An investigation on the effects of a rhenium (V) compound with uracil-derived ligands on markers associated with hepatic, cardiovascular and renal complications in diet-induced prediabetic rats.

by

Angezwa Siboto

Student number:212518628

Supervisor: Prof Andile Khathi

Co-Supervisors: Dr Phikelelani Siphosethu Ngubane

Dr Ntethelelo Hopewell Sibiya

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Preface

Prediabetes is a metabolic disorder that often precedes the onset of type 2 diabetes mellitus. This development of this asymptomatic condition is associated with chronic consumption of high calorie diets and sedentary lifestyles. Prediabetes results in decreased insulin sensitivity in the peripheral tissues resulting in elevated blood glucose levels that are not high enough for a diagnosis of type 2 diabetes. This moderate hyperglycaemia has been shown to lead to trigger complications such as non-alcoholic fatty liver disease, renal dysfunction and cardiovascular disease which are generally only diagnosed during type 2 diabetes. The current management strategy for prediabetes consists of a combination of pharmacological and lifestyle intervention. The pharmacological agents such as metformin while lifestyle intervention involves dietary modification to lower calorie diets. Studies show that prediabetic patients tend to be more dependent pharmacological intervention and struggle with changing diets thus lowering the efficacy of drugs such as metformin. This often leads to the eventual development of prediabetes. Therefore, there is a need for new pharmacological agents that can remain effective in both the presence and absence of dietary intervention. In our laboratory we have synthesised a novel rhenium (v) compound with uracil-derived ligands that has shown promising biological activities that include anti-hyperglycaemic effects in diet-induced prediabetic rats. This compound was shown to improve insulin sensitivity in peripheral tissues in prediabetic rats. To advance from this knowledge, this study sought to investigate the effects of the rhenium (V) compound with uracil-derived ligands on markers associated with hepatic, cardiovascular and renal complications in diet induced prediabetic rats model.

Declaration

I, **Angezwa Siboto,** student number **212518628** hereby declare that the dissertation entitled: "An investigation on the effects of a rhenium (V) compound with uracil-derived ligands on markers associated with hepatic, cardiovascular and renal complications in diet-induced prediabetic rats" 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. The research done in this study was carried out under the supervision of Dr A. Khathi, Dr P.S. Ngubane and Dr N.H. Sibiya.

Where use of the work of others was made, it is duly acknowledged in the text.

[redacted] Date 31/March/2023

Student: A. Siboto (212518628)

_____ Date_____

Supervisor: Dr A Khathi

_____ Date _____

Co-Supervisor: Dr PS Ngubane

_____Date_____

Co-Supervisor: Dr NH Sibiya

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Plagiarism declaration

I, Angezwa Siboto declare that

i. The research reported in this thesis, expect where otherwise indicated, is my original work.

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Presentations

Symposium presentation

- **Siboto A**, Ngubane S.P, Khathi A. INVESTIGATING THE PROTECTIVE EFFECTS OF A RHENIUM (V) COMPOUND WITH URACIL-DERIVED LIGANDS ON LIVER DAMAGE ASSOCIATED WITH PREDIABETES IN DIET-INDUCED PREDIABETIC RATS. School of Laboratory Medicine and Medical Science Annual Research Symposium **30 NOVEMBER 2022**. University of Kwazulu-Natal, South Africa.
- Siboto A, Ngubane S.P, Khathi A. EFFECTS OF RHENIUM (V) COMPOUND WITH URACIL DERIVED LIGANDS ON SELECTED MARKERS ASSOCIATED WITH CARDIOVASCULAR FUNCTION IN DIET INDUCED PREDIABETIC RATS. School of Laboratory Medicine and Medical Science Annual Research Symposium 15 AUGUST 2023. University of Kwazulu-Natal, South Africa.
- Siboto A, Ngubane S.P, Khathi A. EFFECTS OF RHENIUM (V) COMPOUND WITH URACIL DERIVED LIGANDS ON SELECTED MARKERS ASSOCIATED WITH CARDIOVASCULAR FUNCTION IN DIET INDUCED PREDIABETIC RATS. School of Laboratory Medicine and Medical Science research day 04 October 2023. University of Kwazulu-Natal, South Africa.

Dedication

This dissertation is dedicated to my late father, my loving mom, my daughter and my siblings.

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List of abbreviations

Abbreviation	Name
ADA	American Diabetes Association
ALT	Alanine transaminase
ANOVA	One-way analysis of variance
ARE	Antioxidant response element
AREC	Animal Research Ethics Committee
AST	Aspartate transaminase
AMPK	Adenosine monophosphate-activated protein kinase
Akt	Protein kinase B
AGE	Advanced glycation end products
AREC	Animal research ethics committee
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
BMI	Body mass index
BRU	Biomedical Research Unit
BP	Blood pressure
CAT	Catalase
DCM	Diabetic cardiomyopathy
CO_2	Carbon dioxide
CKD	Chronic kidney disease
DI	Diet Intervention
DNA	Deoxyribonucleic acid
CVD	Cardiovascular disease

CRP	C-Reactive Protein
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbent assay
Enos	Nitric Oxide synthase
FFA	Free fatty acids
G	Grams
GFR	Glomerular filtration rate
GPx	Glutathione peroxidase
GLUT 2	Glucose transporter 2
GPO-PAP	Glycerine phosphate oxidase peroxidase
HW/BW ratio	Heart weight to body weight ratio
Hr	Hour
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
INF-I	Interferon I
IR	Insulin Resistance
HCl	Hydrochloric acid
HbA1C	Glycated haemoglobin
HDL	High density lipoprotein
HFHC	High fat high carbohydrates
H_2O	Water
IDF	International Diabetes Federation

IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
JNK	c-Jun-N terminal kinase pathway
K+	Potassium
KIM 1	Kidney injury molecule
Kg	Kilogram
KOH	Potassium hydroxide
L	Litre
Na+	Sodium
Na_2SO_4	Sodium sulphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NADPH	Nicotinamide adenine dinucleotide phosphate
NPD	Non-prediabetic control
NOX 1	NAD(P)H oxidase 1
NOX4	NAD(P)H oxidase 4
NC	Normal control
ND	Normal Diet (standard rat chow)
NF -Kb	Nuclear Factor Kb pathway
No.	Number
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2
MAP	Mean arterial pressure
MET	Metformin
MDA	Malondialdehyde

µ	Micro
µg	Micrograms
µl	Microlitre
mg	Milligram
ml	Millilitre
mmol/L	Millimoles per Litre
OGTT	Oral glucose tolerance test
PI3K	Phosphatidylisotol-3-kinase
p.o.	per os (orally)
PBS	Phosphate buffered saline
PKC	Protein kinase C
PD	Prediabetes
PTP	Protein Tyrosine Phosphatase
RAAS	renin–angiotensin–aldosterone system
Re	Rhenium (V) compound
ROS	Reactive oxygen species
SREBP-1	Sterol regulatory element binding protein 1
TBARS	Thiobarbituric acid reacting substance
TC	Total cholesterol
TGs	Triglycerides
TNF α	Tumor necrosis factor-alpha
T2DM	Type 2 diabetes Mellitus
SD	Sprague-Dawley
SEM	standard error of means
SGLT2	Sodium-glucose co-transporter-2

s.c.	Subcutaneously
SOD	Superoxide dismutase
STD	Standard
SD	Standard deviation
UKZN	University of KwaZulu-Natal
USA	United states
VLDL	Very low-density lipoprotein
VLDL-TG	Very-low-density lipoprotein–triglyceride

List of Appendices

Study outline

This dissertation is presented in manuscript format. It consists of 7 sections: dissertation abstract, chapter 1: introduction/literature review, chapter 2: manuscript 1, chapter 3: manuscript 2, chapter 4: manuscript 3, chapter 5: synthesis, conclusion and appendices. The dissertation abstract points out the purpose and summarizes the key findings of the study. Chapter 1 is a brief background and a literature review to highlight the gaps that exist in literature and how the current study aims to fill these gaps.

Chapter 2 is the first novel research paper that investigated the Protective Effects of a Rhenium (V) Compound with Uracil-Derived Ligands on Liver Damage Associated with Prediabetes in Diet-Induced Prediabetic Rats. Angezwa Siboto, Akinjide Moses Akinnuga, Muhammed Bilaal Ismail, Irvin Noel Booysen, Ntethelelo Hopewell Sibiya, Phikelelani Ngubane and Andile Khathi are authors. The manuscript has been published in the Journal of Diabetology.

Chapter 3 is the second research study manuscript which investigated Effect of rhenium (V) compound with uracil derived ligands on selected markers associated with cardiovascular function in diet induced prediabetic rats. Angezwa Siboto, Akinjide Moses Akinnuga, Muhammed Bilaal Ismail, Irvin Noel Booysen, Ntethelelo Hopewell Sibiya, Phikelelani Ngubane and Andile Khathi are authors. The manuscript is currently under review in the Diabetes and Vascular Disease Research Journal.

Chapter 4 is the third research study manuscript which investigated the ameliorative Effects of a Rhenium (V) Compound with Uracil-Derived Ligand Markers Associated with Hyperglycaemia-Induced Renal Dysfunction in Diet-Induced Prediabetic Rats. A Siboto, A Akinnuga AM, BN. Khumalo, MB Ismail, IN Booysen, NH Sibiya, PS Ngubane and A Khathi are authors. The manuscript has been published in the Journal of Molecular Science.

Chapter 5 is the synthesis which discusses the link between the three studies and highlights the key findings for the specific aims of the study. Appendices include the letter of ethical clearance, abstract and certificate of presentations to various conferences: and the guidelines to authors applicable to the three journals where the three manuscripts were submitted.

Abstract

Introduction

Prediabetes is a metabolic disorder of decreased insulin sensitivity in peripheral tissues that often precedes the onset of type 2 diabetes mellitus. It is characterized by blood glucose levels higher than normal but not high enough for a diagnosis of type 2 diabetes. The prevalence of prediabetes is increasing exponentially in developing countries due to urbanization, adapting to high calorie diets and normalized sedentary lifestyle. The increasing prevalence of this condition has been difficult to manage due to its' asymptomatic nature. The moderate hyperglycaemia in prediabetic condition has been shown to lead to the development of hepatic, cardiovascular and renal complications. The current management strategy for prediabetes consists of a combination of pharmacological and lifestyle interventions. The pharmacological intervention involves pharmacological agents such as metformin while lifestyle intervention involves dietary modification to lower calorie diets. Studies show that prediabetic patients tend to depend more on pharmacological interventions but struggle adapting to consuming low calorie diets thus lowering the efficacy of pharmacological agents such as metformin. This often leads to the eventual development of type 2 diabetes mellitus. Therefore, there is a need for new pharmacological agents that can remain effective in both the presence and absence of dietary intervention. This study investigated the effects of a novel rhenium (V) compound on prediabetes-associated complications such NAFLD, CVD and renal injury in a diet-induced pre-diabetic rat model.

Methods

Thirty-six (36) male Sprague Dawley rats (150–180 g) were obtained from the Biomedical Research Unit at the University of KwaZulu Natal, South Africa. Prediabetes was induced in thirty (30) of the rats by allowing chronic consumption on a high fat high carbohydrate (HFHC) diet with drinking water supplemented with 15% fructose (HFHC + fructose). The other six (6) rats were kept as non-prediabetic controls. After prediabetes induction, twelve (12) of the rats were treated with the rhenium (V) compound for 12 weeks in both the presence and absence of dietary modification. Another twelve (12) were treated with metformin for 12 weeks in both the presence and absence of dietary modification to serve as treated controls. The last six (6) animals serve as untreated prediabetic controls. For the study on liver function, parameters including fasting blood glucose (FBG) concentration and body weights were monitored every 4 weeks during the treatment period. Following sacrifice, hepatic glycogen, plasma

triglycerides, tumor necrosis factor alpha (TNF α) and liver function enzymes were measured. Liver tissue malondialdehyde (MDA) concentration, antioxidant markers: glutathione peroxidase (GPx) and superoxide dismutase (SOD) were also quantified. For the cardiovascular study cardiac blood glucose concentration, food intake, body weights, body mass index (BMI), mean arterial pressure (MAP), blood pressure (BP) and heart rate were monitored every 4 weeks of the treatment period. Following sacrifice cardiac tissue MDA concentration, antioxidant markers GPx and SOD, the markers of inflammation cardiac C-reactive protein (CRP), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF α), plasma nitric oxide (NO) as well as the plasma lipid [high-density lipoproteins (HDL) and triglycerides (TGs)] profile were quantified. Lastly for the renal study, blood glucose concentration, blood pressure, fluid intake and urine output were monitored every 4 weeks during the treatment period. Following sacrifice oxidative stress and the systemic anti-oxidant response were determined by measuring MDA concentration and GPx and SOD activities. Renal biochemical analysis after treatment period included plasma and urine electrolytes (Na^+ and K^+), , KIM 1, podocin concentration and GFR as well as the plasma creatinine, urea, uric acid and albumin..

Results

In all three studies, the untreated prediabetic control group showed dysregulation in glycaemic control through impaired fasting glucose concentration. Imbalance between oxidative stress and antioxidant profile, dyslipidaemia, high blood pressure and impaired kidney function. **T**he administration of rhenium (V) compound to prediabetic rats in both the presence and absence of dietary modification ameliorated metabolic dysfunction and complications associated with prediabetes progression. In the liver study, the administration rhenium (V) compound, in both the presence and absence of dietary intervention, resulted in reduced liver glycogen and plasma triglycerides. The treatment with this metal-based compound resulted in improved liver health as evidenced by reduced levels of plasma markers of liver damage and MDA but with increased antioxidant enzymes activities In the cardiovascular study, the administered of the rhenium (V) compound, in both the presence and absence of dietary intervention, resulted a significant reduction of total cholesterol, LDL, TGs and significant increase in HDL production. Additionally, the administered rhenium (V) compound resulted in reduced oxidative stress in cardiac tissue, blood pressure and the concentration of plasma markers of inflammation. In the renal study, the administered rhenium (V) compound, in both the presence and absence of dietary intervention, improved kidney function as evidenced by improved GFR, reduced KIM

1, podocin and aldosterone concentration. The Rhenium (V) compound also resulted in a significantly decreased in plasma creatinine, plasma urea and uric acid levels, signifying its nephroprotective potential.

Conclusion

The treatment of diet-induced prediabetic rats with the rhenium (V) compound with uracil-derived ligands in both the presence and absence of a diet intervention did not only markedly improve insulin sensitivity but also effectively decreased prediabetes-associated hepatic, cardiovascular and renal disturbances. Taken together, the findings of this study warrant further study into this compound as a potential pharmacological agent in the management of prediabetes and the associated complications.

Chapter 1: literature review

1.1. Background

The global prevalence of type 2 diabetes mellitus (T2DM) is projected to increase to 7079 individuals per 100 000 by 2030, reflecting a continued rise across all regions of the world (1, 2). T2DM is known for co-existing complications such as impaired fasting blood glucose concentration (IFG), insulin resistance in the peripheral tissues, dyslipidaemia, and the development of atherosclerosis (1, 2). These T2DM complications are known to have developed during the prediabetic state (1, 3). Therefore, it crucial that prediabetes is managed, as studies have shown the prevalence of prediabetes is increasing rapidly global (1, 4). Recent studies further reported that 70% of individuals with prediabetes will develop T2DM within their lifetime (1, 4).

There are several factors that are implicated in the increase in the prevalence of T2DM and prediabetes as high calorie diets and sedentary lifestyles (5, 6). Prediabetes is moderate hyperglycaemia which is above the homeostatic range but not high enough for a T2DM diagnosis (3, 7). Moderate insulin resistance and β cell dysfunction are the beginning of complications associated with T2DM (8, 9).

In a prediabetic state there is onset of non-alcoholic fatty liver disease (NAFLD), cardiovascular disease (CVD) and renal dysfunction (10, 11). NAFLD has recently became the most frequent chronic liver disease that occurs across age groups due to the growing prevalence of obesity and prediabetes (11-13). The prevalence of NAFLD is 4.6 times greater in the obese population and up to 74% of obese individuals have NAFLD (14, 15). NAFLD is associated with IFG due to hepatocytes insulin resistance (13, 15). Cardiovascular disease is associated with both IFG, impaired glucose tolerance (IGT) and the development of atherosclerosis (16, 17). The acceleration of atherosclerosis in prediabetes may be explained by several conditions including hyperglycaemia, increased oxidative stress, advanced glycation end products (AGE), dyslipidaemia, hyperinsulinemia, excessive production of compounds that caused inflammation and genetic variables (18, 19). Hyperglycaemia is a well-established risk factor for cardiovascular disease (18, 20).

Insulin resistance is also directly linked with the development of cardiovascular complications as increased blood pressure as a consequence of the inhibition of nitric oxide synthase and decreased nitric-oxide (NO) bioavailability (21). Hypertension, diabetes mellitus and

dyslipidemia are the mutual risk factors between CVD and kidney disease (22, 23). Kidney disease is associated with activated renin angiotensin aldosterone system (RAAS), hyperuricemia, increased levels of albuminuria and low glomerular filtration rate (GFR) (21, 23). Early management of prediabetes is recommended to prevent these co-existing complications and to delay the onset of T2DM (9, 21, 24).

The treatment of prediabetes-related complications involves lifestyle modification to enhance weight loss as well as the use of different insulin-sensitizing agents metformin (15, 16). However, metformin is known to induce hepatotoxicity and cause kidney damage (25, 26). and has also, metformin has been shown to have maximum efficacy when used in combination with lifestyle modifications (27, 28). However, there have been reports of poor patient compliance when it comes to the lifestyle modifications which leads to a reduction of its efficacy (29). Therefore, there is a need of a pharmacological agent that is efficacious in presence and or absence of lifestyle modification (30).

Metal based complexes have been used experimentally to treat prediabetes-related complications but there is concern on their toxicity on organs (16). However, a recent study conducted by Mabuza et al showed that when a ruthenium complex had an organic ligand, it displayed hepatoprotective effects by eliminating the hepatotoxicity associated with the use of metal compounds (31). Therefore, there is a possibility of synthesising more metal based compounds that are not toxic (32, 33). This study focused on rhenium (V) compound {trans-[Re(ddd)(Hduo)(PPh3)2] I (H2ddd = 5,6-diamino-1,3-dimethyluracil and H3duo = N-(2-hydroxybenzylidene)-5-amino-1,3dimethyl uracil which has been found to be not toxic to C2C12 skeletal muscle cells and has promising ability to be used to treat prediabetes. This compound was further shown to have anti-hyperglycaemic effects on a glucose homeostatic study as it also restored insulin sensitivity in skeletal muscle leading to improve glucose handling prediabetic rats (34). However, the protective effects of rhenium (V) compound on NAFLD, CVD and renal dysfunction are not yet known. Therefore, this study sought to evaluate protective role of the rhenium (V) compound with uracil derived ligands on hepatocytes, cardiovascular, and renal complications in diet induced prediabetic rats.

1.2. Glucose metabolism

After ingestion of a meal, the liver and skeletal muscle are responsible for about 70% glucose utilization (35, 36). Insulin secretion is stimulated which results in the activation of glycogen synthesis and storage in both the liver and skeletal muscle (37, 38).

1.2.1. Role of the liver in glucose homeostasis

The liver has the ability to produce glucose that is released to the systemic circulation and used by other tissues, particularly during periods of fasting (39, 40). Hepatic glucose production derives from glycogenolysis and from gluconeogenesis (41, 42). In addition to the liver, the kidney proximal tubule also produces a limited amount from glucose from the carbon skeletons of gluconeogenic amino acids. Glucose synthesis by the kidneys is largely in response to acidosis since the kidney also have physiologically relevant amounts of glucose-6-phosphatase(41, 43).

Gluconeogenesis and glycogenolysis are physiological processes in the liver that contribute to hepatic glucose production (41, 44). During short-term periods of fasting, glycogenolysis is the predominant source of glucose released to the bloodstream (42). However, during prolonged periods of fasting, the glycogen reserves are used up resulting in a decrease in glucose production from glycogenolysis and then gluconeogenesis becomes the predominant source of glucose (45, 46). The contribution of gluconeogenesis to hepatic glucose production increases gradually with prolonged fasting so that after approximately 42 h of fasting, gluconeogenesis accounts for almost all of glucose production in healthy subjects (45, 46).

In healthy individuals, a reduction of fatty acid availability inhibits gluconeogenesis, the rate of gluconeogenesis being positively correlated with the rate of fatty acid oxidation (42, 47). Reduced fatty acid oxidation in the liver suppresses gluconeogenesis due, at least in part, to decreased production of acetyl-CoA, which is an activator of pyruvate carboxylase (42). Insulin inhibits lipolysis and consequently reduces plasma concentration of fatty acids thus reducing fatty acid availability for oxidation and therefore suppressing gluconeogenesis (42). However, the inhibitory effect of insulin on hepatic gluconeogenesis is limited despite its suppressing effect on adipose lipolysis. Insulin infusion to non-diabetic subjects fasted overnight almost completely suppresses fatty acid availability and oxidation, but gluconeogenesis flux is reduced by only 20%. In healthy subjects, insulin reduces hepatic glucose output predominantly by reducing glycogenolysis and enhancing glycogen accumulation (42, 46). The glucose that is produced by the liver is used by cardiomyocytes as the source of energy (19).

1.2.2. Glucose utilization in cardiovascular system.

The cardiovascular is designed to ensure the survival of all cells in the body at every moment by maintaining the immediate chemical environment of each cell (39, 48). It consists of the

heart and the blood vessels running through the entire body. The arteries carry blood away from the heart; the veins carry it back to the heart (48, 49). Since glucose is a vital metabolic fuel for all mammalian cells, the heart is adapted to utilize all classes of substrates to meet the high-energy demand, and it tightly regulates it's substrate utilization in response to environmental changes (19, 39). In order to carry out its' function of delivering oxygen (19). The heart needs a continuous supply of ATP which (ATP) is produced from the metabolism of glucose and lactate (47, 48).

When glucose is released into the bloodstream from the liver, the heart uses endothelial cell-specific HIF-1α–dependent function to utilize glucose and in most cases, glucose uptake occurs primarily by facilitated diffusion, an energy-independent process that uses a carrier protein to transport a substrate across a membrane (39). This facilitated diffusion occurs via GLUT-1 which is widely expressed in endothelial cells (47, 50). The endothelium is a metabolically active organ that maintains both vascular homeostasis and systemic metabolism. The endothelium has the genes insulin receptor substrate 2 (IRS2), peroxisome proliferator-activated receptor-gamma (PPARγ), and fatty acid translocase (FAT)/cluster of differentiation 36 that regulate systemic glucose levels (51).

