-2 inhibitors are also effective in ameliorating glycemia in triple combination with metformin and either sulfonylureas, DPP-4 inhibitors, or glitazones, which have been shown to lower FPG and HbA1c concentrations (Vallianou *et al.*, 2017). The addition of SGLT-2 inhibitors in patients inadequately controlled on insulin and mean HbA1c is related to improved glycaemic control without increasing body weight or the risk of hypoglycaemia (Mudaliar *et al.*, 2015). Improvement in hyperglycaemia downregulates carbohydrate-responsive element-binding protein (ChREBP), a transcription factor responsible for activating the machinery for fatty acid synthesis (Park *et al.*, 2014). Improvement in insulin resistance results in downregulation of SREBP-1c and the blockage of de novo hepatic lipogenesis (Kuchay *et al.*, 2018). Thus, SGLT-2 inhibitors should improve NAFLD and provide a mechanistic rationale to conduct human trials with SGLT-2 inhibitors in patients with NAFLD (Kuchay *et al.*, 2018). Furthermore, inflammation, oxidative stress, and fibrosis are involved in the initiation and progression of kidney disease (Elmarakby and Sullivan, 2012; Sangoi *et al.*, 2016). Experimental studies have linked SGLT-2 inhibitors with reduction in anti-inflammatory, antioxidant and anti-fibrotic markers after treatment with ipragliflozin and empagliflozin (Elmarakby and Sullivan, 2012; Sangoi *et al.*, 2016). A recent study translated these preclinical findings to the human situation and demonstrated that 6 weeks treatment with the SGLT-2 inhibitor dapagliflozin decreased urinary levels of the inflammatory markers interleukin-6 and monocyte-attractive-protein-1 (Dekkers *et al.*, 2018). At present, several preclinical and clinical studies examine the effects of SGLT-2 inhibitors on pre-diabetes and related immune dysregulation, liver and kidney complications (Dekkers *et al.*, 2018).

1.7.2.4 α-Glucosidase inhibitors

α-glucosidase inhibitors reduce the rate of polysaccharide digestion from the proximal small intestine and mainly lower post prandial glucose without causing hypoglycaemia (Knowler *et al.*, 2018). Since their effect on HbA1c is smaller than that of other oral antidiabetic agents, they are seldom used in the treatment of T2DM (Chaudhury *et al.*, 2017). However, in the Study to Prevent Non-Insulin- Dependent Diabetes Mellitus (STOP-NIDDM), acarbose resulted in a 25 % relative-

risk reduction for T2DM in individuals with IGT with a suggested decrease in CVD and hypertension risk (Haw *et al.*, 2017; Holman *et al.*, 2017). However, about a third of the acarbose-treated individuals could not complete the trial because of gastrointestinal side-effects (Knowler *et al.*, 2018). A recent study investigating voglibose, another α-glucosidase inhibitor, reported a 40% reduction in incident T2DM risk during 48 weeks of follow-up in high-risk Japanese individuals with IGT (Kraus *et al.*, 2018). In addition, α-glucosidase inhibitors have also been shown to augment incretin secretion as well as altering gut micobiota flora, which may partially explain their beneficial effects (Chen *et al.*, 2014). Although α-glucosidase inhibitors are commonly used for controlling postprandial blood glucose, α-glucosidase inhibitors-induced liver injuries have been reported (Maximos *et al.*, 2017; Chen *et al.*, 2017). However, the relationship between α-glucosidase inhibitors and liver injuries in advanced chronic kidney disease patients remains unexplored (Kao *et al.*, 2016). Furthermore, oral α-glucosidase inhibitors are poorly absorbed from the gastrointestinal tract and then almost completely eliminated in the urine and renal failure greatly influences pharmacokinetics and drug plasma levels (Wu *et al.*, 2015).

1.7.2.5 Glucagon-like peptide-1 receptor (GLP-1R) agonists

Glucagon-like peptide-1 receptor agonists regulate appetite and hyperglycaemia by activating the GLP-1R, rather than inhibiting the breakdown of glucagon-like peptide-1 (GLP-1) as oppose to DPP-4 inhibitors (Heppner and Perez, 2015). Glucagon-like peptide-1 receptor agonists therapies are approved for the treatment of T2DM and obesity and they have demonstrated anti-inflammatory, lipid-lowering, and anti-fibrotic effects in liver and protects cardiovascular system (Vishal *et al.*, 2014; Heppner and Perez, 2015). Previous studies demonstrated protective effects of GLP-1R analogs in acute renal injury and diabetic dyslipidaemia-induced renal dysfunction in diabetic db/db mice by attenuating oxidative stress, renal lipid accumulation, and inflammation (Fujita *et al.*, 2014; Patel *et al.*, 2018). Thus, GLP-1 signalling can modulate renal dysfunctions through multiple mechanisms (Patel *et al.*, 2018). Furthermore, exenatide is a synthetic analog of exendin-4, a 39-amino-acid agonist of the GLP-1R has shown promising effects in the treatment of liver disease and are under investigation (Patel *et al.*, 2016). In a murine model of fatty liver, administration of exendin-4 significantly reduced glucose levels, improved insulin sensitivity, and reduced hepatic steatosis (Patel *et al.*, 2016; Jin and Weng, 2016). In an open-label, uncontrolled clinical trial using exenatide to assess drug safety in patients with diabetes, patients were noted to have had improved AST, ALT and insulin sensitivity over the 3.5-year follow-up period (van Raalte *et al.*, 2016; Jin and Weng, 2016). Finally, an open-label, prospective case series measuring the effect of exenatide on the hepatic

histology of eight patients with T2DM and biopsy-proven NAFLD demonstrated improvement in serum aminotransferases, although no significant improvement in histopathology was demonstrated (Jin and Weng, 2016; Dong *et al.*, 2017). Moreover, as GLP-1R analogs are effective in reducing IGT conversion to T2DM, improving β-cell function, promoting weight loss, improving cardiovascular risk factors and do not cause hypoglycaemia, they may be beneficial for treating IGT and pre-diabetes related complications (Brill *et al.*, 2016; Lundkvist *et al.*, 2019). However, the major side-effects of GLP-1R agonists are nausea and vomiting and approximately 5 % of individuals cannot tolerate these agents (Brill *et al.*, 2016; Lundkvist *et al.*, 2019). While pancreatitis has been listed as a potential side-effect, an increased incidence of the latter has not been substantiated (Brill *et al.*, 2016; Lundkvist *et al.*, 2019). In considering their potential suitability for use in the prevention of T2DM, the considerable costs of these agents require cost effectiveness studies should they ultimately be approved for this indication (Brill *et al.*, 2016; Lundkvist *et al.*, 2019).

1.7.2.6 Dipeptidyl Peptidase-4 Inhibitors

Dipeptidyl peptidase-4 inhibitors are relatively new incretin-based drugs available for the management of T2DM, like the GLP-1R agonists they act via the incretin pathway (Schnapp *et al.*, 2016; Thomas *et al.*, 2016). Today, several DPP-4 inhibitors are available worldwide, the main action of which is to increase the level of incretin hormones such as GLP-1, thereby stimulating insulin secretion from pancreatic β cells, and many researchers have reported sufficient evidence of effective glucose-lowering action (Schnapp *et al.*, 2016; Thomas *et al.*, 2016). Currently sitagliptin and saxagliptin are the only DPP-4 inhibitors available for the treatment of T2DM and related complications (Schnapp *et al.*, 2016; Thomas *et al.*, 2016). Dipeptidyl peptidase-4 is a ubiquitous enzyme expressed on the surface of most cell types that deactivates a variety of other bioactive peptides, including GLP-1 (Thomas *et al.*, 2016). Therefore, its inhibition could potentially affect glucose regulation through multiple effects. These inhibitors, unlike other GLP-1-based therapies, can be administered orally, making them attractive candidates for liver disease treatment (Joy *et al.*, 2017). Sitagliptin is eliminated almost entirely by the kidneys and have very little hepatic metabolism, making them safe for use in patients with decreased liver function, whilst saxagliptin and vildagliptin are metabolized in the liver (Joy *et al.*, 2017; Lyu *et al.*, 2017). In animal studies, sitagliptin has been shown to attenuate the effects of metformin, through phosphorylation of the AMP-K pathway (Lyu *et al.*, 2017; Jin *et al.*, 2019). In addition, in fructose-fed rats who develop insulin resistance and the metabolic syndrome, administration of sitagliptin decreased liver steatosis, decreased β-cell apoptosis and insulin resistance (Maiztegui *et al.*, 2017). It is hypothesized that in NAFLD the DPP-4 enzymatic activity is increased which

might contribute to the development of T2DM and metabolic deterioration, therefore based on these results, DPP-4 inhibitors might offer prevention of further metabolic deterioration, especially in NAFLD (Schnapp *et al.*, 2016; Lyu *et al.*, 2017). However, there are currently no clinical studies in humans examining the effect of sitagliptin or other DDP-4 inhibitors on liver disease but results in animal studies suggest these may be promising drugs (Thomas *et al.*, 2016; Maiztegui *et al.*, 2017). The renoprotective effect of DPP-4 inhibitors has also been reported and the effects was mainly against diabetic kidney diseases, reducing proteinuria and glomerular sclerosis in some animal models and in clinical studies (Tanaka *et al.*, 2016). In addition, DPP-4 inhibition has the potential to address several impediments associated with intensification of glycaemic control in patients with DN (Thomas *et al.*, 2016; Tanaka *et al.*, 2016). Furthermore, apart from specific hypoglycaemic mechanisms, DPP-4 inhibitors possess an immune modulation profile, through regulation of T cell phenotype and cytokine section offering the potential for extendibility to autoimmune diabetes (Wang *et al.*, 2018). Therefore, agents in this class could in principle be beneficial in prevention of prediabetes progression, although there are no long-term studies available examining the enhancement of insulin secretion into preservation of β-cell function (Sakura *et al.*, 2016).

Pharmacological therapy for pre-diabetes is often combined with dietary intervention. However, there is reported poor patient compliance in terms of dietary intervention as patients tend to heavily rely on the pharmacological treatment, these therefore further worsen the disease and reduce the efficacy of the drug (Bailey and Kodack, 2011; Ahmad *et al.*, 2013). Hence, there is a necessity to investigate alternative antidiabetic drugs like transition metals that will possibly be effective even in the presence or absence of dietary intervention to manage and prevent pre-diabetes and its related complications.

1.8 Alternative management of pre-diabetes

There is a continuous search and evaluation of several synthetic compounds of transitional metals, where most drugs used today are purely organic compounds (Rafique *et al.*, 2010). Recently, interest in metal complexes in the management of diseases has grown (Allardyce and Dyson, 2006). Synthetic organometallic compounds are generally considered to be toxic or non-compatible with biological systems (Rafique *et al.*, 2010). Despite this perception, the medicinal properties of organometallic compounds in particular, organo-transition metal compounds have been investigated for a long period and in the last few years the area has grown considerably (Allardyce and Dyson, 2006). Transition metals have an important place within medicinal biochemistry (Rafique *et al.*, 2010). Transition metals represent the d block element which includes groups 3 - 12 on the periodic table. They have partially filled d-shells in any of their commonly occurring

oxidation state (Levina *et al.*, 2009). A metal complex or coordination compound is a structure consisting of a central metal atom, bonded to a surrounding array of ligands (molecules or anions), which donate an electron pair to the metal (Zhang and Lippard, 2003). Research has shown significant progress in the utilization of transition metal complexes as drugs to treat several human diseases such as carcinomas, lymphomas, neurological disorders and diabetes (Rafique *et al.*, 2010). Transition metals exhibit different oxidation states and can interact with a number of negatively charged molecules (Zhang and Lippard, 2003). This activity of transition metals has started the development of metal-based drugs with promising pharmacological application and may offer unique therapeutic opportunities (Rafique *et al.*, 2010). Therefore, the interest of the current study is on the transition metal ruthenium(II) complex.

1.8.1 Ruthenium(II) uracil-derived diimine complex [Ru^{II}(H$_3$ucp)Cl(PPh$_3$)]

Ruthenium compounds emerge as the most promising metal complexes with biological features including, i) ability to adopt numerous oxidation states (most commonly II, III, and IV); ii) octahedral geometry, an improvement over the platinum drugs, allowing for more intensive tuning of the complexes electronic and steric properties; iii) facility to exchange with O- and N- donor molecules in a way similar to platinum drugs; iv) slow rate of ligand exchange in comparison with other transition metal complexes; v) transport by binding to serum albumin and transferrin, as ruthenium mimic iron in binding to proteins (Allardyce and Dyson, 2006; Rafique *et al.*, 2010). Currently there are three ruthenium (III) complexes entering clinical trials: NAMI-A [ImH][trans-RuCl4(DMSO)(Im)], KP1019–[InH][trans- RuCl4(In)2] and NKP3019-Na[trans-RuCl4(In)2] (Im = imidazole, In = indazole) (Alessio and Messori, 2019). Recent literature trends have shown significant progress in the utilization of transition metal complexes as metal-based drugs to manage diabetes (Rafique *et al.*, 2010; Naik *et al.*, 2014). Ruthenium complex have been synthesised and complexed with a nitric oxide (NO) ligand as NO donor and scavenger for the treatment of diabetes and cardiovascular complications (Komers and Anderson, 2003). NO released from the ruthenium induces vascular smooth muscle relaxation via activation of cGMP production and K$^+$ channel activation, as well as decreased cytosolic Ca^{2+} concentrations (Komers and Anderson, 2003). Antonyan *et al.* (2014) demonstrated that the coordination of ruthenium to the natural product, curcumin and the resultant ruthenium(II)-curcumin complexes are promising for the development of dipeptidyl peptidase-4 (DPP-IV) inhibitors, thus the use of DPP-IV inhibitors increases the circulating levels of endogenous glucagon like pepdite-1 (GLP-1), leading to increased insulin secretion biosynthesis and inhibiting glucagon release (Komers and Anderson, 2003; Antonyan *et al.*, 2014). Ruthenium compounds have been also shown to exhibit effective anti-inflammatory properties via inhibiting

inflammatory mediators (NO and iNOS) and pro-inflammatory cytokines (TNF-α and IL-1β) (Hsia *et al.*, 2018). Resulting in blocking lipopolysaccharides (LPS)-induced p38 MAPK/p65 phosphorylation, IκBα degradation and p65 nuclear translocation in RAW 264.7 cells (Hsia *et al.*, 2018). There has also been an upsurge in the interest of ruthenium polypyridyl nitrogen-donor complexes in exploring their fibril inhibitory capabilities of the human islet amyloid polypeptide (hIAPP) (He *et al.*, 2013; Ma *et al.*, 2015; Gong *et al.*, 2017). Aggregation of the hIAPP are associated with the development of T2DM and this phenomenon leads to pancreatic β cell apoptosis. The inhibitory activities of these ruthenium complexes are initiated by their covalent attachment to intracellular bodies of the pancreatic β-cells which in turn modulate insulin production (He *et al.*, 2013; Ma *et al.*, 2015; Gong *et al.*, 2017).

Our leading candidate, anti-diabetic ruthenium(II) Schiff base complex, [Ru^{II}(H$_3$ucp)Cl(PPh$_3$)] (H$_4$ucp = 2,6-*bis*-((6-amino-1,3-dimethyluracilimino)methylene)pyridine) contains the H$_3$ucp monoanionic tridentate diimine which comprises of two derivatives of the RNA nucleotide base, uracil. The presence of these moieties could promote target specificity, physiological compatibility and cell permeability (Booysen *et al.*, 2014). Furthermore, this complex has been recently shown to improve glycaemic control and insulin sensitivity as evidence by the amelioration of HbA1c levels, which in turn ameliorated risk metabolic factors associated with CVD in pre-diabetes diet-induced rats (Mabuza *et al.*, 2018; Mabuza *et al.*, 2019). However, it is not known how this metal-based complex will influence prediabetes-related hepatic, renal and immune dysregulation complications in a diet-induced pre-diabetic rat model. Thus, the goal of this study was to investigate the effects of a ruthenium(II) uracil-derived diimine complex on markers associated with hepatic, renal and immune dysregulation in the presence and absence of dietary intervention in a diet-induced pre-diabetes male Sprague-Dawley rat.

1.9 Experimental pre-diabetic rat models

There are various diabetic models used to study diabetic-related pathology and to investigate the effects of various therapeutic modalities in management of diabetes (Srinivasan and Ramarao, 2007). Experimental diabetes studies can be conducted *in vivo* and *in vitro* using animals and cell lines, respectively. Although both do not perfectly mimic human diabetes, they are valuable tools for evaluating novel anti-diabetic agents (Srinivasan and Ramarao, 2007). However, the use of animal models has contributed a great impact in the study of diabetes. Animal models allow researchers to control *in vivo*, genetic, and environmental factors that may influence the development of the disease and its secondary complications, therefore gaining useful information on its management and treatment in humans (Lu *et al.*, 2010). There

are many animal models used to study T2DM (Lu *et al.*, 2010). Some of these models show a genetic predisposition to the disease, while others may develop the disease spontaneously or in a diet-induced manner (Liu *et al.*, 2002; Dourmashkin *et al.*, 2005). The most commonly used non-genetic animal models of type 1 diabetes are those induced by streptozotocin or alloxan, or models obtained by partial pancreatectomy which leads to insulin deficiency, hyperglycaemia, and ketosis (Lenzen, 2008; Lu *et al.*, 2010). Although these models are useful for the study of diabetes, however, they are not fully representative of diet-induced human metabolic syndrome and pre-diabetes as these models skip the pre-diabetes state.

Diet composition has been considered an important factor in the impairment of insulin activity (Lozano *et al.*, 2016; Dooley and Ryan, 2019; Kalita *et al.*, 2019). Literature evidence has shown that the administration of a high-fat diet (HFD) or high carbohydrates (HCD) and fructose to rats for 2 months is a fast and easy way to induce pre-diabetes associated with metabolic and oxidative disorders (Lenzen, 2008; Auberval *et al.*, 2014; Lozano *et al.*, 2016). However, recent epidemiological studies of sugar consumption and diabetes prevalence suggest that a diet rich in fat as well as carbohydrates is a greater risk factor for these disorders than a diet that is rich in either fats or sugars (Basu *et al.*, 2013).

Of interest in this study was the diet-induced pre-diabetic rat model by the combination of HFD and HCD supplemented with 15% fructose. This preliminary diabetic animal model has shown to display complications and aetiology of pre-diabetes, which is insulin resistance, hyperglycaemia and glucose intolerance over time (Luvuno *et al.*, 2018). In addition, a study by Mzimela *et al.,* showed the changes in immune response during the progression of pre-diabetes to T2DM in a high fat high carbohydrate diet-induced prediabetic rat model (Mzimela *et al.*, 2019). However, the effects of this mononuclear metal-based complex on markers associated with hepatic, renal and immune dysregulation in diet-induced pre-diabetic rats are yet to be investigated. Moreover, the use of this diabetic animal model that best displays the clinical manifestations of the disease is advantageous in developing therapeutic novel treatments in managing pre-diabetes and related complications (Basu *et al.*, 2013; Auberval *et al.*, 2014).

1.10   Justification of the present study

Pre-diabetes, an intermediate hyperglycaemic state that often precedes the onset of T2DM, is best characterised with hyperglycaemia, insulin resistance and glucose intolerance (Tabák *et al.*, 2012; Hostalek, 2019). Insulin resistance is a key component of pre-diabetes and encompasses a wide spectrum of disorders that drives the development of atherogenic dyslipidaemia, generates a low-grade inflammatory state and increases the release of pro-inflammatory markers and ROS

(Akash *et al.*, 2013; Rehman and Akash, 2016). In addition, insulin resistance also affects blood pressure, endothelial cells and macrophages, eventually leading to prediabetes-related complications (Donath, 2014; Chen *et al.*, 2015). Lifestyle and dietary interventions are recognized as one of the bases in preventing pre-diabetes, managing existing pre-diabetes, and delaying the rate of development of diabetes complications through improved glycaemic control and insulin sensitivity (Hu *et al.*, 2010; Shrestha and Ghimire, 2012; Venkataraman *et al.*, 2013). Therefore, dietary intervention is important at all levels of diabetes prevention. However, these preventative strategies should be implemented as soon as the diagnosis of pre-diabetes or diabetes is made and beginning treatment during the prediabetic state could be used as a therapeutic target in the management and prevention of pre-diabetes (Hu *et al.*, 2010). However, patients still take the conventional drugs without lifestyle intervention, such as dietary intervention and these lead to further poor patient compliance and exacerbate the disease (Bailey and Kodack, 2011; Ahmad *et al.*, 2013). Therefore, novel therapeutic agent with less undesirable limitation coupled with dietary intervention should be developed to overcome these issues. Research has shown significant progress in utilization of transition metal complexes as drugs to treat several human diseases (Zhang and Lippard, 2003). The biological activities of transition metals have started the development of metal-based drugs with promising pharmacological application and may offer unique therapeutic opportunities (Sekhon, 2011; Bagchi *et al.*, 2015). Organoruthenium compounds emerge as the most promising with biological features including their low toxicity, mechanism of action and a preference for protein binding (Naik *et al.*, 2014; Hsia *et al.*, 2018). Furthermore, the ruthenium(II) uracil-derived diimine complex has been shown to improve glycaemic control and insulin sensitivity while ameliorating risk factors associated with CVD through its anti-inflammatory, anti-oxidative and vasodilative properties (Mabuza *et al.*, 2018; Mabuza *et al.*, 2019). Therefore, the literature evidence described in the preceding sections indicates that this ruthenium compound has the potential in managing pre-diabetes and its related complications. Therefore, of interest in this study, are the effects of a ruthenium(II) uracil-derived diimine complex on markers associated with hepatic, renal and immune dysregulation complications in diet-induced pre-diabetic rat. The outcome of the study may justify further evaluations of this metal-based complex on pre-diabetic complications.

1.11   Aims

The aims of the study were to investigate the effects of a ruthenium(II) uracil-derived diimine complex on risk markers associated with hepatic, renal and immune dysregulation complications in both the presence and absence of dietary intervention in diet-induced pre-diabetic male Sprague-Dawley rats.

1.12 Objectives

The objectives of the study were:

➢ To investigate the hepatoprotective effects of a ruthenium(II) uracil-derived diimine complex in diet-induced pre-diabetic rats, by evaluating liver and body weight, liver function enzymes, SREBP 1c concentration and histopathological analysis of the liver.

➢ To investigate the renoprotective effects of a ruthenium(II) uracil-derived diimine complex in diet-induced pre-diabetic rats, by evaluating electrolytes, antioxidants activity, renal function biomarkers, KIM-1 concentration, histopathological analysis of the kidney and expression of mRNA podocin concentration in urine.

➢ To investigate the effects of a ruthenium(II) uracil-derived diimine complex on immune dysregulation in diet-induced pre-diabetic rats, by evaluating platelets activation markers, immune cell counts, pro-inflammatory cytokines and VLDL.

References

1   ADA. 2019. 2. Classification and diagnosis of diabetes: standards of medical Care in Diabetes-2019. *Diabetes Care;* 42**:** 13-28.

2   Adiels M, Olofsson S, Taskinen M and Borén J. 2008. Overproduction of very low–density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome. *Arteriosclerosis, thrombosis, and vascular biology;* 28(7)**:** 1225-1236.

3   Ahmad N, Ramli A, Islahudin F and Paraidathathu T. 2013. Medication adherence in patients with type 2 diabetes mellitus treated at primary health clinics in Malaysia. *Patient preference and adherence;* 7**:** 525.

4   Ahmed K, Muni S and Ismail I. 2010. Type 2 diabetes and vascular complications: a pathophysiologic view. *Biomedical Research;* 21(2)**:** 147-155.

5   Akash M, Rehman K and Chen S. 2013. Role of inflammatory mechanisms in pathogenesis of type 2 diabetes mellitus. *Journal of cellular biochemistry;* 114(3)**:** 525-531.

6   Akash M, Rehman K and Liaqat A. 2018. Tumor Necrosis Factor-Alpha: Role in Development of Insulin Resistance and Pathogenesis of Type 2 Diabetes Mellitus. *Journal of cellular biochemistry;* 119(1)**:** 105-110.

7   Al-Aubaidy H and Jelinek H. 2011. Oxidative DNA damage: antioxidant response in postprandial hyperglycaemia in type 2 diabetes mellitus. *The British Journal of Diabetes & Vascular Disease;* 11(2)**:** 87-91.

8   Alessio E and Messori L. 2019. NAMI-A and KP1019/1339, Two Iconic Ruthenium Anticancer Drug Candidates Face-to-Face: A Case Story in Medicinal Inorganic Chemistry. *Molecules;* 24(10)**:** 1995.

9   Allardyce C and Dyson P. 2006. Medicinal Properties of Organometallic Compounds, In: . *Journal of Bioorganometallic Chemistry (Topics in Organometallic Chemistry);* 17**:** 177-210.

10   Anil Kumar P, Welsh G, Saleem M and Menon R. 2014. Molecular and cellular events mediating glomerular podocyte dysfunction and depletion in diabetes mellitus. *Frontiers in endocrinology;* 5**:** 151.

11   Anjana R, Deepa M, Pradeepa R, Mahanta J, Narain K, Das H, Adhikari P, Rao P, Saboo B and Kumar A. 2017. Prevalence of diabetes and prediabetes in 15 states of India: results from the ICMR–INDIAB population-based cross-sectional study. *The lancet Diabetes & endocrinology;* 5(8)**:** 585-596.

12   Antonyan A, De A, Vitali L, Pettinari R, Marchetti F, Gigliobianco M, Pettinari C, Camaioni E and Lupidi G. 2014. Evaluation of (arene) Ru (II) complexes of curcumin as inhibitors of dipeptidyl peptidase IV. *Biochimie;* 99**:** 146-152.

13   Auberval N, Dal S, Bietiger W, Pinget M, Jeandidier N, Maillard-Pedracini E, Schini-Kerth V and Sigrist S. 2014. Metabolic and oxidative stress markers in Wistar rats after 2 months on a high-fat diet. *Diabetology & metabolic syndrome;* 6(1)**:** 130.

14   Avramoglu R, Basciano H and Adeli K. 2006. Lipid and lipoprotein dysregulation in insulin resistant states. *Clinica chimica acta;* 368(1)**:** 1-19.

15   Bagchi A, Mukhergee P and Raha A. 2015. A Review on Transition Metal Complex-Mordern Weapon in Medicine. *International Journal of Advances in Pharmaceutical Sciences;* 5**:** 171-180.

16   Bailey C and Day C. 2010. SGLT2 inhibitors: glucuretic treatment for type 2 diabetes. *The British Journal of Diabetes & Vascular Disease;* 10(4)**:** 193-199.

17   Bailey C and Kodack M. 2011. Patient adherence to medication requirements for therapy of type 2 diabetes. *International journal of clinical practice;* 65(3)**:** 314-322.

18   Barter P, Rye K, Tardif J, Waters D, Boekholdt S, Breazna A and Kastelein J. 2011. Effect of torcetrapib on glucose, insulin, and hemoglobin A 1c in subjects in the Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial. *Circulation;* 124(5)**:** 555-562.

19   Basu S, Yoffe P, Hills N and Lustig R. 2013. The relationship of sugar to population-level diabetes prevalence: an econometric analysis of repeated cross-sectional data. *PloS one;* 8(2)**:** 57873.

20   Bergman M. 2013. Pathophysiology of prediabetes and treatment implications for the prevention of type 2 diabetes mellitus. *Endocrine;* 43(3)**:** 504-513.

21   Booysen I, Maikoo S, Akerman M and Xulu B. 2014. Novel ruthenium (II) and (III) compounds with multidentate Schiff base chelates bearing biologically significant moieties. *Polyhedron;* 79**:** 250-257.

22   Boren J, Taskinen M, Olofsson S and Levin M. 2013. Ectopic lipid storage and insulin resistance: a harmful relationship. *Journal of internal medicine;* 274(1)**:** 25-40.

23   Bouby N and Fernandes S. 2003. Mild dehydration, vasopressin and the kidney: animal and human studies. *European journal of clinical nutrition;* 57(S2)**:** S39.

24   Brannick B, Wynn A and Dagogo-Jack S. 2016. Prediabetes as a toxic environment for the initiation of microvascular and macrovascular complications. *Experimental biology and medicine;* 241(12)**:** 1323-1331.

25   Brill A, Wisinski J, Cadena M, Thompson M, Fenske R, Brar H, Schaid M, Pasker R and Kimple M. 2016. Synergy between Gαz deficiency and GLP-1 analog treatment in preserving functional β-cell mass in experimental diabetes. *Molecular Endocrinology;* 30(5)**:** 543-556.

26   Broedbaek K, Weimann A, Stovgaard E and Poulsen H. 2011. Urinary 8-oxo-7, 8-dihydro-2′-deoxyguanosine as a biomarker in type 2 diabetes. *Free Radical Biology and Medicine;* 51(8)**:** 1473-1479.

27   Brown D and Griendling K. 2015. Regulation of signal transduction by reactive oxygen species in the cardiovascular system. *Circulation research;* 116(3)**:** 531-549.

28   Brownlee M. 2005. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes;* 54**:** 1615–1625.

29   Bugianesi E, McCullough A and Marchesini G. 2005. Insulin resistance: a metabolic pathway to chronic liver disease. *Hepatology;* 42(5)**:** 987-1000.

30   Byrne C and Targher G. 2014. Ectopic fat, insulin resistance, and nonalcoholic fatty liver disease: implications for cardiovascular disease. *Arteriosclerosis, thrombosis, and vascular biology;* 34(6)**:** 1155-1161.

31   Carlsson A, Calamia M, Risérus U, Larsson A, Helmersson-Karlqvist J, Lind L and Ärnlöv J. 2014. Kidney injury molecule (KIM)-1 is associated with insulin resistance: Results from two community-based studies of elderly individuals. *Diabetes research and clinical practice;* 103(3)**:** 516-521.

32   Ceriello A. 2008. Thiazolidinediones as anti-inflammatory and anti-atherogenic agents. *Diabetes/metabolism research and reviews;* 24(1)**:** 14-26.

33   Chaudhury A, Duvoor C, Dendi R, Sena V, Kraleti S, Chada A, Ravilla R, Marco A, Shekhawat N and Montales M. 2017. Clinical review of antidiabetic drugs: implications for type 2 diabetes mellitus management. *Frontiers in endocrinology;* 8**:** 6.

34   Chen J, Chang C, Lin Y, Horng J and Sheu W. 2014. Acarbose treatment and the risk of cardiovascular disease in type 2 diabetic patients: a nationwide seven-year follow-up study. *Journal of diabetes research;* 2014.

35   Chen L, Chen R, Wang H and Liang F. 2015. Mechanisms linking inflammation to insulin resistance. *International journal of endocrinology;* 2015**:** 9.

36   Chen S, Tsai S, Jhao J, Jiang W, Tsao C and Chang L. 2017. Liver fat, hepatic enzymes, alkaline phosphatase and the risk of incident type 2 diabetes: a prospective study of 132,377 adults. *Scientific reports;* 7(1)**:** 1-9.

37   Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg A and Obin M. 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of lipid research;* 46(11)**:** 2347-2355.

38   Cooper M, Menta B, Perez-Sanchez C, Jack M, Khan Z, Ryals J, Winter M and Wright D. 2018. A ketogenic diet reduces metabolic syndrome-induced allodynia and promotes peripheral nerve growth in mice. *Experimental neurology;* 306**:** 149-157.

39   Curtis L. 2007. New technologies and therapies in the management of diabetes. *American journal of managed care;* 13**:** 47-54.

40   Danaei G, Finucane M, Lu Y, Singh G, Cowan M, Paciorek C, Lin J, Farzadfar F, Khang Y and Stevens G. 2011. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *The Lancet;* 378(9785)**:** 31-40.

41   De Nicola L, Conte G and Minutolo R. 2016. Prediabetes as a precursor to diabetic kidney disease. *American Journal of Kidney Diseases;* 67(6)**:** 817-819.

42   DeFronzo R, Eldor R and Abdul-Ghani M. 2013a. Pathophysiologic approach to therapy in patients with newly diagnosed type 2 diabetes. *Diabetes care;* 36(Supplement 2)**:** 127-138.

43   DeFronzo R, Hompesch M, Kasichayanula S, Liu X, Hong Y, Pfister M, Morrow L, Leslie B, Boulton D and Ching A. 2013b. Characterization of renal glucose reabsorption in response to dapagliflozin in healthy subjects and subjects with type 2 diabetes. *Diabetes care;* 36(10)**:** 3169-3176.

44   Dekkers C, Ron T and Hiddo J. 2018. New therapies and diabetic kidney disease progression: the role of SGLT-2 inhibitors. *Current diabetes reports;* 18(5)**:** 27.

45   Donath M and Shoelson S. 2011. Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology;* 11(2)**:** 98.

46   Donath M. 2014. Targeting inflammation in the treatment of type 2 diabetes: time to start. *Nature reviews Drug discovery;* 13(6)**:** 465.

47   Dong X, Park S, Lin X, Copps K, Yi X and White M. 2006. Irs1 and Irs2 signaling is essential for hepatic glucose homeostasis and systemic growth. *Journal of Clinical Investigation;* 116**:** 101–114.

48   Dong Y, Lv Q, Li S, Wu Y, Li L, Li J, Zhang F, Sun X and Tong N. 2017. Efficacy and safety of glucagon-like peptide-1 receptor agonists in non-alcoholic fatty liver disease: A systematic review and meta-analysis. *Clinics and research in hepatology and gastroenterology;* 41(3)**:** 284-295.

49   Dooley C and Ryan A. 2019. Role of Dietary Macronutrients and Fatty Acids in Obesity and Metabolic Risk in Older Adults (P08-026-19). *Current Developments in Nutrition;* 3(Supplement_1)**:** P08-026-019.

50   Dourmashkin J, Chang G, Gayles E, Hill J, Fried S, Julien C and Leibowitz S. 2005. Different forms of obesity as a function of diet composition. *International journal of obesity;* 29(11)**:** 1368-1378.

51   Drew B, Duffy S, Formosa M, Natoli A, Henstridge D, Penfold S, Thomas W, Mukhamedova N, de Courten B and Forbes J. 2009. High-density lipoprotein modulates glucose metabolism in patients with type 2 diabetes mellitus. *Circulation;* 119(15)**:** 2103-2111.

52   Drew B, Rye K, Duffy S, Barter P and Kingwell B. 2012. The emerging role of HDL in glucose metabolism. *Nature Reviews Endocrinology;* 8(4)**:** 237-245.

53   El-Ashmawy N, El-Zamarany E, Khedr N, El-Fattah A and Eltoukhy S. 2015. Kidney injury molecule-1 (Kim-1): an early biomarker for nephropathy in type II diabetic patients. *International Journal of Diabetes in Developing Countries;* 35(3)**:** 431-438.

54   Elmarakby A and Sullivan J. 2012. Relationship between Oxidative Stress and Inflammatory Cytokines in Diabetic Nephropathy. *Cardiovascular Therapeutics;* 30(1)**:** 49-59.

55   Erem C, Ozbas H, Nuhoglu I, Deger O, Civan N and Ersoz H. 2014. Comparison of effects of gliclazide, metformin and pioglitazone monotherapies on glycemic control and cardiovascular risk factors in patients with newly diagnosed uncontrolled type 2 diabetes mellitus. *Experimental and Clinical Endocrinology & Diabetes;* 122(05)**:** 295-302.

56   Eringa E, Serne E, Meijer R, Schalkwijk C, Houben A, Stehouwer C, Smulders Y and van Hinsbergh V. 2013. Endothelial dysfunction in (pre) diabetes: characteristics, causative mechanisms and pathogenic role in type 2 diabetes. *Reviews in Endocrine and Metabolic Disorders;* 14(1)**:** 39-48.

57   Erion D, Park H and Lee H. 2016. The role of lipids in the pathogenesis and treatment of type 2 diabetes and associated co-morbidities. *BMB reports;* 49(3)**:** 139.

58   Esterson Y, Zhang K, Koppaka S, Kehlenbrink S, Kishore P, Raghavan P, Maginley S, Carey M and Hawkins M. 2013. Insulin sensitizing and anti-inflammatory effects of thiazolidinediones are heightened in obese patients. *Journal of Investigative Medicine;* 61(8)**:** 1152-1160.

59   Evert A, Boucher J, Cypress M, Dunbar S, Franz M, Mayer-Davis E, Neumiller J, Nwankwo R, Verdi C and Urbanski P. 2014. Nutrition therapy recommendations for the management of adults with diabetes. *Diabetes care;* 37(Supplement 1)**:** S120-S143.

60   Ferrannini E. 2014. The target of metformin in type 2 diabetes. *New England Journal of Medicine;* 371(16)**:** 1547-1548.

61   Ferreiro J, Gómez-Hospital J and Angiolillo D. 2010. Platelet abnormalities in diabetes mellitus. *Diabetes and vascular disease research;* 7(4)**:** 251-259.

62   Fiorentino V, Prioletta A, Zuo P and Folli F. 2013. Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Current pharmaceutical design;* 19(32)**:** 5695-5703.

63   Franz M. 2003. The glycemic index. *Diabetes Care;* 26(8)**:** 2466-2468.

64   Fritsche L, Weigert C, Haring H and Lehmann R. 2008. How insulin receptor substrate proteins regulate the metabolic capacity of the liver– implications for health and disease. *Current Medicinal Chemistry;* 15**:** 1316–1329.

65   Frost J, Garside R, Cooper C and Britten N. 2014. A qualitative synthesis of diabetes self-management strategies for long term medical outcomes and quality of life in the UK. *BMC health services research;* 14(1)**:** 348.

66   Fujita H, Morii T, Fujishima H, Sato T, Shimizu T, Hosoba M, Tsukiyama K, Narita T, Takahashi T and Drucker D. 2014. The protective roles of GLP-1R signaling in diabetic nephropathy: possible mechanism and therapeutic potential. *Kidney international;* 85(3)**:** 579-589.

67   Gaiz A, Mosawy S, Colson N and Singh I. 2017. Thrombotic and cardiovascular risks in type two diabetes; Role of platelet hyperactivity. *Biomedicine & pharmacotherapy;* 94**:** 679-686.

68   Gastaldelli A and Ferrannini E. 2014. Pathophysiology of Prediabetes: Role of Lipotoxicity? *Global Health Perspectives In Prediabetes And Diabetes Prevention;* 38**:** 31.

69   Ghody P, Shikha D, Karam J and Bahtiyar G. 2015. Identifying prediabetes–Is it beneficial in the long run? *Maturitas;* 81(2)**:** 282-286.

70   Giacco R, Vetrani C, Griffo E and Rivellese A. 2013. Role of Diet and Diet Interventions in Diabetic Patients: Physiological and Metabolic Changes and Reduction in Morbidity and Mortality. *Current Nutrition Reports;* 2(4)**:** 174-180.

71   Girona J, Ibarretxe D, Plana N, Guaita-Esteruelas S, Amigo N, Heras M and Masana L. 2016. Circulating PCSK9 levels and CETP plasma activity are independently associated in patients with metabolic diseases. *Cardiovascular diabetology;* 15(1)**:** 107.

72   Goh Y, Henderson N, Heredia J, Eagle A, Odegaard J, Lehwald N, Nguyen K, Sheppard D, Mukundan L and Locksley R. 2013. Eosinophils secrete IL-4 to facilitate liver regeneration. *Proceedings of the National Academy of Sciences;* 110(24)**:** 9914-9919.

73   Gong G, Wang W and Du W. 2017. Binuclear ruthenium complexes inhibit the fibril formation of human islet amyloid polypeptide. *RSC Advances;* 7(30)**:** 18512-18522.

74   Grossmann V, Schmitt V, Zeller T, Panova-Noeva M, Schulz A, Laubert-Reh D, Juenger C, Schnabel R, Abt T and Laskowski R. 2015. Profile of the immune and inflammatory response in individuals with prediabetes and type 2 diabetes. *Diabetes Care;* 38(7)**:** 1356-1364.

75 Guven A, Yavuz O, Cam M, Ercan F, Bukan N, Comunoglu C and Gokce F. 2006. Effects of melatonin on streptozotocin-induced diabetic liver injury in rats. *Acta histochemica;* 108(2)**:** 85-93.

76 Hamad E, Taha S, Dawood A, Sitohy M and Abdel-Hamid M. 2011. Protective effect of whey proteins against nonalcoholic fatty liver in rats. *Lipids in health and disease;* 10(1)**:** 57.

77 Hamden K, Carreau S, Boujbiha M, Lajmi S, Aloulou D, Kchaou D and Elfeki A. 2008. Hyperglycaemia, stress oxidant, liver dysfunction and histological changes in diabetic male rat pancreas and liver: Protective effect of 17β-estradiol. *Steroids;* 73(5)**:** 495-501.