In the cardiomyocytes, insulin promotes glucose and fatty acid uptake, but inhibits the use of fatty acids as an energy source (39, 50). The protective effects of insulin are related to endothelial nitric oxide synthase (eNOS) activation via the PI3K/Akt pathway. The deleterious effects involve the induction of vascular smooth muscle cell (VSMC) proliferation, vasoconstriction and proinflammatory activity. These vascular effects are mediated through the mitogen-activated protein kinase (MAPK) pathway, which is involved only in the mitogenic effects of insulin, but not in its metabolic effects (47, 50). The cardiovascular system delivers oxygen, metabolic substrates as well as hormones to other parts of the body including the kidney (39, 50). The kidney can produce glucose that can be used by the heart as a form of energy (48).

1.2.3. The role of kidneys in glucose homoeostasis.

The kidney plays an important role in glucose homeostasis via gluconeogenesis, glucose utilization and glucose reabsorption from the renal glomerular filtrate (50). After an overnight fast, 20–25% of glucose that is released into the circulation originates from the kidneys through gluconeogenesis (41, 52). In this post-absorptive state, the kidneys utilize about 10% of all glucose utilized by the body (38). Each day, the kidneys filter approximately 180 g of glucose and virtually all of this is reabsorbed into the circulation (21, 23). Hormones most insulin and catecholamines, substrates, enzymes and glucose transporters are some of the various factors influencing the kidney's role (53, 54).

The renal medulla is an obligate user of glucose via glycolysis and produces no glucose but the renal cortex in contrast does not use much glucose and instead is the site of gluconeogenesis (38). Insulin has been shown to suppress renal glucose release by inhibiting gluconeogenesis and to stimulate renal glucose uptake (41). Furthermore, it also increases the sodium re-absorptive activity of renal sodium transporter proteins in nearly every cell type from the proximal tubule through to the collecting duct (41). In animal models, approximately 90% of glucose is reabsorbed by sodium-glucose co-transporter-2 (SGLT2), a high-capacity low-affinity glucose transporter located on the luminal surface of the epithelial cells lining the S1 and S2 segments of the proximal tubule. Transport of sodium and glucose via this transporter occurs in a 1:1 ratio. The remaining ∼10% of glucose reabsorption is via sodium-glucose co-transporter-1 (SGLT1), a high-affinity, low-capacity glucose/galactose transporter with sodium: glucose coupling ratio of 2:1(38, 41). Also GLUT 2 is involved glucose uptake in kidneys (52, 55).

1.3. Risk factors associated with the development of prediabetes.

Many developing countries such as South Africa are presently experiencing rapid urbanization and economic development which has led to a transition in nutrition patterns and levels of physical activity (56, 57). An increase in consumption of high calorie diets and sedentary lifestyles has been positively correlated with increases in average Body Mass Index (BMI), fasting plasma glucose and insulin resistance (56, 57). The high calorie diets include foods rich in saturated fatty acids, refined grains, red meat and fizzy drinks, has been reported to increase the risk of diabetes (24, 56).

1.3.1. Role of high calorie diets in the development of prediabetes.

Pathological changes that occur in the progression from normal glucose to T2DM have been associated with unhealthy dietary choices and sedentary lifestyles (36, 58). T2DM, a disease whose onset is often preceded by prediabetes. Both prediabetes and T2DM have been linked with diets that are high in fats and carbohydrates (5, 59). The presence of trans fats, saturated fats, refined grains and high-sweetened sugars in foods have shown as the major drivers of in the increase in the prevalence of T2DM (36, 60). Several studies have shown that chronic ingestion of high fructose-sweetened beverages also leads to insulin resistance which can manifest with in elevated plasma glucose concentrations (5, 36). The high glycaemic load of the high calorie diets and absorption of fructose into the system may account for this elevation in blood glucose concentration (59, 60). Overtime uncontrolled elevated plasma glucose may lead to impaired fasting blood glucose or impaired glucose tolerance and glucose-mediated non-enzymatic glycation of as haemoglobin causing an increase in glycated haemoglobin (HbA1c) (5, 36, 58). Intracellular hyperglycaemia has been linked to the activation of four toxic pathways that can lead to tissue damage: increased flux through the polyol pathway, formation of advanced glycosylation end products, increased hexosamine pathway activity, and protein kinase C (PKC) activation (58, 61).

Consumption of high caloric diets alters glucose homeostasis and energy balance (5, 58). During overfeeding, excess fat intake is stored as fat, whereas excess carbohydrate is mostly oxidized in the short term but can lead to substantial gain in fat stores because of reduced fat oxidation and considerable *de novo* lipogenesis in the long term (59, 60). Studies have shown that chronic consumption of high fructose diets in the form of sweetened beverages exposes the liver to high-fructose amounts resulting in rapid stimulation of lipogenesis and accumulation of triglycerides which in turn contribute to reduced insulin sensitivity, hepatic insulin resistance or glucose intolerance (58, 62). It has also been shown that increased chronic consumption of trans or saturated fats results in decreased expression of apo-B which leads to the accumulation of low-density lipoproteins (LDLs) and consequently elevated triglyceride concentration in circulation which result in insulin resistance (36, 58, 59). A high fat high carbohydrate (HFHC) diet supplemented with 15% fructose in drinking water was used to induce prediabetes. Luvuno et al, 2018 discovered that chronic exposure of Sprague-Dawley rats to HFHC diet leads to the onset of prediabetes with metabolic disorders, vascular complications and oxidative stress (5).

1.3.2. Peripheral tissue insulin resistance.

The physiological effect of insulin varies dramatically from tissue to tissue and different pathways within the same tissue vary in their degree of insulin resistance (40, 63). Insulin resistance is primarily an acquired condition related to excess body fat (40, 63). Muscle , liver and adipose tissue are the main organs that are affected by insulin resistance (63, 64). The skeletal muscle is the primary site for glucose disposal, accounting for up to 70% of tissue glucose uptake (40, 59). However, with excess calorie loads, glucose uptake by skeletal muscle exceeds capacity and excess glucose returns to the liver where it triggers *de novo* lipogenesis (DNL) (63). Increased DNL increases triglyceride and free fatty acids (FFA) production, causing ectopic fat deposition into the liver, skeletal muscle and adipose tissue (63-65). which mediates the development of insulin resistance as well as the production of markers of inflammation. (61). Increased lipolysis in adipocytes as a consequence of failure of insulin to suppress lipolysis in insulin-resistant visceral adipose tissue, thus increasing circulating FFA (45, 63). Higher levels of circulating FFAs directly effects on liver promoting gluconeogenesis and skeletal muscle FFA causes insulin resistance blocking glucose utilization (63).

Following the ingestion of a meal, insulin reduces hepatic glucose production via inhibition of glycogenolysis, limiting the postprandial rise in glucose (30, 46). In the presence of insulin resistance, this feedback mechanism is impaired and hepatic glucose production continues to rise, even as postprandial glucose rises (46, 54). In a previous study on glucose homeostasis, Siboto *et al*, 2020 used OGT, HOMAR index and glycated haemoglobin to assess for insulin resistance in skeletal muscle of prediabetic rats and to diagnose prediabetes (34). After treating the prediabetic rats with rhenium (V) compound they concluded that the compound exhibited glucose lowering effects by improving insulin resistance on skeletal muscle (34). However, this study sought to investigate effects of rhenium (V) compound on metabolic complications associated with prediabetes.

1.4. Prediabetes and the development of non-alcoholic fatty liver disease .

Prediabetes is a state characterized by impaired fasting blood glucose and impaired glucose tolerance (15, 35). Insulin resistance in the adipose and skeletal muscles tissue results in hyperglycaemia, hyperinsulinemia and dyslipidaemia (6, 15). Hyperglycaemia and hyperinsulinemia have different effects on hepatocytes, hyperglycaemia promotes glycogenesis leading to excessive glycogen storage in the hepatocytes whereas hyperinsulinemia stimulates gluconeogenesis in the liver as well as stimulates the transcription factor Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) resulting in elevated production and storage of TGs (9, 66).

Non-alcoholic fatty liver disease is characterised by excessive hepatic fat content of more than 5% of the liver weight, mainly in the form of triglycerides, in the absence of excessive alcohol consumption (6, 43). Under normal physiological conditions, insulin decreases circulating lipids through inhibition of adipose tissue lipolysis and by directly suppressing hepatic production of very low-density lipoproteins (VLDL) (44, 67). However, in an insulin resistant state these actions are impaired (40, 62). NAFLD develops when the rate of hepatic triglyceride (TG) synthesis, mainly due to increased hepatic non-esterified fatty acids (NEFA) uptake and *de novo* lipogenesis, exceeds the rate of hepatic TG catabolism due to NEFA oxidation and TG export as VLDL particles (44, 67).

1.4.1. Excess glucose utilization by the liver.

Liver plays a key role in glucose metabolism as it can utilise glucose independently of insulin though GLUT 2 transporters (10, 46). This organ stores glucose in the form of glycogen and also endogenously produces glucose (13, 66). However, due to systemic insulin resistance, excess glucose is shunted to the liver resulting in elevated activation of glucokinase which phosphorylates glucose into glucose-6-phosphate which is further metabolised by glycogen synthase into glycogen and stored in the hepatocytes (13, 14). Humans with insulin resistance exhibit reduced muscle glycogen synthesis, doubling of both liver triglyceride levels and hepatic *de novo* lipogenesis without any changes in circulating adipocytokines (13). Muscle insulin resistance shifts postprandial energy storage from muscle glycogen to hepatic lipid storage (13). Impaired fasting blood glucose (IFG) is characterized by reduced hepatic insulin sensitivity (68). Increase in hepatic glucose production (HGP) during fasting in the prediabetic state is primarily the result of an increase in gluconeogenesis and that glycogenolysis remains

unchanged (68). Plasma fasting glucose was measured as a parameter of glycaemic control and further investigated the effects of rhenium (V) compound on regulating plasma fasting glucose level and hepatic glycogen storage in diet induced prediabetic rats.

1.4.2. Effects of hyperinsulinemia on the liver.

The liver is also a key site of insulin action, it is the main source of endogenous hepatic glucose production as well as a major site for the synthesis and disposal of lipids and the primary site of insulin extraction from plasma (30, 46). Endogenous hepatic glucose production and gluconeogenesis in particular are largely controlled by transcriptional regulation of key rate limiting enzymes in the gluconeogenic pathway, specifically phosphoenolpyruvate carboxykinase and glucose 6 phosphatase (G-6-Pase) (49, 68). However, in both insulin resistant and hyperinsulinemic states, there is impairment of glucose anabolism and catabolism (5, 63). The resistance of adipose tissue and muscle to insulin leads to a subsequent increase in lipolysis and a divergence of glucose to the liver, respectively. As a result, *de novo* lipogenesis and gluconeogenesis in the liver are increased (12, 40).

Under normal physiological conditions, free fatty acids (FFA) and glycerol contribute to liver triglyceride synthesis via hepatocellular long-chain fatty acids bound to coenzyme A to form fatty acyl coenzyme A (CoA) (43, 46). The hepatocellular concentration of fatty acyl CoA results from the balance between FFA formation, and triglyceride breakdown (43, 46). About 25% of FFA entering the liver are taken up mainly via arteriovenous supply from subcutaneous adipocytes (68). Insulin resistance in the adipose tissue causes uncontrolled fat accumulation in the liver because there is excessive lipid availability resulting increased triglyceride synthesis the development of NAFLD (30, 62). *De novo* lipogenesis can be stimulated both by insulin, via sterol regulatory element binding-protein 1c (SREBP1c) and by glucose, via carbohydrate response element-binding protein (ChREBP). Thus, hyperinsulinaemia and diets high in fat and carbohydrate will contribute to elevated *de-novo* lipogenesis in prediabetes and NAFLD (10, 43). The hepatocellular FFA pool can be further increased by impaired export of VLDL cholesterol in insulin-resistant patients with non-alcoholic steatohepatitis NASH (15). This study investigated the effect of rhenium (V) compound on TGs concentrations and relative liver weights in a prediabetic rats.

1.4.3. Hyperglycaemia-induced oxidative stress and hepatic damage.

Several important pathways have been identified as causing liver damage in T2DM patients (7, 35). The liver is among the primary organs susceptible to the effects of hyperglycaemia-induced oxidative stress, which may lead to liver tissue injury (7, 35). Oxidative stress and inflammatory responses act as damaging agents on liver tissue resulting in a leakage of enzymes resident in liver cells when the cells are damaged by ROS. The leakage then results in the enzymes getting into systemic circulation hence the elevated levels in liver damage. . These enzymes include plasma alanine transaminase (ALT) and aspartate transaminase (AST) (1, 35). Studies have shown that in prediabetic patients with NAFLD, there are high plasma levels of both ALT and AST (12, 13). This study looked at liver enzymes (ALT and AST) as a parameters of liver damage in prediabetic rats and further investigated the effects of rhenium (V) compound on regulating liver enzymes (ALT and AST) levels by increasing antioxidant activity (SOD and GPx) in prediabetic rats.

1.4.4. Hepatocyte dyslipidaemia and of cardiovascular disease .

Insulin resistance, hyperinsulinemia and excess visceral fat have been shown to contribute to the development of NAFLD (10, 30). In return, the insulin resistant fatty liver overproduces glucose and VLDL thus boosting the mechanisms that lead to the development of cardiovascular disease (12, 19). The liver fat content reflects the equilibrium between FFA flux through lipolysis, fatty acid oxidation, *de-novo* lipogenesis and VLDL secretion (12, 68). The hepatic triglyceride accumulation is probably a consequence of saturation of fatty acid oxidation and VLDL secretion (12, 62). Both these pathways are up-regulated rather than decreased in NAFLD (12, 19). Impaired liver function results in secretion and deposition of LDL, TGs and other fatty acid content in the plasma causing the development of CVD (11, 19).

1.5. Development of cardiovascular disease in prediabetes

Cardiovascular disease is the leading cause of death worldwide (69, 70). Hypercholesterolemia has been shown to be one of the most important risk factors in the development of CVD (12, 19). In an insulin resistant state, the decreased ability of insulin to inhibit lipolysis leads to increased FFA generation and lower lipoprotein lipase activity (19, 68). Impairment in lipogenesis causes the generation of a chylomicron remnant rich in TGs caused by elevated

hepatic FFAs and VLDL TG-rich particles secretion (19, 61, 71). These processes affect HDL-C metabolism through the interchange with TG-rich lipoproteins *via* cholesteryl ester transfer protein to produce HDL particles containing high TG concentrations (9, 18).

These HDL-TG particles are hydrolysed by hepatic lipase to TG and HDL (19). This HDL becomes smaller and less antiatherogenic and is more easily removed from the circulation by the kidneys (9, 18). (4). The most atherogenic subfractions of LDL are elevated in circulation of obesity individuals, as a key feature in association with elevated triglyceride and low HDL cholesterol (17). Elevated VLDL concentrations are also found in abdominal obesity patients and demonstrated greater myocardial risk (9, 19). The following mechanisms are related to excess accumulation of abdominal adipose tissues, elevated total cholesterol and LDL-C, consumption of diet rich in saturated-fat, weight gain and obesity (11, 72). This study looked at TGs, HDL, and TC as parameters of dyslipidaemia control in prediabetic rats and further investigated the effects of rhenium (V) compound on regulating lipogenesis on diet induced prediabetic rats.

Dyslipidaemia a metabolic derangement characterised by elevated TG, total cholesterol, small dense LDLs (VLDL) and reduced HDL levels, is a common feature of T2DM and increases the incidence of atherosclerosis and mortality of diabetic patients (19, 73). The development of atherosclerosis begins during the prediabetic state (19). Dyslipidaemia is a characteristic dyslipidaemic profile consisting of elevated TG, total cholesterol, small dense LDLs (VLDL) and reduced HDL levels (19, 73). Oxidation of LDLby free radicals, is considered one of the hallmark risk factors for CVD (49, 74). Oxidized LDL is more reactive with surrounding tissues and can collect within the inner lining of arteries. The build-up of oxidized LDL leads to reduced blood flow in the coronary arteries, endothelial damage and hypertension (19, 75). This study investigated the effect of rhenium (V) compound on preventing the development of atherosclerosis and further investigated its effects on oxidative stress and antioxidants mechanisms on diet induced prediabetic rats.

Hyperglycaemia can mediates markers of chronic inflammation and contribute to increased reactive oxygen species (ROS) generation, which ultimately causes vascular dysfunction (69, 76). The protein kinase C (PKC) pathway can be activated by hyperglycaemia. PKC activation induces tandem alterations in the expression of TGF-b, reactive oxygen species and nuclear factor-kappa B, a master regulator of inflammation (77). These downstream effects of

glycaemic activation of PKC, especially those involving vascular and inflammatory pathways, can account for the worsening β-cell function, insulin resistance, microvascular and macrovascular complications in susceptible persons (69, 77). In prediabetic patients, there is chronic low-grade inflammation which is reflected by high levels of cytokines TNF-α and other inflammatory markers CRP and IL-6 (76, 77).

TNF-a increases insulin resistance via modulation of GLUT 4 and phosphorylation of insulin receptor substrate-1 (76, 77). C-reactive protein is strongly associated with CVD and in prediabetes, early low-level inflammation is present which is reflected by the rise in CRP levels (69, 77) . This study we investigated the effects of rhenium (V) compound in possible preventing inflammation by measuring TNF-α, CRP and IL-6 in diet induced prediabetic rats.

1.5.1. Effect of prediabetes-associated hypertension in CVD development.

Prediabetes is an intermediate state of hyperglycaemia with glycaemic parameters above normal but below the diabetes threshold. Increased body weight in prediabetes patients is associated with IFG, fat accumulation in other organs due to insulin resistant adipose tissue and impaired fat secretion or deposition from the liver (17, 43). Fat accumulation in the arteries reduce blood flow and blocks insulin receptors. The hyperinsulinemia and insulin resistance state in prediabetes contributes to increased mean arterial pressure (MAP) via mediating increased the sympathetic nervous activity(11, 36). Prediabetic patients experience increased peripheral artery resistance caused by vascular remodelling and increased body fluid volume associated with insulin resistance-induced hyperinsulinemia and hyperglycaemia (78, 79). Both mechanisms elevate systemic blood pressure (78, 80). This study looked at MAP, systolic and diastolic blood pressure throughout the treatment process to monitor if rhenium (V) compound can regulate blood pressure in diet induced prediabetic rats.

Dyslipidaemia is not the only risk factor associated with the development of CVD. Body mass index, body weight, hypertension, and compromised nitric oxide (NO) availability are also risk factors (79). Hypertension and dyslipidemias occur earlier, more frequently and more severely in patients with T2DM (68) . In the insulin-resistant state, the insulin-mediated stimulation of the insulin receptor substrate (IRS)-1/PI-3 kinase pathway by insulin is severely impaired, causing a decrease in NO. The later is a potent vasodilator hence compromised NO availability results in increased mean arterial pressure, which if untreated, could result in the development of hypertension (80). Hyperglycaemia also activates the PKC pathway that alters the expression

of nitric oxide synthase, vascular endothelial growth factor, and plasminogen activator inhibitor-1 (80).

The presence of co-existing risk factors hypertension, prediabetes and dyslipidemia promotes more advanced vascular endothelial dysfunction and ultimately mediating organ damage (78, 79). This study monitored the body weight for prediabetic rats and measured NO as possible hypertension risk factors and further investigated effect of rhenium (V) compound on NO bioavailability (17).

The development of heart failure causes activation of two systems, the renin angiotensin aldosterone system (RAAS) which causes retention of Na^+ and water as well as the sympathetic nervous system causing lipolysis (37, 78). When these activated systems combine with a typical western high calorie diet, this can lead to insulin resistance (37, 81). The upregulation of RAAS contributes to renal system impairment (80, 82).

1.6. Renal dysfunction.

Kidney dysfunction is caused by risk factors that include hypertension, arteriosclerosis and T2DM (71, 83). A combination of fat accumulation as a result of insulin resistance in the peripheral tissues and primary hypertension leads to kidney dysfunction, which may cause secondary hypertension and further impairment of renal function (71, 84). Arterial hypertension plays an important role in the development of kidney dysfunction by causing glomerular hyperfiltration, glomerular hypertrophy, followed by expansion of the mesangium and accumulation of extracellular matrix (37, 83). Impaired glomerular function is one of the indicators that the kidney is not functioning optimally(83, 84).

Microalbuminuria represents the first abnormality in individuals suffering from diabetic nephropathy (78, 83). Diabetic nephropathy causes decline in renal function including reduced function of glomerular filtration rate (GFR) that results in electrolyte abnormalities, water imbalance and hypernatremia (83, 84). Under normal physiological conditions, insulin stimulates Na^+/K^+-ATPase from the cytoplasm to the cell membrane to open the Na^+/H^+ channel that passively transports hydrogen ions out of the cell and sodium ions into the cell (84, 85). This process also increases the cellular calcium ion concentration and decreases pH (37, 85). However, in an insulin resistant state, the Na+/K+-ATPase activity is decreased (40, 63). The sodium reabsorption from renal tubules is increased to increased circulating fluid volume due to hyperglycaemia-induced hyperosmolarity leading to high blood pressure (84,

85). This study investigated the potential of rhenium (V) compound to improve GFR function in prediabetic rats by monitoring urine volume, Na^+, K^-, creatinine, glucose protein concentration and plasma albumin and creatinine concentration during 12 weeks of treatment,

Kidney dysfunction is associated with loss of podocytes, mesangiolysis, and glomerular fibrosis (71, 84). Hyperuricemia, which in some instances is secondary to renal dysfunction, can feed into this cycle. Uric acid is pro-inflammatory and induces oxidative stress and activates the RAAS (84, 86). The upregulation of RAAS contributes to impairment of therenal, which manifests with proteinuria and the significantly increased plasma kidney injury molecule (Kim 1) concentration (82, 86). Hyperinsulinemia stimulates sympathetic nervous activity and increases renin secretion (8, 81). The increase in renin activates the sympathetic nervous system and increases cardiac output and peripheral vascular resistance (86, 87). These changes ultimately elevate blood pressure by increasing both the circulatory fluid volume and peripheral vascular resistance (82, 83). Furthermore, insulin due to the hyperinsulinemia state mediates fat accretion leading, to obesity-induced hypertension in association with type 2 diabetes mellitus (84). This study investigated the effect of rhenium (V) compound on kidney injury in a prediabetic rats by determining its effects on. KIM 1 and aldosterone concentration as a markers of kidney injury as well as its effects on the regulation RAAS.