78 Hameed I, Masoodi S, Mir S, Nabi M, Ghazanfar K and Ganai B. 2015. Type 2 diabetes mellitus: from a metabolic disorder to an inflammatory condition. *World journal of diabetes;* 6(4)**:** 598.

79 Hamelet J, Demuth K, Paul J, Delabar J and Janel N. 2007. Hyperhomocysteinemia due to cystathionine beta synthase deficiency induces dysregulation of genes involved in hepatic lipid homeostasis in mice. *Journal of hepatology;* 46(1)**:** 151-159.

80 Haus J, Solomon T, Marchetti C, Edmison J, Gonzalez F and Kirwan J. 2010. Free fatty acid-induced hepatic insulin resistance is attenuated following lifestyle intervention in obese individuals with impaired glucose tolerance. *The Journal of Clinical Endocrinology & Metabolism;* 95(1)**:** 323-327.

81 Haw J, Galaviz K, Straus A, Kowalski A, Magee M, Weber M, Wei J, Narayan K and Ali M. 2017. Long-term sustainability of diabetes prevention approaches: a systematic review and meta-analysis of randomized clinical trials. *JAMA internal medicine;* 177(12)**:** 1808-1817.

82 He L, Wang X, Zhao C, Wang H and Du W. 2013. Ruthenium complexes as novel inhibitors of human islet amyloid polypeptide fibril formation. *Metallomics;* 5(12)**:** 1599-1603.

83 Heppner K and Perez D. 2015. GLP-1 based therapeutics: simultaneously combating T2DM and obesity. *Frontiers in neuroscience;* 9**:** 92.

84 Holman R, Coleman R, Chan J, Chiasson J, Feng H, Ge J, Gerstein H, Gray R, Huo Y and Lang Z. 2017. Effects of acarbose on cardiovascular and diabetes outcomes in patients with coronary heart disease and impaired glucose tolerance (ACE): a randomised, double-blind, placebo-controlled trial. *The lancet Diabetes & endocrinology;* 5(11)**:** 877-886.

85 Holstein A and Beil W. 2009. Oral antidiabetic drug metabolism: pharmacogenomics and drug interactions. *Expert opinion on drug metabolism & toxicology;* 5(3)**:** 225-241.

86 Hostalek U. 2019. Global epidemiology of prediabetes-present and future perspectives. *Clinical diabetes and endocrinology;* 5(1)**:** 5.

87 Hotamisligil G. 2010. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell;* 140(6)**:** 900-917.

88 Hsia C, Velusamy M, Jayakumar T, Chen Y, Hsia C, Tsai J, Teng R and Sheu J. 2018. Mechanisms of TQ-6, a Novel Ruthenium-Derivative Compound, against Lipopolysaccharide-Induced In Vitro Macrophage Activation and Liver Injury in Experimental Mice: The Crucial Role of p38 MAPK and NF-κB Signaling. *Cells;* 7(11)**:** 217.

89 Hu J, Wallace D and Tesh A. 2010. Physical activity, obesity, nutritional health and quality of life in low-income hispanic adults with diabetes. *Journal of community health nursing;* 27(2)**:** 70-83.

90 Huynh F, Neumann U, Wang Y, Rodrigues B, Kieffer T and Covey S. 2013. A role for hepatic leptin signaling in lipid metabolism via altered very low density lipoprotein composition and liver lipase activity in mice. *Hepatology;* 57(2)**:** 543-554.

91   Inzucchi S. 2002. Oral antihyperglycemic therapy for type 2 diabetes. *scientific review;* 3**:** 360-372.

92   Iroz A, Couty J and Postic C. 2015. Hepatokines: unlocking the multi-organ network in metabolic diseases. *Diabetologia;* 58(8)**:** 1699-1703.

93   Jansen P, Danser A, Imholz B and van den Meiracker A. 2009. Aldosterone-receptor antagonism in hypertension. *Journal of hypertension;* 27(4)**:** 680-691.

94   Jesudason D, Dunstan K, Leong D and Wittert G. 2003. Macrovascular risk and diagnostic criteria for type 2 diabetes. *Diabetes care;* 26(2)**:** 485-490.

95   Jialal I, Huet B, Kaur H, Chien A and Devaraj S. 2012. Increased toll-like receptor activity in patients with metabolic syndrome. *Diabetes care;* 35(4)**:** 900-904.

96   Jin S, Lee S, Jeon J, Kim H, Choi M and Song I. 2019. Enhanced Intestinal permeability and plasma concentration of metformin in rats by the repeated administration of Red Ginseng extract. *Pharmaceutics;* 11(4)**:** 189.

97   Jin T and Weng J. 2016. Hepatic functions of GLP-1 and its based drugs: current disputes and perspectives. *American Journal of Physiology-Endocrinology and Metabolism;* 311(3)**:** E620-E627.

98   Joy T, McKenzie C, Tirona R, Summers K, Seney S, Chakrabarti S, Malhotra N and Beaton M. 2017. Sitagliptin in patients with non-alcoholic steatohepatitis: a randomized, placebo-controlled trial. *World journal of gastroenterology;* 23(1)**:** 141.

99   Kahn B. 2002. Facilitative Glucose Transporters: Regulatory Mechanisms and Dysregulation in Diabetes. *Journal of Clinical Investigation;* 89**:** 1367-1374.

100   Kalita H, Hazarika A and Devi R. 2019. Withdrawal of high-carbohydrate high-fat diet alters status of trace elements to ameliorate metabolic syndrome in rat. *Canadian Journal of Diabetes*.

101   Kanasaki K, Kitada M, Kanasaki M and Koya D. 2013. The biological consequence of obesity on the kidney. *Nephrology Dialysis Transplantation;* 28(4)**:** 1-7.

102   Kao C, Wu P, Wu C, Chen L, Chen H, Wu M and Wu V. 2016. Risk of liver injury after α-glucosidase inhibitor therapy in advanced chronic kidney disease patients. *Scientific reports;* 6**:** 18996.

103   Katikireddi S, Morling J and Bhopal R. 2011. Is there a divergence in time trends in the prevalence of impaired glucose tolerance and diabetes? A systematic review in South Asian populations. *International journal of epidemiology;* 40(6)**:** 1542-1553.

104   Kim J and Yoon K. 2011. Glucolipotoxicity in pancreatic β-cells. *Diabetes & metabolism journal;* 35(5)**:** 444-450.

105   Knowler W, Crandall J, Chiasson J and Nathan D. 2018. Prevention of type 2 diabetes. *Diabetes in America, 3rd edn National Institutes of Health, Bethesda, MD***:** 38-31.

106   Kodiatte T, Manikyam U, Rao S, Jagadish T, Reddy M, Lingaiah HK and Lakshmaiah V. 2012. Mean platelet volume in type 2 diabetes mellitus. *Journal of laboratory physicians;* 4(1)**:** 5.

107   Komers R and Anderson S. 2003. Paradoxes of nitric oxide in the diabetic kidney. *American journal of physiology Renal physiology;* 284**:** 1121-1137.

108   Kraus W, Yates T, Tuomilehto J, Sun J, Thomas L, McMurray J, Bethel M and Holman R. 2018. Relationship between baseline physical activity assessed by pedometer count and new-onset diabetes in the NAVIGATOR trial. *BMJ Open Diabetes Research and Care;* 6(1)**:** e000523.

109   Krug A and Ehrhart-Bornstein M. 2008. Adrenocortical dysfunction in obesity and the metabolic syndrome. *Hormone and metabolic research;* 40(08)**:** 515-517.

110   Krysiak R and Okopien B. 2013. The effect of metformin on monocyte secretory function in simvastatin-treated patients with impaired fasting glucose. *Metabolism;* 62(1)**:** 39-43.

111   Kuchay M, Krishan S, Mishra S, Farooqui K, Singh M, Wasir J, Bansal B, Kaur P, Jevalikar G and Gill H. 2018. Effect of empagliflozin on liver fat in patients with type 2 diabetes and nonalcoholic fatty liver disease: a randomized controlled trial (E-LIFT Trial). *Diabetes care;* 41(8)**:** 1801-1808.

112   Kumashiro N, Erion D, Zhang D, Kahn M, Beddow S, Chu X, Still C, Gerhard G, Han X and Dziura J. 2011. Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. *Proceedings of the National Academy of Sciences;* 108(39)**:** 16381-16385.

113   Kutoh E. 2005. Possible metformin-induced hepatotoxicity. *The American journal of geriatric pharmacotherapy;* 3(4)**:** 270-273.

114   Laakso M. 2010. Cardiovascular disease in type 2 diabetes from population to man to mechanisms. *Diabetes care;* 33(2)**:** 442-449.

115   Laakso M and Kuusisto J. 2014. Insulin resistance and hyperglycaemia in cardiovascular disease development. *Nature Reviews Endocrinology;* 10(5)**:** 293.

116   Laaksonen D, Lindström J, Lakka T, Eriksson J, Niskanen L, Wikström K, Aunola S, Keinänen-Kiukaanniemi S, Laakso M and Valle T. 2005. Physical activity in the prevention of type 2 diabetes. *Diabetes;* 54(1)**:** 158-165.

117   Larter CZ and Farrell GC. 2006. Insulin resistance, adiponectin, cytokines in NASH: which is the best target to treat? *Journal of hepatology;* 44(2)**:** 253-261.

118   Leclercq I, Morais A, Schroyen B, Van Hul N and Geerts A. 2007. Insulin resistance in hepatocytes and sinusoidal liver cells: mechanisms and consequences. *Journal of hepatology;* 47(1)**:** 142-156.

119   Lenzen S. 2008. The mechanisms of alloxan-and streptozotocin-induced diabetes. *Diabetologia;* 51(2)**:** 216-226.

120   Levina A, Mitra A and Lay P. 2009. Recent developments in ruthenium anticancer drugs. *Metallomics;* 1**:** 458-470.

121   Lim A. 2014. Diabetic nephropathy–complications and treatment. *International journal of nephrology and renovascular disease;* 7**:** 361.

122   Lindström J, Peltonen M, Eriksson J, Louheranta A, Fogelholm M, Uusitupa M and Tuomilehto J. 2006. High-fibre, low-fat diet predicts long-term weight loss and decreased type 2 diabetes risk: the Finnish Diabetes Prevention Study. *Diabetologia;* 49(5)**:** 912-920.

123   Lindström J, Peltonen M, Eriksson J, Ilanne-Parikka P, Aunola S, Keinänen-Kiukaanniemi S, Uusitupa M and Tuomilehto J. 2013. Improved lifestyle and decreased diabetes risk over 13 years: long-term follow-up of the randomised Finnish Diabetes Prevention Study (DPS). *Diabetologia;* 56(2)**:** 284-293.

124   Liu R, Mizuta M, Kurose T and Matsukura S. 2002. Early events involved in the development of insulin resistance in Zucker fatty rat. *International journal of obesity;* 26(3)**:** 318.

125   Lontchi-Yimagou E, Sobngwi E, Matsha T and Kengne A. 2013. Diabetes mellitus and inflammation. *Current diabetes reports;* 13(3)**:** 435-444.

126   Loomba R, Lutchman G, Kleiner D, Ricks M, Feld J, Borg B, Modi A, Nagabhyru P, Sumner A and Liang T. 2009. Clinical trial: pilot study of metformin for the treatment of non-alcoholic steatohepatitis. *Alimentary pharmacology & therapeutics;* 29(2)**:** 172-182.

127   Lorenzo C, Hartnett S, Hanley A, Rewers M, Wagenknecht L, Karter A and Haffner S. 2013. Impaired fasting glucose and impaired glucose tolerance have distinct lipoprotein and apolipoprotein changes: the insulin resistance atherosclerosis study. *The Journal of Clinical Endocrinology & Metabolism;* 98(4)**:** 1622-1630.

128   Lozano I, Van der Werf R, Bietiger W, Seyfritz E, Peronet C, Pinget M, Jeandidier N, Maillard E, Marchioni E and Sigrist S. 2016. High-fructose and high-fat diet-induced disorders in rats: impact on diabetes risk, hepatic and vascular complications. *Nutrition & metabolism;* 13(1)**:** 15.

129   Lu H, Jian C, Chen S, Chen T, Lee S, Chang C and Weng C. 2010. Hypoglycaemic effects of fermented mycelium of Paecilomyces farinosus (G30801) on high-fat fed rats with streptozotocin-induced diabetes 21**:** 7040-7045.

130   Lucchesi A, Cassettari L and Spadella C. 2015. Alloxan-induced diabetes causes morphological and ultrastructural changes in rat liver that resemble the natural history of chronic fatty liver disease in humans. *Journal of diabetes research;* 2015**:** 494578.

131   Lumeng C and Saltiel A. 2011. Inflammatory links between obesity and metabolic disease. *The Journal of clinical investigation;* 121(6)**:** 2111.

132   Lundkvist P, Pereira M, Kamble P, Katsogiannos P, Langkilde A, Esterline R, Johnsson E and Eriksson J. 2019. Glucagon levels during short-term SGLT2 inhibition are largely regulated by glucose changes in patients with type 2 diabetes. *The Journal of Clinical Endocrinology & Metabolism;* 104(1)**:** 193-201.

133   Luvuno M, Mabandla M and Khathi A. 2018. Voluntary ingestion of a high-fat high-carbohydrate diet: A model for prediabetes. *Ponte international scientific researchs journal;* 74.

134   Lyu X, Zhu X, Zhao B, Du L, Chen D, Wang C, Liu G and Ran X. 2017. Effects of dipeptidyl peptidase-4 inhibitors on beta-cell function and insulin resistance in type 2 diabetes: meta-analysis of randomized controlled trials. *Scientific reports;* 7**:** 44865.

135   Ma L, Fu Y, Yu L, Li X, Zheng W and Chen T. 2015. Ruthenium complexes as inhibitors of human islet amyloid polypeptide aggregation, an effect that prevents beta cell apoptosis. *RSC Advances;* 5(23)**:** 17405-17412.

136   Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P and Khathi A. 2018. Effects of a ruthenium schiff base complex on glucose homeostasis in diet-induced pre-diabetic rats *Molecules (Basel, Switzerland)* 23(7)**:** E1721.

137   Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P and Khathi A. 2019. Cardioprotective effects of a ruthenium (II) Schiff base complex in a diet-induced prediabetic rats. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy;* 12**:** 1-7.

138   Maiztegui B, Boggio V, Román C, Flores L, Zotto H, Ropolo A, Grasso D, Vaccaro M and Gagliardino J. 2017. VMP1-related autophagy induced by a fructose-rich diet in β-cells: its prevention by incretins. *Clinical Science;* 131(8)**:** 673-687.

139   Makki K, Froguel P and Wolowczuk I. 2013. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. *ISRN inflammation;* 2013.

140   Manna P, Das J, Ghosh J and Sil P. 2010. Contribution of type 1 diabetes to rat liver dysfunction and cellular damage via activation of NOS, PARP, IκBα/NF-κB, MAPKs, and mitochondria-dependent pathways: Prophylactic role of arjunolic acid. *Free Radical Biology and Medicine;* 48(11)**:** 1465-1484.

141   Mannucci E, Monami M, Di Bari M, Lamanna C, Gori F, Gensini G and Marchionni N. 2010. Cardiac safety profile of rosiglitazone: a comprehensive meta-analysis of randomized clinical trials. *International journal of cardiology;* 143(2)**:** 135-140.

142   Markiewicz M, Richard E, Marks N and Ludwicka-Bradley A. 2013. Impact of endothelial microparticles on coagulation, inflammation, and angiogenesis in age-related vascular diseases. *Journal of aging research;* 2013.

143   Maschirow L, Khalaf K, Al-Aubaidy H and Jelinek H. 2015. Inflammation, coagulation, endothelial dysfunction and oxidative stress in prediabetes—Biomarkers as a possible tool for early disease detection for rural screening. *Clinical biochemistry;* 48(9)**:** 581-585.

144   Mavrogiannaki A and Migdalis I. 2013. Nonalcoholic Fatty liver disease, diabetes mellitus and cardiovascular disease: newer data. *International Journal of Endocrinology;* 2013**:** 450639.

145   Maximos S, Chamoun M, Gravel S, Turgeon J and Michaud V. 2017. Tissue specific modulation of cyp2c and cyp3a mRNA levels and activities by diet-induced obesity in mice: the impact of type 2 diabetes on drug metabolizing enzymes in liver and extra-hepatic tissues. *Pharmaceutics;* 9(4)**:** 40.

146   May L, Lefkowitch J, Kram M and Rubin D. 2002. Mixed hepatocellular–cholestatic liver injury after pioglitazone therapy. *Annals of Internal Medicine;* 136(6)**:** 449-452.

147   McArdle M, Finucane O, Connaughton R, McMorrow A and Roche H. 2013. Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies. *Frontiers in endocrinology;* 4**:** 52.

148   McLellan K, Wyne K, Villagomez E and Hsueh W. 2014. Therapeutic interventions to reduce the risk of progression from prediabetes to type 2 diabetes mellitus. *Therapeutics and clinical risk management;* 10**:** 173.

149   Michael M, Kulkarni R, Postic C, Previs S, Shulman G, Magnuson M and Kahn C. 2000. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Molecular cell;* 6(1)**:** 87-97.

150   Misra A, Singhal N and Khurana L. 2010. Obesity, the metabolic syndrome, and type 2 diabetes in developing countries: role of dietary fats and oils. *Journal of the American College of Nutrition;* 29(sup3)**:** 289S-301S.

151   Miyazaki Y, Glass L, Triplitt C, Matsuda M, Cusi K, Mahankali A, Mahankali S, Mandarino L and DeFronzo R. 2001. Effect of rosiglitazone on glucose and non-esterified fatty acid metabolism in type II diabetic patients. *Diabetologia;* 44**:** 2210-2219.

152   Mohamed J, Nafizah A, Zariyantey A and Budin S. 2016. Mechanisms of diabetes-induced liver damage: the role of oxidative stress and inflammation. *Sultan Qaboos University Medical Journal;* 16(2)**:** 132–141.

153   Moriwaki Y, Yamamoto T, Shibutani Y, Aoki E, Tsutsumi Z, Takahashi S, Okamura H, Koga M, Fukuchi M and Hada T. 2003. Elevated levels of interleukin-18 and tumor necrosis factor-α in serum of patients with type 2 diabetes mellitus: relationship with diabetic nephropathy. *Metabolism;* 52(5)**:** 605-608.

154   Moscatiello S, Manini R and Marchesini G. 2007. Diabetes and liver disease: an ominous association. *Nutrition, Metabolism and Cardiovascular Diseases;* 17(1)**:** 63-70.

155   Mudaliar S, Polidori D, Zambrowicz B and Henry R. 2015. Sodium–glucose cotransporter inhibitors: effects on renal and intestinal glucose transport: from bench to bedside. *Diabetes care;* 38(12)**:** 2344-2353.

156   Musso G, Gambino R, Cassader M and Pagano G. 2010. A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology;* 52(1)**:** 79-104.

157   Mzimela N, Ngubane P and Khathi A. 2019. The changes in immune cell concentration during the progression of pre-diabetes to type 2 diabetes in a high-fat high-carbohydrate diet-induced pre-diabetic rat model. *Autoimmunity;* 52(1)**:** 27-36.

158   Naik N, Hirya K, Bhimareddy A and Naik N. 2014. Ruthenium metal complexes and their biological approach-a short review. *International Journal of Pharmacy & Life Sciences;* 5(3).

159   Nannipieri M, Gonzales C, Baldi S, Posadas R, Williams K, Haffner S, Stern M and Ferrannini E. 2005. Liver enzymes, the metabolic syndrome, and incident diabetes: the Mexico City diabetes study. *Diabetes care;* 28(7)**:** 1757-1762.

160   Navarro-González J, Mora-Fernández C, De Fuentes M and García-Pérez J. 2011. Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. *Nature Reviews Nephrology;* 7(6)**:** 327-340.

161   Navarro J, Mora C, Muros M and García J. 2006. Urinary tumour necrosis factor-α excretion independently correlates with clinical markers of glomerular and tubulointerstitial injury in type 2 diabetic patients. *Nephrology Dialysis Transplantation;* 21(12)**:** 3428-3434.

162   Nishizawa T and Bornfeldt K. 2012. Diabetic vascular disease and the potential role of macrophage glucose metabolism. *Annals of medicine;* 44(6)**:** 555-563.

163   Norton L, Shannon C, Fourcaudot M, Hu C, Wang N, Ren W, Song J, Abdul-Ghani M, DeFronzo R and Ren J. 2017. Sodium-glucose co-transporter (SGLT) and glucose transporter (GLUT) expression in the kidney of type 2 diabetic subjects. *Diabetes, Obesity and Metabolism;* 19(9)**:** 1322-1326.

164   Nuttall F, Ngo A and Gannon M. 2008. Regulation of hepatic glucose production and the role of gluconeogenesis in humans: is the rate of gluconeogenesis constant? *Diabetes Metabolism Research and Reviews;* 24**:** 438–458.

165   Obstfeld A, Sugaru E, Thearle M, Francisco A, Gayet C, Ginsberg H, Ables E and Ferrante A. 2010. CC chemokine receptor 2 (CCR2) regulates the hepatic recruitment of myeloid cells that promote obesity-induced hepatic steatosis. *Diabetes;* 59(4)**:** 916-925.

166   Okada R, Yasuda Y, Tsushita K, Wakai K, Hamajima N and Matsuo S. 2014. The number of metabolic syndrome components is a good risk indicator for both early-and late-stage kidney damage. *Nutrition, Metabolism and Cardiovascular Diseases;* 24(3)**:** 277-285.

167   Oliver M. 2015. Fatty acids and the risk of death during acute myocardial ischaemia. *Clinical Science;* 128(6)**:** 349-355.

168   Opie L. 2014. Lifestyle and diet. *Cardiovascular journal of Africa;* 25(6)**:** 298.

169   Palsamy P, Sivakumar S and Subramanian S. 2010. Resveratrol attenuates hyperglycemia-mediated oxidative stress, proinflammatory cytokines and protects hepatocytes ultrastructure in streptozotocin–nicotinamide-induced experimental diabetic rats. *Chemico-biological interactions;* 186(2)**:** 200-210.

170   Parajuli J, Saleh F, Thapa N and Ali L. 2014. Factors associated with nonadherence to diet and physical activity among Nepalese type 2 diabetes patients; a cross sectional study. *BMC research notes;* 7(1)**:** 758.

171   Parhofer K. 2015. Interaction between glucose and lipid metabolism: more than diabetic dyslipidemia. *Diabetes & metabolism journal;* 39(5)**:** 353-362.

172   Park M, Kim D, Lim S, Choi J, Han H, Yoon K and Park S. 2014. High Glucose-induced O-GlcNAcylated Carbohydrate Response Element-binding Protein (ChREBP) Mediates Mesangial Cell Lipogenesis and Fibrosis: The possible role in the development of diabetic nephropathy. *Journal of Biological Chemistry;* 289(19)**:** 13519-13530.

173   Patel V, Joharapurkar A, Kshirsagar S, Patel K, Bahekar R, Shah G and Jain M. 2016. Central GLP-1 receptor activation improves cholesterol metabolism partially independent of its effect on food intake. *Canadian journal of physiology and pharmacology;* 94(2)**:** 161-167.

174   Patel V, Joharapurkar A, Kshirsagar S, Sutariya B, Patel M, Patel H, Pandey D, Bahekar R and Jain M. 2018. Coagonist of glucagon-like peptide-1 and glucagon receptors ameliorates kidney injury in murine models of obesity and diabetes mellitus. *World journal of diabetes;* 9(6)**:** 80.

175   Pitocco D, Tesauro M, Alessandro R, Ghirlanda G and Cardillo C. 2013. Oxidative stress in diabetes: implications for vascular and other complications. *International journal of molecular sciences;* 14(11)**:** 21525-21550.

176   Plantinga L, Crews D, Coresh J, Miller E, Saran R, Yee J, Hedgeman E, Pavkov M, Eberhardt M and Williams D. 2010. Prevalence of chronic kidney disease in US adults with undiagnosed diabetes or prediabetes. *Clinical Journal of the American Society of Nephrology;* 5(4)**:** 673-682.

177   Pongwecharak J, Tengmeesri N, Malanusorn N, Panthong M and Pawangkapin N. 2009. Prescribing metformin in type 2 diabetes with a contraindication: prevalence and outcome. *Pharmacy world & science;* 31(4)**:** 481-486.

178   Pradhan A, Manson J, Rifai N, Buring J and Ridker P. 2001. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *Jama;* 286(3)**:** 327-334.

179   Prieur X, Mok C, Velagapudi V, Núñez V, Fuentes L, Montaner D, Ishikawa K, Camacho A, Barbarroja N and O'Rahilly S. 2011. Differential lipid partitioning between adipocytes and tissue macrophages modulates macrophage lipotoxicity and M2/M1 polarization in obese mice. *Diabetes;* 60(3)**:** 797-809.

180   Rachek L. 2013. Free fatty acids and skeletal muscle insulin resistance. *Glucose Homeostatis and the Pathogenesis of Diabetes Mellitus;* 121**:** 267.

181   Rafique S, Idrees M, Nasim A, Akbar H and Athar A. 2010. Transition metal complexes as potential therapeutic Agents. *Biotechnology and Molecular Biology Reviews;* 5**:** 1538-2273.

182   Rahati S, Shahraki M, Arjomand G and Shahraki T. 2014. Food pattern, lifestyle and diabetes mellitus. *International journal of high risk behaviors & addiction;* 3(1).

183   Raskin P. 2013. Sodium–glucose cotransporter inhibition: therapeutic potential for the treatment of type 2 diabetes mellitus. *Diabetes/metabolism research and reviews;* 29(5)**:** 347-356.

184   Regnell S and Lernmark A. 2011. Hepatic steatosis in type 1 diabetes. *The review of diabetic studies: RDS;* 8(4)**:** 454.

185   Rehman K and Akash M. 2016. Mechanisms of inflammatory responses and development of insulin resistance: how are they interlinked? *Journal of biomedical science;* 23(1)**:** 87.

186   Remuzzi G, Perico N, Macia M and Ruggenenti P. 2005. The role of renin-angiotensin-aldosterone system in the progression of chronic kidney disease. *Kidney International;* 68**:** S57-S65.

187   Ritz E. 2008. Metabolic syndrome and kidney disease. *Blood purification;* 26(1)**:** 59-62.

188   Roumen C, Feskens E, Corpeleijn E, Mensink M, Saris W and Blaak E. 2011. Predictors of lifestyle intervention outcome and dropout: the SLIM study. *European journal of clinical nutrition;* 65(10)**:** 1141.

189   Sakura H, Hashimoto N, Sasamoto K, Ohashi H, Hasumi S, Ujihara N, Kasahara T, Tomonaga O, Nunome H and Honda M. 2016. Effect of sitagliptin on blood glucose control in patients with type 2 diabetes mellitus who are treatment naive or poorly responsive to existing antidiabetic drugs: the JAMP study. *BMC endocrine disorders;* 16(1)**:** 70.

190 Saltiel A and Pessin J. 2003. Insulin signaling in microdomains of the plasma membrane. *Traffic;* 4**:** 11-16.

191 Sangoi M, de Carvalho J, Haussen B, Bollick Y, Londero S and Comim F. 2016. Urinary inflammatory cytokines as indicators of kidney damage in type 2 diabetic patients. *Clinica chimica acta;* 460**:** 178-183.

192 Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, Kopp A, Schoelmerich J and Falk W. 2009. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-κB pathway in adipocytes links nutritional signalling with innate immunity. *Immunology;* 126(2)**:** 233-245.

193 Schnapp G, Klein T, Hoevels Y, Bakker R and Nar H. 2016. Comparative analysis of binding kinetics and thermodynamics of dipeptidyl peptidase-4 inhibitors and their relationship to structure. *Journal of medicinal chemistry;* 59(16)**:** 7466-7477.

194 Schöndorf T, Musholt P, Hohberg C, Forst T, Lehmann U, Fuchs W, Löbig M, Müller J and Pfützner A. 2011. The fixed combination of pioglitazone and metformin improves biomarkers of platelet function and chronic inflammation in type 2 diabetes patients: results from the PIOfix study. *Journal of diabetes science and technology;* 5(2)**:** 426-432.

195 Schwartz E and Reaven P. 2012. Lipolysis of triglyceride-rich lipoproteins, vascular inflammation, and atherosclerosis. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids;* 1821(5)**:** 858-866.

196 Sekhon B. 2011. Inorganics/bioinorganics: Biological, medicinal and pharmaceutical uses. *Journal of Pharmaceutical Education and Research;* 2(1)**:** 1.

197 Sena C, Pereira A and Seiça R. 2013. Endothelial dysfunction—a major mediator of diabetic vascular disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease;* 1832(12)**:** 2216-2231.

198 Sequeira I and Poppitt S. 2017. HbA1c as a marker of prediabetes: a reliable screening tool or not? *Insights in Nutrition and Metabolism* 1(1)**:** 21-29.

199 Shepherd P. 2005. Mechanisms regulating phosphoinositide 3-kinase signaling in insulin-sensitive tissues. *Acta Physiologica Scandinavica;* 18**:** 3-12.

200 Shrestha P and Ghimire L. 2012. A review about the effect of life style modification on diabetes and quality of life. *Global journal of health science;* 4(6)**:** 185.

201 Simmgen M, Knauf C, Lopez M, Choudhury A, Charalambous M, Cantley J, Bedford D, Claret M, Iglesias M and Heffron H. 2006. Liver-specific deletion of insulin receptor substrate 2 does not impair hepatic glucose and lipid metabolism in mice. *Diabetologia;* 49**:** 552–561.

202 Spoto B, Pisano A and Zoccali C. 2016. Insulin resistance in chronic kidney disease: a systematic review. *American Journal of Physiology-Renal Physiology;* 311(6)**:** 1087-1108.

203 Srinivasan K and Ramarao P. 2007. Animal models in type 2 diabetes research. *Indian Journal of Medical Research;* 125**:** 451–472.

204 Stefan N, Sun Q, Fritsche A, Machann J, Schick F, Gerst F, Jeppesen C, Joost H, Hu F and Boeing B. 2014. Impact of the adipokine adiponectin and the hepatokine fetuin-A on the development of type 2 diabetes: prospective cohort-and cross-sectional phenotyping studies. *PloS one;* 9(3)**:** e92238.

205 Stiegler R, Zimmet P, Cameron A and Shaw J. 2009. Lifestyle management: preventing Type 2 diabetes and cardiovascular complications. *Diabetes Care;* 6**:** 489-496.

206 Stolar M. 2010. Glycemic control and complications in type 2 diabetes mellitus. *The American Journal of Medicine;* 123**:** 1-11.

207   Suh J and Miner J. 2013. The glomerular basement membrane as a barrier to albumin. *Nature Reviews Nephrology;* 9(8)**:** 470.

208   Sullivan S and Ratner R. 2011. Should the metabolic syndrome patient with prediabetes be offered pharmacotherapy? *Current diabetes reports;* 11(2)**:** 91-98.

209   Syed I. 2011. Glycated haemoglobin; past, present, and future are we ready for the change. *diabetes;* 11**:** 12.

210   Tabák A, Herder C, Rathmann W, Brunner E and Kivimäki M. 2012. Prediabetes: a high-risk state for diabetes development. *The Lancet;* 379(9833)**:** 2279-2290.

211   Tagawa A, Yasuda M, Kume S, Yamahara K, Nakazawa J, Chin-Kanasaki M, Araki H, Araki S, Koya D and Asanuma K. 2016. Impaired podocyte autophagy exacerbates proteinuria in diabetic nephropathy. *Diabetes;* 65(3)**:** 755-767.

212   Taguchi A and White M. 2008. Insulin-like signaling, nutrient homeostasis and life span. *Annual Review of Physiology* 70**:** 191–212.

213   Talukdar S, Bandyopadhyay G, Li D, Xu J, McNelis J, Lu M, Li P, Yan Q, Zhu Y and Ofrecio J. 2012. Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nature medicine;* 18(9)**:** 1407.

214   Tanaka Y, Kume S, Chin-KM, Araki H, Araki S, Ugi S, Sugaya T, Uzu T and Maegawa H. 2016. Renoprotective effect of DPP-4 inhibitors against free fatty acid-bound albumin-induced renal proximal tubular cell injury. *Biochemical and biophysical research communications;* 470(3)**:** 539-545.

215   Taniguchi C, Ueki K and Kahn R. 2005. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *Indian Journal of Medical Research;* 115**:** 718–727.

216   Taskinen M. 2003. Diabetic dyslipidaemia: from basic research to clinical practice. *Diabetologia;* 46(6)**:** 733-749.

217   Thomas M, Paldánius P, Ayyagari R, Ong S and Groop P. 2016. Systematic literature review of DPP-4 inhibitors in patients with type 2 diabetes mellitus and renal impairment. *Diabetes Therapy;* 7(3)**:** 439-454.

218   Tran H, Silva D and Petrovsky N. 2004. Case study: potential pitfalls of using hemoglobin A1c as the sole measure of glycemic control. *Clinical Diabetes;* 22(3)**:** 141-143.

219   Tseng E, Greer R, O'Rourke P, Yeh H, McGuire M, Clark J and Maruthur N. 2017. Survey of primary care providers' knowledge of screening for, diagnosing and managing prediabetes. *Journal of general internal medicine;* 32(11)**:** 1172-1178.

220   Undas A, Wiek I, Stêpień E, Zmudka K and Tracz W. 2008. Hyperglycemia is associated with enhanced thrombin formation, platelet activation, and fibrin clot resistance to lysis in patients with acute coronary syndrome. *Diabetes care;* 31(8)**:** 1590-1595.

221   Vallianou N, Geladari E and Kazazis C. 2017. SGLT-2 inhibitors: their pleiotropic properties. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews;* 11(4)**:** 311-315.

222   van Raalte D, Bunck M, Smits M, Hoekstra T, Corner A, Diamant M and Eliasson B. 2016. Exenatide improves β-cell function up to 3 years of treatment in patients with type 2 diabetes: a randomised controlled trial. *Eur J Endocrinol;* 175(4)**:** 345-352.

223   Venkataraman K, Wee H, Leow M, Tai E, Lee J, Lim S, Tavintharan S, Wong T, Ma S and Heng D. 2013. Associations between complications and health-related quality of life in individuals with diabetes. *Clinical endocrinology;* 78(6)**:** 865-873.

224 Viscogliosi G, Cipriani E, Liguori M, Marigliano B, Saliola M, Ettorre E and Andreozzi P. 2013. Mediterranean dietary pattern adherence: associations with prediabetes, metabolic syndrome, and related microinflammation. *Metabolic syndrome and related disorders;* 11(3)**:** 210-216.

225 Vishal J, Amit A, Gaurang B and Mukul R. 2014. Effect of GLP-1 based therapies on diabetic dyslipidemia. *Current diabetes reviews;* 10(4)**:** 238-250.

226 Vizioli L, Muscari S and Muscari A. 2009. The relationship of mean platelet volume with the risk and prognosis of cardiovascular diseases. *International journal of clinical practice;* 63(10)**:** 1509-1515.

227 Wang X, Zheng P, Huang G, Yang L and Zhou Z. 2018. Dipeptidyl peptidase-4 (DPP-4) inhibitors: promising new agents for autoimmune diabetes. *Clinical and experimental medicine;* 18(4)**:** 473-480.

228 Watson R, Kanzaki M and Pessin J. 2004. Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. *Endocrine Society Journals* 25**:** 177-204.

229 Weil E, Lemley K, Mason C, Yee B, Jones L, Blouch K, Lovato T, Richardson M, Myers B and Nelson R. 2012. Podocyte detachment and reduced glomerular capillary endothelial fenestration promote kidney disease in type 2 diabetic nephropathy. *Kidney international;* 82(9)**:** 1010-1017.

230 Whaley J, Tirmenstein M, Reilly T, Poucher S, Saye J, Parikh S and List J. 2012. Targeting the kidney and glucose excretion with dapagliflozin: preclinical and clinical evidence for SGLT2 inhibition as a new option for treatment of type 2 diabetes mellitus. *Diabetes, metabolic syndrome and obesity: targets and therapy;* 5**:** 135.

231 White M. 2003. Insulin signaling in health and disease. *Science;* 302**:** 1710–1711.

232 Wilding JP. 2014. The role of the kidneys in glucose homeostasis in type 2 diabetes: clinical implications and therapeutic significance through sodium glucose co-transporter 2 inhibitors. *Metabolism;* 63(10)**:** 1228-1237.

233 Winer S and Winer D. 2012. The adaptive immune system as a fundamental regulator of adipose tissue inflammation and insulin resistance. *Immunology and cell biology;* 90(8)**:** 755-762.

234 Wu P, Wu C, Lin C and Wu V. 2015. Long-term risk of upper gastrointestinal hemorrhage after advanced AKI. *Clinical Journal of the American Society of Nephrology;* 10(3)**:** 353-362.

235 Yang J, Zhou Y and Guan Y. 2012. PPARγ as a therapeutic target in diabetic nephropathy and other renal diseases. *Current opinion in nephrology and hypertension;* 21(1)**:** 97-105.

236 Yip W, Sequeira I, Plank L and Poppitt S. 2017. Prevalence of pre-diabetes across ethnicities: a review of impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) for classification of dysglycaemia. *Nutrients;* 9(11)**:** 1273.

237 Zhang C and Lippard S. 2003. New metal complexes as potential therapeutics Current. *Journal of Chemical Biology;* 7**:** 1367-5931.

238 Zhao Y, He X, Huang C, Fu X, Shi X, Wu Y, Han Y, Li N, Wu Y and Heng C. 2010. The impacts of thiazolidinediones on circulating C-reactive protein levels in different diseases: a meta-analysis. *diabetes research and clinical practice;* 90(3)**:** 279-287.

Prologue 1: Manuscript 1

"Hepatoprotective effects of a ruthenium(II) Schiff base complex in rats with diet-induced prediabetes"

The relation between glucose and lipid toxicity with insulin resistance and β cell toxicity has been shown to impair liver function and leads to a vicious cycle. Lipid accumulation in the liver seems to be a major mechanism associated with prediabetes-related insulin resistance and T2DM progression. The pharmacological treatment of pre-diabetes-related liver complications aid in reducing body weight and improving insulin sensitivity in the liver and periphery in conjunction with dietary intervention. Previously, we have shown that treatment with ruthenium (II) complex (15 mg/kg) (extrapolated from previous studies) in both the presence and absence of dietary intervention significantly improved markers associated with the risk of progression to T2DM in a diet-induced pre-diabetic rat model. However, the effects of this compound on liver injury enzymes markers and NAFLD-associated complications in a diet-induced pre-diabetic rat model remain unknown. Hence, manuscript 1, investigated the hepato-protective effects of a ruthenium(II) Schiff base complex in diet-induced prediabetic rats in both the presence and absence of dietary intervention.

This manuscript has been accepted for publication in the journal "**Current Therapeutic Research"** and has been formatted according to the journal's guidelines for authors (Appendix 7).

This work was authored by **L.P Mabuza**, **M.W Gamede**, **S Maikoo**, **IN Booysen**, **P.S Ngubane** and **A Khathi.**

Abstract

Background: Progressive insulin resistance in a pre-diabetic state has been reported to be the predominant causative factor for the development of non-alcoholic fatty liver disease (NAFLD). The combination of dietary modification and pharmacotherapy have been recommended to manage diabetic liver complications. However, poor patient compliance and toxicity of current drug therapy on liver function still results, therefore newer alternative drugs are required.

Materials and methods: Prediabetic rats were randomly allocated to respective treatment groups. The ruthenium-based compound (15 mg/kg) was administered to the pre-diabetic rats in both the presence and absence of dietary intervention once a day every third day for 12 weeks.

Results: The administration of the ruthenium compound in both the presence and absence of dietary intervention resulted in the restoration of liver and body weights. This treatment also reduced liver damage enzyme biomarkers, bilirubin and sterol regulatory element binding protein 1c (SREBP-1c) concentrations in the plasma.

Conclusion: The ruthenium(II) complex showed beneficial effects as it ameliorated and prevented the progression of diabetes-related liver derangements while eliminating the hepatotoxicity associated with the use of metal compounds. However, further studies are still required to further determine the physiological mechanisms behind this effect.

Keywords: Ruthenium(II) complex, Non-alcoholic fatty liver disease (NAFLD), Pre-diabetes, Hepatic insulin resistance, Dietary intervention

1. Introduction

The liver plays an important role in the maintenance of systemic lipid and glucose homeostasis. [1] However, liver damage leads to dysregulation of both lipid and glucose metabolism and is a serious complication among pre-diabetic and diabetic patients. [2] Liver damage is associated with several abnormalities, such as abnormal glycogen deposition, NAFLD and elevated plasma concentrations of liver damage biomarkers. [3, 4] In pre-diabetes, insulin resistance and compensatory hyperinsulinaemia have been shown to be the predominant causative factors of liver pathology. [5-7] The insulin resistance is said to impair the anti-lipolytic action of insulin in adipose tissue, which leads to increased release of free fatty acids (FFA). [8-11] The elevated plasma concentrations of insulin, glucose and fatty acids then impairs the β-oxidation of fatty acids and enhances *de novo* lipogenesis in the liver under the control of a specific transcription factor SREBP-1c. [4, 12, 13] The presence of abnormal plasma levels of liver enzymes, such as alanine transaminase (ALT) and aspartate transaminase (AST) levels resemble liver injury and are found to be increased in pre-diabetic patients. [4, 14] In addition, studies have shown that bilirubin, which has been shown to have protective effects against cardiovascular complications, is lowered in diabetes. [15, 16] Furthermore, the histopathological changes in diabetic patients include increased hepatic glycogen concentration, accumulation of hepatic fat and increased liver size. [13, 17, 18] Dietary modifications and pharmacotherapy have been used to manage NAFLD as well as non-alcoholic steatohepatitis (NASH) liver complications which aid in reducing body weight and improving insulin sensitivity in the liver and periphery. [7, 19, 20] However, the clinical value of metformin, pioglitazone, thiazolidinediones together with other treatments such as betaine, atorvastatin, losartan and orlistat is very subjective. Patients taking these drugs should be closely monitored due to possible contra-indications with diabetes mellitus medications and the vulnerable condition of the liver during the drug detoxification process. [20, 21] Ruthenium (II)-derived complex has been shown to possess anti-inflammatory properties and exhibited protective effects against lipopolysaccharide-induced liver injury in mice through mediation of the inhibition of NF-κB signaling pathways. [22] Furthermore, recent studies have reported that ruthenium(II) complex with a diimine uracil chelating ligand possesses anti-diabetic properties which improved glycaemic control, insulin sensitivity and decreased risk of developing diabetes-related cardiovascular diseases in diet-induced pre-diabetic rats. [23, 24] However, the effects of this metal complex on hepatic complications in the pre-diabetic state remain unknown. Hence, this study sought to investigate the hepatoprotective effects of the ruthenium(II) Schiff base complex in the presence and absence of dietary intervention in a diet-induced pre-diabetic rat model.

2. Materials and Methods

2.1 Chemicals and drugs

All chemicals and drugs were of analytical grade and purchased from standard commercial suppliers. Previous studies showed that the dose of the ruthenium complex used in this study was non-toxic. [23, 24]

2.2 Synthesis of ruthenium(II) Schiff base complex

The synthesis of ruthenium(II) Schiff base complex, $[Ru^{II}(H_3ucp)Cl(PPh_3)]$ (H_4ucp = 2,6-*bis*-((6-amino-1,3-dimethyluracilimino)methylene)pyridine) has been done in our laboratory as previously reported. [25]

2.3 Animals and housing

In this study, 36 male Sprague-Dawley rats (150-180 g) were used. The animals were housed in a room with a 12-hour light/12hour dark cycle, at room temperature (25°C), for the duration of the study. The animals in each group had access to food and water *ad libitum*. All animal procedures and housing conditions were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (ethical clearance number: AREC/038/016M).

2.3.1 Induction of pre-diabetes

The animals were randomly assigned to the following diet groups; Normal diet with drinking water (ND) and high-fat high-carbohydrate diet with drinking water supplemented with 15% fructose (HFHC) (AVI Products (Pty) Ltd, Waterfall, South Africa). Pre-diabetes was induced by allowing the animals to feed on the HFHC diet for 20 weeks as previously described. [26] Glucose tolerance was evaluated 5 days after the 20 weeks of induction with a well-known established laboratory protocol, the oral glucose tolerance test (OGTT), to determine pre-diabetes according to the American Diabetes Association criteria. [23] The rats with fasting blood glucose (FBG) of more than 5.6 mmol/L were considered pre-diabetic and grouped further for pharmacological studies. The animals that were fed the normal diet were also tested and were found to be normoglycemic and without pre-diabetes. The treatment started on the subsequent day and this was considered as the first day of treatment.

2.3.2 Experimental design

The study consisted of two main groups, the non-prediabetic animals (NPD, n=6) and the pre-diabetic animals (PD, n=30). After 20 weeks of induction, the pre-diabetic animals were divided into the following five groups (n=6): the first group

pre-diabetic (PD) were fed a high fat high carbohydrate (HFHC) diet without treatment. The second group (metformin [MTF] + HFHC) were fed on high fat high carbohydrate diet and treated with an oral dose of metformin (500 mg/kg, Sigma-Aldrich, St Louis, Missouri, United State of America). The third group (MTF + ND) were fed a normal diet (ND) and treated with an oral dose of metformin (500 mg/kg, Sigma-Aldrich, St Louis, Missouri, USA). The fourth group (ruthenium [RU] + HFHC) were fed a high fat high carbohydrate diet and treated with subcutaneous injection of ruthenium complex (15 mg/kg) while the fifth group (RU + ND) were fed a normal diet and treated with subcutaneous injection of ruthenium complex (15 mg/kg). The animals were treated once a day every third day at 09:00 am for 12 weeks. For 12 weeks treatment period parameters such as, body weight and FBG were monitored every 4 weeks.

2.3.3   Blood collection and tissue harvesting

All animals were anaesthetised with Isofor (100 mg/kg)) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) using a gas anaesthetic chamber (Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa) and allowed to inhale for 3 minutes. Blood was collected by cardiac puncture and then injected into individual pre-cooled heparinized containers. The blood was then centrifuged for plasma collection (Eppendorf centrifuge 5403, Germany) at 4 °C, 503 g for 15 minutes. Thereafter, the liver was removed, weighed and halved into two liver tissue. One liver tissue was snap frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until biochemical analysis, and the other liver tissue was kept in 10 % formalin buffer for histopathological examination.

2.4   Biochemical analysis

Plasma AST, ALT and Total bilirubin were measured using the Catalyst One Chemistry Analyzer (IDEXX Laboratories, United State of America). The concentration of SREBP-1c was analysed using specific ELISA kits in accordance with the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China). The kits included a micro-ELISA plate that was coated with antibodies specific to SREBP-1c. Standards and samples were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min at 37 °C. This was followed by the addition of the relevant biotinylated detection antibody (100 μl). After 60 min incubation at 37 °C, avidin–horseradish peroxidase conjugate (100 μl) was added to each micro-plate well. After a further 30 min incubation at 37 °C, the unbound components were washed away using the wash buffer provided. Substrate solution (100 μl) was added to each micro-plate well and, after 15 min incubation at 37 °C, the stop solution (50 μl) was added. Optical density was measured using a nano spectrophotometer

(BMG Labtech, Ortenburg, Germany) at 450 nm. The concentrations of SREBP-1c in the samples were extrapolated from a standard curve.

2.5 Glycogen Assay

Glycogen analysis was performed in liver tissues. Glycogen assay was conducted using a well-established laboratory protocol. [23] Liver tissues (50 mg) were weighed and heated with KOH (30%, 2 ml) at 100 °C for 30 min. Thereafter, Na_2SO_4 (10%, 0.194 ml) was added to stop the reaction and allowed to cool. For glycogen precipitation, the cooled mixture (200 µl) was aspirated and mixed with ethanol (95%, 200 µl). The precipitated glycogen was pelleted, washed and redissolved in H_2O (1 ml). Thereafter, anthrone (0.5 g dissolved in 250 ml of sulphuric acid, 4 ml) was added and boiled for 10 min. After cooling the absorbance was read using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Württernberg, Germany) at 620 nm. The glycogen concentrations were calculated from a glycogen standard curve.

2.6 Hepatic histology examination

Liver histology was analysed using a previously described protocol. [27] Liver tissues were immediately fixed in 10% formalin buffer after removal from the animals. The tissue samples were embedded in paraffin wax after alcohol dehydration process. Using microtome, 5 µm sections of liver tissue were taken and were stained by haematoxylin and eosin (H&E) stain. The processed tissue sections were then visualized and captured using a Leica Scanner, SCN400 and Slide Path Gateway LAN software for analysis (Leica Microsystems CMS, Wetzlar, Germany). Two paraffin-embedded liver samples from each group were sectioned (4 kidney sections per slide). Liver tissue was evaluated for histopathological changes using a semi-quantitative assessment. Each liver section was assessed for the presence of the following changes: lipid droplet (LD) accumulation, hepatocytes ballooning (H) and lobular disarray. The observed changes were graded from zero to three, with zero indicating normal tissue, one indicating mild change, two indicating moderate change, and three indicating severe change.

2.7 Statistical Analysis

Data were reported as mean ± standard error of mean (SEM). GraphPad Prism Software (version 5) was used to conduct statistical analysis. The differences between control and treated groups were analysed using One-way analysis of variance (ANOVA) followed by Tukey-Kramer. Values of $p < 0.05$ show statistical significance between the compared groups.

3. Results

3.1 Liver glycogen

By comparison with the non-prediabetic (NPD) group, the pre-diabetic (PD) group showed a significant increase in liver glycogen concentration ($p < 0.05$; Table 1). The ruthenium(II) complex and high fat high carbohydrate (RU+HFHC) and the metformin and high fat high carbohydrate (MTF+HFHC) groups had significant high glycogen concentrations when compared with the NPD group ($p < 0.05$; Table 1).However, the administration of RU+ND and MFT+ND significantly ($p<0.05$) reduced liver glycogen concentration when compared with the PD group (Table 1).

Table 1: Effects of the ruthenium(II) complex on liver glycogen concentration of pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means $\pm$ SEM (n=6) in each group.

Groups	Hepatic Glycogen (nmol/g protein)
NPD	1.88±0.40
PD	2.25±0.51*
MTF+HFHC	2.38±1.12*
MTF+ND	1.41±0.12α
RU+HFHC	2.18±0.72*
RU+ND	1.62±0.23α

* $p < 0.05$ compared to non-prediabetic (NPD), **α** $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

3.2 Liver and body weight

By comparison with the NPD group, the PD group showed a significant increase in both liver and body weight ($p<0.05$; Table 2). The administration of RU+HFHC and MTF+HFHC showed a significant increase in both liver and body weight when compared with the NPD group ($p < 0.05$; Table 2). However, when compared with the PD group, administration of ruthenium complex in the presence and absence of dietary intervention showed a significant decrease in body weight with

further decrease in liver weight in the ruthenium-treated pre-diabetic animals (p<0.05; Table 2). In addition, the metformin-treated animals displayed similar results when compared with the PD groups (p<0.05; Table 2).

Table 2: The influences of the ruthenium(II) complex on liver and body weight of pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means ± SEM (n=6) in each group.

Groups	Initial body weight (g)	Final body weight (g)	Liver weight (g)
NPD	173±1.45	384±6.30	11.53±0.14
PD	176±1.73	680±8.08*	24.33±1.23*
MTF+HFHC	172±1.22	501±5.06*α	17.34±0.86*α
MTF+ND	177±0.98	443±3.90α	13.35±1.37α
RU+HFHC	172±1.45	490±5.09*α	16.23±0.65*α
RU+ND	175±1.67	435±2.61α	13.20±0.91α

* $p < 0.05$ compared to non-prediabetic (NPD), **α** $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

3.3 Plasma liver function enzymes AST and ALT concentration

The untreated pre-diabetic group showed a significant increase in plasma AST concentration when compared with the non-prediabetic group over the treatment period (p<0.05; Fig. 1A). However, when compared with the PD group, both the groups treated with the metal-based drug resulted in a significant decrease in AST concentration (p<0.05; Fig. 1(A)). The metformin-treated groups showed a restored AST concentration when compared with the PD group (p<0.05; Fig. 1(A)). In addition, there were no significant changes in ALT concentration in the PD group when compared with the NPD group (p>0.05; Fig. 1(B)). Interestingly, administration of ruthenium complex in the presence and absence of dietary intervention showed a significant decrease in ALT concentration when compared to both the NPD and the PD groups, with the RU+ND group showing more effective results when compared to NPD group (p<0.05; Fig 1(B)). However, the metformin-treated groups showed a significant increase in ALT concentration when compared with both the NPD and the PD groups (p<0.05; Fig. 1(B)).

49

Fig. 1. The effects of the ruthenium(II) complex on (A) AST and (B) ALT concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM (n = 6) in each group. * $p < 0.05$ compared to non-prediabetic (NPD), α $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

3.4 Plasma total bilirubin concentration

The pre-diabetic group showed a significantly decreased plasma total bilirubin concentration when compared with the non-prediabetic group after the treatment period ($p<0.05$; Fig. 2). Interestingly, both RU+HFHC and RU+ND treated groups resulted in a significant increase in plasma total bilirubin concentration when compared with PD group to within the NPD group ($p<0.05$; Fig. 2). Remarkably, the metformin-treated groups showed a significant increase in plasma total bilirubin concentration when compared with both the NPD and the PD groups ($p<0.05$; Fig. 2).
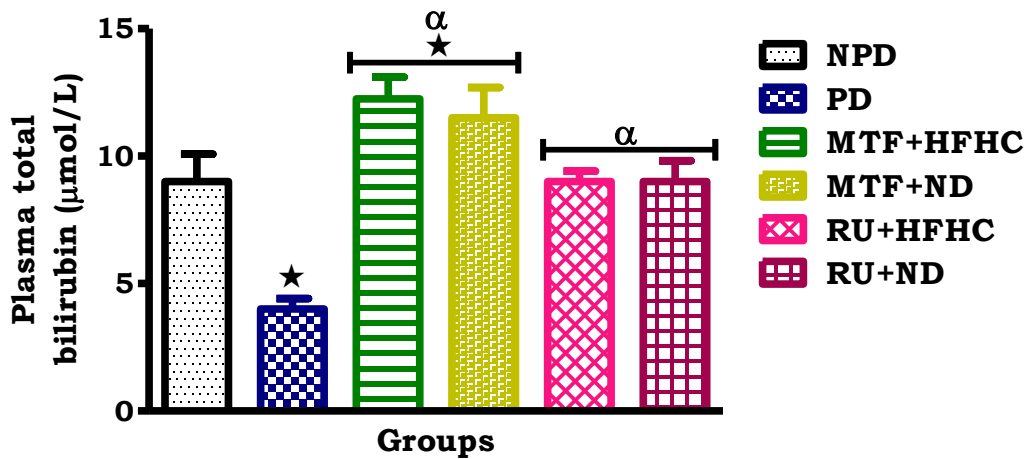
Fig. 2. The effects of the ruthenium(II) complex on total bilirubin concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM (n = 6) in each group. ***** $p < 0.05$ compared to non-prediabetic (NPD), **α** $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

3.5 Plasma SREBP-1c concentration

The pre-diabetic group showed significantly increased plasma SREBP-1c concentration when compared with the non-prediabetic group after the treatment period (p<0.05; Fig. 3). The RU+HFHC group had significant high plasma SREBP-1c concentration when compared with the NPD group (p<0.05; Fig. 3). Interestingly, when compared with the PD group, the administration of RU+ND reduced plasma SREBP-1c concentration to within that of NPD group (p<0.05; Fig. 3). The metformin-treated groups showed significant decrease in plasma SREBP-1c concentration when compared with the PD group (p<0.05; Fig. 3).
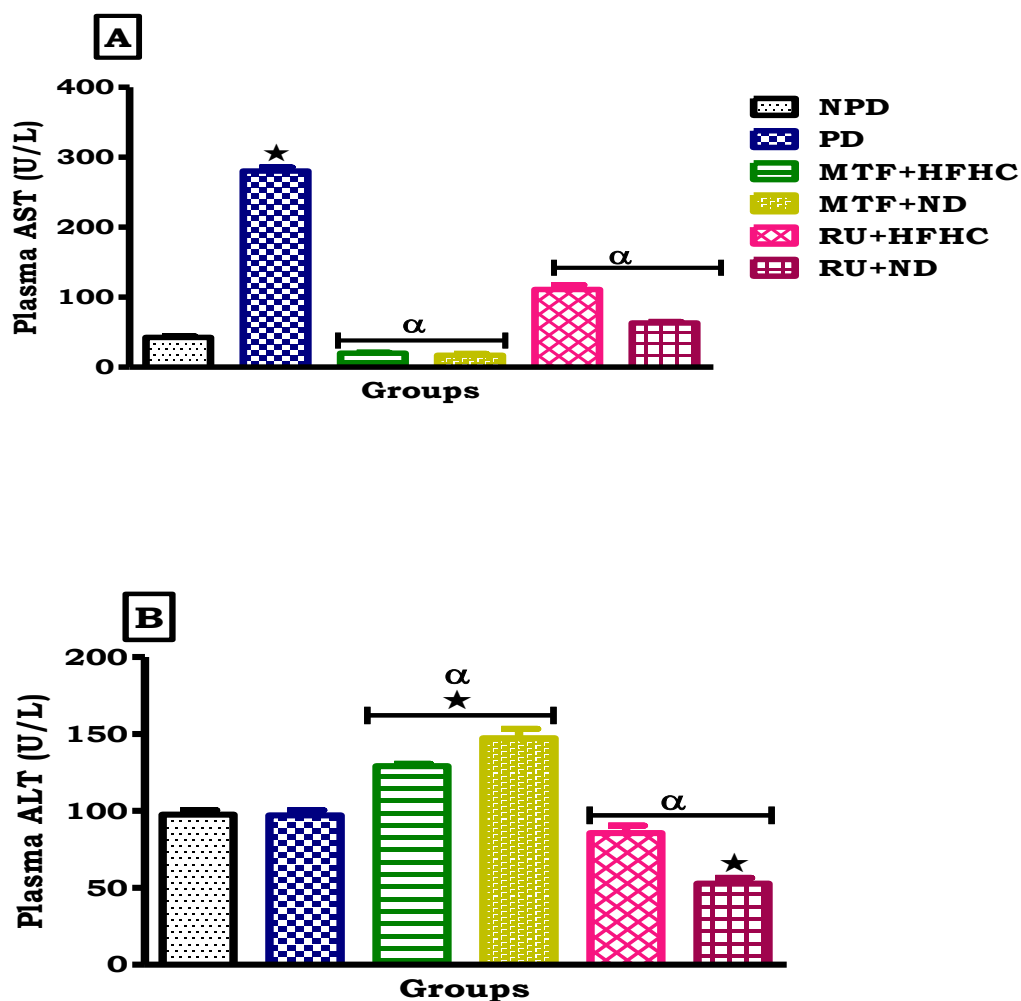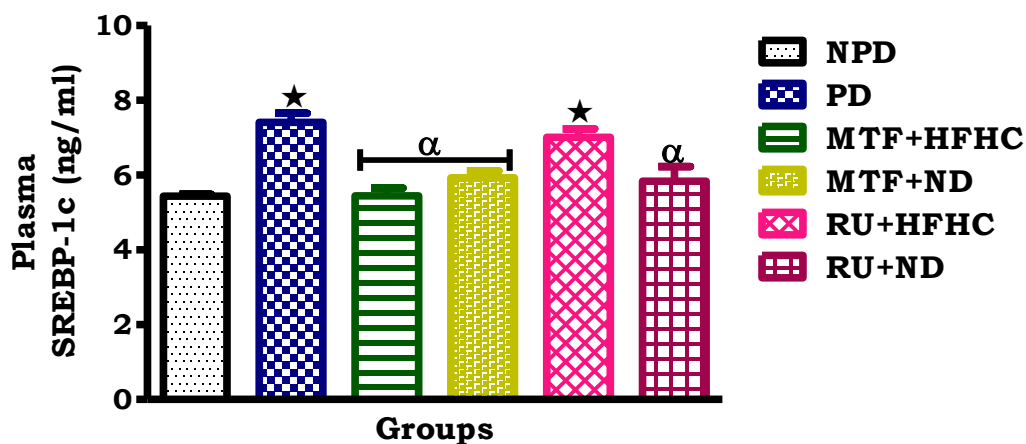
Fig. 3. The effects of the ruthenium(II) complex on SREBP-1c concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM (n = 6) in each group. **∗** $p < 0.05$ compared to non-prediabetic (NPD), **α** $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

3.6 Histology of the liver

Liver histology analysis revealed that there was increased lipid droplet (LD) accumulation, hepatocytes ballooning (H) and lobular disarray in PD group animals (Fig. 4(B)) as compared to NPD animals (Fig. 4(A)). The administration of ruthenium compound to the pre-diabetic animals (Fig. 4(E)-(F)) remarkably reduced lipid accumulation and restored hepatocytes size and shape in the pre-diabetic metal complex-treated animals as compared to PD control animals (Fig. 4(B)). Results showed that there was no remarkable difference between RU+HFHC and RU+ND treated animals in liver histological sections (Fig. 4(E)-(F)). In addition, the metformin-treated animals showed similar observation (Fig. 4(C)-(D)).
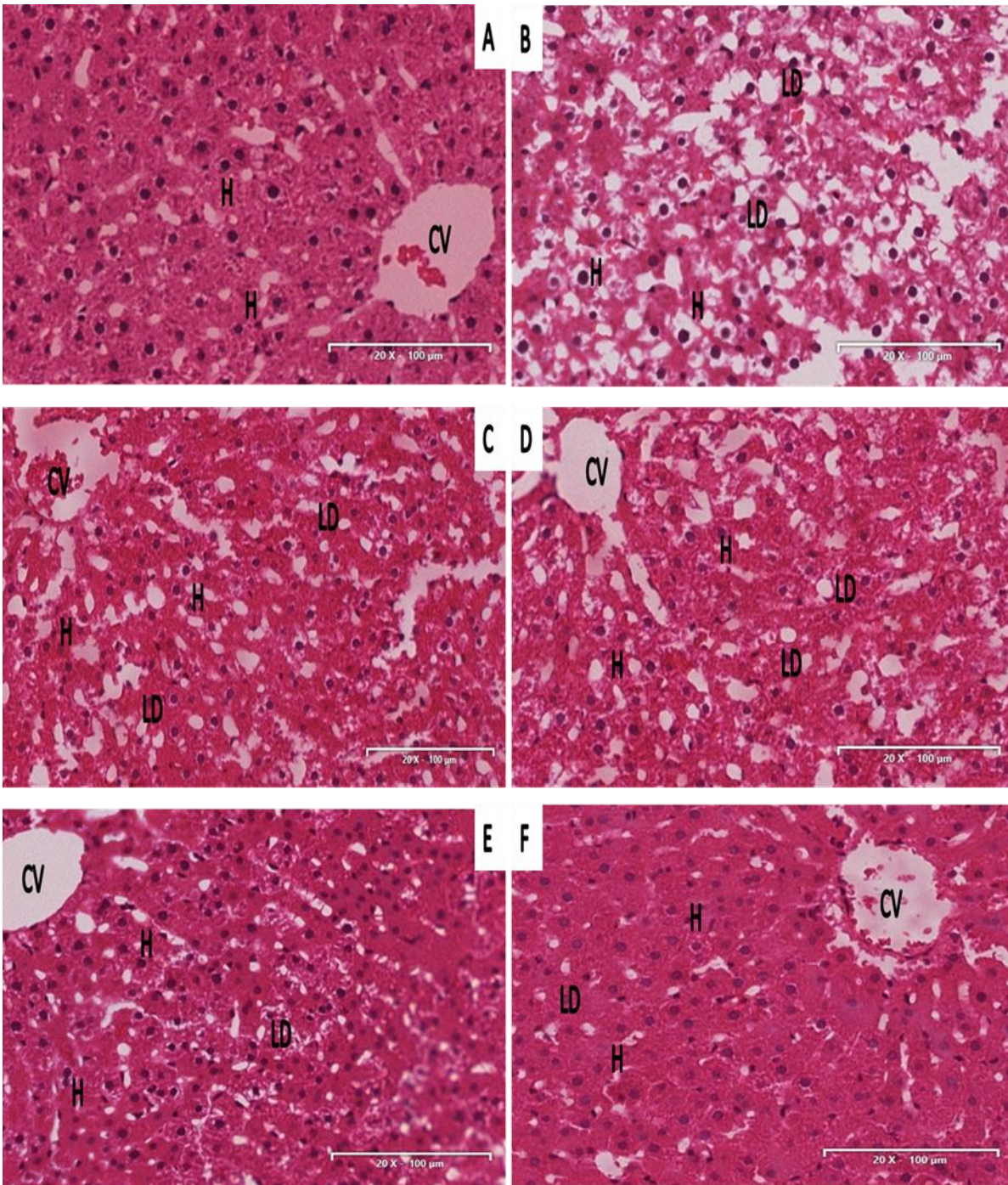
Fig. 4: The effects of the ruthenium(II) complex on liver histopathological analyses (A) NPD group, (B) PD group, (C) MFT+HFHC group, (D) MFT+ND group, (E) RU+HFHC group and (F) RU+ND group of pre-diabetes treated animals during the treatment period (H&E) stain. Magnification 20X (100 µm). CV=Central vein, H=Hepatocyte, LD=Lipid droplets.

4. Discussion

In the current study, we investigated the effects of a ruthenium(II) Schiff base complex on diabetes-related hepatic complications in the presence and absence of dietary intervention. The liver plays a key role in regulating glucose metabolism and becomes compromised during derangements, such as NAFLD and pre-diabetes. [28-30] The results obtained in this study showed a significant increase in hepatic glycogen content in the PD group by comparison with the NPD control group. Studies show that the increased glycogen content in obese and pre-diabetic patients is associated with decreased peripheral insulin sensitivity. [31, 32] The administration of the ruthenium(II) complex in conjunction with dietary intervention showed effective effects in reducing hepatic glycogen concentration in the pre-diabetic treated animals. Although, we have not come up with a conclusive mechanism for this observation. At this point we can only speculate. We speculate that the observed results on hepatic glycogen concentration could, in part, be mediated via dietary carbohydrates. The decrease in dietary carbohydrates accompanied with improved peripheral insulin sensitivity, decreased hepatic glycogen concentration. [23] Excessive accumulation of glycogen and fatty acids in the hepatocytes have been reported to lead to hepatomegaly with steatosis being the major causative factor in diabetic patients. [33] This study has shown that the administration of this ruthenium(II) complex in both the presence and absence of dietary intervention ameliorated body weight gain in pre-diabetic rats. A reduction in body weight of as low as 8% is associated with reduced steatosis and improved insulin tolerance. [30] The results of the present study further revealed that administration of the ruthenium(II) complex resulted in a significant decrease in the liver weights as compared to pre-diabetic group. Ruthenium(II) Schiff base complex has been shown to ameliorate caloric intake through the reduction of plasma ghrelin levels in diet-induced pre-diabetes rats. [23] Furthermore, several studies suggest that increased liver weights in prediabetes and NAFLD are associated with increased hepatic lipid accumulation. [8-11] Indeed, histological analysis showed that there was increased hepatic lipid droplet accumulation in the untreated prediabetic rats. However, the administration of the ruthenium(II) complex in both the presence and absence of dietary intervention resulted in a decrease in lipid accumulation. Taken together, these results could suggest that the ability of this compound to restore insulin sensitivity in the pre-diabetic state restores body weights while reducing hepatic accumulation of glycogen and fats. This would then result in a reduction in liver weights and the prevention of hepatomegaly which is associated with NAFLD. [34]

Plasma AST and ALT are enzyme biomarkers used to monitor the liver's structural integrity and aids in the clinical diagnosis of NAFLD and other liver toxicity conditions. [35] Generally, high caloric diets increase plasma concentration of these enzymes through the induction of oxidative stress in the liver. [35] Indeed, the results of the present study showed that

the PD group had significantly increased plasma levels of AST as compared to NPD control group. These results suggest that diet-induced pre-diabetes can has deleterious effects on the liver due to production of free radicals and reactive oxygen species (ROS) which further trigger the inflammatory response mechanism. [36] On the other hand, there was no significant change in plasma ALT levels in the PD group when compared to the NPD group. However, due to the age of the rats, which were 36 weeks old at the end of the experiment, we speculate that the observed result on ALT level may be due to age. Literature evidence has shown that oxidative stress increases with age which then trigger the inflammatory response mechanism and reduces cellular antioxidant capacity, thus leading to mutation and DNA damage that can be a predisposing factor to impair liver function. [37, 38] Furthermore, the ND has a considerable amount of carbohydrates. Ingested carbohydrates are a major stimulus for hepatic de novo lipogenesis and more likely to directly contribute to NAFLD and impair liver function enzymes than dietary fat intake. [39] However, we do acknowledge that further mechanisms are needed to elucidate the increase ALT levels in the non-prediabetic rats. The administration of metal complexes is often associated with increased plasma AST and ALT levels suggesting liver toxicity. [40] Pharmacotherapy for diabetes-related liver disorders is often combined with dietary intervention which involves the consumption of low-caloric diets. [30] However, there is reported low patient compliance in terms of dietary intervention as patients tend to heavily rely on the pharmacological treatments and thus reduce the efficacy of the drugs. [41]

However, the administration of the ruthenium(II) complex, in both the absence and presence of dietary intervention led to decreased plasma AST and ALT levels in the pre-diabetic treated rats. In addition, treatment with the metal compound with dietary intervention displayed more effective results in terms of lowering ALT levels in pre-diabetic rats. A recent study by Hsia *et al.,* has demonstrated the anti-inflammatory properties of a novel ruthenium compound, via inhibiting inflammatory mediators (nitric oxide and nitric oxide synthase) and pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1β)in RAW 264.7 cells. [22] Additionally, the metal compound also displayed protective effect against Lipopolysaccharide-induced liver injury in mice, thus protecting progressive liver damage. [22] Moreover, the findings of the study could suggest that the integration of the Schiff base ligand within the coordination sphere of this ruthenium(II) complex could possibly protect the liver from toxic and proinflammatory effects as metal compounds complexed with organic ligands have been found to be less toxic. [25] Notably, the metformin-treated pre-diabetic animals showed decrease in AST levels in both the presence and absence of dietary intervention. In contrast, there was an increase in plasma ALT levels in metformin-treated animals as compared to both NPD and PD control groups. Metformin has been reported to improve liver injury but could not prevent fibrosis in patients with steatosis. [42] As metformin is not metabolized *via* the

hepatic CYP450 system, its pharmacokinetic characteristics do not expose patients to drug–drug interactions. [43] However, there have only been a few reported cases of the hepatotoxic side effects of metformin, but there may be an increased risk of developing lactic acidosis in the setting of diabetes-induced impaired liver function. [43-45] Therefore, the findings of the present study suggest that metformin treatment is not therapeutic in terms of reducing ALT levels in prediabetes related NAFLD complications. However, the relationship between these liver enzymes and pre-diabetes may be more complex than generally appreciated. Future studies are warranted to help provide insight into the nature of these processes and determine the joint role of these liver enzymes in the pathophysiology of pre-diabetes.

Studies have reported that continuously low bilirubin levels are strongly correlated to the risk of development of diabetes-related cardiovascular complications. [16, 46, 47] Additionally, bilirubin has been reported to be inversely associated with NAFLD and steatohepatitis. [48] In this study, the induction of prediabetes resulted in a significant reduction of plasma total bilirubin concentration. However, the administration of the ruthenium complex resulted in a significant increase in plasma bilirubin levels . We speculate that these findings may be due to the metal complex activating several key reactions, in particular those catalyzed by heme oxygenase 1, biliverdin reductase and UDP- glucuronosyltransferase enzymes, which play an important role in bilirubin homeostasis. [47, 49] While we have not investigated these reactions in this study, this metal compound has been shown to have cardio-protective properties in diet-induced pre-diabetes rats and we speculate that these effects could be, in part, attributed to its effects on the synthesis of bilirubin in the liver. [24] Recent studies have identified bilirubin to be a major contributor to the total antioxidant capacity in blood. [47] Indeed, ruthenium(II) Schiff base complex has been shown to possess antioxidant ability as it reduced tissue lipid peroxidation and increased the concentration of antioxidant markers such as glutathione peroxidase and superoxide dismutase. [24] Interestingly, metformin-treated animals also showed increased plasma total bilirubin when compared with both the NPD and the PD control groups further explaining the reported cardio-protective effects of this compound.

Studies show that insulin resistance plays a key role in hepatic lipid accumulation and the subsequent increase of adipose tissue lipolysis. [50] The resultant fatty acid accumulation disturbs the β-oxidation system in the hepatic mitochondria and leads to further infiltration of fats in the liver. [50] In the liver, the molecular basis for this mechanism was shown to involve SREBP-1c, an important lipogenic transcription factor. [4, 12, 13] In the present study, the plasma level of SREBP-1c in the PD control animals was significantly higher than that in the NPD control animals. The administration of the ruthenium(II) complex coupled with dietary intervention tended to reduce plasma SREBP-1c levels in pre-diabetic treated animals.

Suggesting a potent effect of the metal in conjunction with dietary modification. Numerous Schiff base ruthenium(II) complexes have been isolated and their structural diversity have culminated into a wide spectrum of structure-activity of relationships. [25, 51] For instance, the stereo-electronic features of the organic chelating ligands have been closely correlated to DNA binding studies and cytotoxicity while the redox properties of the metal centre typically dictate their radical scavenging capabilities. [25, 51, 52] We speculate that the insulin-sensitizing effect of this compound may relate to the decreased SREBP-1c levels in ruthenium-treatment group. It might be, therefore speculated that administration of the ruthenium(II) complex in the presence of dietary intervention ameliorated the lipid accumulation in liver. Furthermore, decrease SREBP-1c levels were observed in the metformin-treated pre-diabetic rats. Metformin has been shown to activate AMPK phosphorylation which inhibited SREBP-1c activity and attenuated hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. [53] Indeed, histological analysis showed that the administration of the ruthenium(II) Schiff base complex resulted in reduced hepatic lipid droplet accumulation as well as reduced hepatocyte ballooning and lobular disarray by comparison to the PD group, with the metformin-treated pre-diabetic rats with similar observation. This possibly indicates that this compound restored the balance between hepatic lipid storage and removal thus resulting in a reduction in triacylglycerol accumulation. [50] Although more studies are needed to elucidate the mechanism by which this occurs, these results further suggest the ability of this compound to protect the liver against NAFLD.

5. Conclusion

The results of this study suggest that the administration of the ruthenium(II) Schiff base complex normalized the concentration of liver damage biomarker enzymes, significantly reduced hepatic lipid accumulation, decreased SREBP-1c plasma levels and prevented the onset of hepatomegaly observed in pre-diabetic animals, although the underlying mechanisms are yet to be determined. This warrants further studies into this ruthenium(II) Schiff base complex as a potential therapeutic alternative for pre-diabetes associated NAFLD.

ALT	Alanine transaminase
AST	Aspartate transaminase
CV	Central vein
FBG	Fasting blood glucose
FFA	Free fatty acid
H	Hepatocyte
H&E	Haematoxylin and eosin
HFHC	High fat high carbohydrate
LD	Lipid droplets
MTF	Metformin
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
ND	Normal diet
NPD	Non-prediabetic
OGTT	Oral glucose tolerance test
PD	Pre-diabetes
ROS	Reactive oxygen species
RU	Ruthenium
SREBP-1c	Steroid regulatory elementary binding protein-1c

Authors' contributions: LPM and MWG were involved in study design, *in vivo* studies, data analysis and assisted in preparing the manuscript. SM and INB synthesised and spectroscopically characterized the free-ligand and its ruthenium complex which was utilised as the metal-based drug. INB proof-read the manuscript. AK and PSN were involved in conceptualization and design of the study, execution of animal studies, data analysis, provided funding and assisted in writing the manuscript. All authors have read and approved the manuscript.

Conflicts of Interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial in the subject matter or materials discussed in this manuscript.

References

1. Hamelet J, Demuth K, Paul J, Delabar J, Janel N. Hyperhomocysteinemia due to cystathionine beta synthase deficiency induces dysregulation of genes involved in hepatic lipid homeostasis in mice. *Journal of hepatology*. 2007;46(1):151-159.

2. Loria P, Lonardo A, Anania F. Liver and diabetes. A vicious circle. *Hepatology research*. 2013;43(1):51-64.

3. Guven A, Yavuz O, Cam M, Ercan F, Bukan N, Comunoglu C*, et al.* Effects of melatonin on streptozotocin-induced diabetic liver injury in rats. *Acta histochemica*. 2006;108(2):85-93.

4. Mavrogiannaki A, Migdalis I. Nonalcoholic Fatty liver disease, diabetes mellitus and cardiovascular disease: newer data. *International Journal of Endocrinology*. 2013;2013:450639.

5. Larter CZ, Farrell GC. Insulin resistance, adiponectin, cytokines in NASH: which is the best target to treat? *Journal of hepatology*. 2006;44(2):253-261.

6. Leclercq I, Morais A, Schroyen B, Van Hul N, Geerts A. Insulin resistance in hepatocytes and sinusoidal liver cells: mechanisms and consequences. *Journal of hepatology*. 2007;47(1):142-156.

7. Bugianesi E, McCullough A, Marchesini G. Insulin resistance: a metabolic pathway to chronic liver disease. *Hepatology*. 2005;42(5):987-1000.

8. Manna P, Das J, Ghosh J, Sil P. Contribution of type 1 diabetes to rat liver dysfunction and cellular damage via activation of NOS, PARP, IκBα/NF-κB, MAPKs, and mitochondria-dependent pathways: Prophylactic role of arjunolic acid. *Free Radical Biology and Medicine*. 2010;48(11):1465-1484.

9. Palsamy P, Sivakumar S, Subramanian S. Resveratrol attenuates hyperglycemia-mediated oxidative stress, proinflammatory cytokines and protects hepatocytes ultrastructure in streptozotocin–nicotinamide-induced experimental diabetic rats. *Chemico-biological interactions*. 2010;186(2):200-210.

10. Stefan N, Sun Q, Fritsche A, Machann J, Schick F, Gerst F*, et al.* Impact of the adipokine adiponectin and the hepatokine fetuin-A on the development of type 2 diabetes: prospective cohort-and cross-sectional phenotyping studies. *PloS one*. 2014;9(3):e92238.

11. Iroz A, Couty J, Postic C. Hepatokines: unlocking the multi-organ network in metabolic diseases. *Diabetologia*. 2015;58(8):1699-1703.

12. Regnell S, Lernmark A. Hepatic steatosis in type 1 diabetes. *The review of diabetic studies: RDS*. 2011;8(4):454.

13. Moscatiello S, Manini R, Marchesini G. Diabetes and liver disease: an ominous association. *Nutrition, Metabolism and Cardiovascular Diseases*. 2007;17(1):63-70.

14. Nannipieri M, Gonzales C, Baldi S, Posadas R, Williams K, Haffner S*, et al.* Liver enzymes, the metabolic syndrome, and incident diabetes: the Mexico City diabetes study. *Diabetes care*. 2005;28(7):1757-1762.

15. Fujii M, Inoguchi T, Sasaki S, Maeda Y, Zheng J, Kobayashi K*, et al.* Bilirubin and biliverdin protect rodents against diabetic nephropathy by downregulating NAD (P) H oxidase. *Kidney international*. 2010;78(9):905-919.

16. Fukui M, Tanaka M, Yamazaki M, Hasegawa G, Nishimura M, Iwamoto N*, et al.* Low serum bilirubin concentration in haemodialysis patients with Type 2 diabetes. *Diabetic Medicine*. 2011;28(1):96-99.

17. Hamden K, Carreau S, Boujbiha M, Lajmi S, Aloulou D, Kchaou D*, et al.* Hyperglycaemia, stress oxidant, liver dysfunction and histological changes in diabetic male rat pancreas and liver: Protective effect of 17β-estradiol. *Steroids*. 2008;73(5):495-501.

18. Lucchesi A, Cassettari L, Spadella C. Alloxan-induced diabetes causes morphological and ultrastructural changes in rat liver that resemble the natural history of chronic fatty liver disease in humans. *Journal of diabetes research*. 2015;2015:494578.

19. DeFronzo R, Eldor R, Abdul-Ghani M. Pathophysiologic approach to therapy in patients with newly diagnosed type 2 diabetes. *Diabetes care*. 2013;36(Supplement 2):127-138.

20. Hamad E, Taha S, Dawood A, Sitohy M, Abdel-Hamid M. Protective effect of whey proteins against nonalcoholic fatty liver in rats. *Lipids in health and disease*. 2011;10(1):57.

21. Mohamed J, Nafizah A, Zariyantey A, Budin S. Mechanisms of diabetes-induced liver damage: the role of oxidative stress and inflammation. *Sultan Qaboos University Medical Journal*. 2016;16(2):132–141.