Glomerulonephritis is known as the injury to glomerulus. Hyperglycaemia contributes to scarring of the glomeruli, increases the rate of blood flow through the nephrons and can cause oxidative stress (71, 88). Advanced glycation end products and oxidative stress are critical factors in the progression of diabetic nephropathy and are augmented by inflammatory mechanisms of injury in the kidney (83, 88). Glomerular cells produce a multitude of inflammatory mediators in T2DM, especially as glomerular injury proceeds that can augment inflammatory damage and even lead to systemic effects (84). A previous study on rhenium (V) compound, showed that it can lower hyperglycaemia (34, 89). However, the effect of rhenium (V) compound on injury caused by oxidative stress in the glomerular are yet to be discovered. Therefore, this study, also investigated the effect of rhenium (V) compound on glomerular injury caused by oxidative stress in the prediabetic state. Furthermore, the effects of the compound on oxidative stress induced inflammation were evaluated by measuring TNF-α, and IL-6 GPX, SOD activities in diet induced prediabetic rats. Prediabetes complications such as NAFLD, CVD and renal dysfunction co-exist in individuals with prediabetes therefore there is a need for a management strategy that can target these complications collectively (11, 68).

2. Management of prediabetes complications and T2DM development.

In prediabetic patients, the combination of diet and exercise is arguably the single most important factor that could delay the progression towards type 2 diabetes (68, 90). Early and effective glycaemic control reduces the likelihood of developing insulin resistance and prediabetes (11, 91). Vigorous control of hypertension may prevent ventricular hypertrophy and thereby limit both cardiac and renal damage (61, 75).

2.1. Dietary intervention.

Many guidelines highlight the importance of reducing the intake of foods high in sodium and trans-fat in order ensure maintenance of cardiovascular health (91, 92). Other studies also recommend transitioning to diets that have low amounts of carbohydrates as well as avoiding fructose sweetened drinks in the management of both prediabetes and T2DM (68, 79). Foods that improve glycaemic control are recommended for prediabetic patients (92). However, the challenge still remains in implementing these methods in a community setting in developing countries (11, 68). Therefore, there is a need of pharmacotherapy to help to manage prediabetes (11, 68).

2.2. Management of prediabetes with metformin.

Metformin has been shown to reduce the incidence of new onset diabetes by 40% (9, 68). It inhibits the mitochondrial respiratory chain in the liver and thus activating adenosine monophosphate-activated protein kinase (AMPK), this enhances insulin sensitivity and lowers cAMP, which then reduces the expression of gluconeogenic enzymes. AMPK-independent effects of metformin include inhibition of fructose-1,6- bisphosphatase by AMP (9, 68). Metformin has been used to treat diabetes associated NAFLD, cardiovascular and kidney diseases.

2.2.1. Effects of metformin on NAFLD.

Metformin has a central role in the treatment of T2DM. It lowers blood glucose by decreasing hepatic gluconeogenesis in the liver, stimulating muscle glucose uptake , and increasing fatty acid oxidation in adipose tissue (27). In the liver, AMPK reduces hepatic gluconeogenesis by inducing the phosphorylation of CREB-binding protein (CBP) and, as consequence, the dissociation of the gluconeogenic CREB-CBP-TORC2 transcriptional complex (26). This

event, mediated by atypical protein kinase C (PKC), triggers the disassembly of the transcription machinery and the inhibition of the expression of gluconeogenesis enzyme genes including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (26, 53).

The activation of AMPK by metformin also exerts beneficial effects on lipid metabolism (27). Metformin-induced AMPK inactivates acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, decreases fatty acid synthase (FAS) expression, and activates malonyl-CoA carboxylase (26). The final effect is a decrease in fatty acid and cholesterol synthesis (27).Furthermore, AMPK suppresses sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor responsible for genes related to fatty acid synthesis (26). SREBP-1c levels rise due to excessive glucose and insulin, and this increase is inappropriately high in individuals with non-alcoholic fatty liver disease (NAFLD) (26, 27). Metformin reduces accumulation of TGs in liver and plasma TGs which is a positive effect for cardiovascular system health (53).

2.2.2. Effects of metformin on cardiovascular system.

Metformin has the following effects on cardiovascular system insulin-dependent and insulin-independent vasodilatory actions and probably also central antihypertensive effects [69]. Several authors have shown that metformin improves lipoprotein profiles with a decrease in low-density lipoprotein cholesterol levels, triglycerides, and high-density lipoprotein cholesterol levels (27).

Metformin has been reported to reduce markers of inflammation and to lessen hypercoagulation and increase fibrinolysis by decreasing levels of plasminogen activator inhibitor-1 and increasing tissue plasminogen activator activity (26, 27). It also improves functional and biochemical markers of endothelial reactivity as well as surrogate indexes of coronary atherosclerosis (27). Furthermore, it mediates areduction of circulating advanced glycated end products (AGEs), which causes oxidative stress that result in endothelial dysfunction. Moreover, metformin is able to stimulate intracellular AMPK and to activate the endothelial isoform of NOSs in human aortic endothelial cellsINCOMPLETE (26, 53). Metformin was also demonstrated to reduce the major adverse cardiovascular events (53).

2.2.3. Effects of metformin on renal dysfunction.

Metformin has been shown to possess reno-protective properties. It acts primarily in the liver by promoting the reduction of glucose production via inhibition of hepatic glucose production and glycogenolysis (27, 84). In the kidney, metformin inhibits oxidative stress and apoptosis by regulating AMPK and mTOR pathways as well as Akt activity.It restores nephrin expression via AMPK-SIRT1-PGC-1α axis and elevates TRPC6 level by activating AMPKα1, thus decreasing podocyte loss, it has been shown that Podocyte loss plays an important role in the development of glomerulosclerosis under diabetic conditions (53). Moreover, metformin was shown to reduce mesangial cell disorders by inhibiting apoptosis partly via upregulating mesangial glucagon-like peptide-1 recepter (GLP-1R) expression, as well as ameliorating oxidative stress. It was also found to promote autophagy, inhibit abnormal cell proliferation through AMPK/SIRT1/FoxO1 pathway and decrease albuminuria inT2DM patients (27).

However, , the therapeutic efficacy of metformin was shown to be optimal effects when combined with lifestyle interventions such as increased physical activity and dietary intervention (9, 68). There are growing reports of low patient compliance to lifestyle modifications but to be heavily reliant on pharmacotherapy thus reducing the efficacy of the metformin (27). Therefore, there is a need for a pharmacological agent that can be effective in both the presence and absence of lifestyle intervention. The potential use of metal complexes to treat prediabetes is growing (32, 33).

3. Metal complexes and Management of prediabetes.

The pathophysiological defects underlying prediabetes include insulin resistance, β-cell dysfunction, increased lipolysis, inflammation and possibly hepatic glucose overproduction (4, 9, 93). In recent years, an increased interest in bioactive metal complexes has led to a multitude of studies describing the synthesis, biological activity of transition metal complexes and their use in treatment of diabetes complications due to their possible anti-diabetic properties (32, 90). Metal based compound including zinc-diosmin complex, oxovanadium (IV), and ruthenium compounds have been used experimentally to manage prediabetes (32, 94).

Oxovanadium (IV) inhibits protein tyrosine phosphatases (PTPs) and it showed higher potency in lowering elevated blood glucose levels in diabetic rats (94-96). A recent study revealed that anti-diabetic ruthenium complexes induce upregulation of insulin expression by various modes (97). Experimental data demonstrates that anti-diabetic ruthenium-based pharmacological agents can target selected proteins or enzymes that are associated with glucose homeostasis

(31, 97). Ruthenium-based pharmacological agents also targets PTP 1B and glycogen synthase kinase 3 (GSK-3) to manage hyperglycaemia and it displayed potent cardioprotective as well as hepatoprotective effects accompanied by the rejuvenation of glucose homeostasis in diet induced prediabetic rats (31, 98).

Studies on ruthenium complexes together with vanadium compounds showed that these metal-based compounds and complexes can reduce inflammation and increase insulin sensitivity as well as improve glucose control in insulin-resistant patients with both prediabetes and T2DM (93, 98). Furthermore, ruthenium compound with a Schiff base was shown to be beneficial in several studies on prediabetes complications such as CVD, renal dysfunction, and hepatic insulin resistance in diet-induced prediabetic rats (31, 93, 98).

Lastly, the ruthenium compound with a Schiff base was shown to be effective in both the presence and the absence of diet intervention in diet induced prediabetic rats (31, 97). Therefore, we need more metal-based compounds that can be effective in both the presence and the absence of diet intervention in managing prediabetes. The rhenium (V) compound is the metal compound of interest in this study, and it shares the same uracil derivative as the ruthenium compound (99).

3.1. Rhenium(V) compound.

This study is on [3+1] oxo- free rhenium (V) compound with uracil-derived ligands (Figure 1 below) in the treatment of prediabetes in diet induced prediabetic rats. Rhenium has been used in many medical applications such as photo-releasing therapeutic, photoluminescence diagnostic, anti-microbial agents and radiopharmaceuticals (100, 101).

Rhenium complexes have been shown to have anticancer, anti-inflammatory and antioxidant properties (100, 102). Studies have shown that metallo-pharmaceuticals require organic biological active ligand systems to provide stability and promote bio-availability of the metal complex (34, 102). A study conducted by Maisuls et al (2017) reported that rhenium (V) compound {trans-[Re(ddd)(Hduo)(PPh3)2] I (H2ddd = 5,6-diamino-1,3-dimethyluracil and H3duo = N-(2-hydroxybenzylidene)-5-amino-1,3- dimethyl uracil allow for a great deal of structure and chemical variability and can be fine-tuned to meet the requirements of a wide range of biological applications including among man, fluorescence markers and/or antitumor pharmacological agents (100, 102).

The [3+1] oxo- free rhenium (V) compound with uracil-derived ligands was shown to have a positive effect on glucose handling in both insulin resistant skeletal muscle cell lines and on diet induced prediabetic rats (34). The metal-based pharmacological agents improved the insulin signalling pathway on insulin resistant cells and promoted glucose uptake. In addition, dosage-dependent reduced lipid peroxidation and enhanced total antioxidant levels, with no accompanied observable cell toxicity (89). In a previous study treating prediabetic rats with rhenium (V) compound improved glycaemic control as evidenced by improved HOMA-IR-index, Hba1c concentration and increased GLUT 4 expression in skeletal muscle tissue (34).

Due to these pharmacological properties, this rhenium (V) compound is a promising candidate as an antidiabetic drug to manage prediabetes. However, the effects of this compound on prediabetes complications are yet not known. Furthermore, the effect of this compound on NAFLD, CVD and renal injury have not yet been conducted. Therefore, the aim of this study was to investigate the effects of a novel rhenium (V) compound on prediabetes associated complications NAFLD, CVD and renal injury in a diet-induced pre-diabetic rat model.

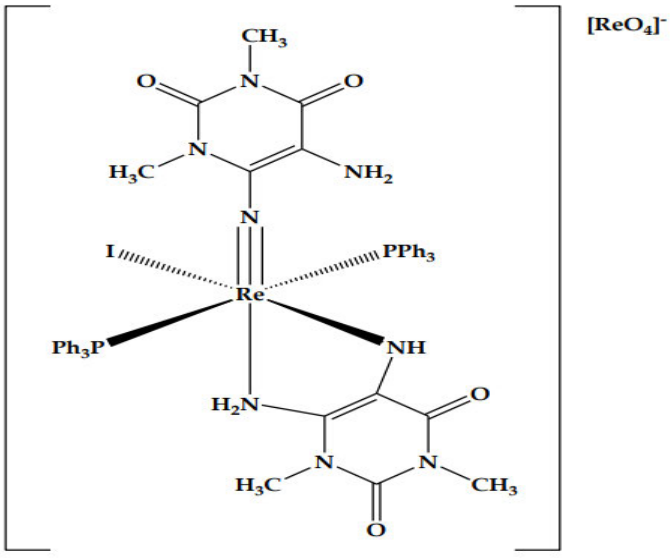

Figure 1: Showing rhenium (V) compound structure. Adapted from Ismail MB *et al*,2015 (103)

4. Justification of the study.

Literature has defined prediabetes as a metabolic disorder that can be recognised by metabolic induces higher than normal but not high enough to for a T2DM diagnosis (11, 78). These metabolic induces include HOMA-IR index, blood glucose concentration, lipids TGs and VLDL and HbA1c (1, 69). Prediabetic patients are therefore at an increased risk of CVD and renal injury (68, 87).

The development of insulin resistance is associated with increased consumption high carbohydrate and high fat diets (16, 73). Busy lifestyles have resulted in increased consumption of unbalanced, high calorie diets in an environment of decreased physical activity has resulted in obese and overweight individuals that struggle with prediabetes (11, 30). Prediabetes patients with suffer from complications including NAFLD, CVD, hypertension, and renal dysfunction (2, 69). In order to prevent the progression of prediabetes to T2DM lifestyle modification inclusive of better dietary choices and increased physical activity are recommended (104, 105). These lifestyle modifications facilitate weight loss and have been found to be beneficial in managing prediabetes and restoring glycaemic control (28, 106).

Currently, metformin is the pharmacologic agent recommended for the prevention or delay of T2DM (25, 28). However, the success of this pharmacological agents is often dependent on it being used in conjunction with lifestyle modifications (28, 107). Patients choose to not follow the recommendation for lifestyle intervention thus this causes the loss of pharmacological agents effective (25, 33).

There is therefore a need for novel pharmacological agents that can remain at its optimal effects in the management of prediabetes even in both the presence and absence of dietary intervention (28, 33). There is significant progress in utilization of transition metal complexes as pharmacological agents to treat T2DM (32, 93, 108). Metal-based pharmacological agents are produced with promising pharmacological application and may offer unique therapeutic opportunities (32, 108).

The [3+1] oxo- free rhenium (V) compound with uracil-derived ligands has been shown to have antioxidant, anti-inflammation and insulin sensitivity improvement in diet induced prediabetic rats REF. The literature evidence described in the above sections indicates that the rhenium (V) compound can be potentially used in management of prediabetes-associated complications. However, it is not known whether rhenium (V) compound will mitigate against

prediabetes associated effect NAFLD, CVD and renal injury in a HFHC diet induced pre-diabetic rat model.

5. Aim:

To investigate the effects of a novel rhenium (V) compound on the prediabetes associated complications NAFLD, CVD, and renal injury in a diet induced pre-diabetic rat model.

6.1. Objectives of the study:

The overall study objective was to investigate the novel rhenium (V) compound on prediabetes treatment. with three specific objectives each associated with a particular sub-stud presented in a manuscript.

6.1.1. Specific objective of sub-study 1.

To determine in prediabetic rats the effects of rhenium (V) compound treatment on fasting blood glucose concentration, insulin sensitivity on insulin resistant hepatocytes, body weight and relative liver weight, TGS concentration, oxidative stress and antioxidants enzymes (SOD and GPx) response, liver enzymes (AST and ALT) activities, TGS concentration, oxidative stress and antioxidant enzymes (SOD and GPx) response, liver enzymes (AST and ALT) activities.

6.1.2. Specific objective of sub-study 2.

To determine in prediabetic rats the effects of rhenium (V) compound treatment on regulating lipogenesis, blood pressure, preventing the development of atherosclerosis and inflammation.

6.1.3. Specific objective of sub-study 3.

To determine in prediabetic rats the effects of rhenium (V) compound treatment on kidney injury and improving GFR function.

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Chapter 2

Prologue 1

Manuscript 1

Prediabetes has been shown to lead to the development of NAFLD which increases the risk of T2DM development. Untreated NAFLD can lead to tissue damage due to hyperglycaemia-induced oxidative stress and dyslipidaemia. Treatment of prediabetes- associated NAFLD usually involves a combination of anti-hyperglycaemic pharmacological agents and dietary modification. However, patients tend to neglect the dietary modification thus reducing the effectiveness of the drug. Therefore, there is a need for alternative pharmacological agents that can work in both the presence and absence of dietary modification. In a previous study in our laboratory rhenium (V) compound proved efficacious at restoring glycaemic homeostasis from an impaired glycaemic control. This study investigated effects of rhenium (V) compound on markers of NAFLD in the presence and absence of diet intervention in a diet-induced prediabetic rat model. The manuscript titled *"***Investigating the Protective Effects of a Rhenium (V) Compound with Uracil-Derived Ligands on Liver Damage Associated with Prediabetes in Diet-Induced Prediabetic Rats"** and is authored by Siboto A, Akinnuga AM, Ismail MB, Booysen I, Sibiya NH, Ngubane PS and Khathi A. The manuscript is published in the journal **"Diabetology"** and has been formatted according to the journal's guidelines to authors. (See Appendix 2).

Manuscript 1

Article

Investigating the Protective Effects of a Rhenium (V) Compound with Uracil-Derived Ligands on Liver Damage Associated with Prediabetes in Diet-Induced Prediabetic Rats

Angezwa Siboto [1,*], Akinjide Moses Akinnuga [1], Muhammed Bilaal Ismail [2], Irvin Noel Booysen [2], Ntethelelo Hopewell Sibiya [3], Phikelelani 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; akinnugaakinjide@yahoo.com (A.M.A.); ngubanep1@ukzn.ac.za (P.N.); khathia@ukzn.ac.za (A.K.)
[2] School of Chemistry and Physics, College of Agriculture, Engineering Sciences, University of KwaZulu-Natal, Pietermaritzburg 3209, South Africa; bilaal.ismail@gmail.com (M.B.I.); booyseni@ukzn.ac.za (I.N.B.)
[3] Pharmacology Division, Rhodes University, Grahamstown 6140, South Africa; n.sibiya@ru.ac.za
* Correspondence: 212518628@stu.ukzn.ac.za

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Abstract:
Non-alcoholic fatty liver disease (NAFLD) is associated with prediabetes and can be treated by using a combination of metformin and dietary modification. However, people often fail to adhere to dietary modifications and become more dependent on pharmaceutical intervention, and this affects the effectiveness of the meformin. In this study, we investigated the effects of rhenium (V) compound with uracil-derived ligands on liver health in diet-induced prediabetic rats in both the presence and absence of dietary modification. Prediabetic male Sprague Dawley rats were treated with the rhenium (V) compound for 12 weeks in both the presence and absence of dietary modification while monitoring fasting blood glucose levels. Antioxidant enzyme activity, inflammation markers and liver enzymes were measured together with liver glycogen and plasma triglycerides after sacrificing. The administration of rhenium (V) compound to prediabetic rats in both the presence and absence of dietary modification resulted in reduced concentrations of fasting blood glucose and triglycerides. There was also reduced liver glycogen, oxidative stress and liver enzymes while increasing antioxidant enzymes. Altogether, the rhenium (V) compound ameliorated liver injury and prevented hepatotoxicity.

Keywords:
prediabetes; liver enzymes; rhenium (V) compound; triglycerides; NAFLD; fructose

1. Introduction:

Non-alcoholic fatty liver disease (NAFLD) has recently become the most frequent chronic liver disease that occurs across all age groups due to the growing prevalence of obesity and prediabetes [1,2]. NAFLD is strongly associated with insulin resistance, dyslipidemia and hypertriglyceridemia [10,11]. Diets that are high in carbohydrates and saturated fats have been shown to predispose individuals to developing both prediabetes and NAFLD [3,4]. Additionally, fizzy drinks that are high in fructose activate lipogenesis in the hepatocytes, resulting in a fatty liver [5–7]. Prediabetes is linked with moderate levels of insulin resistance and has been shown to play a primary role in the pathogenesis of NAFLD [8,9]. Liver dysfunction is associated with hepatic insulin resistance and an increase in hepatic glycogen production, whereas liver injury is shown by abnormal liver enzymes such as alanine transaminase (ALT), aspartate transaminase (AST) and lactic dehydrogenase [10,11]. These may be due to alternations in the permeability of the cell membrane and damage in the liver tissue [5,12].

The treatment of NAFLD involves a combination of dietary modification to enhance weight loss, along with the use of different insulin-sensitizing agents such as metformin [13–16]. However, studies have shown that patients become more dependent on the pharmaceutical interventions while neglecting the lifestyle modification, resulting in a reduction in the efficacy of metformin [17,18]. Therefore, there exists a need for novel pharmacological compounds that can work in both the presence and absence of lifestyle modifications. Transition metals have been used to try and manage diabetes and other metabolic conditions, but they have been shown to disrupt lipid and protein metabolism as well as induce oxidative stress in the liver [5]. However, recent studies from our laboratory have shown that the incorporation of organic ligands to the transition metals results in reduced cellular toxicity [19,20]. We have previously shown that our novel rhenium (V) compound with uracil-derived ligands improves glucose homeostasis in high-fat–high-carbohydrate diet-induced prediabetic animals in both the absence and presence of dietary modifications [20,21]. In this study, we sought to further investigate the effects of the rhenium (V) compound on selected liver function markers in diet-induced prediabetic rats.

2. Methods and Materials.

2.1. Animals,

Thirty-six (36) male Sprague Dawley rats (150–180 g) obtained from Biomedical Research Unit, University of KwaZulu-Natal (UKZN), were kept under standard environmental conditions, i.e., constant humidity (55 ± 5%), temperature (22 ± 2 °C), 12 h day:12 h night cycle. The rats were acclimatized for 2 weeks with free access to a standard rat chow (Meadow Feeds, South Africa) and water ad libitum before being fed on the experimental high-fat–high-carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, South Africa) to induce prediabetes. The HFHC diet consists of carbohydrates (55% kcal/g), fats (30% kcal/g) and proteins (15% kcal/g), as described in our previous study [3,20]. All the experimental designs and procedures were carried out according to the ethics and guidelines of the Animal Research Ethics Committee (AREC, ethical clearance code: AREC/039/018M) of the UKZN, Durban, South Africa.

2.1.1. Induction of Prediabetes on Male Sprague Dawley Rats.

Sprague Dawley rats (n = 6 per group) were divided into groups based on the diet they received: a standard rat chow with normal drinking water (ND + H_2O), high-fat–high-carbohydrate diet with drinking water supplemented with 15 % fructose (HFHC + fructose). Prediabetes induction took 20 weeks using a previously established protocol [3,22]. Rats with fasting blood levels that were higher than 5.6 mmol/L were considered prediabetic and grouped further for pharmacological studies [23,24]. The treatment commenced on the subsequent day, and this was considered as day 1 of treatment.

2.1.2. Study Design for Experiments.

In this study, rats were randomly divided into 6 groups of 6 animals in each (30 prediabetic persisting, 6 normal). Group 1: normal healthy control rats received vehicle (NC); Group 2: prediabetic control rats

continued with HFHC diet and received vehicle (PD); Group 3: prediabetic treated rats switched to the STD diet and received metformin (MET + DI); Group 4: prediabetic treated rats continued with HFHC diet received metformin (MET + HFHC); Group 5: prediabetic rats switched to the STD diet and received rhenium (V) compound (Re + DI); Group 6: prediabetic treated rats continued with HFHC diet received rhenium (V) compound (Re + HFHC). See Figure 1.

2.1.3. Treatment of Prediabetic Animals.

After 20 weeks of inducing prediabetes, the treatment period started and lasted an additional 12 weeks. During the treatment period, the animals were treated once every third day at 9:00 a.m., where metformin (500 mg/kg) was given through oral dosing to the MET + HFHC and MET + DI, while rhenium (V) compound (15 mg/kg) was given via subcutaneous injection to the Re + HFHC and Re + DI groups. Fasting blood glucose (FBG) concentration, food intake and body weights were monitored every 4 weeks during the treatment period. See Figure 1.

2.1.4. Blood collection and tissue harvesting.

For blood collection, all animals were anesthetized with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd., Roodeport, South Africa) via a gas anesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for 3 min in line with the guidelines for use of anesthesia. While the rats were unconscious, blood was collected by cardiac puncture into individual pre-cooled heparinized blood collection tube. The blood was then centrifuged (Eppendorf centrifuge 5403, Germany) at 4 °C, 503× *g* for 15 min. Plasma was collected and stored at −80 °C in a Bio Ultra freezer (Snijers Scientific, Holland, Netherlands) pending for biochemical analysis. Following blood collection, liver was removed, weighed and rinsed with cold normal saline solution and snap frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at −80 °C until biochemical analysis.

2.1.5. Relative Liver Weight.

The relative liver weights of all the animals in each experimental group were determined from the percentage of the ratio of liver weight to the body weight using the formula below:

$$\text{Relative liver weight} = \frac{\text{liver weight}}{\text{body weight}} \times 100\% \quad (1)$$

The experimental protocol for the study is shown in Figure 1 below.

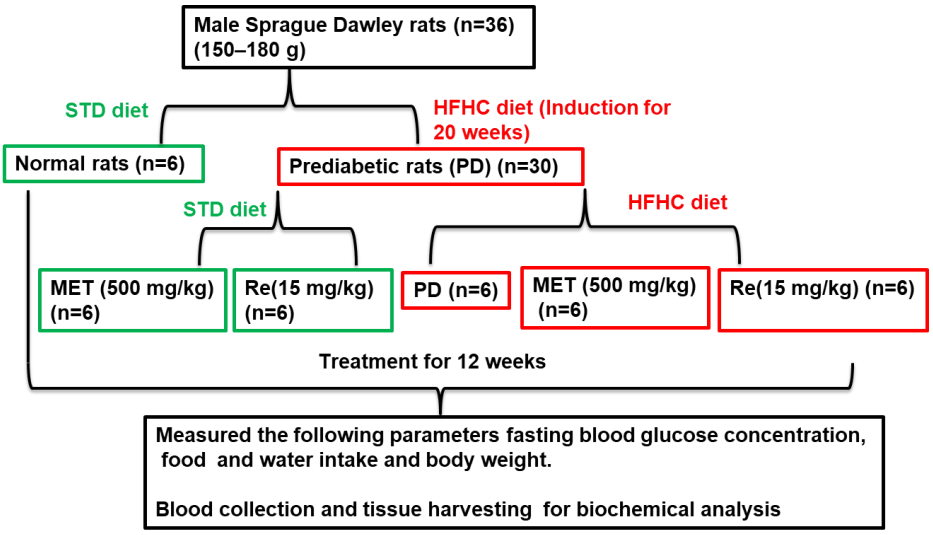

Figure 1. Experimental design.

2.2. *Biochemical Analysis.*

2.2.1. Quantification of Hepatic Glycogen, Plasma Triglycerides, TNF α and Liver Function Enzymes

Glycogen concentration analysis was performed on liver tissues. The glycogen assay was conducted using a well-established laboratory protocol [20].

Triglycerides (TGs) were measured using a colorimetric assay kit (catalog No: E-BC-K238; Manufacturer: Elabscience), single reagent, GPO-PAP method. Briefly, 50 mg of liver tissue was homogenized in 0.9% saline in a ratio of 9:1. The tissue was homogenized on ice for 2 min, and then the sample was centrifuged at 1000× *g* for 10 mins. After centrifugation, the aqueous phase extraction was used in the assay. The protein quantification was conducted using Bradford assay. The assay was carried out according to the manufacturer's instructions. Furthermore, for TNF α measurements, 50 mg of liver tissue was homogenized, and the concentration was determined using an ELISA kit following the manufacturer's instructions (catalog no.: E-EL-R2856; manufacturer: Elabscience). Liver AST and ALT concentrations were measured using the Catalyst One Chemistry Analyzer (IDEXX Laboratories, Westbrook, ME, USA).

2.2.2. Antioxidant Activity Profile.

Antioxidant activity in the liver was measured on selected markers. Glutathione peroxidase (GPx) (catalog no.: E-EL-R2491; manufacturer: Elabscience) and superoxide dismutase (SOD) (catalog no.: E-EL-R1424;

manufacturer: Elabscience) concentrations were determined using assay kits as per manufacturer's instructions.

2.3. Statistical Analysis.

All data are expressed as means ± SD. Statistical analysis were performed with GraphPad InStat Software (version 5.00, GraphPad Software, Inc., San Diego, CA, USA) using two-way analysis of variance (ANOVA) followed by eman comparisons using the Tukey–Kramer multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

3. Results.

3.1. Fasting Blood Glucose Concentration.

Figure 2 shows fasting blood glucose concentrations in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. By comparison with the NC, there was a significant increase in fasting blood glucose concentration in the PD group ($p < 0.05$, Figure 2). The administration of the rhenium (V) compound in both the HFHC and diet intervention groups resulted in significant reduction in fasting blood glucose concentration by comparison to PD ($p < 0.05$, Figure 2). A similar observation was shown by the MET + DI treated group as compared to the PD group (see Figure 2).

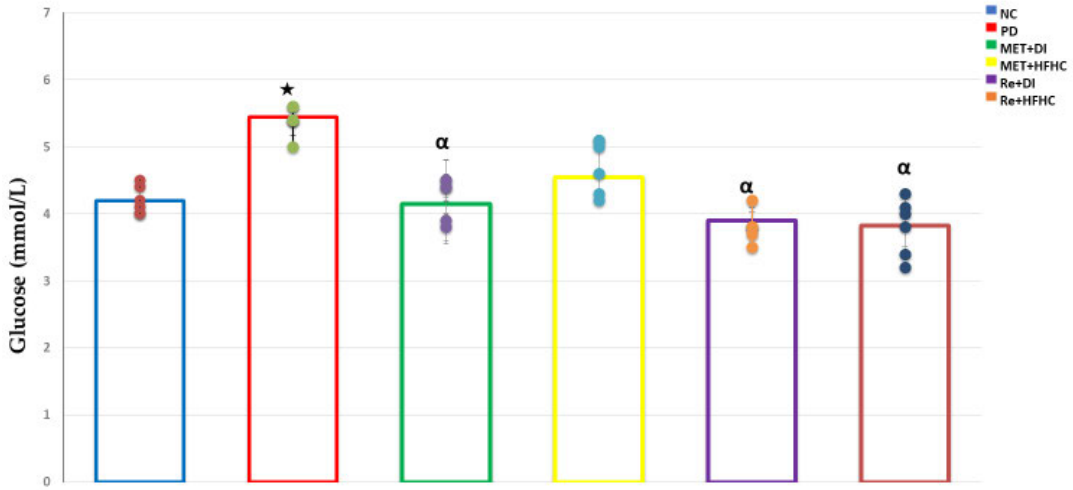

Figure 2. Fasting blood glucose in NC, PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC at 12 weeks of treatment. Values are presented as means ± SD ($n = 6$). * $p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

3.2. Liver Glycogen Concentration.

Figure 3 shows liver glycogen levels in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. By comparison with the NC group, the PD group showed a significant increase in liver glycogen concentration ($p < 0.05$) (Figure 3). The administration of rhenium (V) compound in both diet intervention and high fat–high carbohydrate groups resulted in a significant decrease of liver glycogen concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$) (see Figure 3).

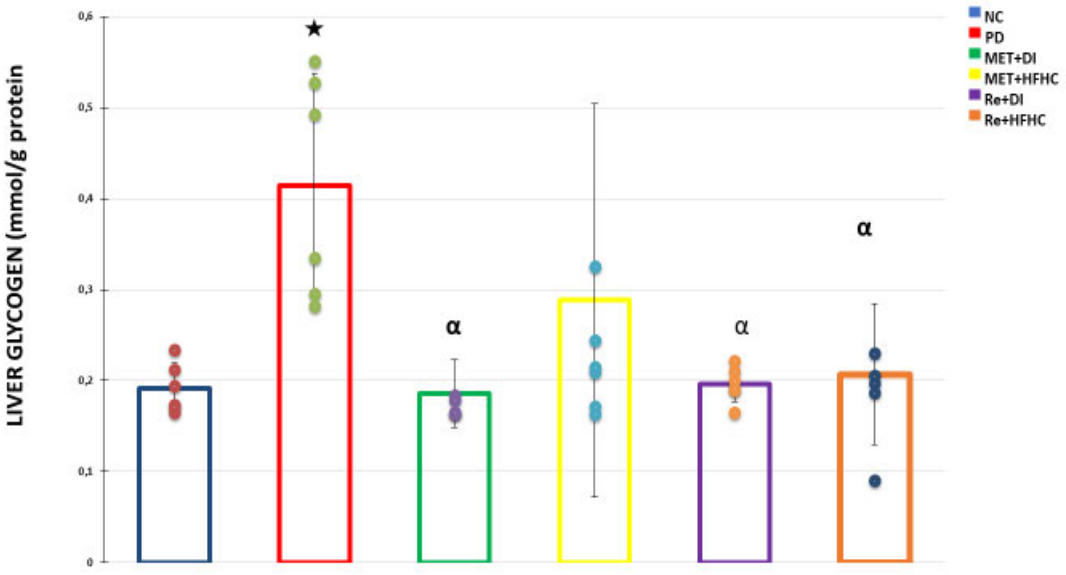

Figure 3. Liver glycogen concentration in NC, PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC after 12 weeks of treatment. Values are presented as means ± SD and individual data points ($n = 6$). * $p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

3.3. Liver Triglycerides (TGs) Concentration.

Figure 4 shows liver triglyceride concentration in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. By comparison with the NC group, the PD group showed a significant increase in liver triglycerides concentration ($p < 0.05$) (Figure 4). The administration of rhenium (V) compound in both diet intervention and high fat–high carbohydrate groups resulted in a significant decrease of liver triglycerides concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$) (see Figure 4).

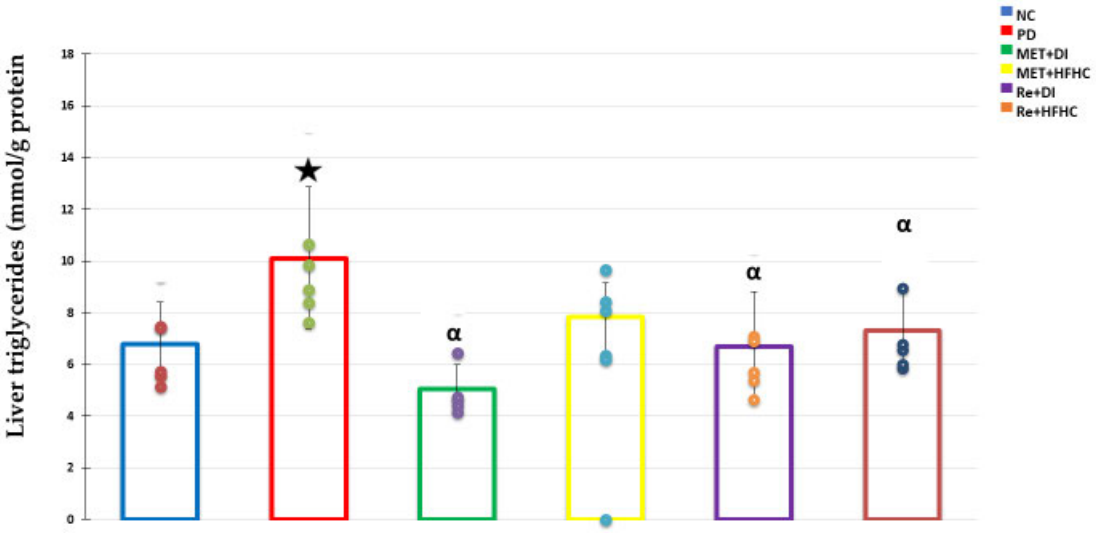

Figure 4. Liver triglycerides concentration in NC, PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC after 12 weeks of treatment. Values are presented as means ± SD and individual data points ($n = 6$). * $p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

3.4. Relative Liver Weight.

Figure 5 shows relative liver weight in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant increase in relative liver weight ($p < 0.05$) (Figure 5). The administration of rhenium (V) compound in both diet intervention and high fat–high carbohydrate groups resulted in a significant decrease of relative liver weight when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$) (see Figure 5).

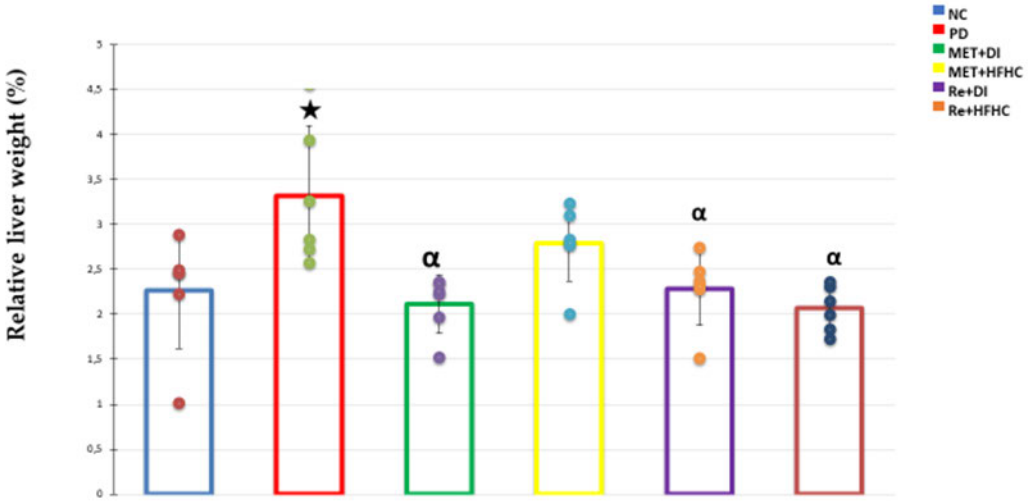

Figure 5. Relative liver weight in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SD and individual data points ($n = 6$). *$p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

3.5. Liver Antioxidant Activity.

Figure 6 shows SOD activity in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant decrease in SOD activity ($p < 0.05$) (Figure 6). The administration of rhenium (V) compound in both diet intervention and high fat–high carbohydrate groups resulted in a significant increase of SOD activity when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$) (see Figure 6).

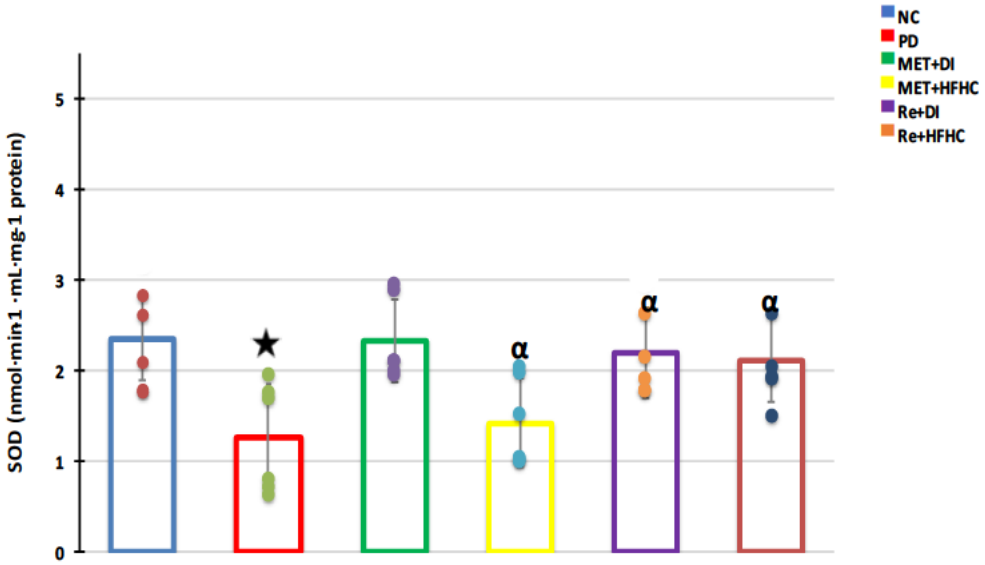

Figure 6. SOD activity in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SD and individual data points ($n = 6$). * $p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 7 shows GPx activity in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant decrease in GPx activity ($p < 0.05$) (Figure 7). The administration of rhenium (V) compound in both diet intervention and high fat–high carbohydrate groups resulted in a significant increase of GPx activity when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$) (see Figure 7).

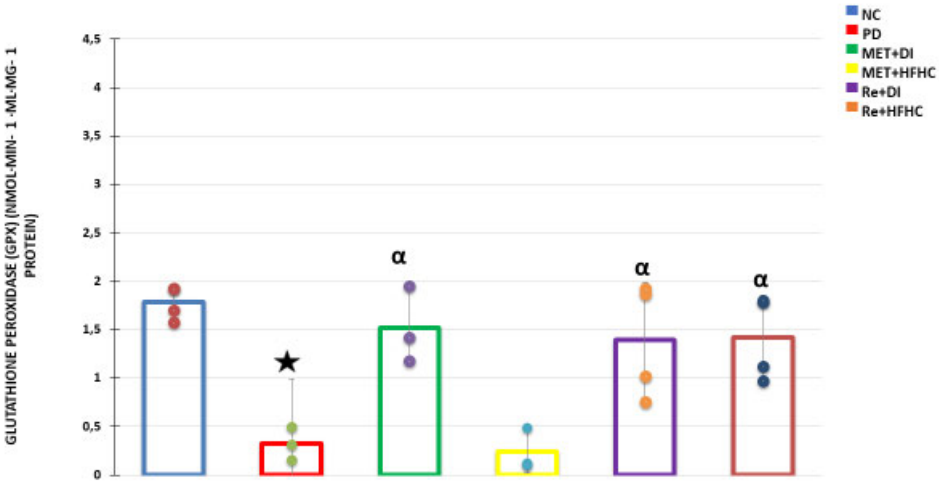

Figure 7. GPx activity in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SD and individual data points ($n = 6$). *$p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

3.6. Liver TNF α Concentration.

Figure 8 shows TNF α concentrations in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant increase in TNF α concentration ($p < 0.05$) (Figure 8). The administration of rhenium (V) compound in both diet intervention and high fat–high carbohydrate groups resulted in a significant decrease of TNF α concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$) (see Figure 8).

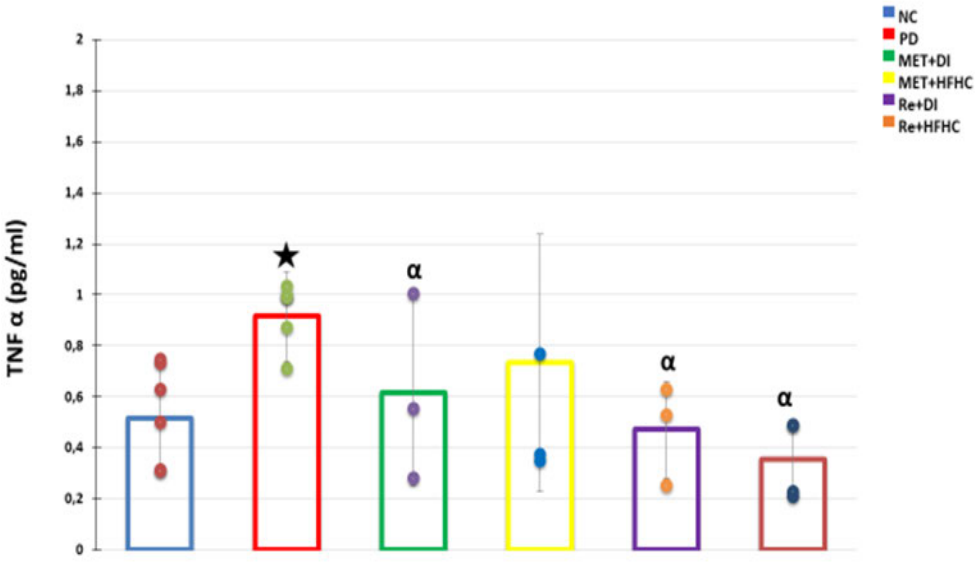

Figure 8. TNF α concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SD and individual data points ($n = 6$). *$p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

3.7. Plasma ALT Concentration.

Figure 9 shows plasma ALT concentration in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant increase in plasma ALT concentration ($p < 0.05$) (Figure 9). The administration of rhenium (V) compound in both diet intervention and high fat–high carbohydrate groups resulted in a significant decrease of plasma ALT concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$) (see Figure 9).