22. Hsia C, Velusamy M, Jayakumar T, Chen Y, Hsia C, Tsai J*, et al.* Mechanisms of TQ-6, a Novel Ruthenium-Derivative Compound, against Lipopolysaccharide-Induced In Vitro Macrophage Activation and Liver Injury in Experimental Mice: The Crucial Role of p38 MAPK and NF-κB Signaling. *Cells*. 2018;7(11):217.

23. Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P, Khathi A. Effects of a Ruthenium Schiff Base Complex on Glucose Homeostasis in Diet-Induced Pre-Diabetic Rats. *Molecules (Basel, Switzerland)*. 2018;23(7):1721.

24. Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P, Khathi A. Cardioprotective effects of a ruthenium (II) Schiff base complex in a diet-induced prediabetic rats. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 2019;12:1-7.

25. Booysen I, Maikoo S, Akerman M, Xulu B. Novel ruthenium (II) and (III) compounds with multidentate Schiff base chelates bearing biologically significant moieties. *Polyhedron*. 2014;79:250-257.

26. Luvuno M, Mabandla M, Khathi A. Voluntary ingestion of a high-fat high-carbohydrate diet: A model for prediabetes. *Ponte international scientific researchs journal*. 2018;74.

27. Mzimela N, Ngubane P, Khathi A. The changes in immune cell concentration during the progression of pre-diabetes to type 2 diabetes in a high-fat high-carbohydrate diet-induced pre-diabetic rat model. *Autoimmunity*. 2019;52(1):27-36.

28. Hsieh P, Hsieh Y. Impact of liver diseases on the development of type 2 diabetes mellitus. *World journal of gastroenterology: WJG*. 2011;17(48):5240.

29. Speliotes E, Massaro J, Hoffmann U, Vasan R, Meigs J, Sahani D, *et al.* Fatty liver is associated with dyslipidemia and dysglycemia independent of visceral fat: the Framingham Heart Study. *Hepatology*. 2010;51(6):1979-1987.

30. Hamed A, Abas B, Shaltout I, Esmt G, Gomez R. Managing diabetes and liver disease association, guidelines (consensus) development. *Journal of Endocrinology Diabetes Obesesity*. 2015;3:1-19.

31. Ferrer J, Favre C, Gomis R, Fernández-Novell J, García-Rocha, de la Iglesia N, *et al.* Control of glycogen deposition. *FEBS letters*. 2003;546(1):127-132.

32. Allick G, Sprangers F, Weverling G, Ackermans M, Meijer A, Romijn J, *et al.* Free fatty acids increase hepatic glycogen content in obese males. *Metabolism*. 2004;53(7):886-893.

33. Messeri S, Messerini L, Vizzutti F, Laffi G, Marra F. Glycogenic hepatopathy associated with type 1 diabetes mellitus as a cause of recurrent liver damage. *Annals of hepatology*. 2012;11(4):554-558.

34. Wani F, Albahrawy A, Rahiman S. Hypolipidemic activity of olive oil (olea europaea) against high fat diet-induced nonalcoholic fatty liver disease (NAFLD) in mice. *Open Journal of Pathology*. 2015;5(03):73.

35. Amin A, Hamza A. Oxidative stress mediates drug-induced hepatotoxicity in rats: a possible role of DNA fragmentation. *Toxicology*. 2005;208(3):367-375.

36. Donnelly K, Smith C, Schwarzenberg S, Jessurun J, Boldt M, Parks E. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *The Journal of clinical investigation*. 2005;115(5):1343-1351.

37. Hamden K, Carreau S, Ayadi F, Masmoudi H, Abdelfattah E. Inhibitory effect of estrogens, phytoestrogens, and caloric restriction on oxidative stress and hepato-toxicity in aged rats. *Biomedical and Environmental Sciences*. 2009;22(5):381-387.

38. Kim H, Kisseleva T, Brenner D. Aging and liver disease. *Current opinion in gastroenterology*. 2015;31(3):184.

39. Basaranoglu M, Basaranoglu G, Bugianesi E. Carbohydrate intake and nonalcoholic fatty liver disease: fructose as a weapon of mass destruction. *Hepatobiliary surgery and nutrition*. 2015;4(2):109.

40. Jan A, Azam M, Siddiqui K, Ali A, Choi I, Haq Q. Heavy metals and human health: mechanistic insight into toxicity and counter defense system of antioxidants. *International journal of molecular sciences*. 2015;16(12):29592-29630.

41. López-Miranda J, Pérez-Jiménez F, Ros E, De Caterina R, Badimón L, Covas M, *et al.* Olive oil and health: summary of the II international conference on olive oil and health consensus report, Jaén and Córdoba (Spain) 2008. *Nutrition, metabolism and cardiovascular diseases*. 2010;20(4):284-294.

42. Loomba R, Lutchman G, Kleiner D, Ricks M, Feld J, Borg B, *et al.* Clinical trial: pilot study of metformin for the treatment of non-alcoholic steatohepatitis. *Alimentary pharmacology & therapeutics*. 2009;29(2):172-182.

43. Holstein A, Beil W. Oral antidiabetic drug metabolism: pharmacogenomics and drug interactions. *Expert opinion on drug metabolism & toxicology*. 2009;5(3):225-241.

44. Kutoh E. Possible metformin-induced hepatotoxicity. *The American journal of geriatric pharmacotherapy*. 2005;3(4):270-273.

45. Saadi T, Waterman M, Yassin H, Baruch Y. Metformin-induced mixed hepatocellular and cholestatic hepatic injury: case report and literature review. *International journal of general medicine*. 2013;6:703.

46. Bao W, Song F, Li X, Rong S, Yang W, Zhang M, *et al.* Plasma heme oxygenase-1 concentration is elevated in individuals with type 2 diabetes mellitus. *PLoS One*. 2010;5(8):e12371.

47. Vítek L. The role of bilirubin in diabetes, metabolic syndrome, and cardiovascular diseases. *Frontiers in pharmacology*. 2012;3:55.

48. Kwak M, Kim D, Chung G, Kang S, Park M, Kim Y, *et al.* Serum bilirubin levels are inversely associated with nonalcoholic fatty liver disease. *Clinical and molecular hepatology*. 2012;18(4):383.

49. Zhou M, Wang A, Yu H. Link between insulin resistance and hypertension: What is the evidence from evolutionary biology? *Diabetology & metabolic syndrome*. 2014;6(1):12.

50. Bugianesi E, Moscatiello S, Ciaravella M, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. *Current pharmaceutical design*. 2010;16(17):1941-1951.

51. Booysen I, Adebisi A, Akerman M. Formation, electrochemical and radical scavenging properties of novel ruthenium compounds with N, X-donor (X= O, N) heterocyclic chelators. *Inorganica Chimica Acta*. 2015;433:13-20.

52. Raja G, Butcher R, Jayabalakrishnan C. Studies on synthesis, characterization, DNA interaction and cytotoxicity of ruthenium (II) Schiff base complexes. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. 2012;94:210-215.

53. Li Y, Xu S, Mihaylova M, Zheng B, Hou X, Jiang B, *et al.* AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell metabolism*. 2011;13(4):376-388.

Tables

Table 1: Effects of the ruthenium(II) complex on liver glycogen concentration of pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means ± SEM (n=6) in each group.

Groups	Hepatic Glycogen (nmol/g protein)
NPD	1.88±0.40
PD	2.25±0.51*
MTF+HFHC	2.38±1.12*
MTF+ND	1.41±0.12α
RU+HFHC	2.18±0.72*
RU+ND	1.62±0.23α

* $p < 0.05$ compared to non-prediabetic (NPD), **α** $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

Table 2: The influences of the ruthenium(II) complex on liver and body weight of pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means ± SEM (n=6) in each group.

Groups	Initial body weight (g)	Final body weight (g)	Liver weight (g)
NPD	173±1.45	384±6.30	11.53±0.14
PD	176±1.73	680±8.08*	24.33±1.23*
MTF+HFHC	172±1.22	501±5.06*α	17.34±0.86*α
MTF+ND	177±0.98	443±3.90α	13.35±1.37α
RU+HFHC	172±1.45	490±5.09*α	16.23±0.65*α
RU+ND	175±1.67	435±2.61α	13.20±0.91α

* $p < 0.05$ compared to non-prediabetic (NPD), **α** $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

Figure legends

Fig. 1. The effects of the ruthenium(II) complex on (A) AST and (B) ALT concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM (n = 6) in each group. * $p < 0.05$ compared to non-prediabetic (NPD), α $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

Fig. 2. The effects of the ruthenium(II) complex on total bilirubin concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM (n = 6) in each group. ***** $p < 0.05$ compared to non-prediabetic (NPD), **α** $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

Fig. 3. The effects of the ruthenium(II) complex on SREBP-1c concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM (n = 6) in each group. ***** $p < 0.05$ compared to non-prediabetic (NPD), **α** $p < 0.05$ compared to pre-diabetes (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium(II) complex and high fat high carbohydrate (RU+HFHC); Ruthenium(II) complex and normal diet (RU+ND).

Fig. 4: The effects of the ruthenium(II) complex on liver histopathological analyses (A) NPD group, (B) PD group, (C) MFT+HFHC group, (D) MFT+ND group, (E) RU+HFHC group and (F) RU+ND group of pre-diabetes treated animals during the treatment period (H&E) stain. Magnification 20X (100 μm). CV=Central vein, H=Hepatocyte, LD=Lipid droplets.

Figures
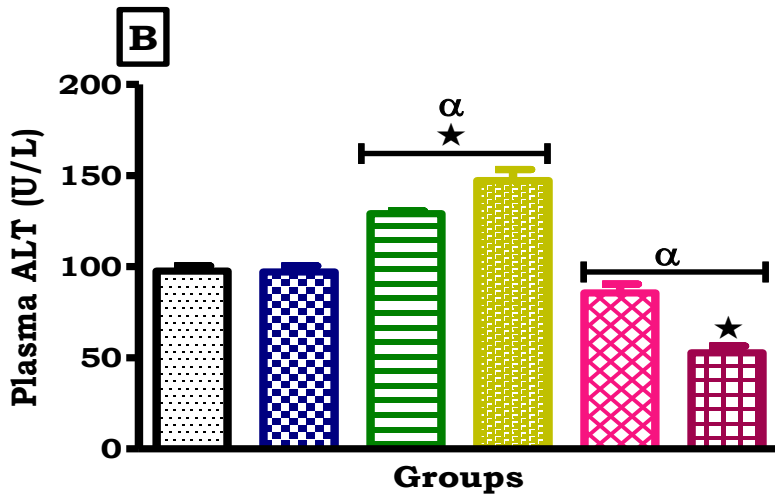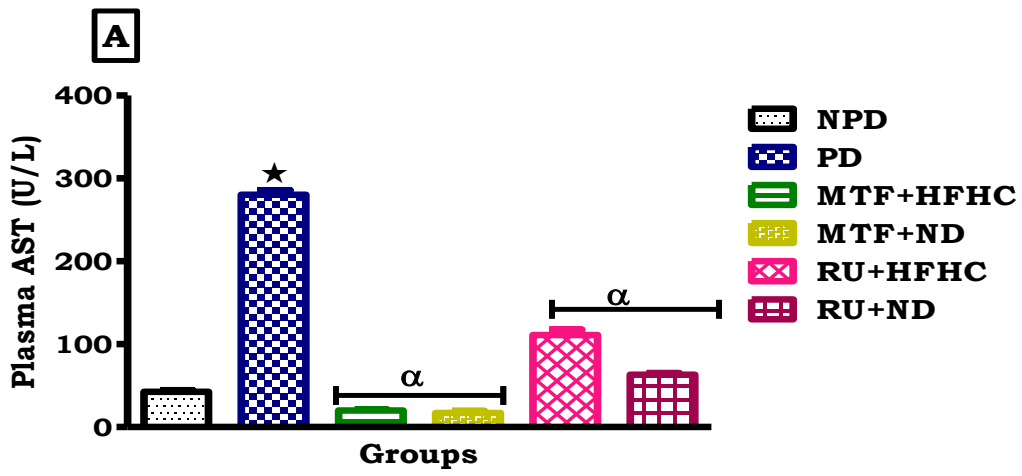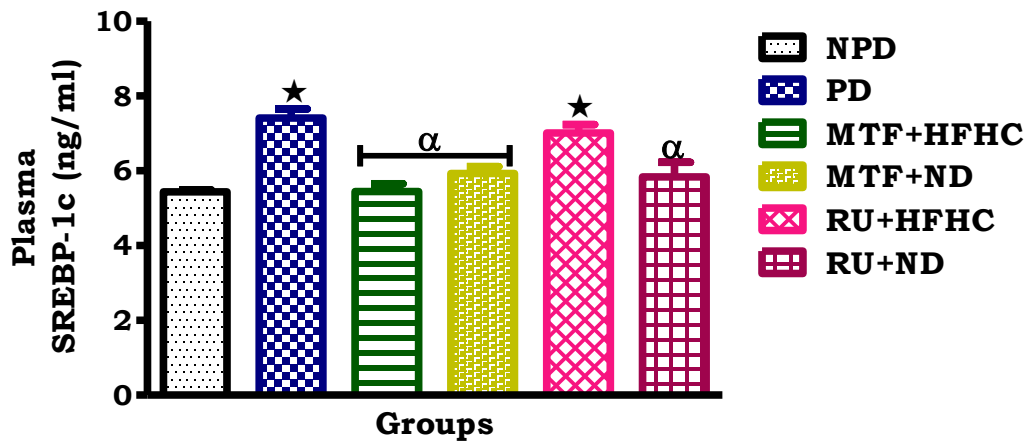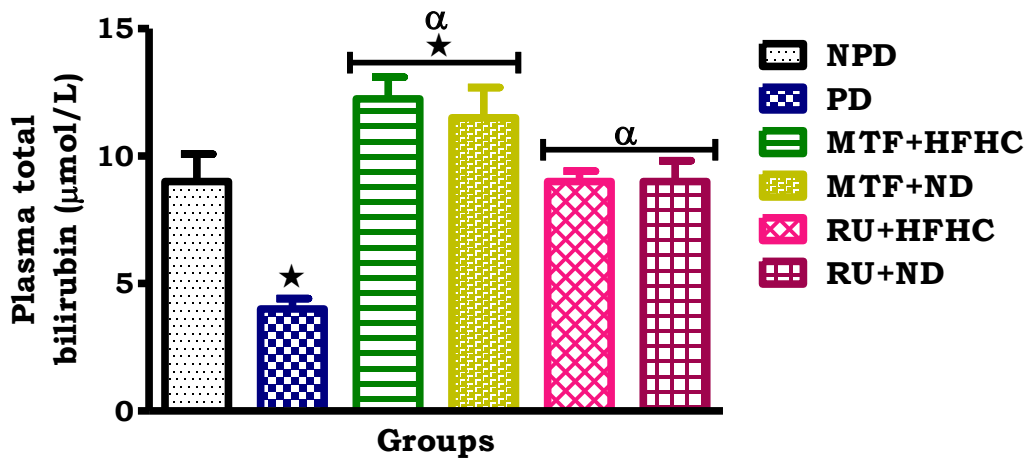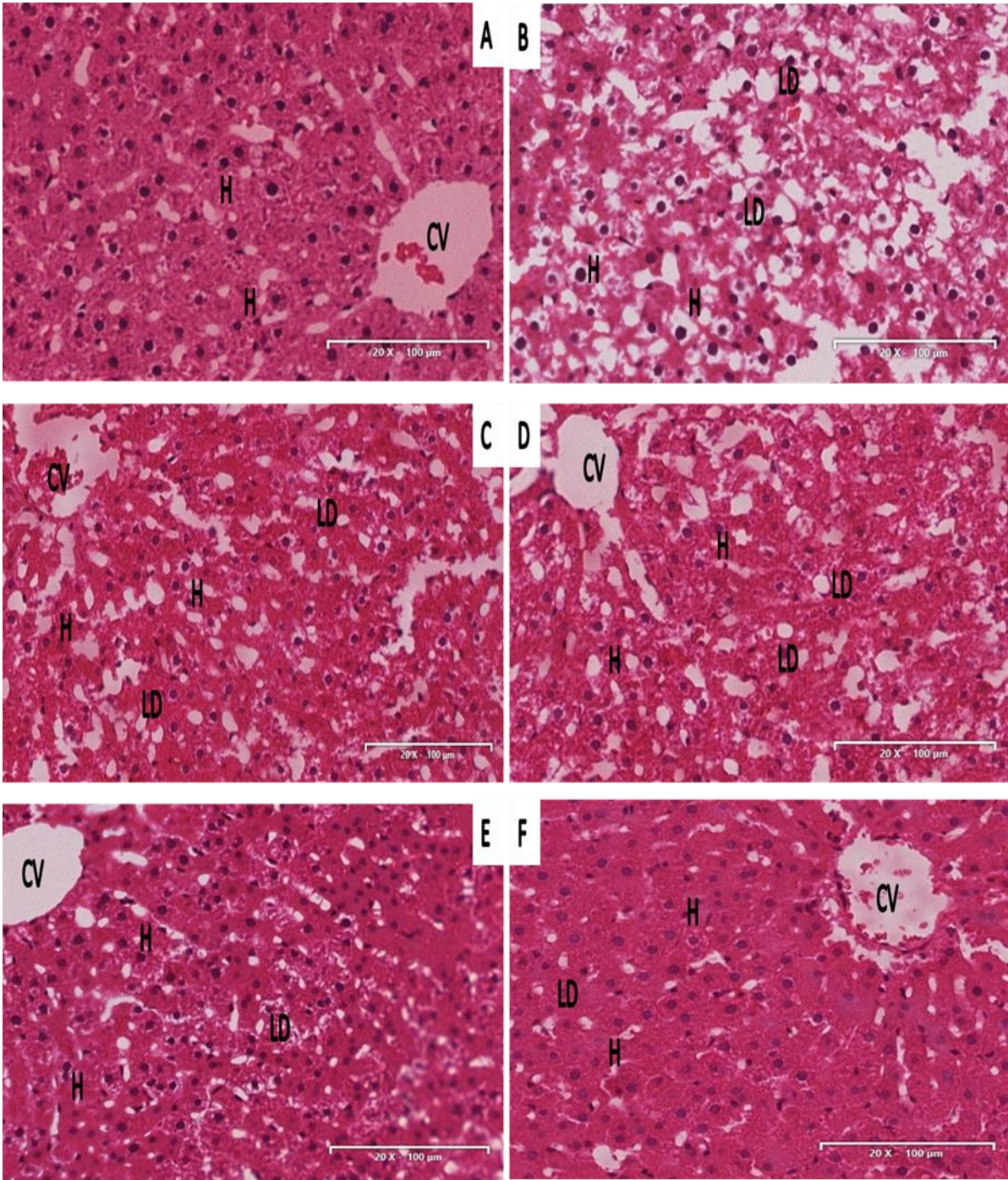
CHAPTER 3

Prologue 2: Manuscript 2

"Amelioration of risk factors associated with diabetic nephropathy in diet-induced pre-diabetic rats by an uracil-derived diimine ruthenium(II) compound"

There is a strong link between hyperglycaemia and the progression of kidney injury. Pre-diabetes is a risk factor for chronic kidney disease and newly diagnosed pre-diabetic patients already have kidney damage. However, whether this prospective association is attributable to the effects of pre-diabetes itself, increased incidence of diabetes, or prediabetic-related complications that contribute to both hyperglycaemia and kidney pathology is unclear. We have previously reported that treatment with ruthenium(II) complex improved glycaemic control as evidenced by observed decreased in HbA1c concentration and decreased body weights, whilst in conjunction with dietary intervention there was reduced plasma ghrelin concentration in the ruthenium(II)-treated rats. In the previous manuscript, we further showed that the administration of this compound resulted in reduced liver weights as well. However, the effects of this compound on kidney-related ROS, impaired electrolytes balance, renal dysfunction biomarkers, mRNA of podocin expression and histopathology analysis of the kidney remain unclear. Hence, manuscript 2, investigated the effects of a ruthenium(II) uracil-derived diimine complex on risk factors associated with DN in a diet-induced pre-diabetic rat model in both the presence and absence of dietary intervention.

The current manuscript has been submitted for publication in the journal "**Nutrients"** and formatted according to journal's guidelines to authors (Appendix 8).

This work was authored by **L.P Mabuza**, **M.W Gamede**, **S Maikoo**, **IN Booysen**, **P.S Ngubane** and **A Khathi.**

Amelioration of risk factors associated with diabetic nephropathy in diet-induced pre-diabetic rats by an uracil-derived diimine ruthenium(II) compound.

Lindokuhle Patience Mabuza[1,3], Mlindeli Wilkinson Gamede[1], Sanam Maikoo[2], Irvin Noel Booysen[2], Phikelelani Siphosethu Ngubane[1] and Andile Khathi [1].

[1]School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[2]School of Chemistry and Physics, College of Engineering and Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa

[3]* Corresponding Author

Email: 211509843@stu.ukzn.ac.za, 213571877@stu.ukzn.ac.za, 208501744@stu.ukzn.ac.za, Booyseni@ukzn.ac.za, Ngubanip1@ukzn.ac.za & khathia@ukzn.ac.za

Ms LP Mabuza

School of Laboratory Medicine & Medical Sciences

University of KwaZulu-Natal

Private Bag X54001

Durban, South Africa 4000

Email: 211509843@stu.ukzn.ac.za

Abstract

Diabetic renal injury advances through different stages of structural and functional changes in the glomerulus, therefore treatment during the pre-diabetic state could be used as therapeutic target in the management and prevention of diabetic nephropathy (DN). Once diagnosed, dietary interventions and pharmacological therapy have been recommended to manage DN and pre-diabetic related complications. However, poor patient compliance still results, therefore newer alternative drugs are required. High fat high carbohydrates (HFHC) diet was used to induce pre-diabetes for 20 weeks. After the induction, pre-diabetic rats were randomly allocated to respective treatment groups. Subcutaneous ruthenium(II) Schiff base complex injection (15 mg/kg) was administered to pre-diabetic rats in both the presence and absence of dietary intervention once a day every third day for 12 weeks. The administration of ruthenium(II) complex resulted in reduced blood glucose, aldosterone, fluid intake and urinary output which correlated with a restoration in plasma and urinary electrolytes along with plasma antioxidants concentration. Furthermore, there was a decrease in kidney injury molecule-1 (KIM-1) concentration, albumin excretion rate (AER) albumin creatinine ratio (ACR) and mRNA expression of podocin in urine in ruthenium-treated pre-diabetic rats. In addition, ruthenium-treated rats showed improved histological structure of renal glomerulus. Ruthenium(II) Schiff base complex ameliorated renal function while preventing the progression of DN in prediabetic-treated rats.

Key words: antioxidants, diabetic nephropathy, dietary intervention, pre-diabetes, ruthenium(II) Schiff base complex

1. Introduction

Several studies have reported that up to one-third of adults with newly diagnosed diabetes mellitus already have kidney damage [1, 2]. These research findings suggest that the onset of diabetic nephropathy (DN) may occur in the early stages of the disease progression [1]. Hyperglycaemia evokes the onset and progression of DN because of its role in causing hemodynamic dysregulation along with abnormal morphological and functional nephron changes, ranging from increased glomerular basement membrane (GBM) thickness, mesangial expansion, extracellular matrix deposition, glomerulosclerosis, overt proteinuria, and decreased glomerular function as well as filtration rate to eventual end-stage renal damage [3, 4]. A significant event that precedes renal injury is an increase in permeability of plasma proteins such as albumin through damage *via* the glomerular filtration barrier [3]. This leads to excessive urinary albumin excretion (UAE) through the nephron where elevated albumin excretion into urine is used as a prominent marker for diabetic kidney disease [5, 6]. Furthermore, the compromised function of the glomerular filtration barrier (GFB) typically escalates into early podocyte loss at the onset of diabetes and in the process initiates increased protein excretion in urine [7, 8]. Kidney injury molecule-1 (KIM-1) is another biomarker for renal proximal tubule injury and has been showed to be substantial in urine samples of diabetic patients [9, 10]. Prediabetes, which is a long lasting condition that precedes the onset of type 2 diabetes (T2DM), is linked to the onset and progression of DN through inappropriate activation of the renin–angiotensin system (RAS), elevated levels of proinflammatory cytokines and oxidative stress in the kidney [11, 12]. Subsequent pathology causes aldosterone excess which emantes in sodium and water retention leading to elevated blood pressure [11, 13, 14]. Thus, beginning treatment during the prediabetic state could be used as therapeutic target in the management and prevention of DN as well as the associated complications. A combination of pharmacological and dietary intervention are recommended for the management of DN progression to result in increased peripheral insulin sensitivity and thus reduce the amount of glucose perfusing the kidney [15, 16]. Our recent studies reported that a ruthenium(II) complex with a diimine uracil chelating ligand exhibited optimal anti-diabetic properties through improvement of glycaemic control, insulin sensitivity and decreased risk of developing diabetes-related cardiovascular diseases in diet-induced pre-diabetic rats [17, 18]. However, the effects of this mononuclear metal-based complex on renal function in diet-induced pre-diabetes are yet to be investigated. Thus, the aim of this research study was to investigate the effects of this potential metallopharmaceutical on renal function in a diet-induced pre-diabetes rat model.

2. Materials and Methods

2.1 Chemicals and drugs

All chemicals and drugs were of analytical grade and purchased from standard commercial suppliers. Previous studies showed that the dose of the ruthenium complex used in this study was non-toxic [17-19].

2.2 Synthesis of ruthenium(II) Schiff base complex

The synthesis of the ruthenium(II) Schiff base complex, $[Ru^{II}(H_3ucp)Cl(PPh_3)]$ (H_4ucp = 2,6-*bis*-((6-amino-1,3-dimethyluracilimino)methylene)pyridine) was conducted according to experimental methods previously reported [20]. The purity of the metal complex was affirmed by nuclear magnetic spectroscopy and Time-Of-Flight (TOF) mass spectrometry.

2.3 Animals and housing

A sample set of 36 male Sprague-Dawley rats (2 weeks old;150-180 g) were used. These animals were housed in room with a 12 hours light/12 hours dark cycle and room temperature (25°C) for the duration of the study. The animals in each group had access to food and water *ad libitum*. All animal procedures and conditions were carried out according to the Animal Research Ethics Committee of the University of KwaZulu-Natal (ethics no: AREC/038/016M).

2.3.1   Experimental design

An established research protocol were used for the induction of pre-diabetes [18]. The study consisted of two main groups, the non-prediabetic animals (NPD, $n = 6$) and the pre-diabetic animals (PD, $n = 30$). After the 20 weeks induction period, the pre-diabetic animals were divided into the following five groups ($n = 6$): the first group (PD) were fed on high fat high carbohydrate (HFHC) diet without treatment; the second group (Metformin [MTF]+HFHC) were fed on HFHC diet and treated with an oral dose of metformin (500 mg/ kg, Sigma-Aldrich, St Louis, Missouri, USA); the third group (MTF+ND) were fed on normal diet (ND) and treated with an oral dose of metformin (500 mg/ kg, Sigma-Aldrich, St Louis, Missouri, USA); the fourth group (Ruthenium [RU]+HFHC) were fed on HFHC diet and treated with subcutaneous injection of ruthenium complex (15 mg/ kg) while the fifth group (RU+ND) were fed on ND and treated with subcutaneous injection of ruthenium complex (15 mg/ kg). The animals were treated once a day every third day at 09:00 am for 12 weeks. Once after 4 weeks, parameters such as body weight, food intake, fasting blood glucose, fluid intake and urinary output were monitored for 12 weeks treatment period.

2.3.2 Blood collection and tissue harvesting

All animals were anaesthetised with Isofor (100 mg/ kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) using a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for 3 minutes. Blood was collected by cardiac puncture and then injected into individual pre-cooled heparinized containers. The blood was then centrifuged for plasma collection (Eppendorf centrifuge 5403, Germany) at 4 °C, 503 g for 15 minutes. Thereafter, kidney was removed, weighed and halved into two kidney tissue. One kidney tissue was snap frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until biochemical analysis, and the othe kidney tissue was kept in 10 % formalin buffer for histolopathological examination.

2.3.3 Biochemical analysis

Plasma and urine sodium (Na^+), potassium (K^+), urea, creatinine and albumin concentrations were measured by Global Clinical and Viral Laboratory (Amanzimtoti, South Africa). Plasma aldosterone and KIM-1, kidney tissue superoxide dismutase (SOD) and glutathione peroxidase (GPx) (respectively) were analysed using separate, specific ELISA kits in accordance with the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China). Kidney tissue malondialdehyde (MDA) levels were measured using a previously described protocol [17], whilst AER, creatinine clearance (CC) and were calculated according to the following:

24 hr urine albumin excretion (mg/ 24 hr) = 24 hr urine volume × albumin

AER (µg/min) = Urinary Albumin (mg/dl) × Urine Volume (dl) × 1000 / Period of urine collected (min)

CC (ml/min) = [(Urinary Creatinine in mg/ dl) × (Urinary volume in ml)]/[(Serum Creatinine in mg/ dl) × 1440 mim]

ACR (mg/g) = [Urinary Albumin (mg/dl)/Urinary Creatinine (mg/dl)] × 1000

2.3.4 Urine RNA isolation

RNA was isolated from urine (4 ml) by using ZR Urine RNA Isolation Kit™ (Zymo Research Corp, Irvine, USA) according to the manufacturer's protocol. The purity of the RNA was confirmed by the relative absorbance of ratio 260/280 nm via Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Urine RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) by using iScript™ cDNA Synthesis Kit (Bio Rad, California, USA) through incubation in a thermal cycler (SimpliAmp Thermal Cycler, Applied biosystems, Life technologies).

2.3.5 Urine complementary DNA (cDNA) synthesis

For cDNA synthesis, urine RNA (2 µl) was mixed with 5X iScript reaction (4 µl), iScript reverse transcriptase enzyme (1µl) (Bio Rad, USA) and nuclease-free water to a final volume of 20 µl. The mixture was incubated in the thermal cycler (SimpliAmp Thermal Cycler, Applied biosystems, Life technologies) at 25 °C for 5 minutes, 42 °C for 30 minutes and finally at 85 °C for 5 minutes. Thereafter, the synthesized cDNA was stored at -80 °C until use for real-time PCR (Polymerase chain reaction).

2.3.6 Real-time quantitative PCR

The urinary mRNA level of podocin was quantified by real-time PCR lightcycler (Roche LightCycler 96, USA). cDNA template (2 µl), SYBR Green PCR master mix (5 µl) (Bio Rad, USA), podocin forward primer (1 µl), podocin reverse primer (1 µl) and nuclease-free water were mixed to a final volume of 10 µL. Thereafter, the sample mixtures were cycled 40 times at 95 °C for 10 seconds, 60 °C for 20 seconds and 72 °C for 20 seconds in the lightcycler (Roche LightCycler 96, USA). All the samples were run in duplicate and β-actin mRNA levels were used as a housekeeping gene to normalize the podocin mRNA level. The sequences of the used oligonucleotide primers (Metabion International AG, Planegg, Germany) were as followed: podocin forward 5`-TGG AAG CTG AGG CAC AAA GA-3`, podocin reverse 5`-AGA ATC TCA GCC GCC ATC CT-3`.

2.3.7 Kidney histology examination

Kidney histology was analysed using a previously described protocol [16]. Kidney tissues were immediately fixed in 10% formalin buffer after removal from the animals. The tissue samples were embedded in paraffin wax after alcohol dehydration process. Using microtome, 5 µm longitudinally sections of kidney tissue were taken and were stained by hematoxylin and eosin (H&E) stain. The processed tissue sections were then visualized and captured using a Leica Scanner, SCN400 and Slide Path Gateway LAN software for analysis (Leica Microsystems CMS, Wetzlar, Germany). Two paraffin-embedded kidney samples from each group were sectioned (4 kidney sections per slide). Kidney tissue was evaluated for histopathological changes using a semi-quantitative assessment. Each kidney section was assessed for the presence of the following changes: Glomerular thickening, interstitial fibrosis and vacuolar degeneration. The observed changes were graded from zero to three, with zero indicating normal tissue, one indicating mild change, two indicating moderate change, and three indicating severe change.

2.4 Statistical Analysis

Data were reported as mean ± standard error of mean (SEM). GraphPad Prism Software (version 5) was used to conduct statistical analysis. The differences between control and treated groups were analysed using One-way analysis of variance (ANOVA) followed by Tukey-Kramer. Values of $p < 0.05$ show statistical significance between the compared groups.

3. Results

3.1 Effects of ruthenium complex on blood glucose level, 24 h fluid intake and 24 h urine output

Induction of pre-diabetes resulted in a significant increase in blood glucose, fluid intake and urine output levels throughout the 12-week treatment period when compared to the NPD group ($p < 0.05$; Figure 1). However, the administration of the ruthenium complex significantly reduced blood glucose levels which correlated with a significant decreased fluid intake and urine output levels after the 12-week treatment period. Interestingly, the animal group treated with the established oral-administered drug, metformin showed analogous *in vivo* activities to the PD group ($p < 0.05$; Figure 1).
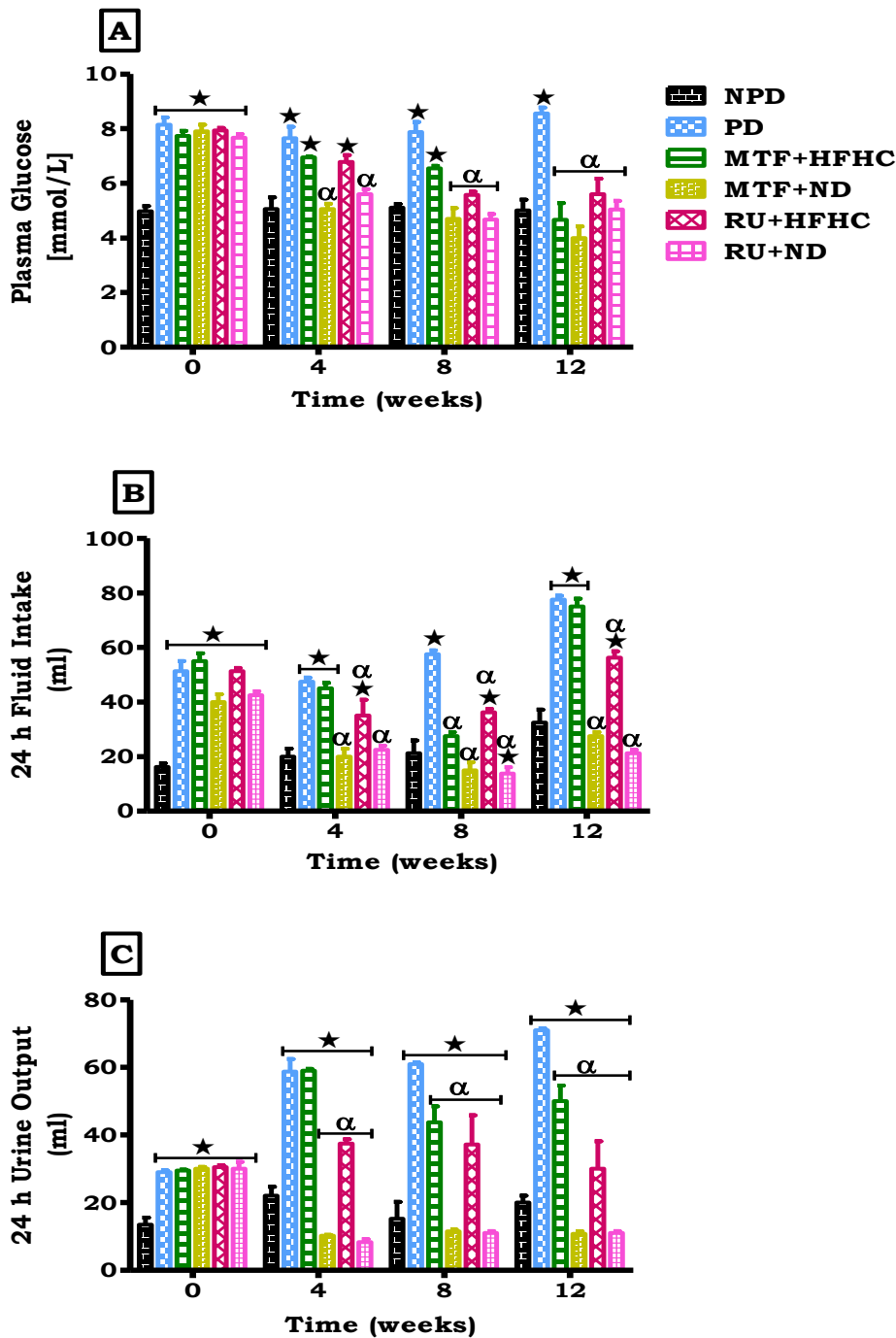
Figure 1: The effects of the metal-based drug on A) blood glucose, B) fluid intake and C) urine output levels of pre-diabetic animals during the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. $*$ $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.2 Effects of ruthenium complex on electrolyte handling, albumin, creatinine and urea levels

Upon pre-diabetes, the PD group showed significant decreases in urinary Na^+, K^+, creatinine and urea levels as well as increase urinary albumin levels when compared to the NPD group ($p < 0.05$; Table 1). When compared with the PD group, the RU+HFHC group showed a significant increased urinary Na^+, K^+ and creatinine concentrations whilst significantly decreasing urinary albumin with no significant change in urea concentration ($p < 0.05$; Table 1). Appreciable increased concentrations of urinary Na^+, K^+, creatinine and urea concentrations whilst significantly decreasing urinary albumin levels for the animals that were treated with the ruthenium(II) complex and dietary intervention in comparison to the PD group ($p < 0.05$; Table 1). The observed effect was comparable to the results obtained from the metformin-treated group when compared to the PD group. In addition, the PD animal group revealed a significant increase in plasma Na^+, creatinine and urea concentrations with a significant decrease in albumin levels when compared to the NPD group ($p < 0.05$; Table 1). Notably, there was no significant difference in plasma K^+ levels amongst the treatment groups when compared to NPD group ($p > 0.05$; Table 1). However, the administration of the ruthenium complex restored plasma Na^+, albumin and creatinine concentrations to within those found in the NPD control group, whilst treatment with ruthenium and dietary intervention showed a significant decrease plasma urea levels when compared with the PD group ($p < 0.05$; Table 1). Furthermore, the animals treated with the established organopharmaceutical, metaformin exhibit data analogous when compared to the PD animal group ($p < 0.05$; Table 1).

Table 1: Influences of the ruthenium compound on electrolytes handling, albumin, creatinine and urea levels of pre-diabetic animals for a treatment period of 12 weeks. Urine collected over 24h. Values are presented as means ± SEM (n = 6) in each group.