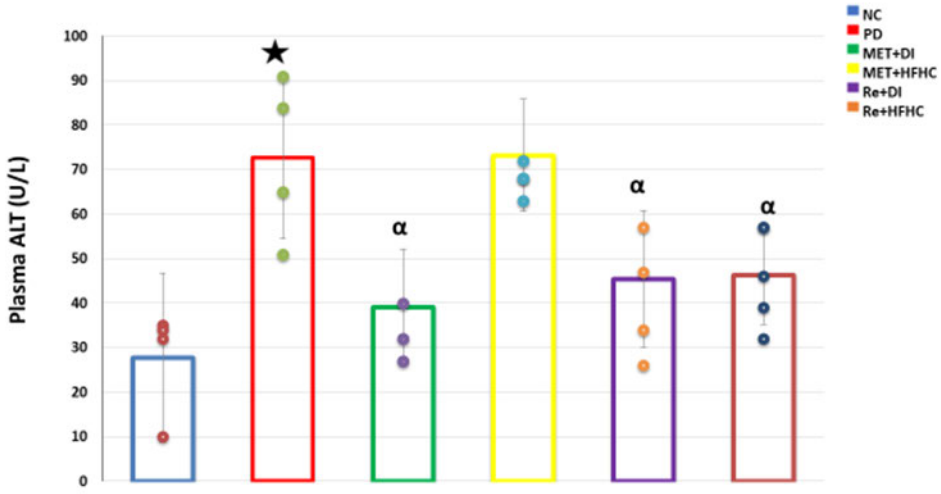

Figure 9. Plasma ALT concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SD and individual data points ($n = 6$). * $p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

3.8. Plasma AST Concentration.

Figure 10 shows plasma AST concentration in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant increase in plasma AST concentration ($p < 0.05$) (Figure 10). The administration of rhenium (V) compound in both diet intervention and high fat–high carbohydrate groups resulted in a significant decrease of plasma AST concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$) (see Figure 10).

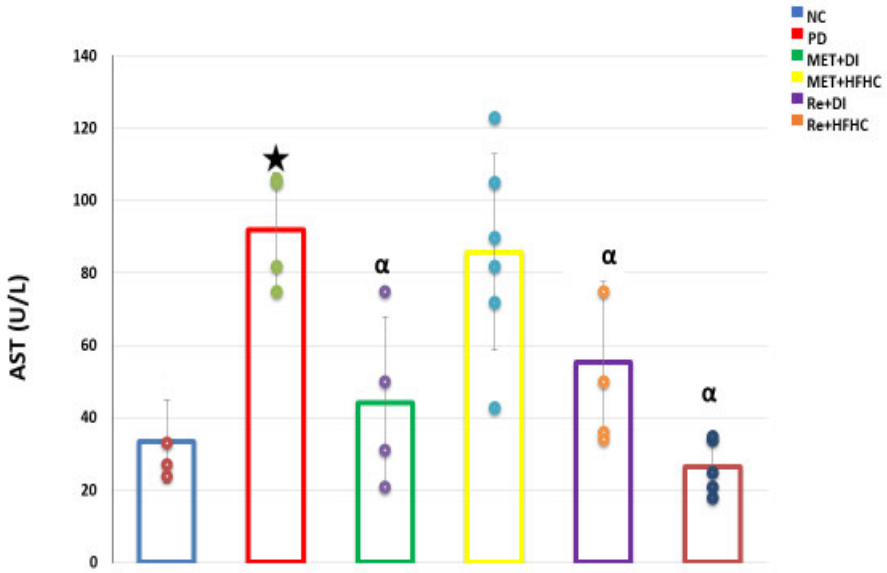

Figure 10. Plasma AST concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high fat–high carbohydrate (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat–high carbohydrate (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SD and individual data points ($n = 6$). *$p < 0.05$ by comparison with NC, α $p < 0.05$ by comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

4. Discussion

There is growing evidence that associates prediabetes with disorders that were previously thought to co-exist with type 2 diabetes [27,28]. These disorders include impaired glucose homeostasis, dyslipidemia, NAFLD and cardiovascular disease [27,28]. The development of NAFLD is strongly linked with chronic consumption of high calorie diets and is considered the most frequent liver disease in developing countries [28]. Prediabetes is associated with insulin resistance, which can increase peripheral lipolysis, triglyceride synthesis and hepatic uptake of free fatty acids, which ultimately leads to NAFLD [27,29]. Individuals with prediabetes and NAFLD have a higher risk of progressing to T2DM [27].

Studies have shown that anti-diabetic pharmacological agents can be used to manage NAFLD and to prevent it from developing into non-alcoholic steatohepatitis (NASH) [12,30]. Metformin has been used to treat NAFLD, as it is known to increase insulin action and reduce plasma glucose levels [31]. However, metformin has been shown to reach optimal efficacy when combined with lifestyle modifications that increase physical activity and lower caloric intake [30,32]. It has been that shown patients tend to over rely on pharmacological interventions leading to a neglect on the lifestyle modification that results in reduced efficacy of the conventional pharmacological agents [33]. Therefore, there is a great need for pharmacological agents which could perhaps assist those individuals who struggle with lifestyle modification [15,18].

There is a growing interest in using transition metal compounds to treat complications associated with metabolic disorders due to their high biological activity [9,34]. Our novel transition metal compound, rhenium (V) compound with uracil-derived ligands, has been previously shown to have anti-diabetic and antioxidant effects [20]. However, the effects of this metal-based compound on prediabetes induced NAFLD are yet to be investigated. In this study, we sought to further investigate the effects of this rhenium (V) compound on selected liver function markers in diet-induced prediabetic rats.

To maintain an individual's health, processes such as glucose homeostasis are tightly regulated to meet the energy needs for important organs. The liver plays an important role in the maintenance of glucose homeostasis through the glucose metabolism pathways glycogenesis, glycogenolysis, glycolysis and gluconeogenesis [6,35]. During fasting , the liver produces glucose through gluconeogenesis and the glucose is then used by brain, red blood cells and muscles [35]. The main function of insulin is to inhibit glycogenolysis and gluconeogenesis in hepatocytes [33]. However, in the prediabetic state, there is increased energy demand due to insulin resistance in some tissues, while there is also dysregulation of gluconeogenesis [36,37]. Gluconeogenesis is increased in the liver due to peripheral insulin resistance [38]. Insulin-resistant muscle and other tissues require energy; therefore, signals are sent to the liver to produce glucose [39,40]. Gluconeogenesis is activated, resulting in glucose producted in the liver being exported to the plasma, whilst hepatic steatosis leads to increased hepatic gluconeogenesis [41,42]. Indeed, the untreated prediabetic group in this study showed high fasting plasma glucose levels. This could be due to unregulated gluconeogenesis and energy demand from insulin-resistant peripheral tissues [32,43]. Furthermore, the groups treated with rhenium (V) compound were shown to have reduced plasma glucose in both the presence and absence of dietary intervention. The rhenium (V) compound has been previously

shown to restore peripheral insulin sensitivity through increased GLUT 4 expression, thus ameliorating insulin sensitivity and improving glycemic control [37]. Another transition metal with an organic ligand such as the dioxidovanadium complex (V) was shown to lower plasma glucose by increasing glucose transport and insulin-receptor tyrosine-kinase activity in NAFLD [44].

Hepatocytes play a vital role in regulating carbohydrate metabolism [45]. The storage of glycogen in the liver during the fed state provides a storage form of glucose that can be used during fasting periods. Normally, stored glycogen is critical for maintaining glucose homeostasis in individuals during an overnight fast [11,45]. However, in the prediabetic state, the liver has to store glucose due to insulin resistance in muscle and adipose tissues [11]. GLUT 2 transports glucose independently of insulin, and this results in a higher rate of hepatic glycogenesis occurring due to the shunting of glucose during the prediabetic state [46]. In this study, the untreated prediabetic group was shown to have high hepatic glycogen concentration. This could be due to high glucose uptake through GLUT 2. Further observations show that rhenium (V) compound treated groups in both the presence and absence of dietary intervention had reduced glycogen concentration. A possible reason for the observed reduction in glycogen levels in the rhenium (V) compound treated group may be because this compound has been shown to improve insulin sensitivity by increasing the expression of GLUT4 in the skeletal muscle and fat tissue, as shown in a study conducted by Siboto, A. et al., 2020 [20]. This reduction in the amount of glucose being shunted to the liver could result in reduced storage of hepatic glycogen [34]. Other studies on transition metals on NAFLD treatment showed that metal complexes including ruthenium and vanadium reduce hepatic glycogen concentration by channeling excess glucose to be metabolized in skeletal muscle and adipose tissue [19,44]. Glucose from excess dietary carbohydrates goes through glycolysis in hepatocytes and is later converted into fatty acids to be esterified into triglycerides (TGs), which are eventually secreted via very low-density lipoproteins [45].

In NAFLD patients with insulin resistance, the increased lipolysis in adipose tissue causes enhanced liver glucose synthesis, which further activates de *novo* lipogenesis, resulting in hepatic fat deposition [12,38]. Enhanced de *novo* lipogenesis and reduced fatty acid oxidation had been reported in patients with insulin resistance and contribute to a critical biochemical pathway for the pathogenesis of NAFLD [47,48].

Consumption of diets high in fructose also results in the activation of the de *novo* lipogenesis pathway. PPARγ-coactivator-1β (PGC-1β), which acts as a co-activator of SREBP1c, can be stimulated by fructose(45). Moreover, fructose inhibits hepatic fatty acid β-oxidation, which mainly occurs by inhibiting the transcriptional activities of PPARα [7,49]. Thus, the shift towards lipogenesis over fatty acid oxidation contributes to hepatic steatosis. In a hyper-insulinemic state, insulin continues to drive lipogenesis via the SREBP1 pathway in addition to failing to suppress gluconeogenesis, contributing to exacerbating hepatic steatosis. Due to hyperinsulinemia and hyperglycemia, SREBP-1c is activated resulting in high storage of TGs observed in the untreated prediabetic group. However, the administration of the rhenium (V) compound in both the presence and absence of diet intervention resulted in reduced hepatic TGs storage on the treated prediabetic rats. This may be because the rhenium (V) compound facilitated weight loss through suppressing the secretion

of ghrelin thus reducing food intake (45, 65). The food consumption results are on a glucose homeostasis paper by Siboto, A. et al., 2020 [20].

Due to a decrease in hepatic TGs concentration in the treated groups, we can also speculate that the rhenium (V) compound may decrease free fatty deposition to the liver by divergence of the substrates to other tissues for metabolism, increased β oxidation of fat or increased triglyceride disposal via very low-density lipoprotein (VLDL) exportation from the liver. Other metals for example dioxidovanadium lowered plasma TGs in diabetic rats via improving insulin sensitivity in adipose tissue and skeletal muscle [44]. Ruthenium (ii) complex facilitated weight loss in prediabetic rat resulting in less fatty acid deposition in the liver [19].

The administration of rhenium (V) compound in both the presence and the absence of diet intervention on treated prediabetic rats resulted in both reduction of hepatic glycogen concentration and hepatic TGs storage. This positive effect resulted in reduced liver weight as compared to the untreated prediabetic group. Several studies suggest that increased liver weights in prediabetes and NAFLD are associated with increased hepatic lipid accumulation [33]. Furthermore, this may imply that the rhenium (V) compound manages NAFLD delaying from becoming NASH. The current study findings on relative liver weights may also be a reflection of triacylglycerol and liver weights due to hepatic very-low-density lipoprotein–triglyceride (VLDL-TG) secretion rates. Studies have shown that liver weight is directly related to hepatic VLDL-TG secretion, independently of body weight. Therefore, since there is reduction of liver TGs in the prediabetic treated group, the liver weight is also reduced. We speculate that with improved insulin sensitivity on the adipose tissue, fat molecules are metabolized, resulting in enhanced HDL reducing the risk of hepatic steatosis associated with insulin resistance.

Oxidative stress is balanced by a number of antioxidant enzymes [50]. Antioxidant enzymes are ROS scavenging agents that protect cells against oxidative stress under physiological conditions [4]. Uncontrolled oxidative stress can result in liver injury [51]. Increased oxidative stress is independently associated with NAFLD [50]. NAFLD is characterized by insulin resistance, which results in elevated concentrations of free fatty acids, providing substrates for triglyceride formation and subsequent progression of the disease in the liver [51]. It is suggested that increased accumulation of liver triglycerides leads to increased oxidative stress in the hepatocytes [52,53]. NAFLD is also associated with mitochondrial dysfunction, and an increase of mitochondrial β-oxidation activity, due to a lipid overload, may induce an impairment of electron transport chain, resulting in electron leakage and increased ROS [54]. Oxidative stress causes hepatocellular damage through many different mechanisms, including lipid peroxidation, that can directly stimulate cell necrosis and activation of apoptosis [41,56]. Oxidation stress can directly lead to the synthesis of reactive oxygen species (ROS) that are usually removed by antioxidant pathways; however, in the prediabetic state, antioxidant activity is reduced. Indeed, this is also observed in the untreated prediabetic group. The administration of the rhenium (V) compound in both the presence and the absence of diet intervention in the prediabetic rats showed reduced lipid peroxidation and improved antioxidant activity in both SOD and GPx. We speculate that since the rhenium (V) compound reduces fat deposition in the liver, this prevents mitochondrial dysfunction. Therefore, the rhenium (V) compound can

prevent liver injury that can be caused by oxidative stress. Vanadium and ruthenium complexes have also been shown to prevent oxidative stress through enhanced glycemic control [19,21,44].

Inflammation, oxidative stress and insulin resistance are involved in NAFLD. The progression of NAFLD to NASH is identified through increased inflammation. NASH patients with increased serum TNF-α concentrations also show higher levels of interleukin (IL)-6 [54]. The administration of the rhenium (V) compound in both the presence and the absence of diet intervention was shown to reduce oxidative stress and improve antioxidant activity, reducing liver injury. The rhenium (V) compound has been shown to have anti-inflammatory properties by reducing plasma TNF α in the treated prediabetic group, protecting progressive liver damage.

Liver enzymes, AST and ALT, are used as clinical biomarkers to identify the degree of hepatocyte damage occurring in the liver [45]. The reason the liver enzyme levels better reflect the presence of injury is that these enzymes are components of hepatocytes that are released into circulation upon hepatocyte damage [45]. Literature trends have variably shown that high AST and ALT occur in blood due to necrosis of the hepatocyte during liver damage [33,55]. Metals are strongly known to be toxic on the liver [39]. The rhenium (V) compound was administered via subcutaneous injection in order for the pharmacological agent to bypass liver metabolism and be absorbed in the bloodstream. This allows the pharmacological agent to avoid first-pass metabolism in the liver, as the rhenium complex is known to be relatively harmless. The literature has shown that metal complexes are often associated with increased plasma AST and ALT levels, suggesting liver toxicity [33,55]. However, the rhenium (V) compound is synthesized with uracil-derived ligands that increase bioactivity and uracil-derived ligands. This has been shown to have the capability of coordination through a variety of donor atoms [56]. These uracil-derived ligands make the rhenium (V) compound have reduced cellular toxicity [56,57].

Liver enzymes such as AST and ALT are released into the bloodstream whenever hepatocytes are damaged, and this has been reported to occur during prediabetes [55]. The untreated prediabetic group had a high level of liver AST and ALT [27]. This observation of increased liver enzymes in the plasma suggested that liver cells are damaged through oxidative stress and increased hepatic lipogenesis or glycogenesis. However, administration of the rhenium (V) compound in prediabetic rats resulted in a decreased concentration of liver enzymes. The rhenium (V) compound seems to improve hepatic health via its antioxidant, antilipidemic and anti-inflammatory effects. Studies have shown that metal-based compounds including ruthenium (II) compounds ameliorated liver disarrangement and prevented hepatotoxicity [19,58].

5. Conclusions

The administration of the rhenium compound is associated with an improved liver health as evidenced by decreased liver triglyceride, glycogen and fasting blood glucose concentrations. Additionally, the observations suggest that this compound may attenuate liver injury associated with prediabetes, as evidenced by the reduction of oxidative stress, reduced plasma ALT and AST activities, as wells as reduced markers of inflammation in the liver. Given the beneficial effects of the

rhenium (V) compound presented in this study, further investigations are warranted to fully assess its therapeutic value in the management of prediabetes.

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Chapter 3

Prologue 2

Manuscript 2

In manuscript 2 we investigated the effects of rhenium (V) compound on prediabetes-induced liver damage. Both prediabetes and impaired liver function is strongly linked with the development of cardiovascular disease. The results in manuscript 1 showed an amelioration of both glucose homeostasis and liver function in both the presence and absence of dietary modification. Therefore, this study sought to find out if the rhenium (V) compound can reduce the risk of cardiovascular disease in prediabetic rats both in the presence and absence of dietary modification. The manuscript is titled "**Effect of rhenium (V) compound with uracil derived ligands on selected markers of cardiovascular function in diet induced prediabetic rats"** the manuscript was authored by Siboto A, Akinnuga AM, Khumalo BN, Ismail MB, Booysen I, Sibiya NH, Ngubane PS and Khathi A.

This manuscript has been accepted for publication in the journal **"Diabetes and Vascular Disease Research"** and has been formatted according to the journal's guidelines to authors. (See Appendix 4)

Manuscript 2

Effect of rhenium (V) compound with uracil derived ligands on selected markers of cardiovascular function in diet induced prediabetic rats.

Angezwa Siboto[1*], Akinjide Moses Akinnuga[1], Muhammed Bilaal Ismail[2], Irvin Noel Booysen[2], Ntethelelo Hopewell Sibiya[3], Phikelelani Siphosethu Ngubane[1], Andile Khathi[1]

1. School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, 4000, South Africa
2. School of Chemistry and Physics, College of Agriculture, Engineering Sciences, University of KwaZulu-Natal, Pietermaritzburg, 3209, South Africa
3. Pharmacology Division, Rhodes University, Grahamstown, 6140, South Africa

* Correspondence: 212518628@stu.ukzn.ac.za

Highlights

The rhenium (V) compound with uracil derived ligaments:

- Restored lipid metabolism in prediabetic rats.
- Oxidative stress and inflammatory markers in the heart of prediabetic rats.
- Increased nitric oxide bioavailability and this promoted vasodilation and resulted in regulated blood pressure in prediabetic rats.

Abstract

Introduction: Prediabetes is closely associated with the development of atherosclerotic lesions and cardiovascular disease (CVD). Metformin has is used to manage prediabetes and CVD complications but has been shown to have reduced efficacy in the absence of lifestyle intervention. A novel rhenium (V) compound has been found to be beneficial in managing prediabetes. This study sought to investigate whether the novel rhenium (V) compound can prevent CVD in diet-induced prediabetic rats in both the presence and absence of dietary intervention.

Methods: Prediabetes was induced in rats using a high fat carbohydrate diet. The rats, lipid profile, blood pressure, cardiac glutathione peroxidase (GPx) and total superoxide dismutase (SOD) activities and cardiac CRP and plasma IL-6 and TNF-α (markers of inflammation) were measured.

Results: The administered of rhenium (V) compound significantly reduced of total cholesterol, low density lipoproteins, and triglycerides. It also ameliorated high blood pressure and increased in nitric oxide and high-density lipoprotein levels.

Conclusion: The cardio-protective effects of the rhenium (V) compound are mediated via the prevention of lipid accretion and storage and modulating the hemodynamic profile in prediabetic rats. These preclinical observations suggest that investigated rhenium compound can attenuate prediabetes associated cardiovascular complications.

Keywords: Body mass index, blood pressure, lipid profile, Rhenium (V) compound

1.1. Introduction

The International Diabetes Federation (IDF) estimates that in 2015 globally, 1 in 11 adults aged 20–79 years (415 million adults) had diabetes mellitus (109, 110). This estimate is projected to rise to 642 million by 2040 and the largest increases will come from the regions experiencing economic transitions from low-income to middle-income levels (110, 111). Living a physically inactive lifestyle and the chronic consumption of high calorie diets increases the susceptibility to developing prediabetes and obesity (112-114). Prediabetes is a metabolic state characterized by higher than normal blood glucose concentrations albeit lower than diabetes thresholds. The prediabetes constitutes a high-risk state for the development of type 2 diabetes diabetes mellitus development (106, 115).Prediabetes is closely associated with a number of diseases such as cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) (116). On the other hand, CVD is associated dyslipidaemia and insulin resistance as risk factors (112, 117). Increased risk of CVD can be associated with both hyperglycaemia and hyperlipidaemia, independently of prediabetes.

Insulin resistance results in the inhibition of lipoprotein lipase activity in adipocytes leading to increased circulatory very-low-density lipoprotein (vLDL) (117, 118). The accumulation of vLDL accompanied by increase oxidative stress can leads to the development of atherosclerosis (118, 119). Persistent atherosclerosis has been shown to be a risk factor for the development of hypertension and cerebrovascular peripheral artery diseases accidents (118, 120, 121). Therefore, that it is important to manage the moderate insulin resistance associated with prediabetes in order to avoid cardiovascular -related complications (122, 123). The first-line management of prediabetes involves lifestyle modifications which include diet modification

and increased physical activity (124, 125). However, there are reports of poor patient-compliance with diet modification, hence there is the need for pharmacotherapy to assist in managing prediabetes (125, 126). Metformin has been used to manage prediabetes although it has been shown to only be effective when combined with lifestyle intervention (124, 127). Therefore, there is a need for pharmacotherapy that can be effective in both the presence and absence of dietary modification.

Complexes involving the transition metals vanadium, copper and ruthenium have been used experimentally to treat prediabetes and its associated complications (128, 129). In such complexes, which have been shown to have antidiabetic and cardio-protective effects the metals are bound to organic ligands. Furthermore, the transition metal based comoplexes have also been shown to restore glycaemic control, protect against the development of atherosclerosis and to have antihypertensive (130-132). In a glucose homeostasis study, rhenium (V) compound improved glucose homeostasis in prediabetic rats by by upregulating GLUT 4 expression and inhibiting the PTP 1B activity (133). To further understand the therapeutic effect of this complex, the effects of the novel rhenium (V) compound on selected markers of cardiovascular function in diet-induced prediabetic rats in the presence and absence of dietary intervention were evaluated.

2. Methods and materials.

Synthesis of rhenium (V) compound {trans-[Re(ddd)(Hduo)(PPh3)2] I (H2ddd = 5,6-diamino-1,3-dimethyluracil and H3duo = N-(2-hydroxybenzylidene)-5-amino-1,3dimethyl uracil) was conducted in our laboratory as previously reported (134). Structural confirmation of the metal compound was done using ^{1}H NMR spectroscopy.

2.1. Animals.

Thirty-six (36) male Sprague Dawley rats (150–180 g; Age = 4-week-old) obtained from Biomedical Research Unit, University of KwaZulu-Natal (UKZN), were kept under standard environmental conditions i.e., constant humidity (55 ± 5%), temperature (22 ± 2°C), 12 h day :12 h night cycle. The rats were acclimatized for 2 weeks and consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* before being fed the experimental high fat-high carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, South Africa) to induce prediabetes. The standard rat chow had of carbohydrates (55% kcal/g), protein (30 % kcal/g), fat (15% kcal/g) The HFHC diet had of carbohydrate (60% kcal/g), fats (25% kcal/g),

and proteins (15% kcal/g); Water supplemented with fructose 15% as described in our previous study (133, 135). All the experimental designs and procedures were carried out according to the ethics and guidelines of the Animal Research Ethics Committee (AREC, ethical clearance code: AREC/00003221/2021) of the UKZN, Durban, South Africa.