Groups	Urinary parameters				
	Na$^+$ (mmol/L)	K$^+$ (mmol/L)	Albumin (mg/dl)	Creatinine (mg/dl/)	Urea (mmol/L)
NPD	124.30±0.37	185.30±0.18	0.73±0.61	114.03±0.92	604.00±1.00
PD	10.00±0.10*	13.33±0.38*	7.64±0.38*	28.62±0.12*	136.00±0.39*
MTF+HFHC	6.50±0.98*	12.33±0.31*	2.06±0.82*α	25.45±0.15*α	140.00±0.54*
MTF+ND	93.00±1.2*α	192.00±0.85α	0.63±0.81*α	141.96±1.44α	890.00±1.00*α
RU+HFHC	28.67±0.53*α	30.67±0.55*α	0.56±0.55α	28.28±1.01*α	163.00±0.97*
RU+ND	89.67±0.12*α	244.50±0.47*α	0.47±0.35*α	126.69±0.578α	802.00±0.35*α
	Plasma parameters				
NPD	128.00±0.21	6.40±0.46	1525.00±0.54	0.36±0.38	4.30±0.66
PD	156.00±0.40*	5.00±0.58	1225.00±0.93*	0.44±0.44*	5.50±0.15*
MTF+HFHC	134.00±0.79α	5.40±0.30	1650.00±0.78	0.38±0.18α	5.60±0.50*
MTF+ND	126.0±0.99α	5.80±0.35	1500.00±0.15α	0.34±1.00α	3.10±0.55α
RU+HFHC	129.70±0.55α	5.20±0.16	1500.00±1.00α	0.36±0.59α	5.20±0.20*
RU+ND	128.0±0.67α	5.70±0.10	1567.00±0.98α	0.34±1.00α	3.40±0.65α

3.3 Effects of ruthenium complex on aldosterone concentration

The PD group illustrated a significant enhancement in averaged aldosterone concentration when compared to the NPD group ($p < 0.05$; Figure 2). Furthermore, a marked increase was observed in aldosterone levels in the MTF+HFHC group when compared to the NPD group ($p < 0.05$; Figure 2). Interestingly, there was a significant reduction in aldosterone concentrations in the ruthenium complex treated groups by comparison to the PD group ($p < 0.05$; Figure 2). The MTF+ND group showed decreased aldosterone concentration when compared with the PD group ($p < 0.05$; Figure 2).
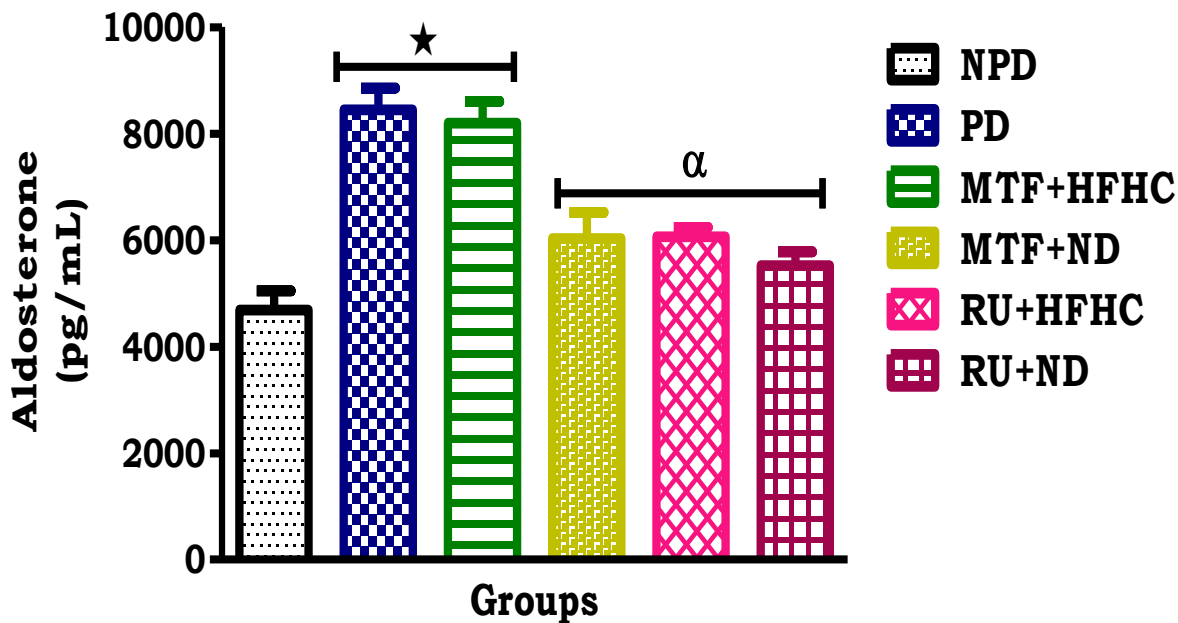
Figure 2: The effects of the metal complex on aldosterone concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. * $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.4 Effects of ruthenium complex on kidney function

The PD group showed significantly increased AER and ACR with a decreased CC and fractional excretion of K^+ when compared to the NPD group (p<0.05; Table 2). There was no significant different in fractional excretion of Na^+ ($FENa^+$) amongst the treatment groups when compared to NPD group ($p > 0.05$; Table 2). Administration of the ruthenium Schiff base complex resulted in significantly decreased AER and ACR whilst restoring fractional excretion of K^+ (FEK^+) when compared to the PD group (p<0.05; Table 2). Of note there was no significant change in CC level in both the treated groups when compared with the PD group (p>0.05; Table 2). The effects of MTF treated groups were similar to those of ruthenium-treated animals (Table 2).

Table 2: Influences of the metallo-drug on measurements of kidney function in pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means ± SEM ($n = 6$) in each group.

Groups	AER (µg/min)	CC (mL/min)	ACR (mg/g)	FENa+ (%)	FEK+ (%)
NPD	0.10±0.021	4.40±0.10	6.40±0.21	0.30±0.0057	9.00±0.033
PD	3.78±0.89*	3.20±0.54*	268.00±1.23*	0.10±0.57	4.00±0.050*
MTF+HFHC	0.72±0.82α	2.30±0.10* α	80.90±0.15*α	0.10±0.13	3.00±0.20*
MTF+ND	0.047±0.022α	3.10±0.12*	4.40±0.29*α	0.20±0.088	8.00±0.050α
RU+HFHC	0.12±0.010α	1.60±0.22*α	19.80±0.058*α	0.30±0.52	7.00±0.050α
RU+ND	0.036±0.012α	2.90±0.21*	3.70±0.050*α	0.20±0.35	11.00±0.70α

* $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.5 Effects of ruthenium complex on renal oxidative stress and antioxidant concentrations

Diet-induced pre-diabetes led to a significant increase in renal MDA level whilst decreasing renal SOD and GPx levels as observed in the PD group relative to that of the NPD control group ($p < 0.05$; Table 3). Treatment with the potential metallopharmaceutical considerably decreased MDA levels and increased renal concentrations of SOD and GPx when

compared to the PD group ($p < 0.05$; Table 3). The MTF+HFHC group showed significant increased MDA levels when compared with the NPD group ($p < 0.05$; Table 3). However, treatment with metformin significantly decreased SOD and GPx levels when compared with the PD group ($p < 0.05$; Table 3).

Table 3: Impacts of the ruthenium complex on oxidative stress and antioxidants concentrations of pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means ± SEM ($n = 6$) in each group.

Groups	MDA (nmol/ g kidney tissue)	SOD (ng/mL)	GPx (ng/mL)
NPD	1.79±0.021	74.92±0.032	1744.13±0.021
PD	2.31±0.089*	65.32±0.023*	1514.24±0.032*
MTF+HFHC	2.01±0.024*	71.56±0.020α	1779.56±0.019α
MTF+ND	1.44±0.037α	72.80±0.034α	1814.24±0.010α
RU+HFHC	1.59±0.018α	70.40±0.02α	1621.74±0.010α
RU+ND	1.34±0.026α	72.98±0.021α	1807.16±0.048α

* $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.6 Consequences of metal-based drug administration on KIM-1 concentration

The PD group showed a significant increase in plasma KIM-1 concentrations when compared to the NPD group ($p < 0.05$; Figure 3). However, there was a significant reduced in KIM-1 levels in the ruthenium complex treated groups when compared to the PD control group ($p < 0.05$; Figure 3). The MTF+ND group showed a similar trend of results (Figure 3).
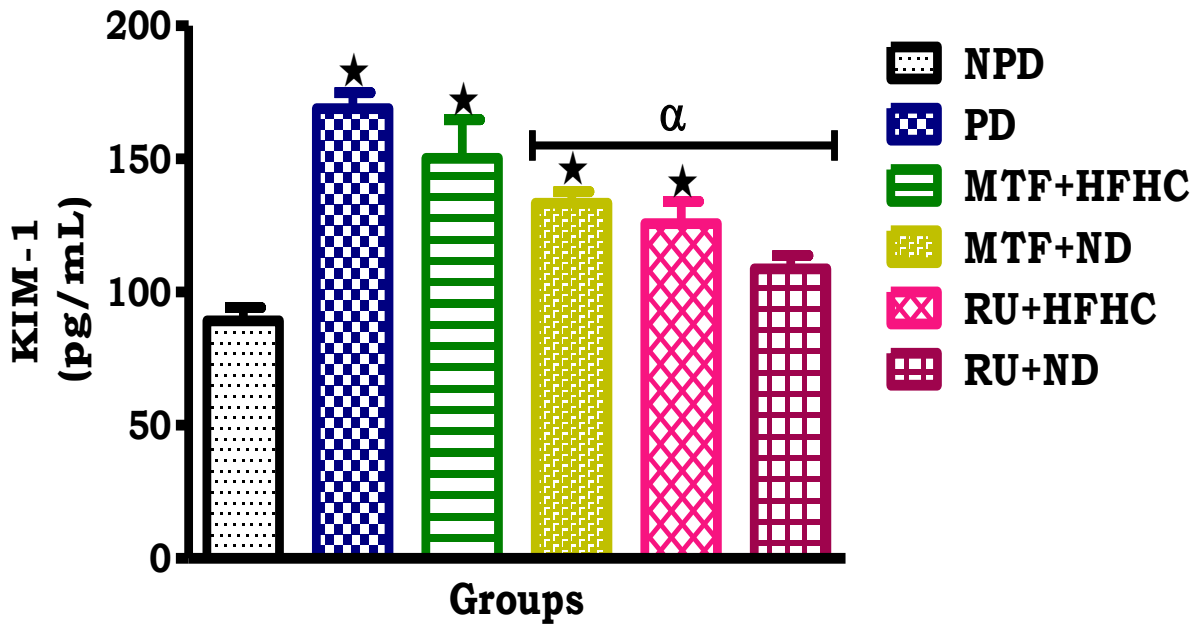
Figure 3: The effects of the metal-based drug on KIM-1 concentration of pre-diabetic animals after the treatment period. Values are presented as means ± SEM (*n* = 6) in each group. ***** *p* < 0.05 compared to the non-prediabetic control (NPD), **α** *p* < 0.05 compared to the pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.7 Real-time quantitative PCR

The expression levels of podocin mRNA was significantly higher in the urine of the PD untreated rats compared with the NPD group (p < 0.05; Figure 4). However, treatment with the ruthenium complex in both the presence and absence of dietary intervention significantly reduced mRNA expression of podocin by comparison with the untreated PD group (p < 0.05; Figure 4). Similar trends were observed in the metformin-treated groups (Figure 4).
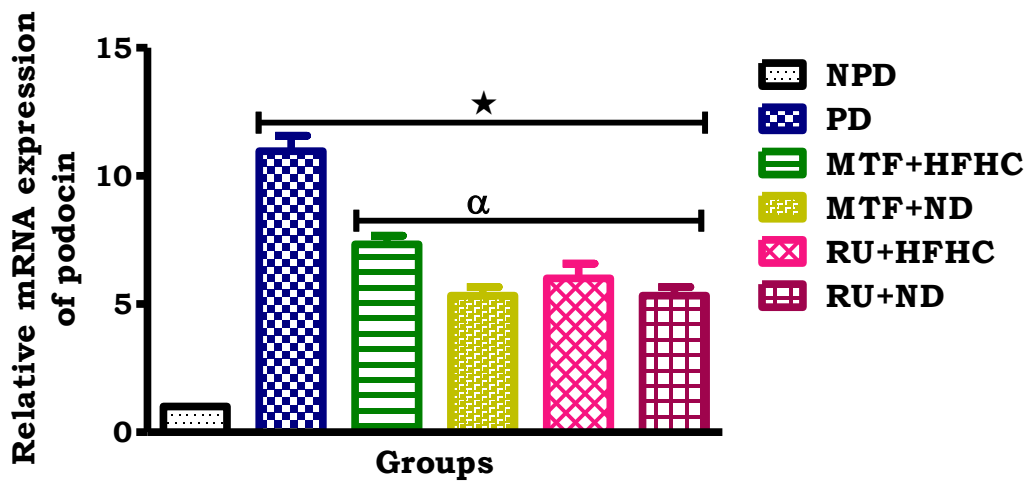
Figure 4: The effects of the metal-based drug on relative mRNA expression of urinary podocin levels of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. * $p < 0.05$ compared to the non-prediabetic control (NPD), **α** $p < 0.05$ compared to the pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.8 Histology of the kidney

Kiney histology analysis revealed that there was glomerular thickening (black arrow) and moderate interstitial fibrosis (blue arrow) in PD group animals (Figure 5 (B)) as compared to NPD animals (Figure 5 (A)). The administration of ruthenium compound in conjunction with dietary intervention to the pre-diabetic animals (Figure 5 (C)-(D)) ameliorated interstitial fibrosis and glomerular thickening in the pre-diabetic metal complex-treated animals as compared to PD control animals (Figure 5 (B)). In addition, the metformin with dietary intervention treated animals showed similar observation (Figure 5 (E)-(F)).
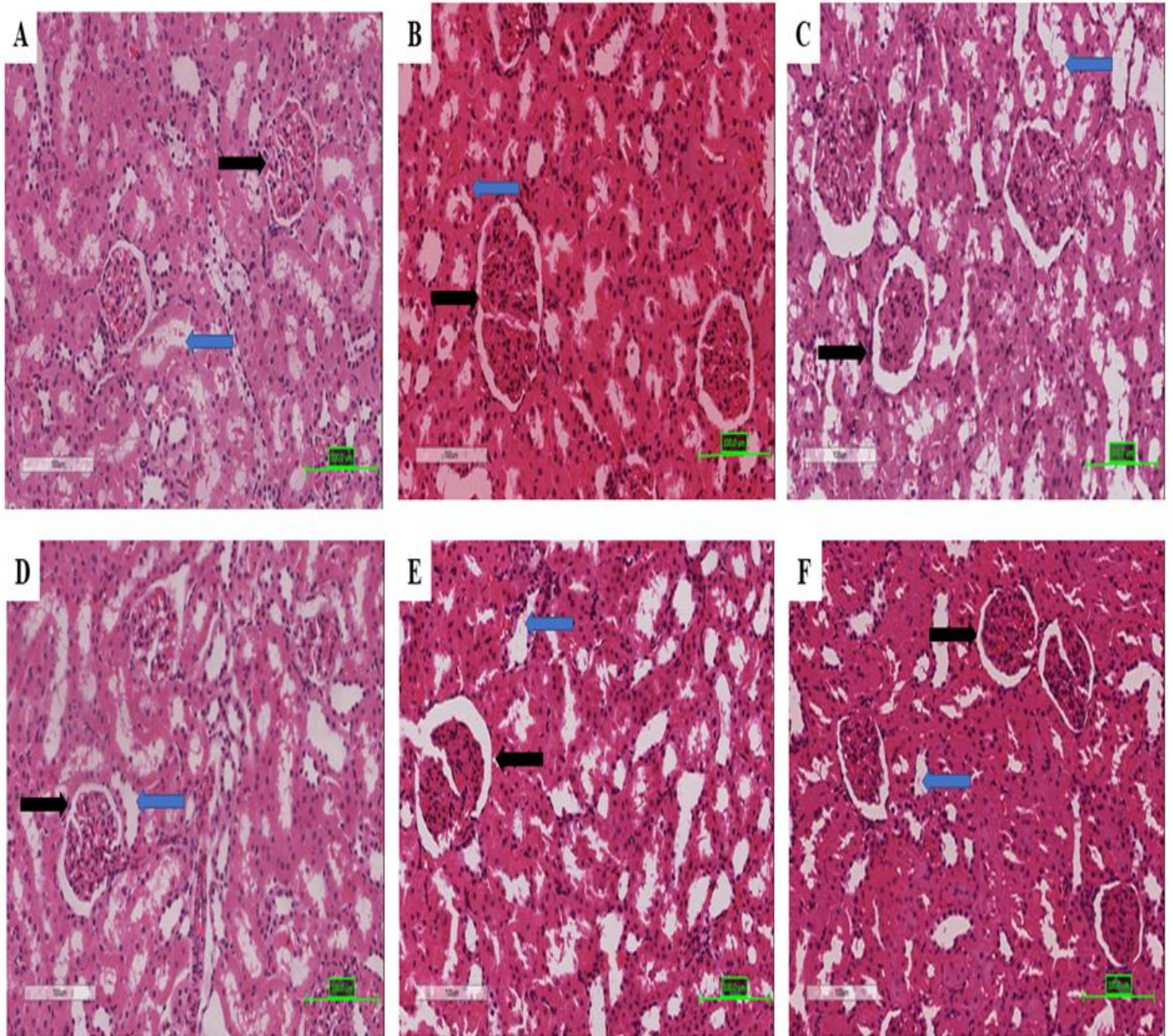
Figure 5: The effects of the ruthenium(II) complex on kidney histopathological analyses (A) NPD group, (B) PD group, (C) RU+HFHC group, (D) RU+ND group, (E) MTF+HFHC group and (F) MTF+ND group of pre-diabetes treated animals during the treatment period (H&E) stain. Magnification 20X (100 µm). Black arrow = glomerular, Blue arrow = interstitial fibrosis.

4. Discussion

Chronic ingestion of a HFHC diet have been shown to lead to the development of pre-diabetes [17, 18, 21]. More specifically, chronic hyperglycaemia is known as a primary contributing factor in the pathogenesis of diabetic complications [22, 23]. In addition, previous studies have shown that hyperglycaemia triggers the excessive generation of reactive oxygen species (ROS) which causes an imbalance in the ability of the body to scavenge the ROS produced due to depletion in its antioxidant defense [24, 25]. The imbalance in ROS production and antioxidant defense status invariably leads to oxidative stress which is involved in several mechanistic pathways that contribute to the pathogenesis and severity of DN [23, 26]. Oxidative stress can also promote the formation of a variety of vasoactive mediators that can affect renal function directly by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient and, thus, reduce GFR [23, 26]. Furthermore, hyperglycaemia-induced ROS and oxidative stress is associated with the accumulation of advanced glycation end-products (AGEs), activation of the polyol and protein kinase C pathways [27]. The results of this study revealed that the prediabetic rats demonstrated increased oxidative stress as indicated by increases in the level of lipid peroxidation marker (MDA) as well as a reduction of antioxidant enzymes SOD and GPx concentrations in the kidney. Administration of the ruthenium(II) Schiff base complex ameliorated oxidative stress by restoring the activities of the antioxidant enzymes SOD and GPx levels and suppressing lipid peroxidation in the treated pre-diabetic rats. We have already illustrated that our leading candidate anti-diabetic drug improved glycaemic control through decrease in glycated haemoglobin levels (HbA1c) and thus increasing insulin sensitivity in pre-diabetic treated rats [18].

The RAAS consists of a group of enzymes and peptides whose main function is to control blood pressure by regulating vasoconstriction, electrolyte balance, and body fluid homeostasis [28]. Activated RAAS has been linked with the development of insulin resistance and diabetes in humans [28]. Activation of the RAAS in pre-diabetic patients has been shown to lead to an increase in aldosterone secretion which results in sodium and water retention leading to elevated blood pressure [11, 13, 14]. Elevations in glucose levels increase plasma tonicity, creating an osmotic driving force that favours the movement of water from the intracellular space to the extracellular space, thereby diluting the extracellular concentration of sodium [28]. In the current study, decreased urinary and increased plasma sodium levels were observed in the pre-diabetic rats. However, administration of the ruthenium(II) Schiff base complex restored both urinary and plasma sodium levels in pre-diabetic rats. The significant increase in the excretion of sodium by ruthenium(II) Schiff base complex may suggest the inhibition of increased proximal reabsorption of sodium which is often seen in diabetes, thus

increasing the sodium-potassium pump activity [29]. The metformin-treated rats showed similar effects; however, this was only when used in conjunction with the normal diet. It has been suggested that the use of metformin as an antidiabetic agent requires dietary modification in order to achieve therapeutic effects [30]. However, patients still neglect lifestyle modification. Therefore, the ruthenium Schiff base complex showed beneficial effects even in the absence of dietary modification.

Elevations in plasma glucose levels observed in pre-diabetes have been found to lead to RAAS activation which can lead to polyuria and polydipsia [16]. In this study, the untreated prediabetic group had increased 24h fluid intake with increased urinary output by comparison to the non-diabetic group. Treatments to delay the progression of DN progression involve adequate blood glucose lowering and control of hypertension [11, 13, 14]. The administration of the ruthenium(II) Schiff base complex, in both the presence and absence of dietary intervention decreased blood glucose, 24h fluid intake and 24h urinary output to within range of the non-prediabetic rats over the treatment period. The ruthenium(II) Schiff base complex has been shown to improve insulin sensitivity and restore glucose homeostasis in diet-induced pre-diabetes [18]. The research results observed in this study may therefore suggest that the renoprotective effect of this complex may, in part, be mediated by its beneficial effects on glucose homeostasis.

In patients with T2DM, the insulin-mediated uptake of glucose is impaired, but the cellular uptake of potassium remains normal [31]. Hyperkalaemia can be caused by an increase in plasma tonicity that results from the redistribution of potassium from the intracellular space to the extracellular space [32]. The efflux of potassium from the cell is thought to be due to intracellular dehydration, which results from the osmotically-induced transcellular movement of water [33]. Potassium and angiotensin II are the major regulators of aldosterone secretion. Interestingly, the pre-diabetic rats showed decreased urinary potassium levels when compared to the NPD group. On the contrary, the administration of the ruthenium(II) Schiff base complex resulted in an increased urinary potassium levels in the pre-diabetes rats. In addition, the research trends obtained may be attributed to the restored sodium reabsorption in exchange with potassium in the proximal tubule [31]. Of note, there was no significant difference in the treated groups in plasma potassium levels when compared to both the NPD and PD groups. However, because redistribution between extracellular and intracellular spaces also influences serum potassium, hypokalaemia should be considered to coexist with normal body potassium levels, therefore further research is required.

Diabetes mellitus is associated with profound changes in protein metabolism and by loss of nitrogen from most organs [34]. Hyperglycaemia triggers elevated levels of urea and creatinine, which are considered as significant markers of renal dysfunction and alter GFR [34]. The HFHC diet-induced pre-diabetic rats markedly decreased urinary creatinine and urea levels with an increased urinary albumin level. Additionally, these correlated with increased plasma urea and creatine levels with decreased plasma albumin levels. Elevated levels in urinary albumin is used as the marker of protein leakage, which is an indicator of kidney damage [35]. The elevated levels of serum creatinine and urea show diminished ability of the kidneys to filter these waste products from the blood and excrete them in the urine [36]. Interestingly, administration of the diamagnetic ruthenium complex in present or absent of dietary intervention resulted in increased urinary creatinine levels with decreased urinary albumin leves in ruthenium-treated pre-diabetic rats. There results were further evidenced by decreased plasma creatinine levels with increased plasma albumin levels in pre-diabetes treated rats. However, treatment with ruthenium complex with dietary intervention resulted in increased urinary urea with decrease plasma urea levels in the pre-diabetes treated rats. The results obtained could be attributed to the metal complex to attenuate the ability of the kidneys to filter these waste products from the blood and excrete them in the urine possibly via the amelioration of hyperglycaemia. Studies have shown that improved glycaemic control is beneficial in improving kidney function [36]. Furthermore, KIM-1 a transmembrane protein serves as a useful biomarker for renal proximal tubule injury facilitating the early diagnosis of the disease [9, 10]. Renal tubular damage, as evidenced by increased levels of KIM-1, is evident even prior to the development of pre-diabetes and overt kidney disease [9, 10]. The plasma levels of KIM-1 were found significantly increased in pre-diabetic animals in comparison with non-prediabetic animals, demonstrating the existence of diabetic tubular damage at the early stage of DN. The administration of the ruthenium(II) Schiff base complex resulted in reduced plasma KIM-1 levels in pre-diabetic treated animals when compared to pre-diabetic animals. Notably, the ruthenium(II) Schiff base complex coupled with dietary intervention showed a significant decreased in plasma KIM-1 levels to within the non-prediabetic KIM-1 levels. The results obtained suggest that the use of ruthenium(II) Schiff base complex is effective in alleviation renal injury in pre-diabetic animals. These observations can be attributed to the influence of the metal complex which improves glucose uptake [18].

The current study showed that pre-diabetes was associated with the subsequent development of glomerular hyperfiltration and abnormal albuminuria. Albuminuria or microalbuminuria, albumin excretion rate ≥ 30 mg/ 24 hours or albumin/creatinine ratio (ACR) ≥ 30 mg/ g (≥ 3 mg/mmol), is used as a marker of renal damage and is used to define

chronic kidney disease along with low estimated glomerular filtration rate (eGFR) [37]. Literature trends have shown that high glomerular plasma flow increases intracapillary pressure; there is a relative constriction of the efferent glomerular arteriole resulting in a flux of plasma proteins into the mesangial [37]. Interestingly, treatment with the metallo-drug in the present or absent of dietary intervention showed decreased AER and ACR in the ruthenium-treated rats. The protective effect of ruthenium complex against DN is also thought to be mediated through its anti-hyperglycaemic action. Of note treatment with both metformin and ruthenium were not able to improve CC levels in the pre-diabetic rats. Podocytes are specialized glomerular epithelial cells that are responsible for preserving the glomerular filtration barrier [38, 39]. In diabetes-induced kidney injury, podocytes loss and injury are associated with marked proteinuria [39]. Previous studies have demonstrated that down regulation in podocin, a key podocyte slit diaphragm protein, is involved in the development of proteinuria in DN [40, 41]. The results obtained showed that podocyte injuries in diabetic kidney could be reversed after treatment with ruthenium complex, through the reduction of mRNA expression of podocin. In addition, the restoration of urinary mRNA expression levels of podocin observed agreed with the decreased in proteinuria in the ruthenium-treated rats. These results show that ruthenium complex protected the rats against podocyte injury by decreasing the expression of podocin in the ruthenium-treated rats. The protective effects of ruthenium(II) complex were further demonstrated by the histopathological analysis of the kidney. The renal tissues of the positive control diabetic rats showed the symptoms of DN illustrated in the hypertrophy of glomerular and tubular elements and increase in the thickness of glomerular basement membranes [3, 4]. These histopathological alterations in the positive control group rats together with the other tests achieved to diagnose diabetes and DN emphasized that DN was onset as one of the diabetic complications [5, 6]. Ameliorated the levels of all these parameters approaching the negative control and restored the normal histology of kidney. Ruthenium complex treatment counteracted the hyperglycaemia-induced oxidative stress as well as renal dysfunction [17, 18]. The renoprotective effects of ruthenium complex seem to result from its inhibitory effect on ROS and lipid peroxidation, which play a role in improving renal dysfunction in diabetes and observed antioxidant effect [17, 18].

5. Conclusion

The candidate anti-diabetic drug, $[Ru^{II}(H_3ucp)Cl(PPh_3)]$ (H_4ucp = 2,6-*bis*-((6-amino-1,3-dimethyluracilimino)methylene)pyridine) exhibited renoprotective effects in HFHC diet-induced pre-diabetic rats by reducing blood glucose, fluid intake and urinary output while ameliorating oxidative stress and antioxidant defence

enzymes, reducing aldosterone and KIM-1 concentrations. These was further evidenced by a decrease in AER, ACR with restoration of electrolytes handling, expression of mRNA podocin levels in urine and histological structure of the renal glomerulus. These results were more potent in conjunction with dietary intervention. Therefore, these research findings substantiate that this mononuclear ruthenium(II) Schiff base complex could be beneficial in the management of DN and prediabetic-related complications. Therefore, this research study warrants further investigations into the mechanism of anti-diabetic activity of the aforementioned compound on renal function.

Authors' contributions: LPM and MWG were involved in study design, *in vivo* studies, data analysis and assisted in preparing the manuscript. SM and INB synthesised and spectroscopically characterized the free-ligand and its ruthenium complex which was utilised as the metal-based drug. INB proof-read the manuscript. AK and PSN were involved in conceptualization and design of the study, execution of animal studies, data analysis, provided funding and assisted in writing the manuscript. All authors have read and approved the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Tabák A, Herder C, Rathmann W, Brunner E, Kivimäki M. Prediabetes: a high-risk state for diabetes development. The Lancet 2012;379:2279-2290.

2. Pecoits-Filho R, Abensur H, Betônico C, Machado A, Parente E, Queiroz M, Salles J, Titan S, Vencio S. Interactions between kidney disease and diabetes: dangerous liaisons. Diabetology & metabolic syndrome 2016;8:50.

3. Weil E, Lemley K, Mason C, Yee B, Jones L, Blouch K, Lovato T, Richardson M, Myers B, Nelson R. Podocyte detachment and reduced glomerular capillary endothelial fenestration promote kidney disease in type 2 diabetic nephropathy. Kidney international 2012;82:1010-1017.

4. Kanwar Y, Wada J, Sun L, Xie P, Wallner E, Chen S, Chugh S, Danesh F. Diabetic nephropathy: mechanisms of renal disease progression. Experimental biology and medicine 2008;233:4-11.

5. De Nicola L, Conte G, Minutolo R. Prediabetes as a precursor to diabetic kidney disease. American Journal of Kidney Diseases 2016;67:817-819.

6.	Tagawa A, Yasuda M, Kume S, Yamahara K, Nakazawa J, Chin-Kanasaki M, Araki H, Araki S, Koya D, Asanuma K. Impaired podocyte autophagy exacerbates proteinuria in diabetic nephropathy. Diabetes 2016;65:755-767.

7.	Anil Kumar P, Welsh G, Saleem M, Menon R. Molecular and cellular events mediating glomerular podocyte dysfunction and depletion in diabetes mellitus. Frontiers in endocrinology 2014;5:151.

8.	Suh J, Miner J. The glomerular basement membrane as a barrier to albumin. Nature Reviews Nephrology 2013;9:470.

9.	Carlsson A, Calamia M, Risérus U, Larsson A, Helmersson-Karlqvist J, Lind L, Ärnlöv J. Kidney injury molecule (KIM)-1 is associated with insulin resistance: Results from two community-based studies of elderly individuals. Diabetes research and clinical practice 2014;103:516-521.

10.	El-Ashmawy N, El-Zamarany E, Khedr N, El-Fattah A, Eltoukhy S. Kidney injury molecule-1 (Kim-1): an early biomarker for nephropathy in type II diabetic patients. International Journal of Diabetes in Developing Countries 2015;35:431-438.

11.	Remuzzi G, Perico N, Macia M, Ruggenenti P. The role of renin-angiotensin-aldosterone system in the progression of chronic kidney disease. Kidney International 2005;68:S57-S65.

12.	Chawla T, Sharma D, Singh A. Role of the renin angiotensin system in diabetic nephropathy. World journal of diabetes 2010;1:141.

13.	Jansen P, Danser A, Imholz B, van den Meiracker A. Aldosterone-receptor antagonism in hypertension. Journal of hypertension 2009;27:680-691.

14.	Lim A. Diabetic nephropathy–complications and treatment. International journal of nephrology and renovascular disease 2014;7:361.

15.	Krug A, Ehrhart-Bornstein M. Adrenocortical dysfunction in obesity and the metabolic syndrome. Hormone and metabolic research 2008;40:515-517.

16.	Bouby N, Fernandes S. Mild dehydration, vasopressin and the kidney: animal and human studies. European journal of clinical nutrition 2003;57:S39.

17.	Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P, Khathi A. Cardioprotective effects of a ruthenium (II) Schiff base complex in a diet-induced prediabetic rats. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy 2019;12:1-7.

18.	Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P, Khathi A. Effects of a ruthenium schiff base complex on glucose homeostasis in diet-induced pre-diabetic rats Molecules (Basel, Switzerland) 2018;23:E1721.

19.	Ciftci O, Beytur A, Vardi N, Ozdemir I. Evaluation of reproductive toxicity in male rats treated with novel synthesized ruthenium(II) and gold(I)-NHC complexes. Drug Development and Industrial Pharmacy 2012;381:40-46.

20.	Booysen I, Maikoo S, Akerman M, Xulu B. Novel ruthenium (II) and (III) compounds with multidentate Schiff base chelates bearing biologically significant moieties. Polyhedron 2014;79:250-257.

21.	Luvuno M, Mabandla M, Khathi A. Voluntary ingestion of a high-fat high-carbohydrate diet: A model for prediabetes. Ponte international scientific researchs journal 2018;74.

22.	Yilmaz S, Canpolat U, Aydogdu S, Abboud H. Diabetic cardiomyopathy; summary of 41 years. Korean circulation journal 2015;45:266-272.

23. Saravanan S, Pari L. Protective effect of thymol on high fat diet induced diabetic nephropathy in C57BL/6J mice. Chemico-biological interactions 2016;245:1-11.

24. Di Marco E, Jha J, Sharma A, Wilkinson-Berka J, Jandeleit-Dahm K, de Haan J. Are reactive oxygen species still the basis for diabetic complications? Clinical Science 2015;129:199-216.

25. Seddon M, Looi Y, Shah A. Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. Heart 2007;93:903-907.

26. Althunibat O, Al-Mustafa A, Tarawneh K, Khleifat K, Ridzwan B, Qaralleh H. Protective role of Punica granatum L. peel extract against oxidative damage in experimental diabetic rats. Process Biochemistry 2010;45:581-585.

27. Kashihara N, Haruna Y, K Kondeti V, S Kanwar Y. Oxidative stress in diabetic nephropathy. Current medicinal chemistry 2010;17:4256-4269.

28. Tran T, Pease A, Wood A, Zajac J, Mårtensson J, Bellomo R, Ekinci E. Review of evidence for adult diabetic ketoacidosis management protocols. Frontiers in endocrinology 2017;8:106.

29. Musso C, Gregori J, Jauregui J, Núñez J. Creatinine, urea, uric acid, water and electrolytes renal handling in the healthy oldest old. World journal of nephrology 2012;1:123.

30. Palmer B, Gates J, Lader M. Causes and management of hyponatremia. Ann Pharmacother 2003;37:1694-1702.

31. Nguyen T, Maalouf N, Sakhaee K, Moe O. Comparison of insulin action on glucose versus potassium uptake in humans. Clinical Journal of the American Society of Nephrology 2011;6:1533-1539.

32. Palmer B. A physiologic-based approach to the evaluation of a patient with hyperkalemia. American Journal of Kidney Diseases 2010;56:387-393.

33. Tzamaloukas A, Ing T, Elisaf M, Raj D, Siamopoulos K, Rohrscheib M, Murata G. Abnormalities of serum potassium concentration in dialysis-associated hyperglycemia and their correction with insulin: review of published reports. International urology and nephrology 2011;43:451-459.

34. Pasupathi P, Chandrasekar V, Kumar U. Evaluation of oxidative stress, enzymatic and non-enzymatic antioxidants and metabolic thyroid hormone status in patients with diabetes mellitus. Diabetes & Metabolic Syndrome: Clinical Research & Reviews 2009;3:160-165.

35. Birn H, Christensen E. Renal albumin absorption in physiology and pathology. Kidney international 2006;69:440-449.

36. Feig D, Mazzali M, Kang D, Nakagawa T, Price K, Kannelis J, Johnson R. Serum uric acid: a risk factor and a target for treatment? Journal of the American Society of Nephrology 2006;17:S69-S73.

37. Sung K, Ryu S, Lee J, Lee S, Cheong E, Hyun Y, Lee K, Kim H, Byrne C. Urine albumin/creatinine ratio below 30 mg/g is a predictor of incident hypertension and cardiovascular mortality. Journal of the American Heart Association 2016;5:e003245.

38. Al-Trad B, Al-Batayneh K, El-Metwally S, Alhazimi A, Ginawi I, Alaraj M, Alkofahi E, Aljumaili O, Kosba A. Nigella sativa oil and thymoquinone ameliorate albuminuria and renal extracellular matrix accumulation in the experimental diabetic rats. Europian Review for Medical and Pharmacological Sciences 2016;20:2680-2688.

39. He B, Ebarasi L, Zhao Z, Guo J, Ojala J, Hultenby K, De Val S, Betsholtz C, Tryggvason K. Lmx1b and FoxC combinatorially regulate podocin expression in podocytes. Journal of the American Society of Nephrology 2014;25:2764-2777.

40. Zheng M, Lv L, Ni J, Ni H, Li Q, Ma K, Liu B. Urinary podocyte-associated mRNA profile in various stages of diabetic nephropathy. PLoS One 2011;6:e20431.

41. Fukuda A, Wickman L, Venkatareddy M, Wang S, Chowdhury M, Wiggins J, Shedden K, Wiggins R. Urine podocin: nephrin mRNA ratio (PNR) as a podocyte stress biomarker. Nephrology Dialysis Transplantation 2012;27:4079-4087.

Table captions

Table 1: Influences of the ruthenium compound on electrolytes handling, albumin, creatinine and urea levels of pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means ± SEM ($n = 6$) in each group.

Table 2: Influences of the metallo-drug on measurements of kidney function in pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means ± SEM ($n = 6$) in each group.

Table 3: Impacts of the ruthenium complex on oxidative stress and antioxidants concentrations of pre-diabetic animals for a treatment period of 12 weeks. Values are presented as means ± SEM ($n = 6$) in each group.

Figure legends

Figure 1: The effects of the metal-based drug on A) blood glucose, B) fluid intake and C) urine output levels of pre-diabetic animals during the treatment period . Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Figure 2: The effects of the metal complex on aldosterone concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Figure 3: The effects of the metal-based drug on KIM-1 concentration of pre-diabetic animals during the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to the non-prediabetic control (NPD), **α** $p < 0.05$ compared to the pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Figure 4: The effects of the metal-based drug on relative mRNA expression of urinary podocin levels of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to the non-prediabetic control (NPD), **α** $p < 0.05$ compared to the pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Figure 5: The effects of the ruthenium(II) complex on kidney histopathological analyses (A) NPD group, (B) PD group, (C) RU+HFHC group, (D) RU+ND group, (E) MTF+HFHC group and (F) MTF+ND group of pre-diabetes treated animals during the treatment period (H&E) stain. Magnification 20X (100 µm). Black arrow = glomerular, Blue arrow = interstitial fibrosis.