2.1.1. Induction of pre-diabetes.

Sprague-Dawley rats were randomly assigned to the following diet groups (n = 6 per group): a standard rat chow with normal drinking water (ND + H_2O), high-fat high-carbohydrate diet with drinking water supplemented with fructose (HFHC + Fructose). The experimental prediabetes induction period was 20 weeks (135, 136). Rats with fasting blood glucose concentration of more than 5.6 mmol/L were considered pre-diabetic and grouped further for pharmacological studies (137, 138). Treatment commenced on the subsequent day, and this was considered as day 1 of treatment.

2.1.2. Experimental design.

The rats were randomly divided into 6 groups of 6 animals in each (30 pre-diabetic persisting, 6 normal). Group 1: non-prediabetic control (NPD); group 2- prediabetic control rats continued with HFHC diet and received vehicle (PD); group 3- prediabetic rats switched into the STD diet and received rhenium (V) compound (Re + DI); group 4- prediabetic treated rats continued with HFHC diet received rhenium (V) compound (Re + HFHC); group 5- prediabetic treated rats switched into the STD diet and received metformin (MET + DI) and group 6: prediabetic treated rats continued with HFHC diet received metformin (MET + HFHC).

2.1.3. Treatment of pre-diabetic rats.

The treatment period which commenced immediately after induction and confirmation of prediabetes and lasted 12 weeks. The animals were treated once every third day at 9:00 a.m., where the MET + DI and MET + HFHC groups received an oral dose of metformin (500 mg/kg), while the Re + DI and Re + HFHC groups received subcutaneous injection of the rhenium (V) compound (15 mg/kg). Blood glucose concentration, food intake, body weights, mean arterial pressure (MAP), blood pressure (BP) and heart rate were monitored every 4 weeks of the treatment period.

2.2. Determination of BMI.

The rats body weights were measured using animal weighing scale available at the Biomedical Research Unit (BRU). The rat body weights were recorded using grams (g) and the body length was measured using a tape measure where the tape measure was placed on the nose-tip to tail-base of the rat. Therefore, BMI was determined using the following formula: BMI is weight in grams divided by length in centimetre squared (139, 140).

$$BMI = \frac{Weight(g)}{Length\ (cm)^2}$$

2.3. Determination of blood pressure and heart rate.

During the prediabetes induction period in addition to being acclimatized to the experimental conditions, the rats were also trained to measurement of recording blood pressures by tail cuff once a week. The blood pressure and heart rate were measured as described in the established protocol (141). Briefly, the non-invasive MRBP IITC Model 31, Life Sciences multichannel tail cuff blood pressure system (Life Sciences, Woodland Hills, CA) was used to monitor the blood pressure and the heart rate by placing the rats in a restrainer (3″ ID (75 mm)–12″ length) and then its tail was attached to the tail cuff. All the rats in the restrainer were placed in a warming chamber (IITC Model 303sc Animal Test Chamber, Life Sciences, Woodland Hills, CA) maintained at 32° C, and the blood pressure as well as the heart rate was measured by occlusion or deflation of the tail cuff which detects alteration of blood flow in the tail artery. An average of three measured sessions consisting of 15 cycles was used for statistical analysis.

2.4. Blood Collection and Tissue Harvesting.

For blood collection, all rats were anaesthetised with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) 4–5% for the induction and 1–2% for maintenance. This is recommended by the anesthesia guidelines and Isofor showed no negative health effects on the rats via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for 3 min. While rats were unconscious, blood was collected by cardiac puncture into individual pre-cooled heparinized blood collection tubes. The blood was then centrifuged (Eppendorf centrifuge 5403, Germany) at 4 °C, 503 g for 15 min. Plasma was collected and stored at −80 °C in a Bio Ultra freezer (Snijers Scientific, Holland) pending biochemical analysis. Following blood collection, heart was removed, weighed, and rinsed with cold normal saline solution and snap frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at −80 °C until biochemical analysis.

2.5. Determination of heart weight: body weight ratio (HW"BW).

Heart weight to body weight ratio (HW"BW) was then calculated by dividing the weight of the heart tissue(g) by the weight of the rat at study termination (g) (142).

3. Biochemical analysis

3.1.1. Oxidative stress, antioxidant activity and inflammation.

In order to estimate the oxidative stress in the cardiac tissue, malondialdehyde (MDA) concentration was measured using previously described protocol (141, 143). The antioxidant activities in the cardiac tissue was determined on glutathione peroxidase (GPx) (GPx: E-EL-R2491) ELISA kit and total superoxide dismutase (SOD) (SOD: E-BC-K020-M; using an assay kit as per manufacturer's instructions (Elabscience Biotechnology Co., Ltd.,). Lastly, the cardiac inflammation was determined on cardiac CRP (Rat CRP: E-EL-R0506), plasma IL-6 (Rat IL-6: E-EL-R0015) and TNF-α (Rat TNF-α: E-EL-R2856) using separate, specific ELISA kits.

3.1.2. Determination of plasma nitric oxide (NO)

Plasma NO levels were measured as an indirect indicator of reactive nitrogen species (RNS) in plasma. To prepare a standard, approximately 10mg sodium nitrite was weighed then it was dissolved in 100ml of water, and serial dilutions (10.0, 7.5, 5.0, 2.0, 1.0 µg/mL) were made. For reagent preparation, approximately 200 ml 0.5 M HCl was pipetted into a reagent bottle and 0.5 g of vanadium (iii) chloride was weighed and added into the reagent bottle. This solution was gentle mixed to allow vanadium (III) chloride to dissolve. Lastly about 0.2 g sulfanilamide and 0.01 g N-(1- naphthyl) ethylenediamine dihydrochloride were weighed and added into the reagent bottle and dissolved. The 45 uL of each dilution of the standard and samples were added into appropriate 24 wells microplate. Thereafter, the1000 ul of reagent was added in each well. The plate was then incubated (37 °C) for 45 min under dark conditions. Subsequently, the optical density was read at 540/690nm using a spectrophotometer (Spectrostarnano, BMG LABTECH, Ortenberg Germany). A standard curve was constructed using the results obtained from the sodium nitrite standard and the resultant NO concentration for each sample was determined by extrapolation (144).

3.1.3. Lipid profile.

Plasma total cholesterol (TC) (catalog No: E-BC-K109S; Manufacturer: Elabscience), high density lipoprotein (HDL) cholesterol (catalog No: E-BC-K221; Manufacturer: Elabscience), and triglycerides (TG) (catalog No: E-BCK238; Manufacturer: Elabscience) were analysed using commercial specialized kits as per manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). The VLDL and LDL were obtained using the following Friedewald equation.

Where: LDL = TC – (HDL + TG/5)

4. Statistical analysis

All data are expressed as means ± SEM. Statistical comparisons were performed with GraphPad In Stat Software (version 5.00, Graph Pad Software, Inc., San Diego, CA, USA) using two-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

5. Results

5.1. Blood glucose, body weights and food intake

5.1.1. Body weight.

Figure 1 shows body weights in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and high fat high carbohydrate (Re + DI) and rhenium (V) compound and diet intervention (Re + HFHC) metformin and high fat high carbohydrate (MET + DI), metformin and diet intervention (MET + HFHC) after every 4th week for 12 weeks. By comparison with the NPD, there was a significant increase in body weight in the PC group throughout the experimental period ($p < 0.05$, fig 1). The administration of the rhenium compound in both the HFHC and diet modification resulted in significant reduction in body weights by comparison to PC ($p < 0.05$, fig 1). A similar observation was shown by the MET+DI treated group as compared to the PC group. See figure 1

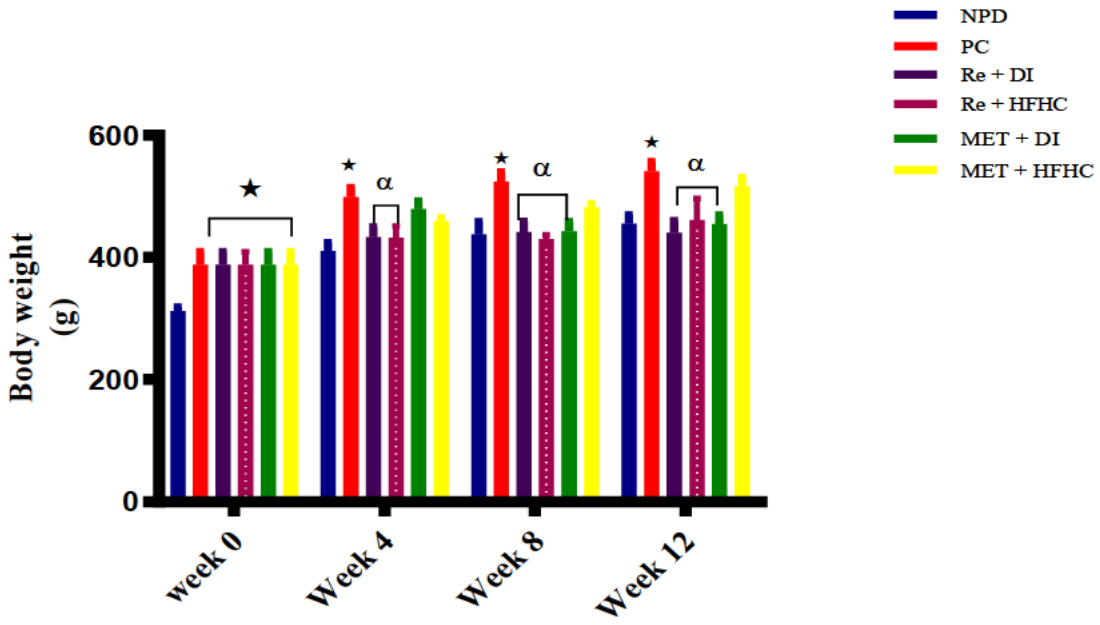

Figure 1: A comparative analysis of the body weights in in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), rhenium (V) compound and diet intervention (Re + DI), metformin and high fat high carbohydrate (MET + HFHC) and metformin and diet intervention (MET + DI) at 12 weeks of treatment. Values are presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI, and. MET + HFHC.

5.1.2. Blood glucose concentration.

Figure 2 shows the glucose levels in the in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and high fat high carbohydrate (Re + DI) and rhenium (V) compound and diet intervention (Re + HFHC) metformin and high fat high carbohydrate (MET + DI), metformin and diet intervention (MET + HFHC) groups after 12 weeks of treatment. In a comparison to with the NPD group, PD showed a significant increase in glucose levels ($p < 0.05$; Figure 2), and in comparison, with the PD control group, the Re + DI and Re + HFHC groups showed a significant decrease in glucose levels ($p < 0.05$). See Figure 2.

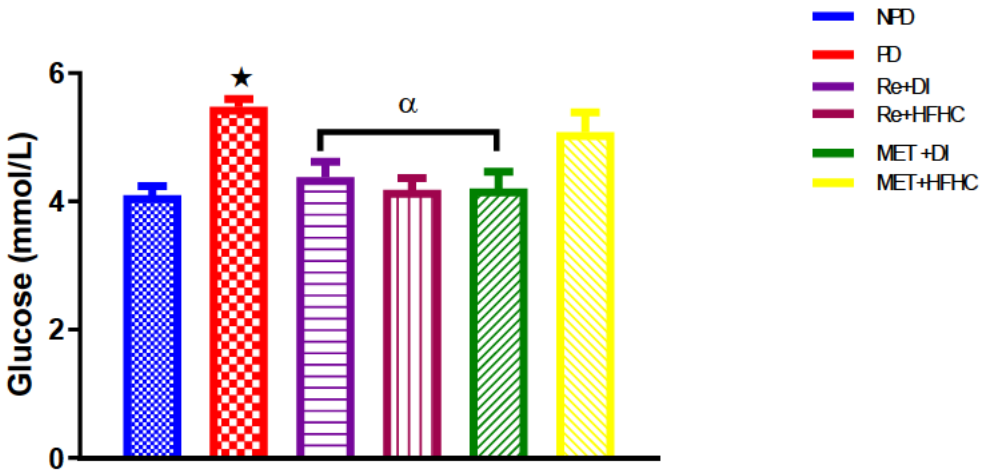

Figure 2. Glucose levels in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), rhenium (V) compound and diet intervention (Re + DI), metformin and high fat high carbohydrate (MET + HFHC) and metformin and diet intervention (MET + DI) at 12 weeks of treatment. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI, and. MET + HFHC.

5.1.3. Caloric intake.

Figure 3: Shows caloric intake in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and high fat high carbohydrate (Re + DI) and rhenium (V) compound and diet intervention (Re + HFHC) metformin and high fat high carbohydrate (MET + DI), metformin and diet intervention (MET + HFHC) at week 12 of treatment period. In comparison with the NPD group, the PC group showed significant increase in caloric intake ($p < 0.05$). However, in comparison with the PC, Re + DI group and Re+ HFHC group it showed a significant decrease in calorie consumption ($p < 0.05$). A similar observation was shown by the MET +DI treated group as compared to PC group ($p < 0.05$, fig 3). See figure 3.

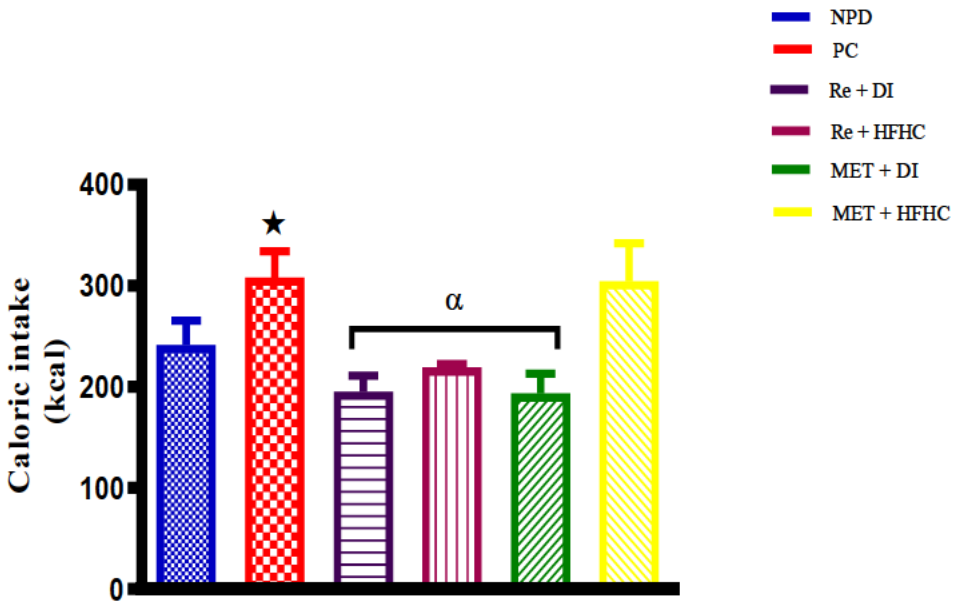

Figure 3: Shows caloric intake in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), rhenium (V) compound and diet intervention (Re + DI), metformin and high fat high carbohydrate (MET + HFHC) and metformin and diet intervention (MET + DI) at 12 weeks of treatment. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by compar0ison with NPD, α $p < 0.05$ by comparison with PD, Re + HFHC, Re + DI, MET + HFHC, and. MET + DI.

5.2. BMI, heart weight: body weight ratio

5.2.1. Body mass index (BMI).

Figure 4 shows BMI in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and high fat high carbohydrate (Re + DI) and rhenium (V) compound and diet intervention (Re + HFHC) metformin and high fat high carbohydrate (MET + DI), metformin and diet intervention (MET + HFHC) measured every 4th week for 12 weeks. By comparison with the NPD, there was a significant increase in BMI in the PD control group throughout the experimental period ($p < 0.05$, Fig. 4). The administration of the rhenium (V) compound in both the HFHC and diet modification resulted in significant reduction in BMI by comparison to PD ($p < 0.05$, Fig. 4). A similar observation was shown by the MET + DI treated group as compared to the PD group. See figure 4.

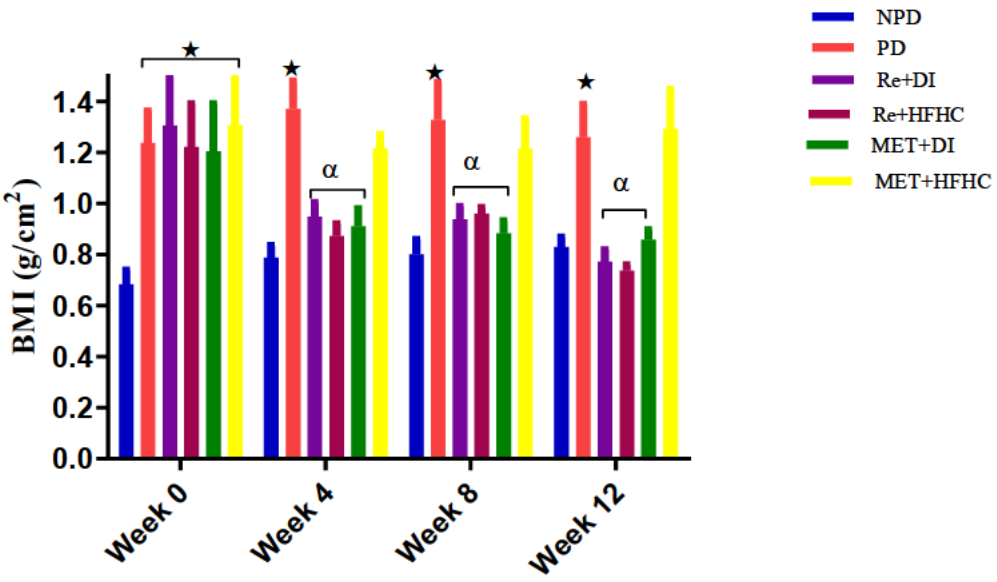

Figure 4: BMI in non-prediabetic control (NPD) prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI and metformin and high fat high carbohydrate (MET + HFHC)) measured every 4th week for 12 weeks. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI and MET + HFHC.

5.2.2. Heart: body weight ratio.

Table 1 shows heart: body ratio in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) after 12 weeks of treatment. In comparison with the NPD group, PD showed a significant increase in heart: body ratio ($p < 0.05$; Table 1). In comparison with the PD control group, Re + DI and Re + HFHC groups showed a significant decrease in heart: body ratio ($p < 0.05$). A similar effect was observed in the MET+DI group with significantly decreased heart: body ratio concentration ($p < 0.05$). see table 1.

Table 1: Shows effects of rhenium (V) compound on heart: body ratio of PD animals for a treatment period of 12 weeks.

GROUPS	BODY WEIGHT	HEART WEIGHT	HEART:BODY WEIGHT RATIO
NPD	378,3 ±0,053	1,242 ± 0,039	0,00204 ± 0,000113
PD	550,5 ± 0,091 ★	1,704 ± 0,035 ★	0,00310 ± 0,000122 ★
Re + DI	440,3 ± 0,051 α	1,127 ± 0,093 α	0,00216 ± 0,000132 α
Re + HFHC	441,3 ± 0,057 α	1,356 ± 0,092 α	0,00215 ± 0,000144 α
MET + DI	454,7 ± 0,049 α	1,179 ± 0,084 α	0,00223 ± 0,000163 α
MET + HFHC	516,0 ± 0,046	1,508 ± 0,012	0,00299 ± 0,000157

★ $p < 0.05$ in comparison with NPD, α $p < 0.05$ by comparison with PD, Re +DI, Re + HFHC, MET + DI and MET + HFHC.

5.3. blood pressure, MAP, nitric oxide (NO).

5.3.1. Systolic blood pressure.

Figure 5 shows systolic blood pressure of NPD, PD, and PD-treated animal groups monitored at week 0 and week 12. The PD and the treated groups started with the similar increased systolic blood pressure at week 0 before treatment (Figure 5). When compared with the NPD group, there was a significant rise in systolic blood pressure of the PD group to the end of the experimental period ($P<0.05$; Fig 5). However, in comparison with the PD group, there was a significant reduction in systolic blood pressure upon administering rhenium (V) compound with both HFHC and diet intervention in the treated animals ($p<0.05$; Fig 5). In addition, a similar effect was observed in the MET + DI treated animals. See figure 5.

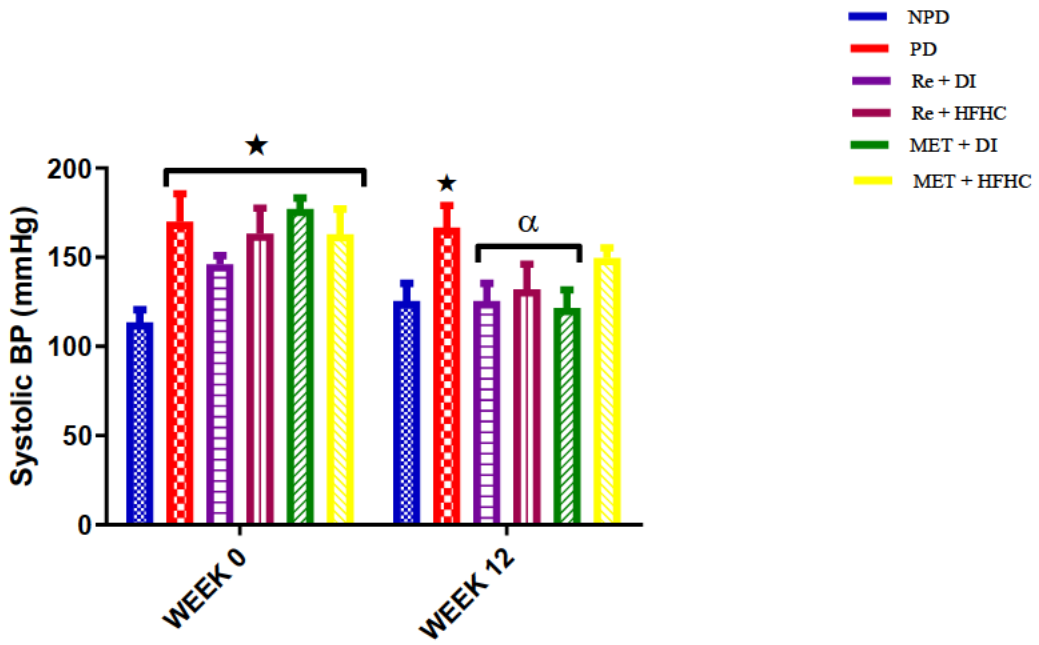

Figure 5: Systolic blood pressure in non-prediabetic control (NPD)prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at week 0 and week 12 of treatment. Data presented as means ± SEM (n = 6). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI and MET + HFHC.