Tables

Groups	Urinary parameters				
	Na$^+$ (mmol/L)	K$^+$ (mmol/L)	Albumin (mg/dl)	Creatinine (mg/dl/)	Urea (mmol/L)
NPD	124.30±0.37	185.30±0.18	0.73±0.61	114.03±0.92	604.00±1.00
PD	10.00±0.10*	13.33±0.38*	7.64±0.38*	28.62±0.12*	136.00±0.39*
MTF+HFHC	6.50±0.98*	12.33±0.31*	2.06±0.82*α	25.45±0.15*α	140.00±0.54*
MTF+ND	93.00±1.2*α	192.00±0.85α	0.63±0.81*α	141.96±1.44α	890.00±1.00*α
RU+HFHC	28.67±0.53*α	30.67±0.55*α	0.56±0.55α	28.28±1.01*α	163.00±0.97*
RU+ND	89.67±0.12*α	244.50±0.47*α	0.47±0.35*α	126.69±0.578α	802.00±0.35*α
Plasma parameters					
NPD	128.00±0.21	6.40±0.46	1525.00±0.54	0.36±0.38	4.30±0.66
PD	156.00±0.40*	5.00±0.58	1225.00±0.93*	0.44±0.44*	5.50±0.15*
MTF+HFHC	134.00±0.79α	5.40±0.30	1650.00±0.78	0.38±0.18α	5.60±0.50*
MTF+ND	126.0±0.99α	5.80±0.35	1500.00±0.15α	0.34±1.00α	3.10±0.55α
RU+HFHC	129.70±0.55α	5.20±0.16	1500.00±1.00α	0.36±0.59α	5.20±0.20*
RU+ND	128.0±0.67α	5.70±0.10	1567.00±0.98α	0.34±1.00α	3.40±0.65α

* $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Groups	AER (μg/min)	CC (mL/min)	ACR (mg/g)	FENa$^+$ (%)	FEK$^+$ (%)
NPD	0.10±0.021	4.40±0.10	6.40±0.21	0.30±0.0057	9.00±0.033
PD	3.78±0.89*	3.20±0.54*	268.00±1.23*	0.10±0.57	4.00±0.050*
MTF+HFHC	0.72±0.82α	2.30±0.10* α	80.90±0.15*α	0.10±0.13	3.00±0.20*
MTF+ND	0.047±0.022α	3.10±0.12*	4.40±0.29*α	0.20±0.088	8.00±0.050α
RU+HFHC	0.12±0.010α	1.60±0.22*α	19.80±0.058*α	0.30±0.52	7.00±0.050α
RU+ND	0.036±0.012α	2.90±0.21*	3.70±0.050*α	0.20±0.35	11.00±0.70α

* $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Groups	MDA (nmol/ g kidney tissue)	SOD (ng/mL)	GPx (ng/mL)
NPD	1.79±0.021	74.92±0.032	1744.13±0.021
PD	2.31±0.089*	65.32±0.023*	1514.24±0.032*
MTF+HFHC	2.01±0.024*	71.56±0.020α	1779.56±0.019α
MTF+ND	1.44±0.037α	72.80±0.034α	1814.24±0.010α
RU+HFHC	1.59±0.018α	70.40±0.02α	1621.74±0.010α
RU+ND	1.34±0.026α	72.98±0.021α	1807.16±0.048α

* $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).
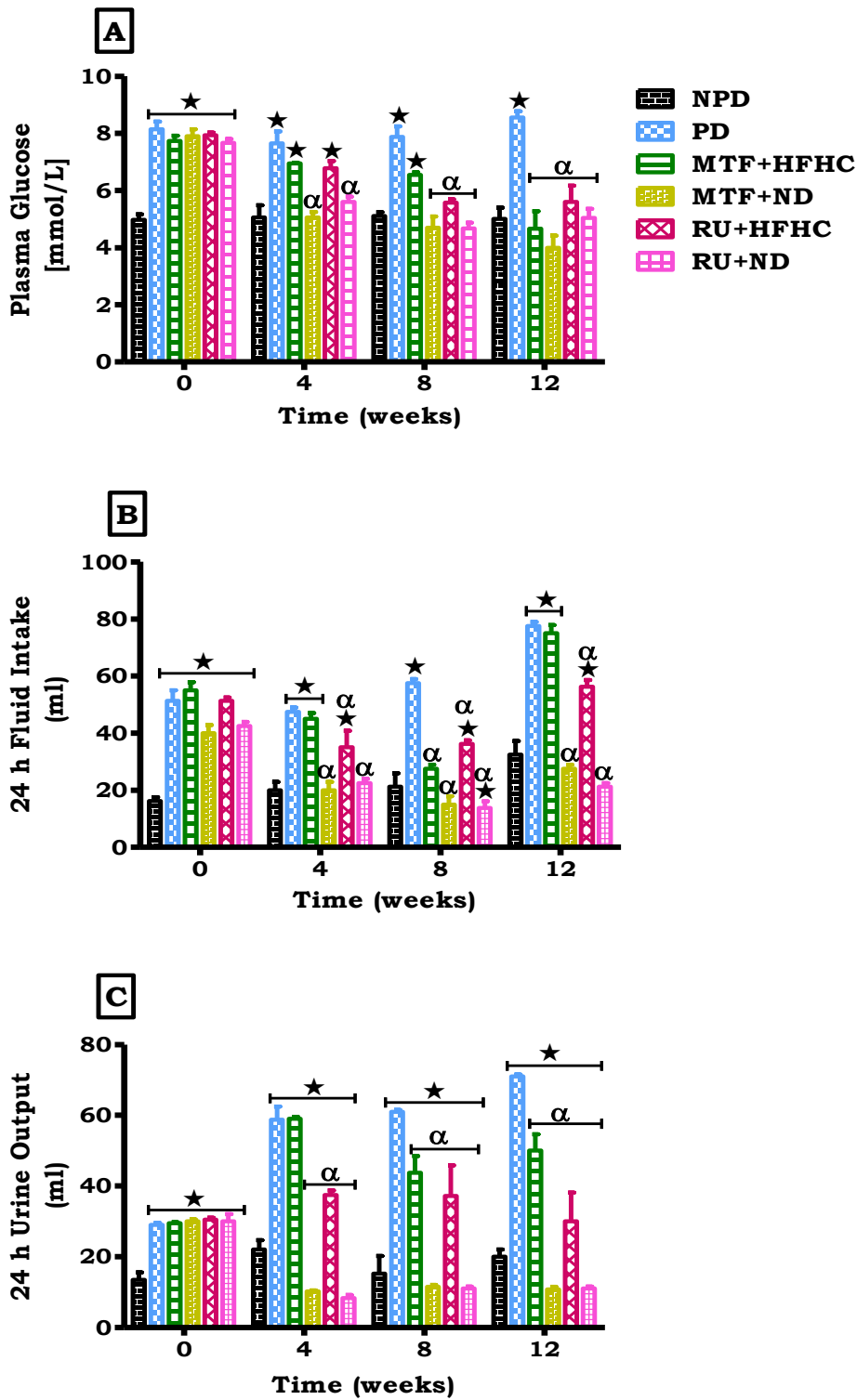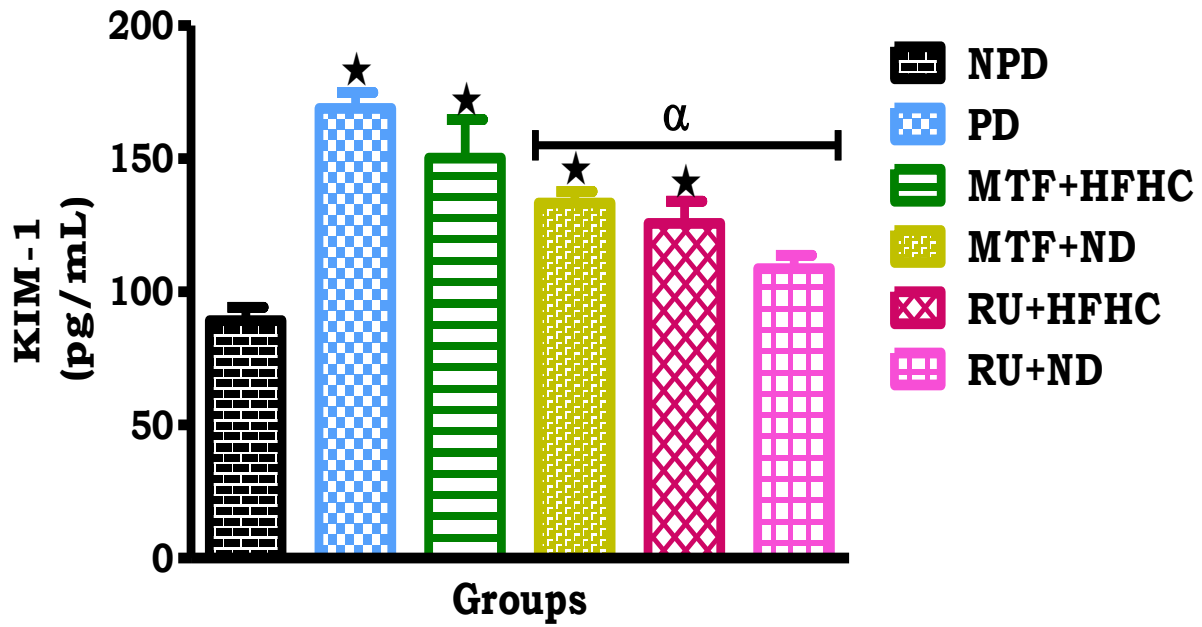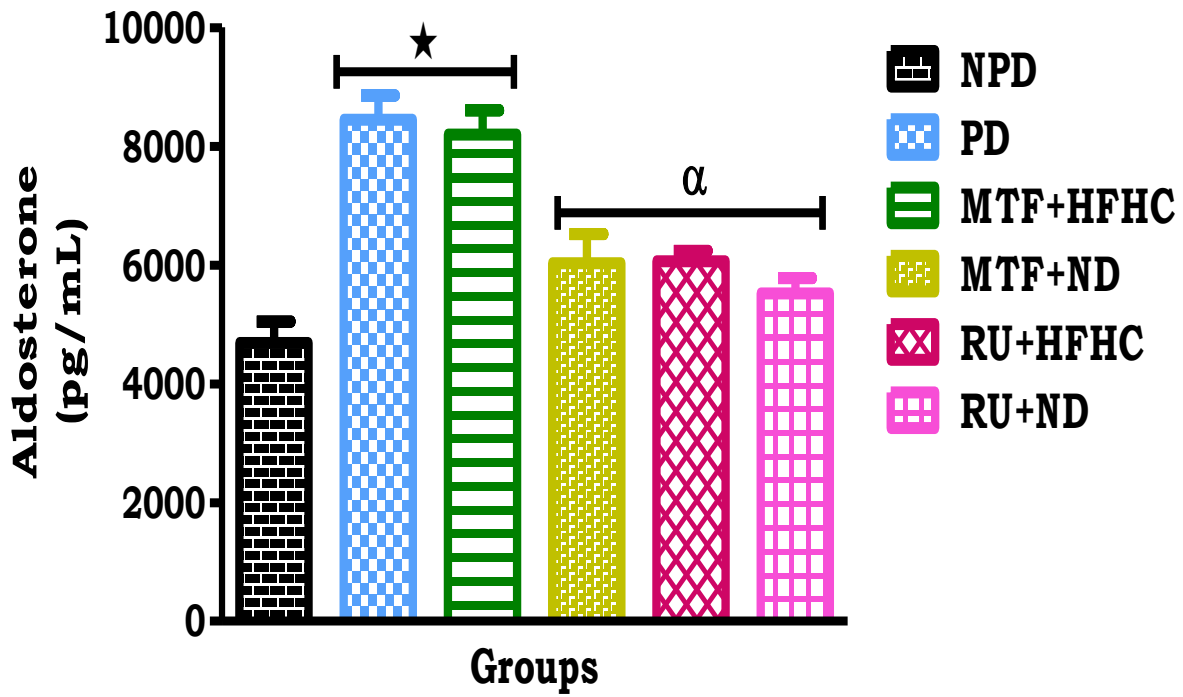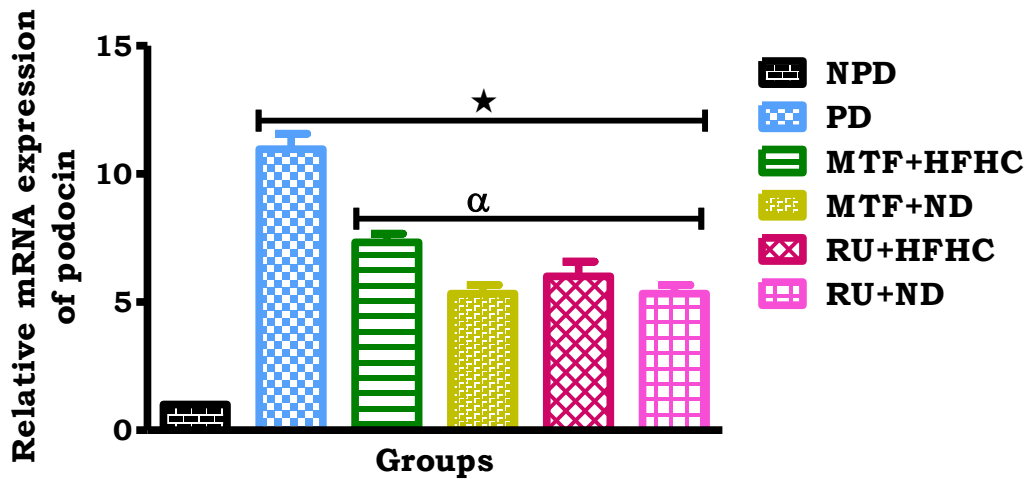
Figures

CHAPTER 4

Prologue 3: Manuscript 3

"Ruthenium(II) Schiff base complex administration ameliorated inflammatory-induced immune response biomarkers in a diet-induced pre-diabetic rat model"

The progression of pre-diabetes to overt T2DM encompasses a complex interaction between metabolism and immunity. This response has been shown to be a precipitating factor sustaining insulin resistance, preceding T2DM progression. Metabolism and immune system are commonly regarded as two distinctive mechanisms regulating nutrient disposal and body defense, respectively. Typically, little is known about the coordination and interplay between these two systems in pre-diabetes progression. Nevertheless, the current study has led to the combination of these distinct entities as studies observe pathological activation of the immune system underlying the pre-diabetic state. We have previously reported that treatment with ruthenium(II) complex improve insulin sensitivity and glycaemic control in a diet-induced pre-diabetic rat model. However, the effects of this compound on pre-diabetes-induced immune dysregulation remain unknown. Hence, manuscript 3, investigated the effects of a ruthenium(II) Schiff base complex on inflammatory-induced immune dysregulation in a diet-induced pre-diabetic rat model in the presence and absence of dietary intervention.

The current manuscript has been submitted for publication in the journal "**Immune Network"** and has been formatted according to journal's guidelines to authors (Appendix 9).

This work was authored by **L.P Mabuza**, **M.W Gamede**, **S Maikoo**, **IN Booysen**, **P.S Ngubane** and **A Khathi.**

Ruthenium(II) Schiff base complex administration ameliorated inflammatory-induced immune response biomarkers in a diet-induced pre-diabetic rat model.

Running tittle: Effects of ruthenium(II) complex on immune dysregulation.

Lindokuhle P Mabuza[1,3], Mlindeli W Gamede[1], Sanam Maikoo[2], Irvin N Booysen[2], Phikelelani S Ngubane[1] and Andile Khathi [1].

[1]School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[2]School of Chemistry and Physics, College of Engineering and Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa

[3]* Corresponding Author

Ms LP Mabuza

School of Laboratory Medicine & Medical Sciences

University of KwaZulu-Natal

Private Bag X54001

Durban, South Africa

Tel: 0312607585

Email: 211509843@stu.ukzn.ac.za

Keywords: pre-diabetes, ruthenium(II) complex, immune dysregulation, inflammation, dietary intervention, platelets activation

Abstract

The coordination and interplay between metabolism and immune response during the progression of pre-diabetes remain unclear. The current study has led to combination of these distinct entities as pathological activation of the immune system and targeting the pre-diabetic state as a therapeutic target in the management of inflammatory-induced immune dysregulation underlying pre-diabetes. Hence, the current study aims to investigate the effects of ruthenium(II) complex on inflammatory-induced immune dysregulation in a diet-induced pre-diabetic rat model. A high fat high carbohydrates (HFHC) diet was given to male Sprague-Dawley rats for 20 weeks to induce pre-diabetes. After the induction, pre-diabetic rats were randomly allocated to respective treatment groups. Subcutaneous injection of ruthenium(II) complex (15 mg/kg) was administered to pre-diabetic rats in both the presence and absence of dietary intervention once a day every third day for 12 weeks. Treatment with ruthenium(II) complex resulted in reduction of platelet activation markers mean platelet volume (MPV) and CD40 Ligand (CD40L) concentrations, which correlated with decreased plasma triglycerides (TG), very low-density lipoproteins (VLDL) and fibrinogen concentrations, these were further evidenced by normalization of immune cell counts. Furthermore, there was a decrease in pro-inflammatory cytokines, tumor necrosis factor α (TNF- α) and interleukin-1β (IL-1β) concentrations in the ruthenium(II)-treated rats. Ruthenium(II) complex exhibited an anti-inflammatory activity and inhibited platelet aggregation thus preventing the progression of immune dysregulation underlying pre-diabetes.

1. Introduction

The progression of pre-diabetes to overt type 2 diabetes mellitus (T2DM) involves a complex interaction between metabolism and immunity response [1]. Metabolic inflammation is regulated by both the innate and adaptive immune cell interactions and has been shown to be a precipitating factor sustaining insulin resistance, preceding T2DM [2]. The initiation events of a proinflammatory response involve synergistic contributions of various mechanisms, which include an increase in nuclear factor κB (NF-κB) and c-Jun NH2-terminal kinase (JNK) activity [3-5]. Adipocytes are an important initiator of inflammatory response and when stressed, they produce various cytokines and chemokines promoting immune cell activation and accumulation in adipose tissue [5]. Increase in adiposity is associated with upregulation of genes encoding pro-inflammatory molecules and accumulation of immune cells [3-5]. The sustained accumulation of lipids in adipose tissues results in switching of macrophages from an anti-inflammatory to a pro-inflammatory phenotype [6]. Elevated concentrations of biomarkers such as C-reactive protein (CRP), TNF- α, white blood cell (WBC) count, IL-1β, interleukin-6 (IL-6) and fibrinogen point to a chronic, often subclinical degree of inflammation and are increased already before the onset of T2DM [7]. The progression of T2DM has been shown to be linked to various immune cells types but the primary sources of inflammatory effectors contributing to insulin resistance are macrophages [8, 9]. Besides macrophages, the representation of several other immune cell populations is also altered in diabetes. These include eosinophils and neutrophils which serve as a negative regulator of adipose tissue inflammation [10, 11]. In addition, various metabolic abnormalities caused by diabetes lead to increased platelet activation and reactivity, evidenced by elevated levels of surface P-selectin and CD40L markers as well as an increased count and MPV [12-14]. Since, T2DM is preceded by an extensive period of disease development and the activation of inflammatory-induced immune response is activated before the onset of the disease, therefore beginning treatment during the pre-diabetic state could be used as therapeutic target in the management and prevention of pre-diabetes inflammatory-induced immune response as well as the associated complications.

A combination of pharmacological and dietary intervention is recommended for the management of pre-diabetes progression to result in increased peripheral insulin sensitivity and improving glycaemic control [15, 16]. Recent studies have reported that a ruthenium(II) Schiff base complex with a diimine uracil chelating ligand possesses anti-diabetic properties as evidence by improved glycaemic control, insulin sensitivity and decreased risk of developing diabetes-related CVDsin diet-induced pre-diabetic rats [17, 18]. In addition, a study by Mzimela *et al.,* has shown the changes in immune cell concentration during the progression of pre-diabetes to T2DM in a high fat high carbohydrate diet-induced

prediabetic rat model [9]. However, the effects of this mononuclear metal-based complex in inflammatory-induced immunity response in diet-induced pre-diabetes are yet to be investigated. Thus, the aim of this study was to investigate the effects of a ruthenium(II) Schiff base complex in inflammatory-induced immune response in the presence and absence of dietary intervention in diet-induced pre-diabetes rat model.

2. Materials and methods

2.1 Chemicals and drugs

All chemicals and drugs were of analytical grade and purchased from standard commercial suppliers. Previous studies showed that the dose of the ruthenium complex used in this study was non-toxic [17-19].

2.2 Synthesis of ruthenium(II) Schiff base complex

The resynthesis of the ruthenium(II) Schiff base complex, [$Ru^{II}(H_3ucp)Cl(PPh_3)$] (H_4ucp = 2,6-*bis*-((6-amino-1,3-dimethyluracilimino)methylene)pyridine) was conducted according to experimental methods previously reported [20]. The purity of the metal complex was affirmed by nuclear magnetic spectroscopy and Time-Of-Flight (TOF) mass spectrometry.

2.3 Animals and housing

In this study, 36 male Sprague-Dawley rats (150-180 g) were used. The animals were housed in room with a 12 hours light/12 hours dark cycle and room temperature (25°C) for the duration of the study. The animals in each group had access to food and water *ad libitum*. All animal procedures and housing conditions were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (ethical clearance number: AREC/038/016M).

2.3.1 Induction of pre-diabetes

The animals were randomly assigned to the following diet groups; Normal diet with drinking water (ND) and high-fat high-carbohydrate diet with drinking water supplemented with 15% fructose (HFHC) (AVI Products (Pty) Ltd, Waterfall, South Africa). Pre-diabetes was induced by allowing the animals to feed on the HFHC diet for 20 weeks as previously described [21]. Glucose tolerance was evaluated 5 days after 20 weeks of induction with oral glucose tolerance test (OGTT) to determine pre-diabetes according to the American Diabetes Association criteria. The rats with fasting blood glucose (FBG) of more than 5.6 mmol/L were considered pre-diabetic and grouped further for pharmacological studies.

The animals that were fed the normal diet were also tested and were found to be normoglycemic and without pre-diabetes. The treatment started on the subsequent day and this was considered as the first day of treatment.

2.3.2 Experimental design

The study consisted of two main groups, the non-prediabetic animals (NPD, n=6) and the pre-diabetic animals (PD, n=30). After 20 weeks of induction, the pre-diabetic animals were divided into the following groups (n=6): Pre-diabetic (PD) group were fed on high fat high carbohydrate (HFHC) diet without treatment. The second group (metformin [MTF] + HFHC) were fed on high fat high carbohydrate diet and treated with oral dose of metformin (500 mg/kg, Sigma-Aldrich, St Louis, Missouri, USA). The third group (MTF + ND) were fed on normal diet (ND) and treated with oral dose of metformin (500 mg/kg, Sigma-Aldrich, St Louis, Missouri, USA). The fourth group (ruthenium [RU] + HFHC) were fed on high fat high carbohydrate diet and treated with subcutaneous injection of ruthenium complex (15 mg/kg) while the fifth group (RU + ND) were fed on normal diet and treated with subcutaneous injection of ruthenium complex (15 mg/kg). The animals were treated once a day every third day at 09:00 am for 12 weeks.

2.3.3 Blood collection and tissue harvesting

All animals were anaesthetised with Isofor (100 mg/kg)) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) using a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) and allowed to inhale for 3 minutes. Blood was collected by cardiac puncture and then injected into individual pre-cooled heparinized containers. The blood was then centrifuged for plasma collection (Eppendorf centrifuge 5403, Germany) at 4 °C, 503 g for 15 minutes. Thereafter, adipose tissue was removed, rinsed with saline and snap frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until biochemical analysis.

2.4 Inflammatory marker measurements

The concentration of adipose tissue TNF-α, plasma IL-1β, fibrinogen and CD40L were analysed using specific ELISA kits (Respectively) in accordance with the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China). Optical density was determined using a Spectrostar nanoplate spectrophotometer (BMG Labtech, Ortenburg, Baden-W€urtternberg, Germany) at 450 nm.

2.5 Immune cell measurements

Monocytes, neutrophils, eosinophils, basophils, lymphocytes, white blood cell and platelets cell count, and MPV were counted using a hemocytometer (COULTER® Ac.T diff™ Analyzer, Beckman Coulter, United State).

2.6 Plasma lipid analysis

Plasma TGs levels were analysed by Global Clinical and Viral laboratories (Amanzimtoti, South Africa). Plasma VLDL concentrations were calculated using the friedewald formula:

$$\mathbf{VLDL} = \frac{Plasma\ TGs}{2.2}$$

2.7 Statistical Analysis

Data is reported as mean ± standard error of mean (SEM). GraphPad Prism Software (version 5) was used to conduct statistical analysis. The differences between control and treated groups were analysed using One-way analysis of variance (ANOVA) followed by Tukey-Kramer. Values of $p < 0.05$ show statistical significance between the compared groups.

3. Results

3.1 Effects of ruthenium complex on platelet activation markers

Induction of diet-induced pre-diabetes resulted in a significant increase in platelet count, MPV and CD40L concentration in the untreated PD group when compared to the NPD group ($p < 0.05$; Table 1). Furthermore, the MTF+HFHC and RU+HFHC treated groups displayed higher platelets count and plasma CD40Llevels after the 12-week treatment period when compared with the NPD group ($p < 0.05$; Table 1). Interestingly, when compared with the PD group, administration of the metal-based drug reduced platelets count which correlated with reduced MPV and plasma CD40L concentration to within that of NPD group after the 12-week treatment period ($p < 0.05$; Table 1). There was no significant difference in CD40L concentration in the MTF+HFHC group when compared with the PD group ($p > 0.05$; Table 1). However, the MTF+ND group displayed reduced platelets count, MPV and CD40L concentration when compared to the PD group ($p < 0.05$; Table 1).

Table 1: Effects of ruthenium complex on platelet count, mean platelet volume and CD40L concentration of pre-diabetic treated animals for 12 weeks period. Values are presented as means ± SEM ($n = 6$) in each group.

Groups	Platelet Count ($10^3/\mu L$)	Mean Platelet Volume (fL)	CD40L (ng/ml)
NPD	360.30±4.20	7.28±0.43	1.94±0.17
PD	1279.00±8.00*	12.90±0.10*	5.90±0.20*
MTF+HFHC	735.50±4.90*$^{\alpha}$	7.35±0.15$^{\alpha}$	4.25±0.38*
MTF+ND	190.00±3.00$^{\alpha}$	6.55±0.050$^{\alpha}$	1.70±0.35$^{\alpha}$
RU+HFHC	641.50±3.40*$^{\alpha}$	7.32±0.18$^{\alpha}$	3.40±0.15*$^{\alpha}$
RU+ND	150.70±3.70$^{\alpha}$	7.40±0.28$^{\alpha}$	1.61±0.26$^{\alpha}$

* $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.2 Effects of ruthenium complex on plasma TGs and VLDL concentrations

Increased plasma TGs and VLDL concentration were observed for the PD control group when compared to the NPD group ($p < 0.05$; Table 2). Furthermore, the MTF+HFHC treated group had displayed higher plasma TGs and VLDL levels after the 12-week treatment period when compared with the NPD group ($p < 0.05$; Table 2). Interestingly, when compared with the PD group, administration of the metal-based drug accompanied with and without dietary modification reduced plasma TGs and VLDL concentrations to within that of NPD group ($p < 0.05$; Table 2). The metformin-treated groups in the presence of dietary intervention showed significant decrease in plasma TGs which correlated with decreased VLDL concentration when compared with the PD group ($p < 0.05$; Table 2).

Table 2: Effects of ruthenium complex on plasma TGs and VLDL concentration of pre-diabetic treated animals for 12 weeks period. Values are presented as means ± SEM ($n = 6$) in each group.

Groups	Plasma TGs (mmol/L)	VLDL (mmol/L)
NPD	1.22±0.31	0.55±0.090
PD	3.34±0.21*	1.52±0.81*
MTF+HFHC	5.62±0.27*	2.55±0.98*
MTF+ND	0.91±0.042$^\alpha$	0.41±0.043$^\alpha$
RU+HFHC	1.87±0.14$^\alpha$	0.85±0.032$^\alpha$
RU+ND	1.17±0.012$^\alpha$	0.53±0.012$^\alpha$

* $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.3 Effects of ruthenium complex on plasma fibrinogen concentration

Upon pre-diabetes, the PD group showed significant increase in plasma fibrinogen concentration after the 12-week treatment period when compared to the NPD group ($p < 0.05$; Figure 1). Of note, treatment with either metformin or ruthenium supplemented with the high fat high carbohydrate diet resulted in higher plasma fibrinogen levels relative to that of the NPD control group ($p < 0.05$; Figure 1). Interestingly, administration of the potential metallopharmaceutical in the presence of dietary intervention considerably decreased plasma fibrinogen levels in pre-diabetic treated rats when compared to the PD group ($p < 0.05$; Figure 1). The MTF+ND group had comparable experimental trends to the PD group ($p < 0.05$; Figure 1).
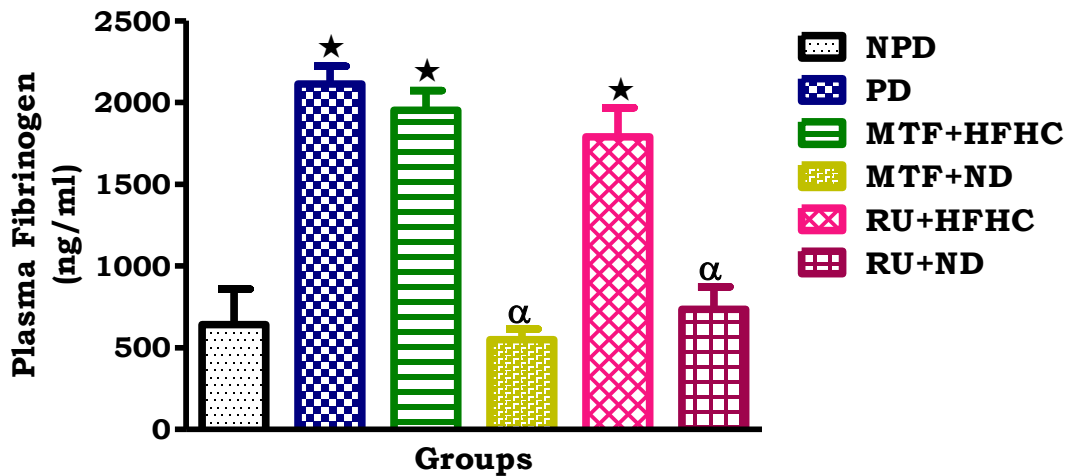
Figure 1: The effects of the ruthenium Schiff-base complex on plasma fibrinogen concentration of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.4 Effects of ruthenium complex on immune cell count

Diet-induced pre-diabetes led to a significant decrease in neutrophils and eosinophils cell count with an increase in WBC, lymphocytes, monocytes and basophils cell count as observed in the PD group in relation to that of the NPD group ($p < 0.05$; Figure 2). In addition, the MTF+HFHC showed significant increased WBC when compared to the NPD group ($p < 0.05$; Figure 2). Interestingly, treatment with ruthenium complex in the presence of dietary intervention considerably increase neutrophils and eosinophils cell count whilst in the presence and absence of dietary intervention the metal-based drug was able to reduce WBC, lymphocytes, monocytes and basophils cell count over the 12-week treatment period when compared to the PD group ($p < 0.05$; Figure 2). Furthermore, the metformin-treated groups showed similar observations when compared to the PD group ($p < 0.05$; Figure 2).
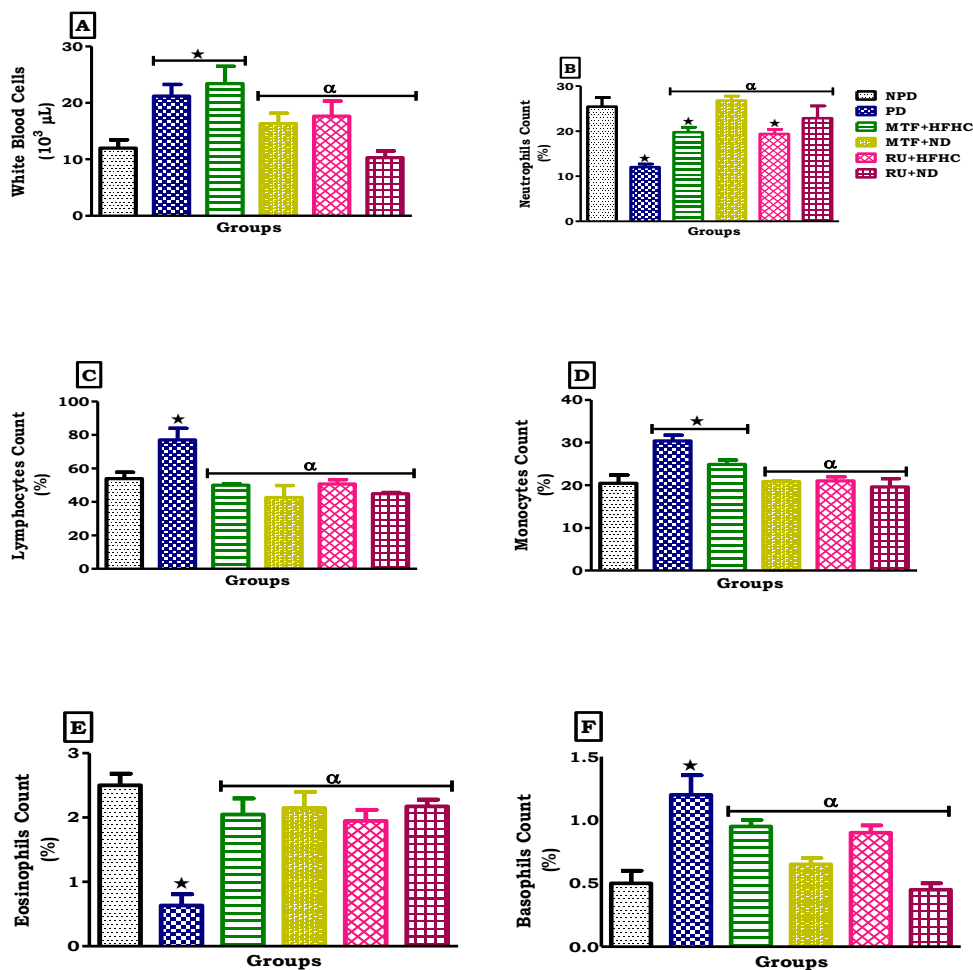
Figure 2: The effects of the ruthenium Schiff-base complex on A) White blood cell B) neutrophils, C) lymphocytes, D) monocytes, E) eosinophils and F) basophils immune cell count of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. * $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.5 Effects of ruthenium complex on adipose tissue tumor necrosis factor-α (TNF-α) concentration

The PD group demonstrated a significant enhancement in averaged adipose tissue TNF-α concentration when compared to the NPD group ($p < 0.05$; Figure 3). Furthermore, a marked increase was observed in adipose tissue TNF-α levels in the MTF+HFHC group when compared to the NPD group ($p < 0.05$; Figure 3). Interestingly, there was a significant reduction in adipose tissue TNF-α concentrations in the ruthenium complex treated groups by comparison to the PD group

($p < 0.05$; Figure 3). However, the MTF+ND group displayed reduced adipose tissue TNF-α level when compared with the PD group ($p < 0.05$; Figure 3).
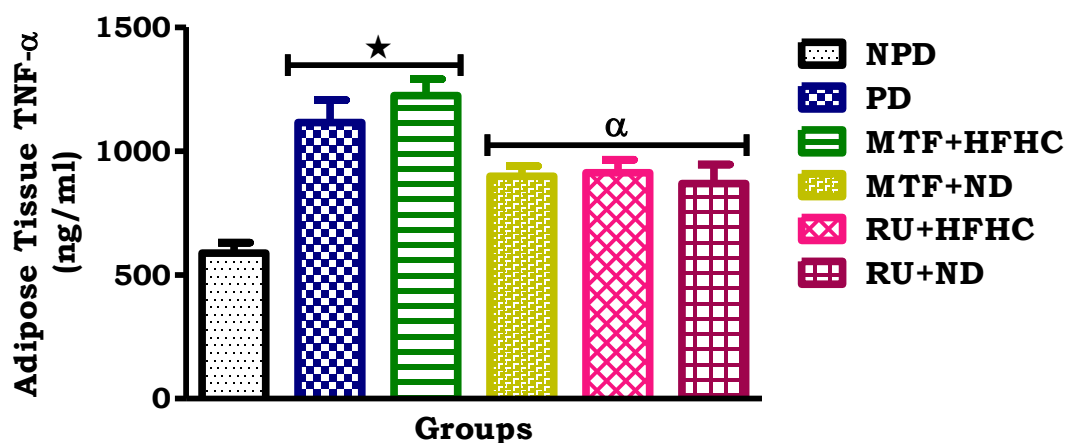

Figure 3: The effects of the metal complex on adipose tissue TNF-α concentration of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

3.6 Effects of ruthenium complex on plasma interleukin-1β (IL-1β) concentration

The PD group showed a significant increase in plasma IL-1β concentrations when compared to the NPD group ($p < 0.05$; Figure 4). Furthermore, the MTF+HFHC and RU+HFHC groups had higher IL-1β concentrations when compared to the NPD group ($p < 0.05$; Figure 4). However, there was a significant reduced in IL-1β levels in the ruthenium complex treated groups coupled with dietary intervention when compared to the PD group ($p < 0.05$; Figure 4). The MTF+ND group showed a similar trend of results when compared to the PD group ($p < 0.05$; Figure 4).
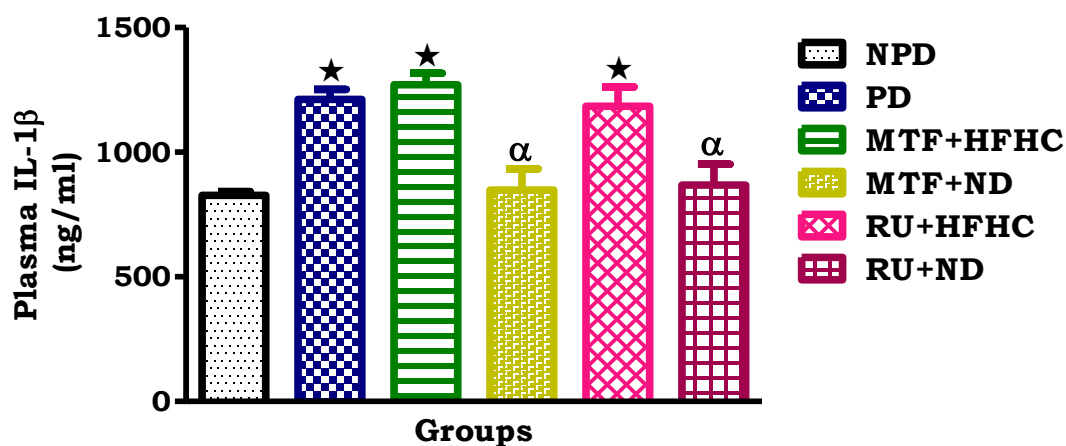
Figure 4: The effects of the ruthenium Schiff-base complex on plasma IL-1β concentration of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

4. Discussion

Metabolism and immune system are commonly regarded as two distinctive mechanisms regulating nutrient disposal and body defense, respectively. Typically, little is known about the coordination and interplay between these two systems in pre-diabetes. However, the current study has led to combination of these distinct entities as studies observe pathological activation of the immune system underlying pre-diabetic [22]. The HFHC diet-induced pre-diabetic rat model was used to assess the effects of a ruthenium(II) Schiff base complex in inflammatory-induced immune response in the presence and absence of dietary intervention.

Platelet functions are directly controlled by insulin through a functional insulin receptor (IR) present on platelet surface [23]. Impaired insulin action is a leading factor for the development of pre-diabetes and has been shown to lead to platelet hyperactivity [24, 25]. Platelet activation is characterized by increased platelets count and MPV [26]. The MPV value is an important marker of platelet activation which gives an indication of platelet size and thus activation of larger platelets represent more activity [13]. Certainly, the results of the current study showed an increased platelet count and MPV in the untreated pre-diabetic rats. These observations were significantly decreased in ruthenium-treated rats. The

ruthenium(II) Schiff base complex has been shown to improve insulin sensitivity and restore glucose homeostasis as evidenced by decrease in glycated haemoglobin levels (HbA1c) in diet-induced pre-diabetes [18]. The research results observed in this study may therefore suggest that the enhanced immune response effect of this complex may, in part, be mediated by its beneficial effects on glucose homeostasis.

Circulatory CD40L (about 95%) is stored in platelets and expressed rapidly on platelet surfaces after activation and thereafter released into the circulation [27]. Expressed CD40L on platelets interacts with cells that constitutively display its receptor CD40 [28]. These cells include endothelial cells, monocytes and macrophages which further provokes a cascade of inflammatory responses, including expression of adhesion factors, expression of tissue factor and release of chemokines and cytokines [28-30]. The pre-diabetic untreated rats had increased plasma CD40L levels compared to the non-prediabetic rats. These findings provide strong evidence of *in vivo* platelet activation and is a major contributor to atherogenesis in diabetic patients [28-30]. The upregulation of CD40L in platelets in our studies could also possibly suggested a mechanism for the increased monocyte tissue factor expression which in turn stimulates thrombosis and platelet release of CD40L, thereby sustaining a vicious cycle of inflammation and thrombosis [30]. Interestingly, treatment with the metallo-drug in the presence or absence of dietary intervention showed decreased plasma CD40L levels in the ruthenium-treated rats. Ruthenium (II)-derived organometallic compound has been reported to potently inhibits platelet aggregation in mice [31]. Therefore, the research results obtained may be attributed to the ameliorated proinflammatory response [17]. The metformin-treated rats displayed similar effects.

Pre-diabetic patients are very susceptible to dyslipidaemia which comprises of increased TGs, elevated low-density lipoprotein cholesterol (LDL-C) and reduced high-density lipoprotein cholesterol (HDL-C) [24, 32]. Hypertriglyceridemia has been reported to increase platelet activation mediated by apolipoprotein E, a content of triglyceride rich very low-density lipoproteins (VLDL) particles [33]. Along with platelet activation, VLDL particles also impair fibrinolysis and disturbs the coagulation cascade, thus resulting in atherothrombotic risk [33]. Taken together, an increase in VLDL concentration was observed in untreated pre-diabetic rats which correlated with an increase in fibrinogen level. Interestingly, the administration of mononuclear complex in the presence and absence of dietary intervention reduced VLDL and TGs levels, whilst in conjunction with dietary intervention there was reduced fibrinogen levels in the ruthenium-treated pre-diabetic rats. Ruthenium complex has been shown to have cardioprotective effects through improved lipid profile in diet-induced prediabetic rats [17]. These results could also possibly suggest that this

metal complex maybe suppressing hormone sensitive lipase which inhibit adipose tissue lipolysis thus reducing circulating TGs and VLDL levels.

White blood cells including granulocytes (neutrophils, eosinophils and basophils), lymphocytes (B and T cells) and monocytes are essential in the innate and adaptive immune response [7]. Monocytes are antigen-presenting cells that possess phagocytic capabilities and play a crucial role in maintaining immune homeostasis and mounting an immune response against infection [34]. The untreated pre-diabetic rats showed increased monocyte count by comparison to the non-prediabetic rats. Monocytes are recruited to adipose tissue where they subsequently mature into adipose tissue macrophages [34]. Once recruited proinflammatory macrophages contribute to secretion of additional chemokines and cytokines thereby initiating a feed-forward loop and potentiating the inflammatory response [34]. Interestingly, treatment with the ruthenium complex in both the presence and absence of dietary intervention resulted in decreased monocyte count. Metal-based complexes have been reported to have an insulin mimic effects [35] Furthermore, we have reported that our ruthenium(II) Schiff base complex improves insulin sensitivity in diet-induced pre-diabetic rats [18]. Insulin influences metabolism of monocytes by increasing oxidized LDL phagocytosis, which causes an increase in glucose utilization and expression of glucose transporters in the plasma membrane [36]. Insulin also regulates monocytes chemokine and cytokine secretion [37]. In addition, insulin also regulates other monocytes activity including superoxide production and expression of tissue factor, thus influencing hypercoagulability [37].

Other immune cell populations in the regulation of inflammatory response in pre-diabetes, includes the granulocytes (neutrophils, eosinophils and basophils) and the lymphocytes (T and B cells) [37]. More than 90% of the granulocytes are neutrophils, which play an important role in the early stages of inflammatory responses [10, 11] . A recent study has provided evidence that neutrophils are involved in insulin resistance through a secreted elastase, which cause reduced insulin signalling, enhanced glucose production and impaired lipid metabolism, thus contributing to insulin resistance [11]. Neutrophils are the first leukocytes to be recruited to the inflammatory site and can eliminate pathogens by multiple mechanisms. Furthermore, stressed adipocytes secrete pro-inflammatory adipokines and cytokines, which attract B cells, T cells and macrophages [3-5]. Both T cells and activated macrophages secrete pro-inflammatory cytokines and chemokines, contributing to the persistence of inflammatory reactions within the tissue and subsequently the underlying potential for autoimmune-mediated β-cell destruction [3-5]. Lymphocytic infiltration in target organs such as the pancreas have been shown to underlines the impression of autoimmune participation in T2DM pathogenesis [3-5]. Furthermore, B

cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies [37]. Diet-induced pre-diabetic rats displayed decreased count of neutrophils and eosinophils with an increased basophils and lymphocytes cell count. Ruthenium complex attenuated the immune response of the granulocyte cells and lymphocytes cell count. The results observed could be attributed to the anti-hypoglycaemic effect of the metal complex [18].

Both clinical and experimental studies have shown that adipose tissue serves as a site of inflammation [1, 3]. Stressed adipocytes produce various cytokines and chemokines promoting immune-cell activation and accumulation in adipose tissue [38]. Continued accumulation of lipids in adipose tissues leads to switching of macrophages from an anti-inflammatory to a pro-inflammatory phenotype [39, 40]. The skew in balance results in an increased secretion of inflammatory molecules that subsequently stimulate the hypertrophied adipocytes resulting into a proinflammatory response [41]. Furthermore, innate immune cells have been reported to secrete chemokines and cytokines such as TNF-α, IL-1β, and IL-6 and further exaggerate the proinflammatory response [7]. Indeed, results of the current study showed a significant increase in adipose tissue TNF-α level in untreated pre-diabetic rats inducing an adipose tissue inflammatory response. However, administration of the ruthenium(II) Schiff base complex in the presence and absence of dietary intervention resulted in reduced adipose tissue TNF-α level in the treated pre-diabetic rats. The metal-base complex has been reported to decrease proinflammatory cytokines such plasma TNF-α, CRP and IL-6 level in diet-induced pre-diabetic rats [17]. Furthermore, ruthenium compounds have been also shown to exhibits effective anti-inflammatory properties via inhibiting inflammatory mediators (NO and iNOS) and pro-inflammatory cytokines (TNF-α and IL-1β) and blocking lipopolysaccharides (LPS)-induced p38 MAPK/p65 phosphorylation, IκBα degradation and p65 nuclear translocation in RAW 264.7 cells [42]. Therefore, these results obtained further correlate with the anti-inflammatory effects of the metal-base complex and its reported antioxidant activity [17, 43].

 IL-1β is another proinflammatory cytokines which mediates auto-inflammatory process resulting in pancreatic β-cell death [44]. Upregulation of IL-1β plays a predominant role as a major cytokine regulating other chemokines and cytokines in islets of T2DM patients [45]. This master cytokine elicits a broader response by recruitment of various immune cells and by induction of IL-1β in β-cells, provoking a vicious inflammatory cycle [46]. Furthermore, several mechanisms like amyloid deposition in islets, presence of long-chain FFAs, and chronic hyperglycaemia have been implicated in β-cell apoptosis [47]. The underlying mechanism for hyperglycaemia-induced β-cell apoptosis is attributed to the glucose-induced IL-1β production that upregulates the Fas receptor [22, 48]. Consequently, induction of pre-diabetes by the HFHC

diet characterised by abundant saturated FFAs that have direct lipotoxic effects on β-cells by inducing ER stress and ROS, resulted in high plasma IL-1β levels in the pre-diabetic rats [22, 48]. The increased plasma IL-1β levels were stabilized in ruthenium-treated pre-diabetic rats in the presence of dietary intervention. These findings can be ascribed to the metal-complex along with dietary intervention enhancing β-cell function thus improving glycaemic control. In addition, studies have shown that blockade of IL-1 has been associated with reduced hyperglycaemia, improved β-cell function and reduced expression of inflammatory markers [49, 50]. Furthermore, ruthenium complexes have been shown to inhibit human islet amyloid polypeptide (hIAPP) aggregation which cause dysfunction and death of pancreatic β-cells thus preventing β-cell apoptosis *in vitro* [51].

5. Conclusion

In this study, it has been demonstrated that the ruthenium(II) diimine uracil complex exhibits effective anti-inflammatory properties via the reduction of pro-inflammatory cytokines (TNF-α and IL-1*β*), platelet activation markers (MPV and CD40L) and fibrinogen. There was also a reduction in TG and VLDL levels as well as improved immune cell counts in HFHC diet-induced pre-diabetic rats. The findings of the current study further demonstrated the potential of this metal-based compound in both the presence and absence of dietary modification. Therefore, ruthenium(II) diimine uracil complex may be a potential therapeutic agent for treating pre-diabetic inflammatory-induced immune dysregulation. However, further studies are still required to find out the exact mechanism behind potential effect of this metal-based compound.

Conflicts of Interest

None

Authors' contributions: Mabuza LP and Gamede MW were involved in study design, *in vivo* studies, data analysis and assisted in preparing the manuscript. Maikoo S and Booysen IN synthesised and spectroscopically characterized the free-ligand and its ruthenium complex which was utilised as the metal-based drug. Booysen IN proof-read the manuscript.

References

1.   Hameed I, Masoodi S, Mir S, Nabi M, Ghazanfar K, Ganai B. Type 2 diabetes mellitus: from a metabolic disorder to an inflammatory condition. *World journal of diabetes* 2015; 6: 598.

2.   Winer S, Winer D. The adaptive immune system as a fundamental regulator of adipose tissue inflammation and insulin resistance. *Immunology and cell biology* 2012; 90: 755-762.

3.   Donath M, Shoelson S. Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology* 2011; 11: 98.

4.   Hotamisligil G. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* 2010; 140: 900-917.

5.   Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg A, Obin M. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of lipid research* 2005; 46: 2347-2355.

6.   Prieur X, Mok C, Velagapudi V, Núñez V, Fuentes L, Montaner D, Ishikawa K, Camacho A, Barbarroja N, O'Rahilly S. Differential lipid partitioning between adipocytes and tissue macrophages modulates macrophage lipotoxicity and M2/M1 polarization in obese mice. *Diabetes* 2011; 60: 797-809.

7.   Grossmann V, Schmitt V, Zeller T, Panova-Noeva M, Schulz A, Laubert-Reh D, Juenger C, Schnabel R, Abt T, Laskowski R. Profile of the immune and inflammatory response in individuals with prediabetes and type 2 diabetes. *Diabetes Care* 2015; 38: 1356-1364.

8.   McArdle M, Finucane O, Connaughton R, McMorrow A, Roche H. Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies. *Frontiers in endocrinology* 2013; 4: 52.

9.   Mzimela N, Ngubane P, Khathi A. The changes in immune cell concentration during the progression of pre-diabetes to type 2 diabetes in a high-fat high-carbohydrate diet-induced pre-diabetic rat model. *Autoimmunity* 2019; 52: 27-36.

10.   Goh Y, Henderson N, Heredia J, Eagle A, Odegaard J, Lehwald N, Nguyen K, Sheppard D, Mukundan L, Locksley R. Eosinophils secrete IL-4 to facilitate liver regeneration. *Proceedings of the National Academy of Sciences* 2013; 110: 9914-9919.

11.   Talukdar S, Bandyopadhyay G, Li D, Xu J, McNelis J, Lu M, Li P, Yan Q, Zhu Y, Ofrecio J. Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nature medicine* 2012; 18: 1407.

12.   Vizioli L, Muscari S, Muscari A. The relationship of mean platelet volume with the risk and prognosis of cardiovascular diseases. *International journal of clinical practice* 2009; 63: 1509-1515.

13.   Kodiatte T, Manikyam U, Rao S, Jagadish T, Reddy M, Lingaiah HK, Lakshmaiah V. Mean platelet volume in type 2 diabetes mellitus. *Journal of laboratory physicians* 2012; 4: 5.

14.   Undas A, Wiek I, Stêpień E, Zmudka K, Tracz W. Hyperglycemia is associated with enhanced thrombin formation, platelet activation, and fibrin clot resistance to lysis in patients with acute coronary syndrome. *Diabetes care* 2008; 31: 1590-1595.

15.   Evert A, Boucher J, Cypress M, Dunbar S, Franz M, Mayer-Davis E, Neumiller J, Nwankwo R, Verdi C, Urbanski P. Nutrition therapy recommendations for the management of adults with diabetes. *Diabetes care* 2014; 37: S120-S143.

16.   Giacco R, Vetrani C, Griffo E, Rivellese A. Role of Diet and Diet Interventions in Diabetic Patients: Physiological and Metabolic Changes and Reduction in Morbidity and Mortality. *Current Nutrition Reports* 2013; 2: 174-180.

17.   Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P, Khathi A. Cardioprotective effects of a ruthenium (II) Schiff base complex in a diet-induced prediabetic rats. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 2019; 12: 1-7.

18.   Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P, Khathi A. Effects of a ruthenium schiff base complex on glucose homeostasis in diet-induced pre-diabetic rats *Molecules (Basel, Switzerland)* 2018; 23: E1721.

19.   Ciftci O, Beytur A, Vardi N, Ozdemir I. Evaluation of reproductive toxicity in male rats treated with novel synthesized ruthenium(II) and gold(I)-NHC complexes. *Drug Development and Industrial Pharmacy* 2012; 381: 40-46.

20.   Booysen I, Maikoo S, Akerman M, Xulu B. Novel ruthenium (II) and (III) compounds with multidentate Schiff base chelates bearing biologically significant moieties. *Polyhedron* 2014; 79: 250-257.

21.   Luvuno M, Mabandla M, Khathi A. Voluntary ingestion of a high-fat high-carbohydrate diet: A model for prediabetes. *Ponte international scientific researchs journal* 2018; 74.

22.   Donath M, Dalmas É, Sauter N, Böni-Schnetzler M. Inflammation in obesity and diabetes: islet dysfunction and therapeutic opportunity. *Cell metabolism* 2013; 17: 860-872.

23.   Kakouros N, Rade JJ, Kourliouros A, Resar JR. Platelet function in patients with diabetes mellitus: from a theoretical to a practical perspective. *International journal of endocrinology* 2011; 2011.

24.   Ferreiro J, Gómez-Hospital J, Angiolillo D. Platelet abnormalities in diabetes mellitus. *Diabetes and vascular disease research* 2010; 7: 251-259.

25.   Gaiz A, Mosawy S, Colson N, Singh I. Thrombotic and cardiovascular risks in type two diabetes; Role of platelet hyperactivity. *Biomedicine & pharmacotherapy* 2017; 94: 679-686.

26.   Buch A, Kaur S, Nair R, Jain A. Platelet volume indices as predictive biomarkers for diabetic complications in Type 2 diabetic patients. *Journal of laboratory physicians* 2017; 9: 84.

27.   Vaidyula V, Rao A, Mozzoli M, Homko C, Cheung P, Boden G. Effects of hyperglycemia and hyperinsulinemia on circulating tissue factor procoagulant activity and platelet CD40 ligand. *Diabetes* 2006; 55: 202-208.

28.   Seijkens T, Kusters P, Engel D, Lutgens E. CD40–CD40L: Linking pancreatic, adipose tissue and vascular inflammation in type 2 diabetes and its complications. *Diabetes and Vascular Disease Research* 2013; 10: 115-122.

29.   Neubauer H, Setiadi P, Günesdogan B, Pinto A, Börgel J, Mügge A. Influence of glycaemic control on platelet bound CD40–CD40L system, P-selectin and soluble CD40 ligand in Type 2 diabetes. *Diabetic Medicine* 2010; 27: 384-390.

30.   Zhang B, Wu T, Chen M, Zhou Y, Yi D, Guo R. The CD40/CD40L system: a new therapeutic target for disease. *Immunology letters* 2013; 153: 58-61.

31. Hsia C, Velusamy M, Sheu J, Khamrang T, Jayakumar T, Lu W, Lin K, Chang C. A novel ruthenium (II)-derived organometallic compound, TQ-6, potently inhibits platelet aggregation: Ex vivo and in vivo studies. *Scientific reports* 2017; 7: 9556.

32. Kaur R, Kaur M, Singh J. Endothelial dysfunction and platelet hyperactivity in type 2 diabetes mellitus: molecular insights and therapeutic strategies. *Cardiovascular diabetology* 2018; 17: 121.

33. Yang Y, Andrews M, Hu Y, Wang D, Qin Y, Zhu Y, Ni H, Ling W. Anthocyanin extract from black rice significantly ameliorates platelet hyperactivity and hypertriglyceridemia in dyslipidemic rats induced by high fat diets. *Journal of agricultural and food chemistry* 2011; 59: 6759-6764.

34. Inzucchi S, Bergenstal R, Buse J, Diamant M, Ferrannini E, Nauck M, Peters A, Tsapas A, Wender R, Matthews D. Management of hyperglycemia in type 2 diabetes, 2015: a patient-centered approach: update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes care* 2015; 38: 140-149.

35. Leung C, Lin S, Zhong H, Ma D. Metal complexes as potential modulators of inflammatory and autoimmune responses. *Chemical science* 2015; 6: 871-884.

36. Sarigianni M, Bekiari E, Tsapas A, Topouridou K, Kaloyianni M, Koliakos G, Paletas K. Effect of glucose and insulin on oxidized low-density lipoprotein phagocytosis by human monocytes: a pilot study. *International journal of endocrinology* 2011; 62(2): 163-166.

37. Winer D, Winer S, Shen L, Wadia P, Yantha J, Paltser G, Tsui H, Wu P, Davidson M, Alonso M. B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. *Nature medicine* 2011; 17: 610.

38. Fang L, Guo F, Zhou L, Stahl R, Grams J. The cell size and distribution of adipocytes from subcutaneous and visceral fat is associated with type 2 diabetes mellitus in humans. *Adipocyte* 2015; 4: 273-279.

39. Ye J. Mechanisms of insulin resistance in obesity. *Frontiers of medicine* 2013; 7: 14-24.

40. Griffin C, Eter L, Lanzetta N, Abrishami S, Varghese M, McKernan K, Muir L, Lane J, Lumeng C, Singer K. TLR4, TRIF, and MyD88 are essential for myelopoiesis and CD11c+ adipose tissue macrophage production in obese mice. *Journal of Biological Chemistry* 2018; 293: 8775-8786.

41. Huang S, Rutkowsky J, Snodgrass R, Ono-Moore K, Schneider D, Newman J, Adams S, Hwang D. Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. *Journal of lipid research* 2012; 53: 2002-2013.

42. Hsia C, Velusamy M, Jayakumar T, Chen Y, Hsia C, Tsai J, Teng R, Sheu J. Mechanisms of TQ-6, a Novel Ruthenium-Derivative Compound, against Lipopolysaccharide-Induced In Vitro Macrophage Activation and Liver Injury in Experimental Mice: The Crucial Role of p38 MAPK and NF-κB Signaling. *Cells* 2018; 7: 217.

43. Antonyan A, De A, Vitali L, Pettinari R, Marchetti F, Gigliobianco M, Pettinari C, Camaioni E, Lupidi G. Evaluation of (arene) Ru (II) complexes of curcumin as inhibitors of dipeptidyl peptidase IV. *Biochimie* 2014; 99: 146-152.

44. Kim J, Yoon K. Glucolipotoxicity in pancreatic β-cells. *Diabetes & metabolism journal* 2011; 35: 444-450.

45. Fang F, Jiang D. IL-1β/HMGB1 signalling promotes the inflammatory cytokines release via TLR signalling in human intervertebral disc cells. *Bioscience reports* 2016; 36: e00379.

46. Mahdi T, Hänzelmann S, Salehi A, Muhammed S, Reinbothe T, Tang Y, Axelsson A, Zhou Y, Jing X, Almgren P. Secreted frizzled-related protein 4 reduces insulin secretion and is overexpressed in type 2 diabetes. *Cell metabolism* 2012; 16: 625-633.

47. Shirakawa J, Fujii H, Ohnuma K, Sato K, Ito Y, Kaji M, Sakamoto E, Koganei M, Sasaki H, Nagashima Y. Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. *Diabetes* 2011; 60: 1246-1257.

48. Wali J, Masters S, Thomas H. Linking metabolic abnormalities to apoptotic pathways in Beta cells in type 2 diabetes. *Cells* 2013; 2: 266-283.

49. Esser N, Legrand-Poels S, Piette J, Scheen A, Paquot N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes research and clinical practice* 2014; 105: 141-150.

50. Vallejo S, Palacios E, Romacho T, Villalobos L, Peiró C, Sánchez-Ferrer C. The interleukin-1 receptor antagonist anakinra improves endothelial dysfunction in streptozotocin-induced diabetic rats. *Cardiovascular diabetology* 2014; 13: 158.

51. Lijuan M, Yuanting F, Lianling Y, Xiaoling L, Wenjie Z, Tianfeng C. Ruthenium complexes as inhibitors of human islet amyloid polypeptide aggregation, an effect that prevents beta cell apoptosis. *Royal Society of Chemistry* 2015; 5: 17405–17412.

Tables

Table 1: Effects of ruthenium complex on platelet count, mean platelet volume and CD40L concentration of pre-diabetic treated animals for 12 weeks period. Values are presented as means ± SEM ($n = 6$) in each group.

Groups	Platelet Count ($10^3/\mu L$)	Mean Platelet Volume (fL)	CD40L (ng/ml)
NPD	360.30±4.20	7.28±0.43	1.94±0.17
PD	1279.00±8.00*	12.90±0.10*	5.90±0.20*
MTF+HFHC	735.50±4.90*$^{\alpha}$	7.35±0.15$^{\alpha}$	4.25±0.38*
MTF+ND	190.00±3.00$^{\alpha}$	6.55±0.050$^{\alpha}$	1.70±0.35$^{\alpha}$
RU+HFHC	641.50±3.40*$^{\alpha}$	7.32±0.18$^{\alpha}$	3.40±0.15*$^{\alpha}$
RU+ND	150.70±3.70$^{\alpha}$	7.40±0.28$^{\alpha}$	1.61±0.26$^{\alpha}$

* $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Table 2: Effects of ruthenium complex on plasma TGs and VLDL concentration of pre-diabetic treated animals for 12 weeks period. Values are presented as means ± SEM ($n = 6$) in each group.

Groups	Plasma TGs (mmol/L)	VLDL (mmol/L)
NPD	1.22±0.31	0.55±0.090
PD	3.34±0.21*	1.52±0.81*
MTF+HFHC	5.62±0.27*	2.55±0.98*
MTF+ND	0.91±0.042[α]	0.41±0.043[α]
RU+HFHC	1.87±0.14[α]	0.85±0.032[α]
RU+ND	1.17±0.012[α]	0.53±0.012[α]

* $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).
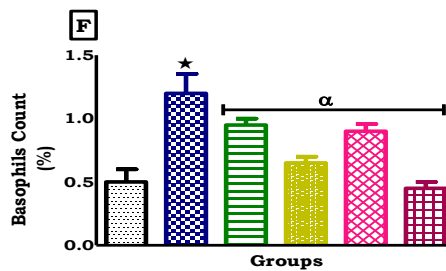
Figure legends

Figure 1: The effects of the ruthenium Schiff-base complex on plasma fibrinogen concentration of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. * $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).
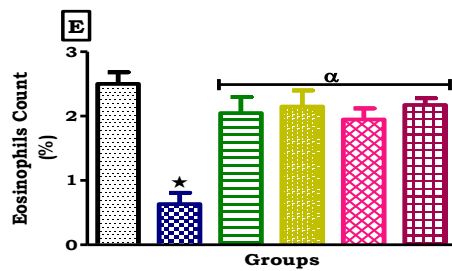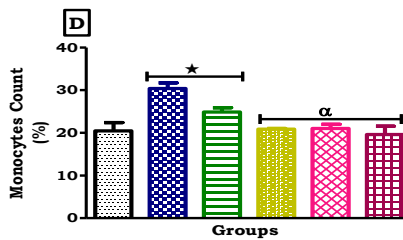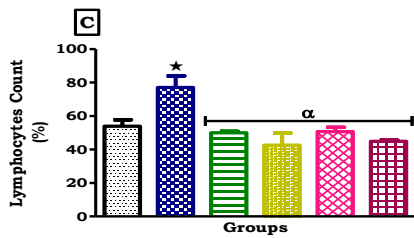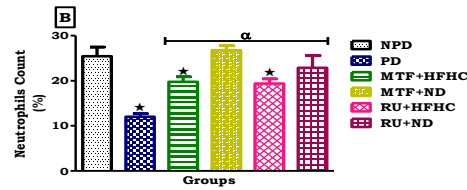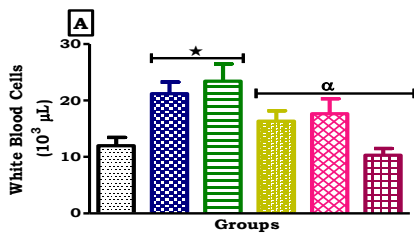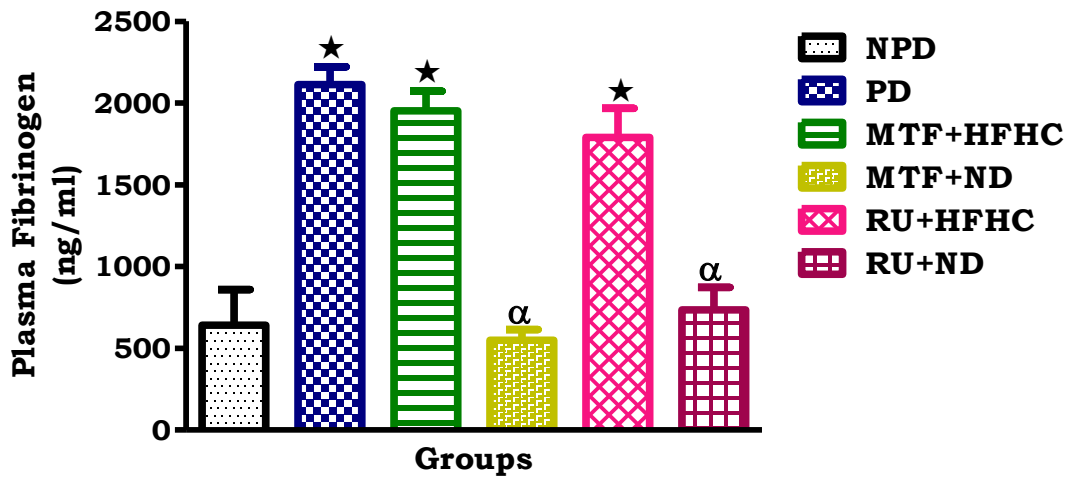
Figure 2: The effects of the ruthenium Schiff-base complex on A) White blood cell B) neutrophils, C) lymphocytes, D) monocytes, E) eosinophils and F) basophils immune cell count of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. * $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Figure 3: The effects of the metal complex on adipose tissue TNF-α concentration of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. * $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Figure 4: The effects of the ruthenium Schiff-base complex on plasma IL-1β concentration of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. * $p < 0.05$ compared to non-prediabetic control (NPD), α $p < 0.05$ compared to pre-diabetic control group (PD); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).
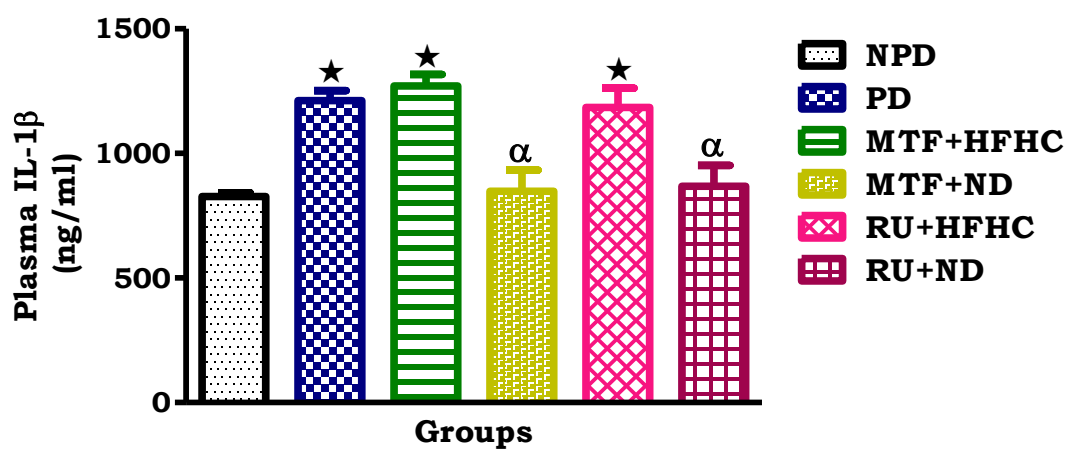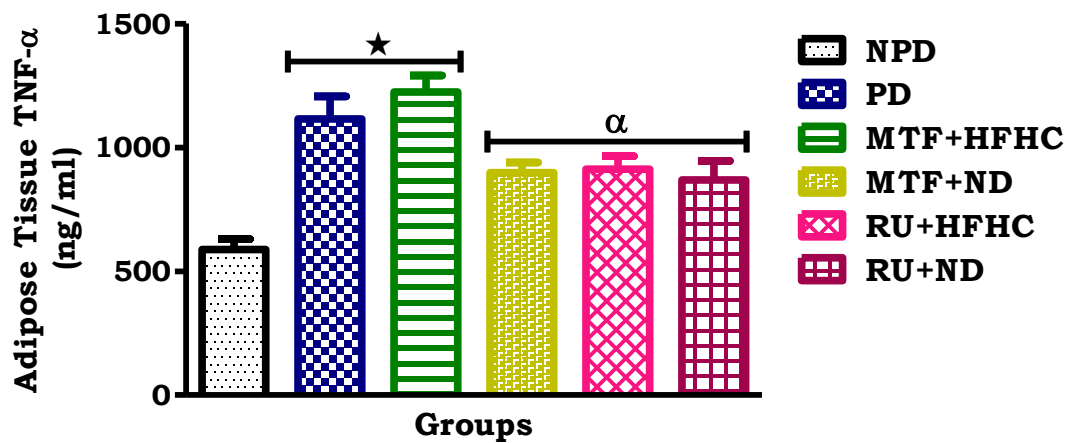
Figures

CHAPTER 5: SYNTHESIS

For every onset of T2DM there is a precedence of pre-diabetes, an intermediate hyperglycaemic state where blood glucose levels are above the upper threshold for normal but below the threshold for a diagnosis for diabetes mellitus (Tabák *et al.*, 2012; Hostalek, 2019). When faced with the hyperglycaemia due to insulin resistance as observed in pre-diabetes (Maschio *et al.*, 2016; Shirakawa and Kulkarni, 2016). The pancreatic pancreatic β cell try to compensate the increased glucose levels by producing increase insulin secretion (hyperinsulinaemia). The increased insulin secretion and expansion of pancreatic β cell mass work together to maintain normal glucose levels under the developed insulin resistance (Maschio *et al.*, 2016; Shirakawa and Kulkarni, 2016). Changes in glucose concentration have long been known to have profound effects upon the rates of insulin secretion and β cell mass. Therefore, these changes eventually lead to β cell exhaustion, causing decreased insulin secretion. Hence, developing T2DM (Maschio *et al.*, 2016; Shirakawa and Kulkarni, 2016). As the disease progresses, tissues or vascular damage ensues leading to severe diabetic macrovascular and microvascular complications (Tabák *et al.*, 2012; Hostalek, 2019). Thus, pre-diabetes covers a wide range of heterogeneous diseases (Laaksonen *et al.*, 2005; Giacco *et al.*, 2013; Evert *et al.*, 2014). Once diagnosed with pre-diabetes, preventative strategies include dietary and lifestyle modification (Giacco *et al.*, 2013). Nevertheless, patients tend to rely on the pharmacotherapy and neglect lifestyle modification and thus reduce the efficacy of the drugs (Bailey and Kodack, 2011; Ahmad *et al.*, 2013). Therefore, there is a necessity to develop novel drugs that can be therapeutic even in the absence of dietary intervention. Current studies have reported that ruthenium(II) uracil-derived diimine complex with a change in dietary intervention had beneficial effects as it restored plasma ghrelin concentrations, leading to a reduction in caloric intake and body weight gain when administered to the diet-induced pre-diabetic rats. Furthermore, these previous findings were accompanied by restored glucose tolerance as well as HbA1c concentration, indicating that the metal complex restores insulin sensitivity in a diet-induced pre-diabetic rat model and delays the onset of T2DM in the presence and absence of dietary intervention (Mabuza *et al.*, 2018). In addition, this metal complex has previously been shown to possess cardioprotective effects through restored lipid profile derangement and cardiac inflammatory cytokines in diet-induced pre-diabetic rats (Mabuza *et al.*, 2019).

In the current study, we investigated the therapeutic properties of a novel ruthenium(II) uracil-derived diimine complex on hepatic, renal and immune dysregulation prediabetic-related complications in both the presence and absence of dietary intervention. We evaluated whether the improved insulin sensitivity following administration of this compound will further ameliorate these prediabetic-related complications in a diet-induced pre-diabetic rat model.

The liver plays a major role in the regulation of glucose and lipid metabolism. During derangements such as NAFLD, the liver becomes compromised (Speliotes *et al.*, 2010; Hsieh and Hsieh, 2011; Hamed *et al.*, 2015). The induction of pre-diabetes by HFHC diet resulted in increased blood glucose levels in the pre-diabetic rats. This in turn resulted in increased hepatic glycogen concentration. Treatment with the ruthenium (II) complex in conjunction with dietary intervention restored hepatic glycogen concentration through the reduction in blood glucose in the ruthenium-treated rats. This metal complex has been previously reported to improve glycaemic control (Antonyan *et al.*, 2014; Mabuza *et al.*, 2018). Increased consumption of the high-fat high carbohydrate (HFHC) diet is known to increase plasma AST and ALT liver enzymes through the induction of ROS which further triggers the inflammatory response mechanism (Mohamed *et al.*, 2016). However, with the results obtained, there was no significant change in ALT levels between the NPD and PD rats. For the first time, the current study shows that age influences liver enzyme ALT. These observations were in line with other research studies who demonstrated that oxidative stress increases with age and induced the inflammatory response mechanism (Hamden *et al.*, 2009; Kim *et al.*, 2015). Moreover, metformin treatment resulted in increased plasma ALT levels inducing liver injury. Administration of ruthenium(II) complex in both the presence and absence of dietary intervention maintained a steady weight gain which resulted in reduced liver weight in the ruthenium-treated rats. These results in turn restored plasma AST and ALT levels in the ruthenium-treated rats. The hepatoprotective effects of the ruthenium(II) uracil-derived diimine complex was further evidenced by a significant increased total bilirubin concentration in the ruthenium-treated rats.

Insulin resistance is a prime factor in the development of pre-diabetes related complications and plays a key role in hepatic lipid accumulation and increased adipose tissue lipolysis (Bugianesi *et al.*, 2010). The induction of pre-diabetes by the HFHC diet resulted in activation of *de novo* lipogenesis and the molecular basis of this mechanism has been shown to involve SREBP-1c (Regnell and Lernmark, 2011; Mavrogiannaki and Migdalis, 2013). The findings of the study showed that the PD group had increased SREBP-1c concentration which was associated with an increase in plasma TGs and VLDL concentrations in the untreated rats. However, administration of the metal complex coupled with dietary intervention decreased SREBP-1c, TGs and VLDL concentrations in the ruthenium-treated rats. These results were further evidenced by histological analysis, showing reduced hepatic lipid droplet accumulation, hepatocyte ballooning and locular disarray in the ruthenium-treated rats, proposing a hepatoprotective effect of ruthenium(II) complex in the treatment of NAFLD complication as seen in manuscript 1, chapter 2 of the study. In contrast, metformin treatment displayed similar results but only when used in conjunction with dietary intervention.

Chronic, low-grade adipose tissue inflammation is a key etiological mechanism linking the increasing incidence of T2DM and pre-diabetes and is found to be activated before the onset of diabetes (Makki *et al.*, 2013). Recent studies have reported the integration between the immune system and metabolism in pre-diabetic state (Makki *et al.*, 2013; Grossmann *et al.*, 2015). The macrophages, in particular, have been identified as critical effector cells in the initiation of inflammation and insulin resistance (Donath and Shoelson, 2011; Hameed *et al.*, 2015). The continuous elevation of lipids accumulation in the adipose tissues, macrophage recruitment and retention to adipose tissue and the participation of other immune cell populations have been shown to lead to the progression of an inflammatory response mechanism indicated by cytokines secretion (Donath and Shoelson, 2011; Hameed *et al.*, 2015). Indeed, the current findings showed that the PD group had significantly increased adipose tissue TNF-α and plasma IL-1β cytokines concentrations. Ruthenium complex ameliorated the increased adipose tissue TNF-α concentration while showing a decreased plasma IL-1β cytokines released in the prediabetic-treated rats in conjunction with a change in the diet. These findings were in line with other research studies, which reported the anti-inflammatory properties of ruthenium via inhibiting these aforementioned cytokines (Hsia *et al.*, 2018; Mabuza *et al.*, 2019). Furthermore, stressed adipocytes have been shown to recruit immune cells to the liver which further secrete chemokines and cytokines, thereby contributing to the development of insulin resistance (Fang *et al.*, 2015). Certainly, diet-induced pre-diabetic rats displayed immune cell count dysregulation and platelet hyperactivity, characterised by increased platelets count, MPV and CD40L concentrations (Ferreiro *et al.*, 2010; Gaiz *et al.*, 2017). Interestingly, ruthenium(II) uracil-derived diimine complex in both the presence and absence of dietary intervention attenuated immune cell count dysregulation and platelet activation markers. A study by Hsia *et al* reported that a ruthenium(II)-derived organometallic compound potently inhibits platelet aggregation in mice (Hsia *et al.*, 2017). Our findings correlated with their findings. In addition, treatment with metformin showed similar results. Furthermore, the observed hypertriglyceridemia and platelet activation in the pre-diabetic rats impaired fibrinolysis and disturbed the coagulation cascade. These observations were demonstrated by the increased fibrinogen concentration in the untreated pre-diabetic rats. Interestingly, the administration of ruthenium(II) uracil-derived diimine complex only with dietary intervention reduced fibrinogen concentration and platelet activation, suggesting that ruthenium complex is effective in reducing fibrinogen level with a change in diet. A comparable trend was observed with metformin treatment. Taken together, administration of the ruthenium(II) complex exhibited an anti-inflammatory activity and inhibited platelet aggregation thus preventing the progression of immune dysregulation underlying pre-diabetes as seen in manuscript 3, in chapter 4 of the study.

Pre-diabetes is a progressive disease to overt T2DM and studies have shown that newly diagnosed pre-diabetic patients already have kidney damage (Tabák *et al.*, 2012; Pecoits-Filho *et al.*, 2016). This suggests that the onset of DN may occur during the early stages of the disease progression. Therefore, the key to prevention of diabetes development and its vascular complications is early detection and treatment of pre-diabetes. The prolonged moderate hyperglycaemia observed in pre-diabetic patients evoked the progression of DN and found to lead to the activation of RAAS and aldosterone secretion which results in sodium and water retention (Yilmaz *et al.*, 2015; Saravanan and Pari, 2016). Indeed, untreated PD group displayed impaired kidney function markers evidenced by increased 24 h fluid intake, urine output and aldosterone levels which correlated with impaired electrolytes balance and fractional excretion of both Na^+ and K^+ throughout the 12 weeks treatment period. However, the increased fluid intake, urine output and aldosterone together with impaired electrolytes balance in the pre-diabetic rats were attenuated by administration of ruthenium(II) complex in both the presence and absence of dietary intervention. In contrast, metformin treatment was effective in reducing aldosterone concentration only in conjunction with dietary intervention. The sustained hyperglycaemia further induced oxidative stress and resulted in decreased antioxidant enzymes activity (Fiorentino *et al.*, 2013). Interestingly, administration of ruthenium(II) complex coupled with dietary intervention afforded to attenuate the induced oxidative stress evidenced by decreased MDA concentration with increased antioxidants enzymes (SOD and GPx) concentrations in the ruthenium-treated rats, protecting the kidneys from oxidative stress. In addition, administration of metformin exhibited comparable results. We further evaluated the AER, ACR, CC as they are used as markers of renal function and used to define kidney disease. As expected, the PD group had increased urinary albumin with decreased urinary creatinine and urea concentrations which correlated with decreased plasma albumin and increased plasma creatinine and urea concentrations. These observations were further demonstrated by the increased AER and ACR levels with decreased CC level, indicating renal injury in the untreated pre-diabetic rats. Interestingly, the metal complex ameliorated this pre-diabetes associated derangements exhibiting a renoprotective effect. Furthermore, the observed renal injury was further evaluated by measuring KIM-1, a useful biomarker for renal proximal tubule injury, histopathological analysis of the kidney and at the expression of mRNA podocin levels in urine as they facilitating the early diagnosis of the disease (Carlsson *et al.*, 2014; El-Ashmawy *et al.*, 2015). Podocyte loss and injury are involved in the development of proteinuria in DN marked with increased urinary albumin (Zheng *et al.*, 2011; Fukuda *et al.*, 2012). Indeed, the observed increased urinary albumin in the untreated prediabetic rats was related to the increased KIM-1 and expression of mRNA podocin levels indicating the impaired function of the glomerular filtration barrier (Zheng *et al.*, 2011; Fukuda *et al.*, 2012). However, the current study

showed for the first time that the attenuation of the pre-diabetic associated increased KIM-1 concentration by the administration of ruthenium(II) complex in both the presence and absence of dietary intervention interrelated with the decreased expression of mRNA podocin level in urine and improved histological structure of the glomerulus in the ruthenium-treated rats. This could be, in part, mediated by the improved insulin sensitivity brought about by the metal complex (Mabuza *et al.*, 2018). Furthermore, metformin treatment showed comparable observation in conjunction of dietary intervention in terms of decreasing KIM-1 concentration in the pre-diabetic treated rats.

CONCLUSION

In conclusion, the administration of ruthenium(II) uracil-derived diimine complex showed beneficial effects as it ameliorated and prevented the progression of diabetes-related liver derangements while eliminating the hepatotoxicity associated with the use of metal compounds, as seen in manuscript 1, ameliorated renal function while preventing the progression of DN in prediabetic-treated rats as seen in manuscript 2 and exhibited an anti-inflammatory activity and inhibited platelet aggregation thus preventing the progression of immune dysregulation underlying pre-diabetes, as seen in manuscript 3. Taken together, the observations of the current study further demonstrated the potential of this metal-based compound in both the presence and absence of dietary modification. Therefore, ruthenium(II) diimine uracil complex may be a potential therapeutic agent for treating pre-diabetic related complication. However, further studies are still required to find out the exact mechanism behind potential effect of this metal-based compound.

SHORTFALLS AND FUTURE STUDIES

Lifestyle modification is not limited to dietary intervention, but it also involves increased physical activity such as exercise. However, the current study only evaluated dietary intervention as one of pre-diabetic preventative strategies. Therefore, we recommend that in the future, we should investigate the effect of physical activity as a strategy in the prevention and management of pre-diabetes progression and link it with dietary intervention. In addition, for future studies, we recommend that we evaluate the gene and molecular protein level behind the therapeutic potential effect of this metal-based compound, ruthenium(II) uracil-derived diimine complex.

References

1 Ahmad N, Ramli A, Islahudin F and Paraidathathu T. 2013. Medication adherence in patients with type 2 diabetes mellitus treated at primary health clinics in Malaysia. *Patient preference and adherence;* 7**:** 525.

2 Antonyan A, De A, Vitali L, Pettinari R, Marchetti F, Gigliobianco M, Pettinari C, Camaioni E and Lupidi G. 2014. Evaluation of (arene) Ru (II) complexes of curcumin as inhibitors of dipeptidyl peptidase IV. *Biochimie;* 99**:** 146-152.

3 Bailey C and Kodack M. 2011. Patient adherence to medication requirements for therapy of type 2 diabetes. *International journal of clinical practice;* 65(3)**:** 314-322.