5.3.2. Diastolic blood pressure.

Figure 6 shows diastolic blood pressure of NPD, PD, and PD-treated animal groups monitored at week 0 and week 12. The PD and the PD-treated groups started with similar increased diastolic blood pressure at week 0 before treatment (Figure 3). When compared with the NPD group, there was a significant rise in diastolic blood pressure of the PD group to the end of the experimental period ($P<0.05$; Fig 6). However, in comparison with the PD group, there was a significant reduction in diastolic blood pressure upon administering rhenium (V) compound with both HFHC and diet intervention in the treated animals ($P<0.05$; Fig 6). In addition, a similar effect was observed in the MET + DI treated animals. See figure 6.

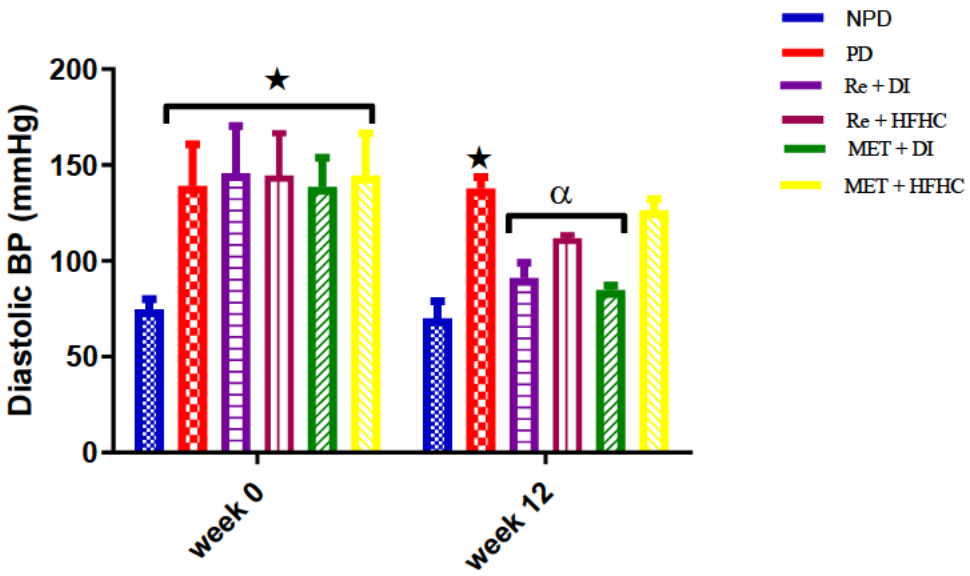

Figure 6: Diastolic blood pressure in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at week 0 and week 12 of treatment. Data presented as means ± SEM (n = 6). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI and MET + HFHC.

5.3.3. Heart rate.

Figure 7 shows heart rate in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC) and metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC), measured every 4th week for 12 weeks. By comparison with the NPD, there was a significant increase in heart rate in the PD group throughout the experimental period ($p < 0.05$, Fig. 7). The administration of the rhenium compound in both the HFHC and diet modification resulted in significant reduction in heat rate by comparison to PD ($p < 0.05$, Fig. 7). A similar observation was shown by the MET + DI treated group as compared to the PD group. See figure 7.

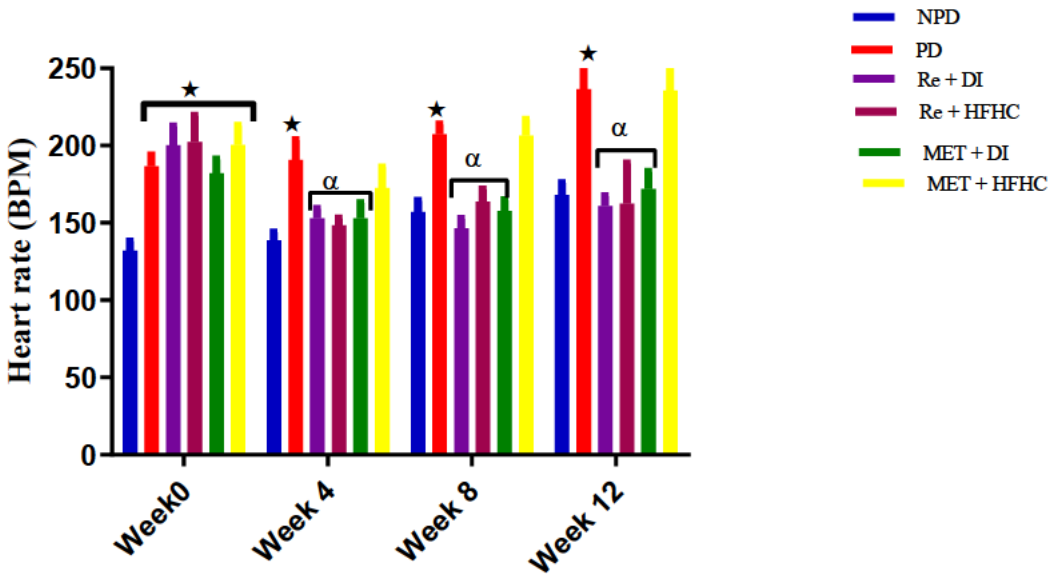

Figure 7: Heart rate in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) measured every 4th week for 12 weeks. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI and MET + HFHC.

5.3.4. Nitric oxide (NO) concentration.

Figure 8 shows nitric oxide concentration in non-prediabetic control (NPD) prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and), metformin and high fat high carbohydrate (MET + HFHC) after 12 weeks of treatment. In comparison with the NPD group, PD showed a significant decrease in NO concentration ($p < 0.05$; fig 5). In comparison with the PD control group, Re + DI and Re + HFHC groups showed a significant increase in NO concentration ($p < 0.05$). A similar effect was observed in the MET+DI group with significantly increase in NO concentration ($p < 0.05$). See figure 8.

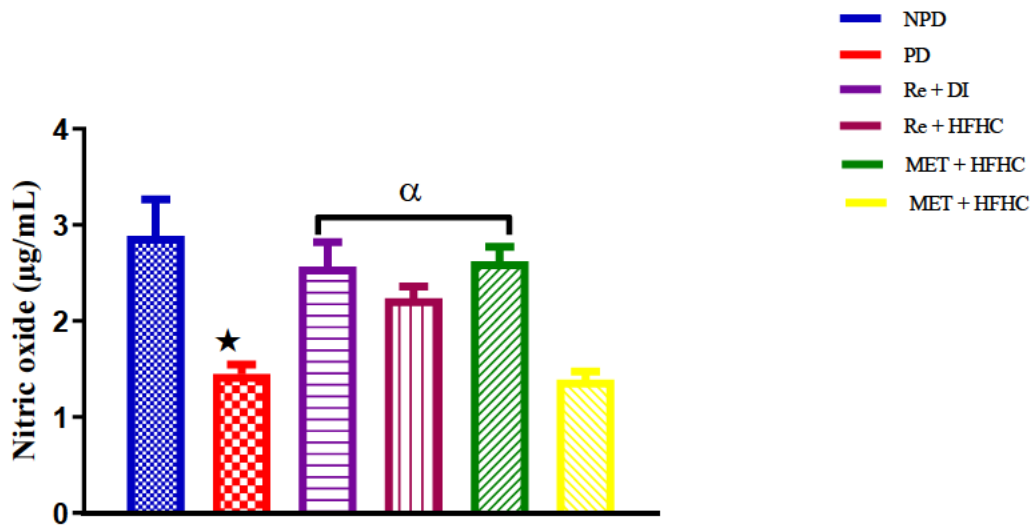

Figure 8: Nitric oxide in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at 12 weeks of treatment. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI, and. MET + HFHC.

5.4. Lipid profile (TGs, TC, HDL, LDL).

5.4.1. Plasma triglycerides (TGs) concentration.

Figure 9 shows plasma triglycerides (TGs) concentration in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI), and metformin and high fat high carbohydrate (MET + HFHC), after 12 weeks of treatment. In comparison with the NPD group, the PD group showed a significant increase in plasma TGs concentration ($p < 0.05$) (fig 9). The administration of rhenium (V) compound in both diet intervention and high fat high carbohydrate groups resulted in a significant decrease of plasma TGs α concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$). see figure 9.

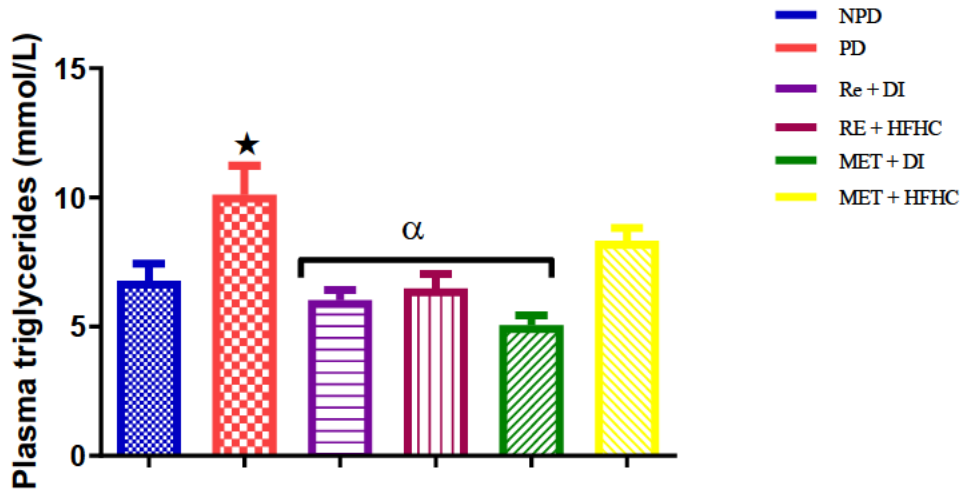

Figure 9: Plasma TGs in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at 12 weeks of treatment. Values are presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI, and. MET + HFHC.

5.4.2. Plasma total cholesterol (TC).

Figure 10 shows plasma total cholesterol (TC) concentration in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI), metformin and high fat high carbohydrate (MET + HFHC), after 12 weeks of treatment. In comparison with the NPD group, the PD group showed a significant increase in plasma TGs α concentration ($p < 0.05$) (fig 10). The administration of rhenium (V) compound in both diet intervention and high fat high carbohydrate groups resulted in a significant decrease of plasma TGs α concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$). see figure 10.

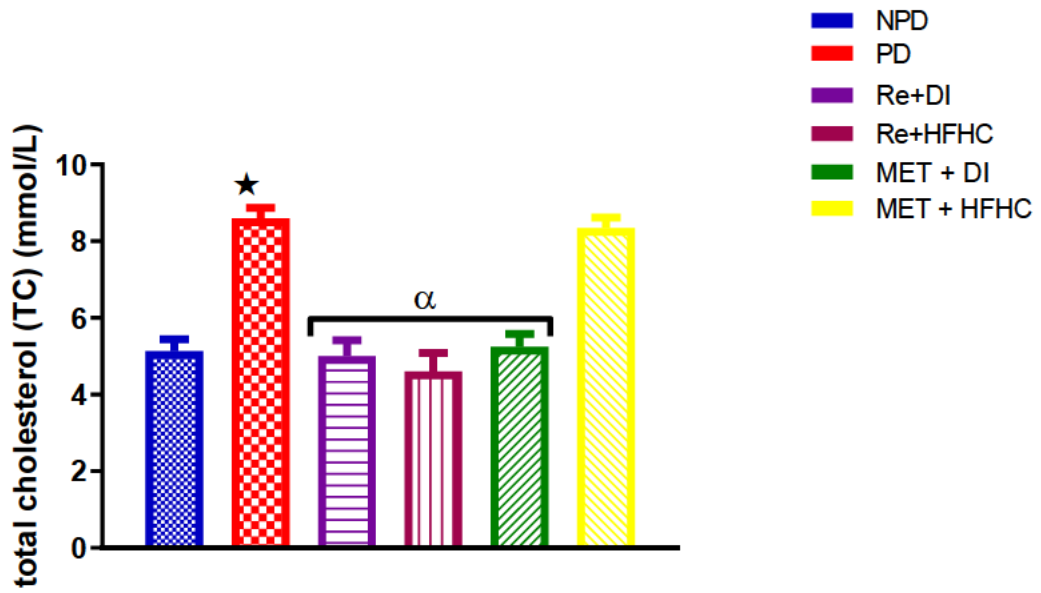

Figure 10: Plasma TC in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at 12 weeks of treatment. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI, and. MET + HFHC.

5.4.3. High-density lipoprotein cholesterol (HDL).

Figure 11 shows high-density lipoprotein cholesterol (HDL) concentration in non-prediabetic control (NPD) prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI), metformin and high fat high carbohydrate (MET + HFHC) after 12 weeks of treatment. In comparison with the NPD group, the PD group showed a significant increase in plasma TGs α concentration ($p < 0.05$) (Fig 11). The administration of rhenium (V) compound in both diet intervention and high fat high carbohydrate groups resulted in a significant decrease of plasma TGs α concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$). see figure 11.

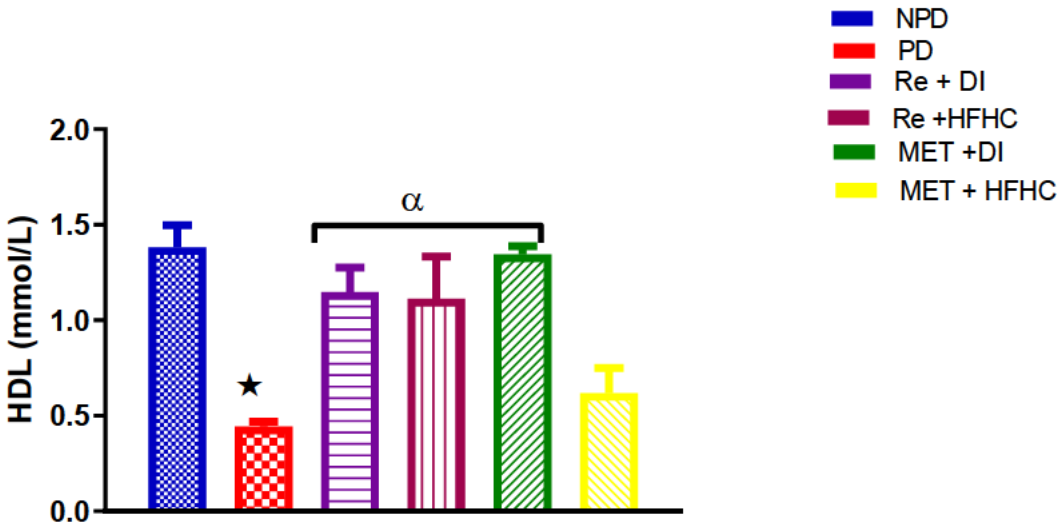

Figure 11: High-density lipoprotein cholesterol (HDL) in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at 12 weeks of treatment. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI, and. MET + HFHC.

5.4.4. Low-density lipoprotein cholesterol (LDL)

Figure 12 shows Low-density lipoprotein cholesterol (LDL) concentration in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI), metformin and high fat high carbohydrate (MET + HFHC), after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant increase in plasma TGs α concentration ($p < 0.05$) (fig 12). The administration of rhenium (V) compound in both diet intervention and high fat high carbohydrate groups resulted in a significant decrease of plasma TGs α concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$). see figure 12.

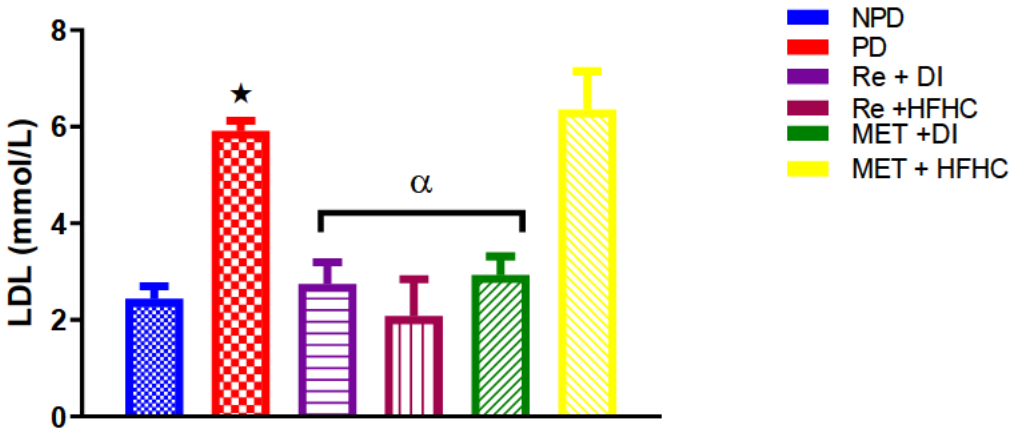

Figure 12: High-density lipoprotein cholesterol (LDL) in non-prediabetic control (NPD) prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at 12 weeks of treatment. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI, and. MET + HFHC.

5.5. Heart oxidative status and antioxidants activities

Table 2 shows MDA concentration and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) antioxidant enzymes in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) after 12 weeks of treatment. For lipid peroxidation, in comparison with the NPD group, PD showed a significant increase in MDA concentration in heart tissue ($p < 0.05$; Table 2). In comparison with the PD control group, Re + DI and Re + HFHC groups showed a significant decrease in MDA concentration ($p < 0.05$). A similar effect was observed in the MET+DI group with significantly decreased MDA concentration ($p < 0.05$). For antioxidant status, in comparison with the NPD group, PD showed a significant decrease in SOD activities in heart tissue ($p < 0.05$; Table 2). In comparison with the PD control group, Re + DI and Re + HFHC groups showed a significant increase in SOD activities ($p < 0.05$). For GPx: in comparison with the NPD group, PD showed a significant decrease in GPx concentration in heart tissue ($p < 0.05$; Table 2). In comparison with the PD control group, Re + DI and Re + HFHC groups showed a significant increase in GPx concentration ($p < 0.05$). A similar effect was observed in the MET+DI group with significantly increase in antioxidant enzyme status ($p < 0.05$). See table 2.

Table 2: The effects of rhenium (V) compound in the presence and absence of dietary intervention on the heart lipid peroxidation and antioxidant enzymes (SOD and GPx) in prediabetic rats. Data presented as mean ± SEM ($n = 6$).

Groups	Malondialdehyde (MDA) (nmol/g protein)	Total superoxide dismutase (SOD) (U/mL)	glutathione peroxidase (GPx) (pg/mL)
NPD	4,01 ± 0,117	44,6 ± 0,91	1,78 ± 0,085
PD	5,36 ± 0,205 ★	28,8 ± 1,58 ★	0,32 ± 0,097 ★
Re + DI	4,06 ± 0,130 α	40,6 ± 1,91 α	1,40± 0,217 α
Re + HFHC	4,09 ± 0,144 α	41,2 ± 1,71 α	1,39 ± 0,296 α
MET + DI	5,26 ± 0,174	30,2 ± 1,43 α	1,51 ± 0,228 α
MET + HFHC	4,16 ± 0,119 α	42,0 ± 1,96	0,24 ± 0,125

★ $p < 0.05$ in comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI and MET + HFHC.

5.6. Inflammatory markers: CRP, TNα and IL-6

5.6.1. Heart CRP concentration

Figure 13 shows heart CRP expression in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI), metformin and high fat high carbohydrate (MET + HFHC) after 12 weeks of treatment. In comparison with the NPD group, the PD group showed a significant increase in CRP concentration ($p < 0.05$) (fig 13). The administration of rhenium (V) compound in both diet intervention and high fat high carbohydrate groups resulted in a significant decrease of CRP concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$). see figure 13.

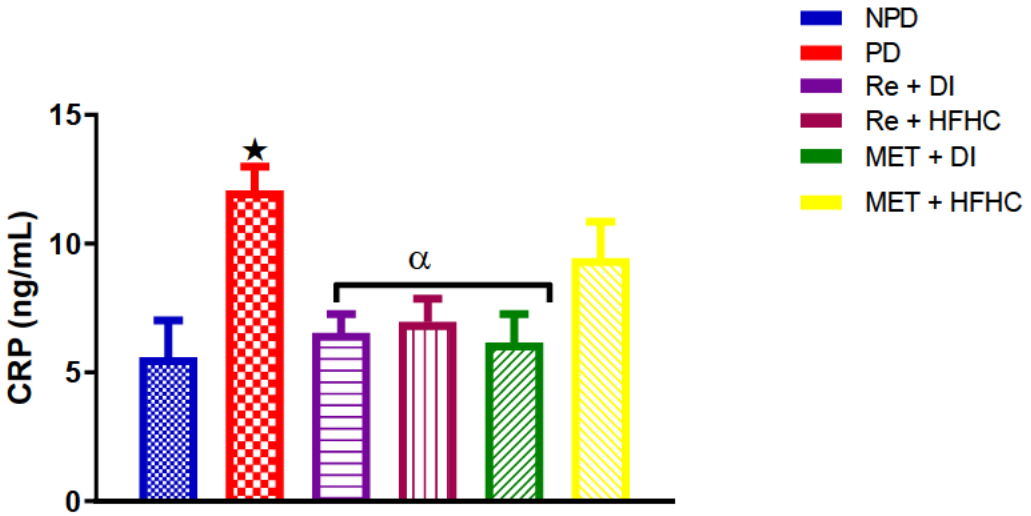

Figure 13: Heart CRP in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at 12 weeks of treatment. Data presented as means

± SEM (n = 6). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI and MET + HFHC.

5.6.2. Heart TNF α concentration

Figure 14 shows TNF α concentration in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI), and metformin and high fat high carbohydrate (MET + HFHC), after 12 weeks of treatment. In comparison with the NPD group, the PD group showed a significant increase in TNF α concentration ($p < 0.05$) (fig 14). The administration of rhenium (V) compound in both diet intervention and high fat high carbohydrate groups resulted in a significant decrease of TNF α concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$). see figure 14.