4 Bugianesi E, Moscatiello S, Ciaravella M and Marchesini G. 2010. Insulin resistance in nonalcoholic fatty liver disease. *Current pharmaceutical design;* 16(17)**:** 1941-1951.

5 Carlsson A, Calamia M, Risérus U, Larsson A, Helmersson-Karlqvist J, Lind L and Ärnlöv J. 2014. Kidney injury molecule (KIM)-1 is associated with insulin resistance: Results from two community-based studies of elderly individuals. *Diabetes research and clinical practice;* 103(3)**:** 516-521.

6 Donath M and Shoelson S. 2011. Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology;* 11(2)**:** 98.

7 El-Ashmawy N, El-Zamarany E, Khedr N, El-Fattah A and Eltoukhy S. 2015. Kidney injury molecule-1 (Kim-1): an early biomarker for nephropathy in type II diabetic patients. *International Journal of Diabetes in Developing Countries;* 35(3)**:** 431-438.

8 Evert A, Boucher J, Cypress M, Dunbar S, Franz M, Mayer-Davis E, Neumiller J, Nwankwo R, Verdi C and Urbanski P. 2014. Nutrition therapy recommendations for the management of adults with diabetes. *Diabetes care;* 37(Supplement 1)**:** S120-S143.

9 Fang L, Guo F, Zhou L, Stahl R and Grams J. 2015. The cell size and distribution of adipocytes from subcutaneous and visceral fat is associated with type 2 diabetes mellitus in humans. *Adipocyte;* 4(4)**:** 273-279.

10 Ferreiro J, Gómez-Hospital J and Angiolillo D. 2010. Platelet abnormalities in diabetes mellitus. *Diabetes and vascular disease research;* 7(4)**:** 251-259.

11 Fiorentino V, Prioletta A, Zuo P and Folli F. 2013. Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Current pharmaceutical design;* 19(32)**:** 5695-5703.

12 Fukuda A, Wickman L, Venkatareddy M, Wang S, Chowdhury M, Wiggins J, Shedden K and Wiggins R. 2012. Urine podocin: nephrin mRNA ratio (PNR) as a podocyte stress biomarker. *Nephrology Dialysis Transplantation;* 27(11)**:** 4079-4087.

13 Gaiz A, Mosawy S, Colson N and Singh I. 2017. Thrombotic and cardiovascular risks in type two diabetes; Role of platelet hyperactivity. *Biomedicine & pharmacotherapy;* 94**:** 679-686.

14 Giacco R, Vetrani C, Griffo E and Rivellese A. 2013. Role of Diet and Diet Interventions in Diabetic Patients: Physiological and Metabolic Changes and Reduction in Morbidity and Mortality. *Current Nutrition Reports;* 2(4)**:** 174-180.

15 Grossmann V, Schmitt V, Zeller T, Panova-Noeva M, Schulz A, Laubert-Reh D, Juenger C, Schnabel R, Abt T and Laskowski R. 2015. Profile of the immune and inflammatory response in individuals with prediabetes and type 2 diabetes. *Diabetes Care;* 38(7)**:** 1356-1364.

16   Hamden K, Carreau S, Ayadi F, Masmoudi H and Abdelfattah E. 2009. Inhibitory effect of estrogens, phytoestrogens, and caloric restriction on oxidative stress and hepato-toxicity in aged rats. *Biomedical and Environmental Sciences;* 22(5)**:** 381-387.

17   Hamed A, Abas B, Shaltout I, Esmt G and Gomez R. 2015. Managing diabetes and liver disease association, guidelines (consensus) development. *Journal of Endocrinology Diabetes Obesesity;* 3**:** 1-19.

18   Hameed I, Masoodi S, Mir S, Nabi M, Ghazanfar K and Ganai B. 2015. Type 2 diabetes mellitus: from a metabolic disorder to an inflammatory condition. *World journal of diabetes;* 6(4)**:** 598.

19   Hostalek U. 2019. Global epidemiology of prediabetes-present and future perspectives. *Clinical diabetes and endocrinology;* 5(1)**:** 5.

20   Hsia C, Velusamy M, Sheu J, Khamrang T, Jayakumar T, Lu W, Lin K and Chang C. 2017. A novel ruthenium (II)-derived organometallic compound, TQ-6, potently inhibits platelet aggregation: Ex vivo and in vivo studies. *Scientific reports;* 7(1)**:** 9556.

21   Hsia C, Velusamy M, Jayakumar T, Chen Y, Hsia C, Tsai J, Teng R and Sheu J. 2018. Mechanisms of TQ-6, a Novel Ruthenium-Derivative Compound, against Lipopolysaccharide-Induced In Vitro Macrophage Activation and Liver Injury in Experimental Mice: The Crucial Role of p38 MAPK and NF-κB Signaling. *Cells;* 7(11)**:** 217.

22   Hsieh P and Hsieh Y. 2011. Impact of liver diseases on the development of type 2 diabetes mellitus. *World journal of gastroenterology: WJG;* 17(48)**:** 5240.

23   Kim H, Kisseleva T and Brenner D. 2015. Aging and liver disease. *Current opinion in gastroenterology;* 31(3)**:** 184.

24   Laaksonen D, Lindström J, Lakka T, Eriksson J, Niskanen L, Wikström K, Aunola S, Keinänen-Kiukaanniemi S, Laakso M and Valle T. 2005. Physical activity in the prevention of type 2 diabetes. *Diabetes;* 54(1)**:** 158-165.

25   Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P and Khathi A. 2018. Effects of a ruthenium schiff base complex on glucose homeostasis in diet-induced pre-diabetic rats *Molecules (Basel, Switzerland)* 23(7)**:** E1721.

26   Mabuza L, Gamede M, Maikoo S, Booysen I, Ngubane P and Khathi A. 2019. Cardioprotective effects of a ruthenium (II) Schiff base complex in a diet-induced prediabetic rats. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy;* 12**:** 1-7.

27   Makki K, Froguel P and Wolowczuk I. 2013. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. *ISRN inflammation;* 2013.

28   Maschio D, Oliveira R, Santos M, Carvalho C, Barbosa-Sampaio H and Collares-Buzato C. 2016. Activation of the Wnt/β-catenin pathway in pancreatic beta cells during the compensatory islet hyperplasia in prediabetic mice. *Biochemical and biophysical research communications;* 478(4)**:** 1534-1540.

29   Mavrogiannaki A and Migdalis I. 2013. Nonalcoholic Fatty liver disease, diabetes mellitus and cardiovascular disease: newer data. *International Journal of Endocrinology;* 2013**:** 450639.

30   Mohamed J, Nafizah A, Zariyantey A and Budin S. 2016. Mechanisms of diabetes-induced liver damage: the role of oxidative stress and inflammation. *Sultan Qaboos University Medical Journal;* 16(2)**:** 132–141.

31   Pecoits-Filho R, Abensur H, Betônico C, Machado A, Parente E, Queiroz M, Salles J, Titan S and Vencio S. 2016. Interactions between kidney disease and diabetes: dangerous liaisons. *Diabetology & metabolic syndrome;* 8(1)**:** 50.

32   Regnell S and Lernmark A. 2011. Hepatic steatosis in type 1 diabetes. *The review of diabetic studies: RDS;* 8(4)**:** 454.

33   Saravanan S and Pari L. 2016. Protective effect of thymol on high fat diet induced diabetic nephropathy in C57BL/6J mice. *Chemico-biological interactions;* 245**:** 1-11.

34   Shirakawa J and Kulkarni R. 2016. Novel factors modulating human β-cell proliferation. *Diabetes, Obesity and Metabolism;* 18**:** 71-77.

35   Speliotes E, Massaro J, Hoffmann U, Vasan R, Meigs J, Sahani D, Hirschhorn J, O'donnell C and Fox C. 2010. Fatty liver is associated with dyslipidemia and dysglycemia independent of visceral fat: the Framingham Heart Study. *Hepatology;* 51(6)**:** 1979-1987.

36   Tabák A, Herder C, Rathmann W, Brunner E and Kivimäki M. 2012. Prediabetes: a high-risk state for diabetes development. *The Lancet;* 379(9833)**:** 2279-2290.

37   Yilmaz S, Canpolat U, Aydogdu S and Abboud H. 2015. Diabetic cardiomyopathy; summary of 41 years. *Korean circulation journal;* 45(4)**:** 266-272.

38   Zheng M, Lv L, Ni J, Ni H, Li Q, Ma K and Liu B. 2011. Urinary podocyte-associated mRNA profile in various stages of diabetic nephropathy. *PLoS One;* 6(5)**:** e20431.

Appendix 1: Ethical clearance

UNIVERSITY OF KWAZIJLU-NATAL

INYUVESI

YAKWAZULU-NATALI

30 August 2016

Ms Lindokuhle Patience Mabuza (211509843)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Ms Mabuza,

Protocol reference number: AREC/038/016M
Project title: Effects of ruthenium complexes on glucose homeostasis in STZ-induced diabetic rats

Full Approval — Research Application

With regards to your revised application received on 13 July 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and FULL APPROVAL for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 30 August 2017.

I take this opportunity of wishing you everything of the best with your study.
Yours faithfully

....
Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Andile Khathi
Cc Acting Academic Leader Research: Dr Michelle Gordon Cc
RegXtrar: Mr Simon Mokoena cc NSPCA: Ms Jessica Light Cc
BRU — Dr Sanil Singh

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001 Durban 4000

Telephone: +27 (0) 31 260 8350 Facsimile: +27 (0) 31 260 4609 Email: animalethics@ukzn.ac.za Website:
http://reseacch.ukzn.ac.za/Research-Ethjcs/Animal-Ethics.aspx

1910 • 2010 too YEARS OF
ACADEMIC EXCELLENCE

C?m.wes•   Edgewood   Howard College   Mediczl School   Pietennaribburg   Westville

Appendix 2: SLMMS research symposium

UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

LMMS RESEARCH SYMPOSIUM 2019

CERTIFICATE OF PRESENTATION

Awarded to

LINDOKUHLE MABUZA

University of KwaZulu-Natal

For presenting the oral:

Hepatoprotective Effects of a Ruthenium (Ii) Schiff Base Complex in Diet-Induced Pre-Diabetic Rats

At the

Annual Laboratory Medicine and Medical Sciences Research Symposium 2019

Which took place in Westville, Durban, South Africa

On the 6th of September 2019

Yours sincerely,

Dr De Gama

Academic Leader Research SLMMS

Appendix 3: CHS symposium

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

COLLEGE OF HEALTH SCIENCES

Annual Research Symposium

1st November 2019

K-RITH Tower Building
Nelson R Mandela School of Medicine Campus

molecules

MDPI

Article

Effects of a Ruthenium Schiff Base Complex on Glucose Homeostasis in Diet-Induced Pre-Diabetic Rats

Lindokuhle Patience Mabuza [1], Mlindeli Wilkinson Gamede [1], Sanam Maikoo [2], Irvin Noel Booysen [2], Phikelelani Siphosethu Ngubane [1] and Andile Khathi [1,*]

[1] School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa; 211509843@stu.ukzn.ac.za (L.P.M.); 213571877@stu.ukzn.ac.za (M.W.G.); Ngubanep1@ukzn.ac.za (P.S.N.)

[2] School of Chemistry and Physics, College of Agricultural and Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg 3209, South Africa; 208501744@stu.ukzn.ac.za (S.M.); booyseni@ukzn.ac.za (I.N.B.)

* Correspondence: khathia@ukzn.ac.za; Tel.: +27-031-260-7585; Fax: +27-031-260-7132

check for updates

Abstract: Pre-diabetes is a condition that precedes type 2 diabetes mellitus (T2DM) that is characterised by elevated glycated haemoglobin (HbA1c). The management of pre-diabetes includes the combination of dietary and pharmacological interventions to increase insulin sensitivity. However, poor patient compliance has been reported with regard to dietary interventions, therefore, new alternative drugs are required that can be effective even without the dietary intervention. In our laboratory, we have synthesised a novel ruthenium complex that has been shown to have elevated biological activity. This study investigated the effects of this complex in both the presence and absence of dietary intervention on glucose handling in a diet-induced pre-diabetes rat model. Pre-diabetic animals were randomly assigned to respective treatment groups. The ruthenium complex was administered to pre-diabetic rats once a day every third day for 12 weeks. The administration of the ruthenium complex resulted in reduced fasting blood glucose, food intake, and body weight gain which was associated with decreased plasma ghrelin, insulin, and HbA1c levels in both the presence and absence of dietary intervention. The administration of the ruthenium complex ameliorated glycaemic control and insulin sensitivity in pre-diabetic rats. The results of this study warrant further investigations as this compound could potentially be able to re-sensitize insulin resistant cells and reduce the incidence of T2DM.

Keywords: pre-diabetes; ruthenium complex; glycated haemoglobin; dietary intervention

1. Introduction

According to the American Diabetes Association (ADA), pre-diabetes is defined as impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and elevated glycated haemoglobin (HbA1c) [1,2]. The measurement of glycated haemoglobin (HbA1c) has been the most widely used test for monitoring glycaemic control in individuals with pre-diabetes [2]. In pre-diabetes, higher amounts of HbA1c indicate poor control of blood glucose levels [1,2]. The regulation of blood glucose levels is a highly integrated process involving the balance of various hormones [3]. Impaired insulin action is identified in the pathophysiology of pre-diabetic abnormalities in glucose, lipid, and protein metabolism [4]. Additionally, the meal-induced decrease of ghrelin levels is impaired in pre-diabetic

Open Access Full Text Article

ORIGINAL RESEARCH

Cardioprotective effects of a ruthenium (II) Schiff base complex in diet-induced prediabetic rats

Lindokuhle Patience
Mabuza[1]
Mlindeli Wilkinson
Gamede[1]
Sanam Maikoo[2]
Irvin Noel Booysen[2]
Phikelelani Siphosethu
Ngubane[1]
Andile Khathi[1]

[1]Department of Human Physiology,
School of Laboratory Medicine
and Medical Sciences, College of
Health Sciences, University of
KwaZulu-Natal, Durban, South Africa;
[2]Department of Chemistry, School
of Chemistry and Physics, College of
Agricultural, Engineering and Sciences,
University of KwaZulu-Natal,
Pietermaritzburg, South Africa

Background: Prediabetes and the onset of cardiovascular diseases (CVD) are strongly related. Prolonged hyperglycemia has been identified as a major contributing factor in the pathogenesis of CVD and diabetic complications. The management of hyperglycemia and prediabetes-associated vascular complications rely on pharmacotherapy and lifestyle intervention strategies. However, patients still take the conventional drugs and neglect lifestyle intervention; therefore, newer alternative drugs are required. The synthesized ruthenium Schiff base complex has been shown to have elevated biological and antidiabetic activity. Thus, the research investigated the cardioprotective effects of ruthenium (II) Schiff base complex in diet-induced prediabetic (PD) rats.

Materials and methods: The rats were randomly allocated to respective groups and treated for 12 weeks. Ruthenium (15 mg/kg) was administered to PD rats once a day every third day. Blood pressure and plasma glucose were monitored throughout the study. Blood and heart tissue were collected for biochemical assays.

Results: Ruthenium complex with dietary intervention lead to reduced mean arterial blood pressure which correlated with a restored heart to body weight ratio. Additionally, there was a significant decrease in tissue malondialdehyde and increased superoxide dismutase and glutathione peroxidase concentration in both the plasma and heart tissue. Furthermore, there was a decrease in plasma triglycerides, low-density lipoprotein with an increased high-density lipoprotein concentration in ruthenium-treated rats. This was further evidenced by reduced plasma tumor necrosis factor-α, IL-6, and cardiac C-reactive protein concentrations in ruthenium-treated rats.

Conclusion: Ruthenium coupled with dietary intervention decreased the risk of developing cardiac injury, thus preventing CVD in prediabetes. Therefore, this complex may be a beneficial therapeutic agent in the prevention of PD cardiovascular complications.

Keywords: prediabetes, cardiovascular complications, ruthenium, dietary intervention, lipid profile, antioxidants, anti-inflammatory

Introduction

Prediabetes and the onset of cardiovascular diseases (CVD) are strongly related.[1–4] Prolonged hyperglycemia has been identified as a primary contributing factor in the pathogenesis of CVD and diabetes complications.[5] Most obese patients are prediabetic (PD) and insulin resistant, which is correlated with subclinical inflammation characterized by overexpression of cytokines by adipose tissue and activated macrophages.[6,7] In PD patients, pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), IL-1, IL-6, leptin, C-reactive protein (CRP), and adiponectin are involved in signaling pathways, insulin mechanism, and endurance of inflammatory response.[7] Pro-inflammatory mediators play a crucial role in inducing insulin resistance and type 2

Correspondence: Lindokuhle Patience
Mabuza
Department of Human Physiology, School
of Laboratory Medicine and Medical
Sciences, University of KwaZulu-Natal,
Room E3-408, Private Bag X54001,
Durban, South Africa
Email 211509843@stu.ukzn.ac.za

Appendix 6: Publication 3

Hepatoprotective Effects of a Ruthenium(II) Schiff Base Complex in Rats with Diet-Induced Prediabetes

Lindokuhle Patience Mabuza [1,*], Mlindeli Wilkinson Gamede [1], Sanam Maikoo [2], Irvin Noel Booysen, PhD [2], Phikelelani Siphosethu Nguban, PhD [1], Andile Khathi, PhD [1]

[1] School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
[2] School of Chemistry and Physics, College of Engineering and Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa

ABSTRACT

Background: Progressive insulin resistance in a prediabetic state has been reported to be the predominant causative factor for the development of nonalcoholic fatty liver disease. The combination of dietary modification and pharmacotherapy has been recommended to manage diabetic liver complications. However, poor patient compliance and toxicity of current drug therapy on liver function still results; thus, newer alternative drugs are required.
Objective: This study sought to investigate the hepatoprotective effects of the ruthenium(II) Schiff base complex in the presence and absence of dietary intervention in a diet-induced pre-diabetic rat model.
Methods: Prediabetic rats were randomly allocated to respective treatment groups. The ruthenium-based compound (15 mg/kg) was administered to the prediabetic rats in both the presence and absence of dietary intervention once a day every third day for 12 weeks.
Results: The administration of the ruthenium compound in both the presence and absence of dietary intervention resulted in the restoration of liver and body weights. This treatment also reduced liver damage enzyme biomarkers, bilirubin, and sterol regulatory element binding protein 1c concentrations in the plasma.
Conclusions: The ruthenium(II) complex showed beneficial effects as it ameliorated and prevented the progression of diabetes-related liver derangements while eliminating the hepatotoxicity associated with the use of metal compounds. However, further studies are still required to further determine the physiological mechanisms behind this effect.

Introduction

The liver plays an important role in the maintenance of systemic lipid and glucose homeostasis.[1] However, liver damage leads to dysregulation of both lipid and glucose metabolism and is a serious complication among patients with prediabetes and diabetes.[2] Liver damage is associated with several abnormalities, such as abnormal glycogen deposition, nonalcoholic fatty liver disease (NAFLD) and elevated plasma concentrations of liver damage biomarkers.[3,4] In pre-diabetes, insulin resistance and compensatory hyperinsulinaemia have been shown to be the predominant causative factors of liver pathology.[5–7] The insulin resistance

is said to impair the antilipolytic action of insulin in adipose tissue, which leads to increased release of free fatty acids.[8–11] The elevated plasma concentrations of insulin, glucose and fatty acids then impairs the β-oxidation of fatty acids and enhances de novo lipogenesis in the liver under the control of a specific transcription factor steroid regulatory elementary binding protein-1c (SREBP-1c).[4,12,13] The presence of abnormal plasma levels of liver enzymes, such as alanine transaminase (ALT) and aspartate transaminase (AST) levels resemble liver injury and are found to be increased in patients with prediabetes.[4,14] In addition, studies have shown that bilirubin, which has been shown to have protective effects against cardiovascular complications, is lowered in diabetes.[15,16] Furthermore, the histopathological changes in patients with diabetes include increased hepatic glycogen concentration, accumulation of hepatic fat, and increased liver size.[13,17,18] Dietary modifications and pharmacotherapy have been used to manage NAFLD as well as nonalcoholic steatohepatitis liver complications

* Address correspondence to: L.P. Mabuza, Lindokuhle Patience Mabuza, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, South Africa.
E-mail address: 211509843@stu.ukzn.ac.za (L.P. Mabuza).

GUIDE FOR AUTHORS

INTRODUCTION

Current Therapeutic Research (CTR) is a Gold Open Access, PubMed/Medline indexed, online-only

journal. CTR focuses on the rapid publication of peer-reviewed original reports of all aspects of

therapeutics, including papers presenting unexpected and/or negative results.

We also encourage the submission of manuscripts presenting preclinical and very preliminary research

that may stimulate further investigation of potentially relevant findings, as well as in-depth review

articles on specific therapies or disease states, and applied health delivery or pharmacoeconomics.

CTR encourages and supports the submission of manuscripts describing: Interventions designed to

understand or improve human health, disease treatment or disease prevention; Studies that focus on

problems that are uncommon in resource-rich countries; Research that is "under-published" because

of limited access to monetary resources such as English language support and Open Access fees (CTR

offers deeply discounted English language editing; See below); Republication of articles previously

published in non-English journals (eg, evidence-based guidelines) which could be useful if translated

into English; Preclinical and clinical product development studies that are not pursued for further

investigation based upon early phase results.

Pre-submission inquiries

Current Therapeutic Research is delighted to respond to pre-submission inquiries for suitability

of manuscript topic, access to author support, etc. Please contact the editorial office at

currtherres@elsevier.com; all queries will be dealt with promptly.

Types of submissions

Published articles cover the entire spectrum of therapeutics, including preclinical, clinical,

postmarketing, outcomes, pharmacoeconomics, alternative medicine, devices, etc. For examples of

some types of studies please click on the appropriate link below.

Original Research

Review Articles and Meta-analyses

Commentaries

Brief Reports

Research Letters

Letters to the Editor

All manuscripts are peer reviewed by independent reviewers for clinical relevance, technical accuracy, methodological rigor, clarity, and objectivity using a double-blind review process. A double-blind peer review process means that neither the author nor the reviewers are aware of each other's identity.

After a technical check by the Managing Editor, new submissions are sent to the Editor-in-Chief to manage the peer review process. Upon receipt of the reviews, the Editor-in-Chief makes a decision to accept, reject, or request revision. The decision is passed to the Managing Editor who sends notification to the corresponding author.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

The work described in your article must have been carried out in accordance with The Code of

Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans

https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/;

EC Directive 86/609/EEC for animal experiments

http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; and Uniform

Requirements for manuscripts submitted to Biomedical journals http://www.icmje.org. This must be

stated at an appropriate point in the article.

Prospective, Observational, or Interventional Pre- and Post-marketing Studies

Pre- or post-marketing studies must undergo review by an institutional review board (IRB) or ethics committee (EC). Patients must give written informed consent unless a waiver of consent is allowed by the IRB/EC. Patients must be informed of any real or potential conflicts of interest, including compensation of the investigator and potential costs to the patient that may result from their participation in the study. The amount of the remuneration of the investigators for their participation in pre- or post-marketing studies must be approved by the IRB/EC. If the design of a prospective pre- or post-marketing study calls for a treatment intervention such as a switch or withdrawal, then criteria must be established a priori for patient selection, the implementation of the intervention, and assessment of success/failure of such intervention. Such criteria must be scientifically justified, documented, uniformly applied and enforced, and clearly reported in the study report. Additionally, the patient or his/her insurance provider will not be required to pay for costs related to prospective interventions, such as those that may result from a drug switch or withdrawal.

All other studies that involve identifiable human subjects, including retrospective studies, chart

reviews, post-marketing surveillance studies, or government mandated phase IV trials require IRB/EC approval or waiver.

In each case, detailed IRB/EC information should be clearly stated in the Methods section.

Studies that only utilize pre-existing, de-identified (according to HIPAA standards) patient data are not required to seek IRB approval.

Declaration of Interest

All authors must disclose any financial or other relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted. 2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches.

Appendix 8: Nutrients Journal author guidelines

Manuscript Preparation

General Considerations

Research manuscripts should comprise:

Front matter: Title, Author list, Affiliations, Abstract, Keywords

Research manuscript sections: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).

Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.

Review manuscripts should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.

Case reports should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.

Graphical abstract: Authors are encouraged to provide a graphical abstract as a self-explanatory image to appear alongside with the text abstract in the Table of Contents. Figures should be a high quality image in any common image format. Note that images displayed online will be up to 11 by 9 cm on screen and the figure should be clear at this size.

Abbreviations should be defined in parentheses the first time they appear in the abstract, main text, and in figure or table captions and used consistently thereafter.

SI Units (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible

Accession numbers of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and of Expression Data.

Equations: If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.

Research Data and supplementary materials: Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about Supplementary Materials and Data Deposit for additional guidelines.

Preregistration: Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.

Guidelines and standards: MDPI follows standards and guidelines for certain types of research. See https://www.mdpi.com/editorial_process for further information.

[Return to top]

Front Matter

These sections should appear in all manuscript types

Title: The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.

Author List and Affiliations: Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.

Abstract: The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.

Keywords: Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

Introduction: The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the workand highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.

Materials and Methods: They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.

Results: Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

Discussion: Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.

Conclusions: This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.

Patents: This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

[Return to top]

Back Matter

Supplementary Materials: Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.

Acknowledgments: All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.

Author Contributions: Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.

For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the CRediT taxonomy for the term explanation. For more background on CRediT, see here. "Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully".

Conflicts of Interest: Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. Nutrients does not publish studies funded by the tobacco industry. Any projects funded by pharmaceutical or food industries must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study".

References: References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, ReferenceManager or Zotero to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.

Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for Endnote and Zotero are available.

References should be described as follows, depending on the type of work:

Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. Abbreviated Journal Name Year, Volume, page range.

Books and Book Chapters:

2. Author 1, A.; Author 2, B. Book Title, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.

3. Author 1, A.; Author 2, B. Title of the chapter. In Book Title, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

Unpublished work, submitted work, personal communication:

4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript in preparation).

5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. Abbreviated Journal Name stage of publication (under review; accepted; in press).

6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.

Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In Title of the Collected Work (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year).

Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:

10. Title of Site. URL (archived on Day Month Year).

See the Reference List and Citations Guide for more detailed information.

[Return to top]

Preparing Figures, Schemes and Tables

File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.

Nutrients can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.

All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, etc.).

All Figures, Schemes and Tables should have a short explanatory title and caption.

All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.

Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

Research and Publication Ethics

Research Ethics

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

Replacement of animals by alternatives wherever possible,

Reduction in number of animals used, and

Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Nutrients endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist.

Manuscripts containing original research on animal subjects must have been approved by an ethical review committee. The project identification code, date of approval and name of the ethics committee or institutional review board must be cited in the Methods section.

For research involving animals, any potentially derived benefits must be significant in relation to harm suffered by participating animals. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

Replacement of animals by alternatives wherever possible,

Reduction in number of animals used, and

Refinement of experimental conditions and procedures to minimize the harm to animals.

 Information for Authors

Manuscript Preparation

1. General Guidelines

Immune Network publishes Full-length manuscripts, Brief

communications, Review articles, and Technical reports. The manuscript should be prepared according to the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals established by ICMJE, 2013 (http://www.icmje.org). In general, manuscripts submitted to Immune Network follow these guidelines:

• Use a 12-point serif font, preferably Times New Roman

• Use a double-space format for the entire manuscript

• Prepare the manuscript in the following order: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, Conflicts of Interest, Author Contributions, References, Tables, Figure Legends, Figures. If necessary, Supplemental Data should be prepared in separate files.

• Begin each section component on a separate page

1) Full-length Manuscripts

(1) Title Page

The title page must include:

• The full title

• A running title (not to exceed 60 characters)

• Each author's full name as it should be published (first name, middle initial, last name)

• The affiliations of all authors and their institutions, departments, or organizations

• Superscript Arabic numbers to designate authors' affiliations

• Email address of the corresponding author with a separate number on the title page

• Up to six key words (the MeSH key words are recommended, but not obligatory, http://www.nlm.nih.gov/mesh/ MBrowser.html)

• Abbreviations: Nonstandard abbreviations used three or more times must be defined on the title page

• Word count is limited to 6,000 words (excluding references)

(2) Abstract

The Abstract must be 250 words or less for full-length type manuscripts. Reference citations should not be included in the Abstract. The species of animals or species of origin of cells used in the manuscript must be clearly stated in the Abstract.

(3) Introduction, Materials and Methods, Results, and Discussion

The Introduction, Materials and Methods, Results, and Discussion sections should begin on separate pages in this order. Use unit names and symbols of measurement according to International System of Units (SI Units based on the meter-kilogram- second [MKS] system).

(4) Acknowledgements

The Acknowledgements section appears immediately after the Discussion section. Grant support must be included in the Acknowledgements. Immune Network allows inclusion of contributors who helped conduct the research.

(5) Conflicts of Interest

Conflicts of Interest should be disclosed following the Acknowledgements section. Disclosure of Conflicts of Interest follows the guidelines described in Ethical Consideration.

(6) Author Contributions

Enter all author contributions in the submission system during submission. The contributions of all authors must be described using the CRediT Taxonomy of author roles.

[Author Contributions (Attachment 2)]

To qualify for authorship, all contributors must meet at least one of the seven core contributions (conceptualization, methodology, software, validation, formal analysis, investigation, data curation), as well as

at least one of the writing contributions (original draft preparation, review and editing).

Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and it is expected that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time.

An example:

Conceptualization: Hong GD (for Gil Dong Hong); Data curation: Kim Y, Kim GD (for Younghee Kim and Gil-Dong Kim); Formal analysis: Kim CS (for Chul-Soo Kim); ...

(7) References

References must be numbered as they appear in the text. If there are ten or less authors in a reference, then all the names of the authors should be listed. If the number of authors is more than ten, list the initial ten authors and then abbreviate the rest of the authors with 'et al'. If citations are included in tables or in figure legends, they must be numbered according to the citation position of the table or figure in the text. Only published papers and papers in press may be included in the references. In press articles, i.e., papers not yet published, must be submitted as online attachments in a PDF format at the time of article submission. The format for references is as follows:

• Periodicals: Antoni MH, Lehman JM, Kilbourn KM, Boyers AE, Culver JL, Alferi SM, Yount SE, McGregor BA, Arena PL, Harris SD, et al. Cognitive-behavioral stress management intervention decreases the prevalence of depression and enhances benefit finding among women under treatment for early-stage breast cancer. Health Psychol 2001;20:20–32.

• Books: McIntyre TM, Strober W. Gut-associated lymphoid tissue: regulation of IgA B-cell development. In: Mucosal Immunology, 2nd ed. Ogra PL, Mestecky J, Lamm E, Strober W, Bienenstock J, McGhee JR, eds. San Diego, CA; Academic Press; 1999.

Appendix 10 List of authors contributions

| Author	Contributions
LP Mabuza	Conceptualized and design of the study, execution of animal studies, conducted all animal and laboratory experimental work, analysis of data, drafting and writing of the thesis.
MW Gamede	Assisted in data analysis of the thesis.
S Maikoo	Synthesised and spectroscopically characterized the free-ligand and its ruthenium complex which was utilised as the metal-based drug.
IN Booysen	Synthesised and spectroscopically characterized the free-ligand and its ruthenium complex which was utilised as the metal-based drug.
PS Ngubane	Co-supervisor, provided funding and assisted in reviewing and editing of the thesis.
A Khathi	Supervisor, provided funding and assisted in reviewing and editing of the thesis.
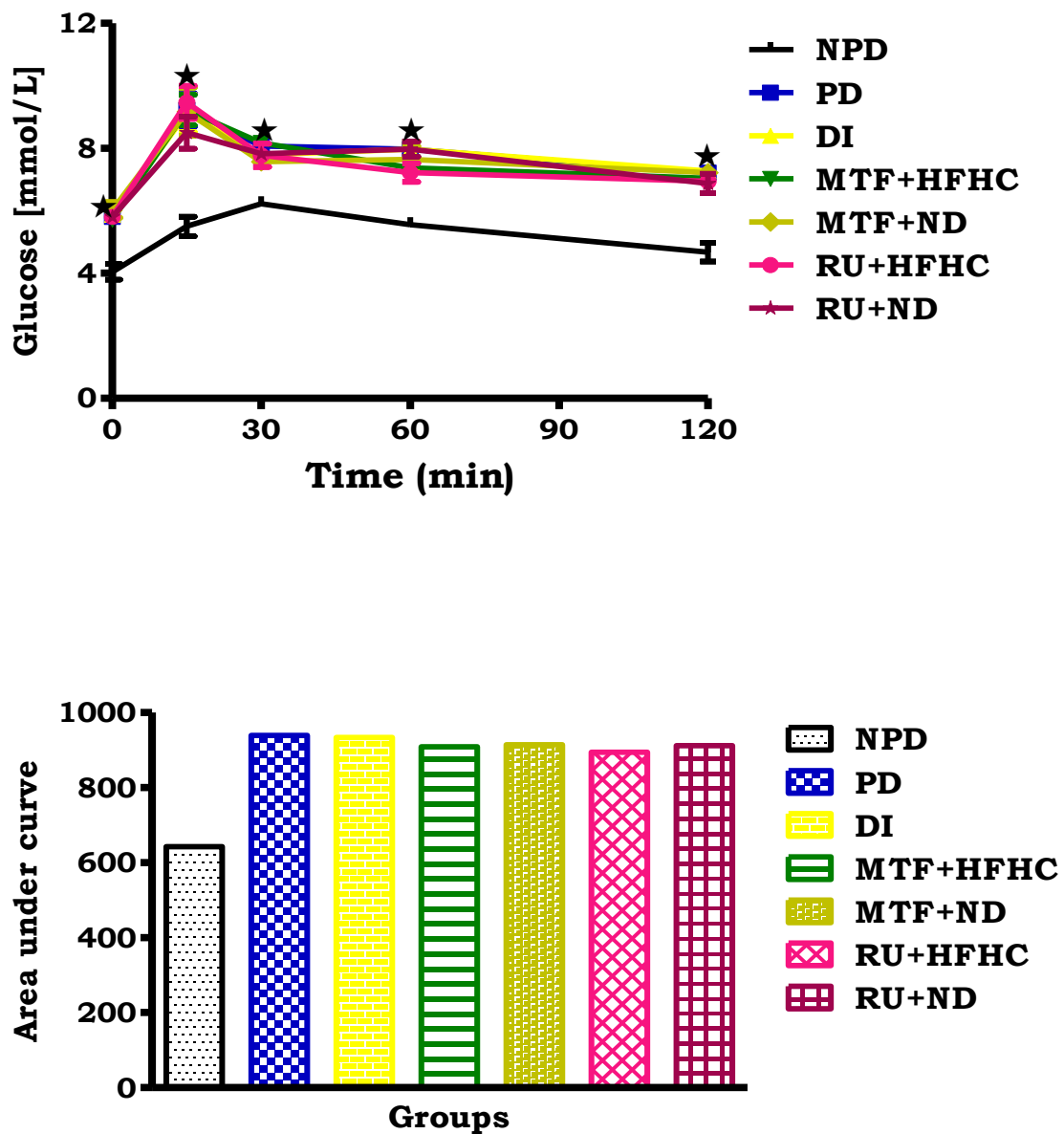
Figure 1: The effects of the ruthenium Schiff-base complex on OGT response of pre-diabetic animals before the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD);Dietary intervention (DI); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).

Figure 2: The effects of the ruthenium Schiff-base complex on OGT response of pre-diabetic animals after the treatment period. Values are presented as means ± SEM ($n = 6$) in each group. ***** $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD); Dietary intervention (DI); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).
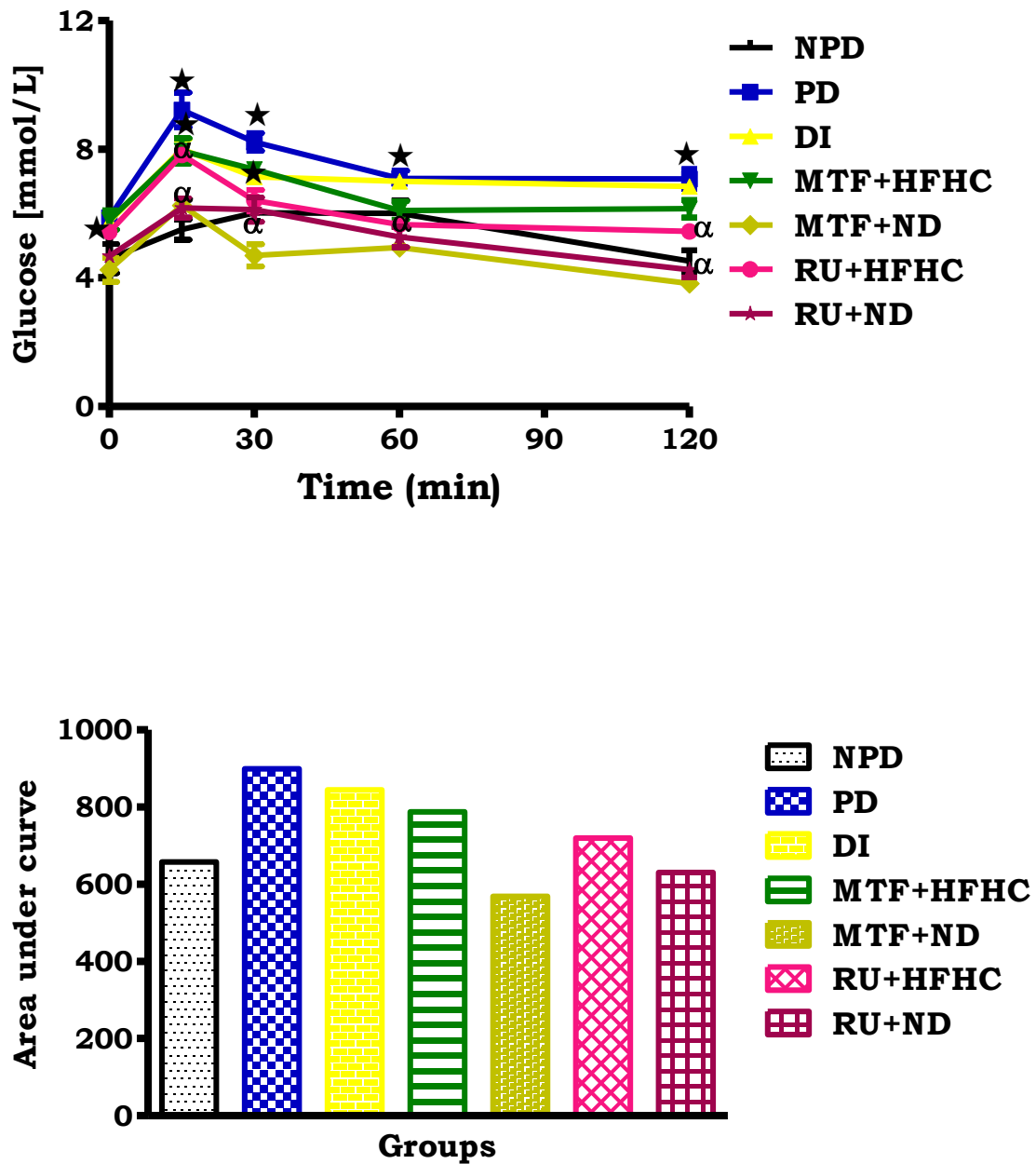
Table 1: Effects of the ruthenium complex on HOMA-IR index of pre-diabetic animals during treatment period. Values are presented as means (n=6) in each group.

Groups	Plasma Glucose (mmol/L)	Plasma Insulin (mlU/L)	HOMA-IR Values
NPD	4.60 ± 0.09	5.89 ± 0.90	1.20 ± 0.61
PD	5.60 ± 0.32*	20.92 ± 3.45*	5.20 ± 2.98*
DI	5.80 ± 1.04*	18.98 ± 2.03*	4.90 ± 1.23*
MTF+HFHC	6.60 ± 0.81*α	18.00 ± 3.12*	5.30 ± 1.02*
MTF+ND	$4.30 \pm 0.90\alpha$	$7.57 \pm 1.32\alpha$	$1.30 \pm 1.15\alpha$
RU+HFHC	5.60 ± 1.02*	16.17 ± 1.88*α	4.00 ± 1.05*
RU+ND	$4.70 \pm 0.82\alpha$	$8.29 \pm 2.00\alpha$	$1.70 \pm 0.99\alpha$

* $p < 0.05$ compared to non-prediabetic control (NPD), **α** $p < 0.05$ compared to pre-diabetic control group (PD); Dietary intervention (DI); Metformin and high fat high carbohydrate (MTF+HFHC); Metformin and normal diet (MTF+ND); Ruthenium complex and high fat high carbohydrate (RU+HFHC); Ruthenium complex and normal diet (RU+ND).