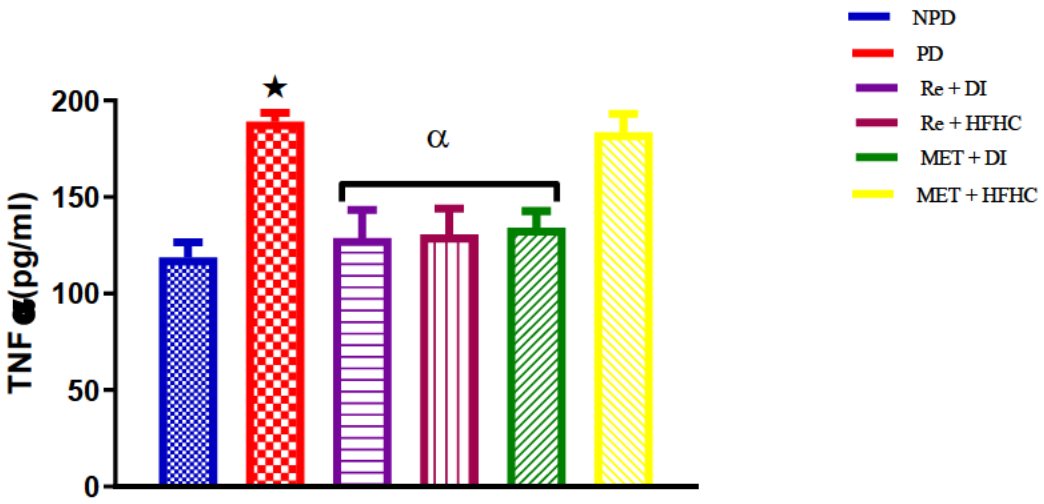

Figure 14: Heart TNF-α in non-prediabetic control (NPD),), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at 12 weeks of treatment. Data presented as means ± SEM (n = 6). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI and MET + HFHC.

5.6.3. IL-6 concentration

Figure 15 shows IL-6 concentration in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI), and metformin and high fat high carbohydrate (MET + HFHC), after 12 weeks of treatment. In comparison with the NPD group, the PD group showed a significant increase in IL-6 concentration ($p < 0.05$) (fig 15). The administration of rhenium (V) compound in both diet intervention and high fat high carbohydrate groups resulted in a significant decrease of IL-6 concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI treated group when compared with the PD group ($p < 0.05$). see figure 15.

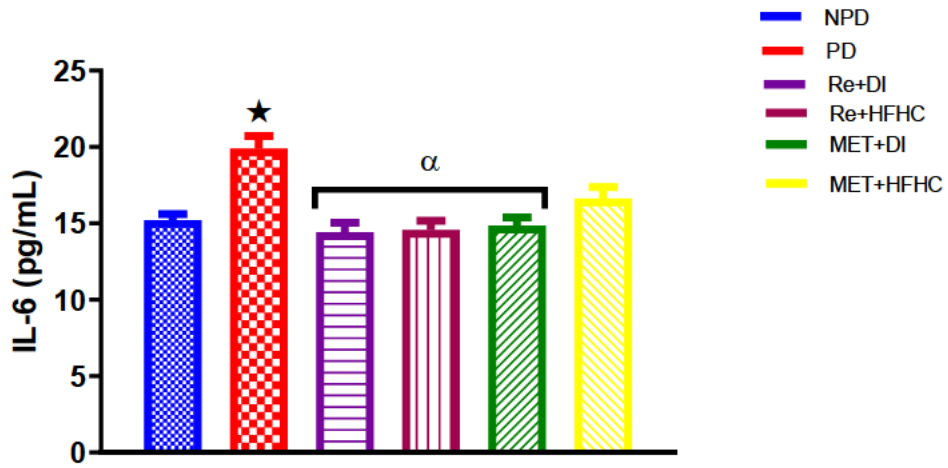

Figure 15: IL-6 in non-prediabetic control (NPD), prediabetic control (PD), rhenium (V) compound and diet intervention (Re + DI), rhenium (V) compound and high fat high carbohydrate (Re + HFHC), metformin and diet intervention (MET + DI) and metformin and high fat high carbohydrate (MET + HFHC) at 12 weeks of treatment. Data presented as means ± SEM ($n = 6$). ★ $p < 0.05$ by comparison with NPD, α $p < 0.05$ by comparison with PD, Re + DI, Re + HFHC, MET + DI and MET + HFHC.

6. Discussion

It has been predicted that by 2030 the incidence of T2DM will rise significantly, with an estimated 69% increase in developing countries, making T2DM the 7th leading cause of mortality (105, 145). Patients with T2DM have macrovascular complications that include cardiovascular disease (CVD), stroke and peripheral vascular disease (146). These complications initiate and progress during the prediabetes stage (147). Patients with prediabetes are advised to change lifestyle and adapt to health living which involves diet modification and increased physical activity (90, 136). However, patient struggle to adapt to healthy living, there is the need for pharmacological agent that may assist in managing prediabetes independently of lifestyle modifications (90, 110). Metformin has been used to manage prediabetes although it has been shown to only be effective when combined with lifestyle intervention (124). Therefore, there is a need for pharmacotherapy that can be effective in both the presence and absence of dietary modification. Therefore, this study sought to investigate whether the novel rhenium (V) compound can induce atherosclerotic lesion reduction in diet-induced prediabetic rats in both the presence and absence of dietary intervention.

Elevated blood glucose levels are the indication of IFG and IGT as a result of reduced insulin sensitivity (66, 80). Uncontrolled hyperglycaemia due to insulin resistance may result in hyperglycaemia-induced oxidative-stress that is implicated in the development of cardiomyopathy (69, 146). Continued consumption of high caloric diets results in increased body weight on individuals due to fat build up. Indeed, the untreated prediabetic rats exhibited high caloric intake, blood glucose, and increased body weight however, when the rats were treated with rhenium (V) compound had reduced blood glucose and caloric intake which facilitated weight loss. More results and in depth discussion of the effect of rhenium (V) compound on glycaemic control are on a published paper by A. Siboto, 2020 (34)

Prediabetes is associated with a higher body mass index (BMI) due to excessive body fat accumulation (146). Previous research studies have also shown that the risk of developing diabetes and its associated cardiovascular diseases rises as body fat, BMI, and waist circumference increase (148). In this study, the prediabetic rats showed an increase in BMI and higher heart: body weight ratio by comparison to the non-prediabetic group. Upon treatment with rhenium (V) compound in both the presence and absence of diet intervention, the prediabetic rats showed a decrease in BMI and higher heart: body weight ratio. This may be

due to the effect of rhenium (V) compound that can improve glucose tolerance and repair impaired fasting blood glucose by improving insulin resistance and upregulating GLUT 4 expression, more of these effects where shown on a paper conducted by A. Siboto *et al*, 2020 (133). Furthermore, in the previous study treatment of prediabetic rats with rhenium (V) compound resulted in reduced hepatic lipogenesis resulting in a concomitant reduction in fat deposition in the plasma and a decrease in BMI (89). Lastly rhenium (V) compound suppressed ghrelin secretion in treated prediabetic rats which regulated caloric intake and resulted in body weight loss (133). It can also be speculated that reduced the risk of prediabetes due to decreased caloric intake facilitated weight loss in prediabetic rats. Prevented the accretion of fat in tissues as evidenced by the decreased heart: body weight ratio in the rhenium treated prediabetic rats. We may further speculate that rhenium (V) compound reduced BMI by reducing the production of TGs and other free fatty acids (FFA) through the suppression of SREBP-1 which is regulated though the AKT pathway (122, 149). It has been shown the impairment in the PI3K/AKT signalling results increase in *de novo* lipogenesis in which excessive fat is deposited in other body parts increasing body weight (45, 65). Indeed, lipid profile in prediabetic animals suggest an upregulation in cholesterol synthesis by the liver.

Insulin resistance in the peripheral tissues results in hyperglycaemia and an increase in the production of free fatty acids (44, 123). Literature trends have shown that in T2DM high blood glucose concentration and FFA are responsible for an increase in ROS production due to metabolic stress resulting in changes within the electron transport chain and overwhelmed antioxidants defence system (74, 123, 150). A study conducted by Gumede *et al*, 2022 showed that prediabetes is associated with myocardial injury through oxidative stress damage (150). Hyperglycaemic environments causes abnormalities the mitochondrial respiratory chain leading to overproduction of superoxide (O_2-) and hydrogen peroxide (H_2O_2). Glutathione peroxidase (GPx) and superoxide dismutase (SOD) are crucial in removing excess free radicals that are produced by mitochondria (74). A decrease in the activities of antioxidant enzymes could be attributed to suppressed NRF2/pathways which are responsible for the upregulating detoxifying enzymes such as antioxidants. Risk factors for CVD include lipid peroxidation, free radicals promotes the oxidation of low-density lipoprotein (49, 74). Oxidized LDL is more reactive with surrounding tissues and can collect within the inner lining of arteries (150). Indeed, untreated prediabetic rats had increased levels of lipid peroxidation, high LDL and reduced antioxidant enzymes activity in comparison to the non-prediabetic rats. However, in comparison to the untreated prediabetic rats the rhenium (V) compound treated groups showed

significant reduction in lipid peroxidation in both the absence and presence of diet intervention. The lowered lipid peroxidation in treated rats is due to that rhenium (V) compound have antioxidant effects through attenuating hyperglycaemia or a direct effect on stimulating the NRF2/ARE pathways (95, 130). By increasing the antioxidant enzymes activities, rhenium (V) compound could therefore reduce the risk of lipid peroxidation, endothelial dysfunction, inflammation and platelet activation. The integrity and function of the endothelium play a crucial role in vascular health by releasing nitric oxide (NO), which aids in vasodilation. However, in a prediabetic state, vascular balance is disrupted due to endothelial dysfunction triggered by oxidative stress. This leads to a decrease in bioactive NO, as it combines with superoxide anion to create peroxynitrite (104, 151). Indeed, untreated prediabetic rats displayed lowered plasma NO levels, indicating an increase in oxidative stress. Interestingly, administering a rhenium complex restored plasma NO concentration. Another explanation for the rise in plasma NO levels could be the ability of the Rhenium (V) compound to enhance insulin sensitivity in skeletal muscle and adipose tissue, consequently improving glycemic control. Studies have indicated that high blood sugar levels reduce endothelium-derived NO by activating protein kinase C (PKC) (95, 152). Since rhenium (V) compound facilitate the expression of GLUT 4 in skeletal muscle this reduces blood glucose concentration and prevent the activation of NOX 1 through PKC-dependent Rac1 activation.

Endothelium dysfunction and other factors including sodium retention result in increased blood pressure (111, 120). This research revealed that prediabetic rats displayed higher heart rates compared to the non-prediabetic control group. Additionally, throughout the 12-week treatment period, the prediabetic rat group consistently showed elevated mean arterial pressure (MAP) and increased blood pressure, both systolic and diastolic, which could be linked to decreased nitric oxide levels. This reduction in nitric oxide might contribute to artery constriction and the deposition of fat within the arteries. However, treatment with rhenium (V) compound in both the absence and presence of diet intervention resulted in a gradual decrease in these parameters throughout the treatment period when compared to untreated prediabetic group. The literature has reported that the metal complexes such as ruthenium(II) Schiff base complex and dioxidovanadium complex cis-[VO2(obz)py] regulate blood pressure through targeting the renin–angiotensin–aldosterone system (RAAS) to reduce blood pressure in prediabetic rats (95, 130). Therefore, it could be suggested that rhenium (V) compound uses the same mechanism to reduce blood pressure. Another potential way the rhenium (V) compound reduces blood pressure is by enhancing NO availability, leading to improved

vasodilation, as mentioned earlier. Additionally, it's likely that the compound's capacity to decrease blood pressure is associated with normalizing the lipid profile.

Patients with acute and chronic heart failure develop manifestations from the liver which has also been shown to be compromised in the prediabetic state (75). Insulin resistance in the hepatocytes and adipocytes result in dyslipidaemia (72, 75). Dyslipidaemia is a common feature of T2DM and increases the incidence of atherosclerosis and mortality of diabetic patients (19, 73). The development of atherosclerosis begins during the prediabetic state (19). Dyslipidemia presents as an altered lipid profile with increased levels of triglycerides, total cholesterol, LDL, and decreased HDL levels (19, 73). . Prior research has established a link between cholesterol accumulation, inflammation due to fat molecules entering artery walls, resulting in endothelial dysfunction and atherosclerosis (73, 87). Treating hyperglycemia associated with diabetes has proven beneficial for numerous cardiovascular disease (CVD) patients globally, largely due to its effectiveness in maintaining vessel patency (73, 104). The untreated prediabetic rats had high levels of plasma TC, TGs and LDL whereas the treated groups with rhenium (V) compound in both the absence and the presence of diet intervention shown to have reduced plasma TC, TGs AND LDL. Normalisation of the lipid profile has also been one way to prevent cardiovascular hazards. rhenium (V) compound ability to reduce triglyceride could be attributed to its ability to promote insulin sensitivity and activation of lipoprotein lipase which can facilitates uptake of free fatty acid into skeletal muscle and adipose tissue. Statin therapy has been shown to lower triglyceride by the similar mechanisms. The treatment with rhenium (V) compound did not only regulated the TGs synthesis, it also significantly improve lipid profile as we observe that the treated prediabetic rats have reduced levels of TC, LDL and increased HDL. Although these could be indirect effect, however, the study of rhenium on rate limiting enzyme (HMG-CoA reductase) for cholesterol synthesis is critical as it could be one the mechanisms to lower cholesterol. Studies have also shown that the treatment of CVD with metal compound such as chromium and ruthenium complexes reduces the risk of atherosclerosis, by improving levels of lipoproteins, such as lowering very-low-density lipoprotein (VLDL) levels and increasing LDL catabolism (153, 154).

Atherosclerosis starts with dysfunctional changes in the endothelium induced by disturbed shear stress which can lead to endothelial and platelet activation, adhesion of monocytes on the activated endothelium (73, 114). Macrophage activation is also associated with an increase in the production of inflammatory cytokines such as interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α) and interferon I (INF-I). IL-6 and TNF-α are the main

inducers of CRP in the heart (87). Prediabetes is associated with low-grade inflammation induced by fat-cell-derived cytokines (105, 155). Prediabetic rats had high levels of TNF- α, IL-6 and CRP when compared with the non-prediabetic control which shows the activation of immunity. However, the rhenium (V) compound treated prediabetic rats had lowered concentration of IL-6, TNF- α and CRP this can be because rhenium (V) compound has anti-inflammatory properties (32). Therefore, we speculate that rhenium (V) compound utilises its anti-inflammatory effects to reduce TNF- α resulting in downregulation of CRP which leads to decrease in tissue factor production and blocking the extrinsic blood coagulation pathway. Other transition metal complexes also have anti-inflammatory properties (97, 130).

7. Conclusion

The administration of rhenium complexes may have cardioprotective effects in prediabetic animal model as demonstrated by a normalised lipid profile, blood pressure and BMI. Additionally, the attenuation of oxidative stress and inflammatory markers was also observed in the heart. Taken together, these observations warrants further research and developments on rhenium complex as a viable alternative pharmacological agent for the management of cardiovascular complications in a prediabetic context.

Ethics approval

The animal study protocol was approved by the Institutional Animal Research Ethics Committee of University of KwaZulu-Natal UKZN (protocol code AREC/00003221/2021 (previous AREC reference number: AREC/039/018M) and date of approval:23 June 2022).

Data Availability

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

Funding Statement

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Authors' contributions

Conceptualization, A.S., A.K. and A.M.A.; methodology, A.S., M.B.I. and A.M.A.; software, A.S.; validation, A.S., A.K. and N.H.S.; formal analysis, A.S. and A.K.; investigation, A.S.; resources, A.K., P.N. and I.N.B.; data curation, A.S.; writing—original draft preparation, A.S.; writing—review and editing, I.N.B., A.K., N.H.S. and P.N.; visualization, A.S.; supervision, N.H.S. and A.K.; project administration A.K.; funding acquisition, A.K. All authors have read and agreed to the published version of the manuscript.

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Chapter 4

Prologue 3

Manuscript 3

Renal function is heavily impacted by glucose homeostasis. Prediabetes, which is associated with insulin resistance, has been shown to result in the early stages of renal dysfunction. Renal dysfunction has also been associated with impaired hepatic and cardiovascular function. In the previous two manuscripts of this dissertation. The rhenium (V) compound was demonstrated to enhance indicators linked to liver and heart complications in prediabetes. Building on this, the current study aimed to explore how this compound affects renal dysfunction associated with prediabetes, following the findings from the previous studies. This was conducted in diet-induced prediabetic rats in the both the presence and absence of dietary modification. The manuscript titled "**Ameliorative Effects of a Rhenium (V) Compound with Uracil-derived Ligand on Markers Associated with Hyperglycaemia-Induced Renal Dysfunction in Diet-Induced Prediabetic Rats"** and is authored by Siboto A, Akinnuga AM, Khumalo BN, Ismail MB, Booysen I, Sibiya NH, Ngubane PS and Khathi A.

This manuscript is published in "**International Journal of Molecular Sciences"** and has been formatted according to the journal's guidelines to authors. See (Appendix 5)

Manuscript 3

Article

Ameliorative Effects of a Rhenium (V) Compound with Uracil-derived Ligand on Markers Associated with Hyperglycaemia-Induced Renal Dysfunction in Diet-Induced Prediabetic Rats

Angezwa Siboto [1,*], Akinjide Moses Akinnuga [1], Bongiwe Khumalo [1], Muhammed Bilaal Ismail [2], Irvin Noel Booysen [2], Ntethelelo Hopewell Sibiya [3], Phikelelani 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; akinnugaakinjide@yahoo.com (A.M.A.); 215080288@stu.ukzn.ac.za (B.K.); ngubanep1@ukzn.ac.za (P.N.); khathia@ukzn.ac.za (A.K.)
[2] School of Chemistry and Physics, College of Agriculture, Engineering Sciences, University of KwaZulu-Natal, Pietermaritzburg 3209, South Africa; bilaal.ismail@gmail.com (M.B.I.); booyseni@ukzn.ac.za (I.N.B.)
[3] Pharmacology Division, Rhodes University, Grahamstown 6140, South Africa; n.sibiya@ru.ac.za
* Correspondence: 212518628@stu.ukzn.ac.za

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Abstract: Kidney disease is characterised by the improper functioning resulting from kidney damage caused by hyperglycaemia-induced oxidative stress. The moderate hyperglycaemia seen in prediabetes can be treated using a combination of metformin and consumption of a low calorie diet lifestyle. However, patients have been reported to over-rely on pharmacological interventions, thus decreasing the efficacy of metformin, which leads to the development of type 2 diabetes mellitus (T2DM). In this study, we investigated the potential of a rhenium (V) compound to ameliorate renal dysfunction in both the presence and absence of dietary modification. Fluid intake and urine output, glomerular filtration rate (GFR), kidney injury molecule (KIM 1), podocin, aldosterone plasma creatinine, urea, albumin and electrolytes, were measured after 12 weeks of treatment. After treatment with the rhenium (V) compound, kidney function was restored, as evidenced by increased GRF and reduced KIM 1, podocin and aldosterone. The rhenium (V) compound ameliorated kidney function by preventing hyperglycaemia-induced oxidative stress in the kidney in both the presence and absence of dietary modification.

Keywords: rhenium (V) compound; prediabetes; dietary modification; kidney dysfunction; oxidative stress

1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) is increasing in African countries due to population growth, urbanisation and sedentary lifestyles **(61, 156)**. Epidemiological studies indicate that 65–75% of the risk of primary hypertension is due to obesity and prediabetes **(61, 81)**. At least 72% of patients with end-stage renal disease have hypertension and T2DM, both driven largely by obesity **(81, 156)**. Kidney dysfunction

as a consequence of T2DM might be reinforced by the presence of other diabetic complications, such as cardiovascular disease and hypertension **(23, 81)**. Kidney dysfunction is associated with albuminuria, a lowered glomerular filtration rate, glomerulosclerosis, electrolyte abnormalities and proteinuria **(157, 158)**. Other factors such as hyperglycaemia, increased oxidative stress, chronic inflammation, impaired insulin signalling, dyslipidaemia, renal polyol formation and the accumulation of advanced glycation end-products can also significantly contribute to the onset and progression of kidney disease **(159, 160)**.

Prediabetes is a progressive disorder that often precedes the onset of T2DM, which is characterised by hyperglycaemia and hyperinsulinaemia **(161, 162)**. Hyperglycaemia-induced oxidative stress leads to kidney damage as a result of the harmful effects of the over-production of reactive oxygen species (ROS) in the mitochondria **(163, 164)**. Kidney disease is characterised by an increase in kidney size, the improper functioning of the kidney, an increased level of albumin in the urine, glomerular basement membrane (GBM) thickening and glomerular hypertrophy **(54, 159)**. Therefore, there is a need for treatments that can ameliorate the hyperglycaemia seen in prediabetes to prevent the onset of kidney disease **(165)**.

Metformin has been used to manage prediabetes and its associated complications (25, 29). However, the use of metformin is more effective when used in conjunction with lifestyle interventions (155). These include the consumption of low-sodium, low-calorie diets and increased physical activity (25, 155). There have been reports of the under-utilisation of lifestyle interventions and an over-reliance on the use of metformin, leading to poor clinical outcomes; therefore, there is a need for alternative pharmacological treatments that are effective with and without lifestyle interventions (29, 155, 165). A previous study in our laboratory showed that a rhenium (V) compound is able to reduce blood glucose by increasing the expression of GLUT 4 in peripheral tissues, such as skeletal muscle (34, 166). Furthermore, the rhenium (V) compound ameliorated liver injury and prevented hepatotoxicity in prediabetic rats in both the absence and presence of a diet intervention (89).

The rhenium (V) compound is a metal-based compound that has a biologically active ligand system (167). A study conducted by Maisuls et al. (2017) reported that rhenium (V) compounds allow for a great deal of structural and chemical variability and can be fine-tuned to meet the requirements of a wide range of biological applications (fluorescence markers and/or antitumor drugs) (100, 168). The ligands provide stability and promote the bio-availability of the metal complex (100, 167). This study sought to investigate whether a rhenium (V) compound with uracil-derived ligands can ameliorate renal dysfunction associated with prediabetes in both the absence and presence of a diet intervention in diet-induced prediabetic rats.

2. Results

2.1. Effects of Rhenium (V) Compound Administration in the Absence and Presence of Diet Intervention on Glucose, Insulin Levels and HOMA2-IR Index

Figure 1 shows the glucose levels in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In a comparative evaluation with the NC group, PD showed a significant increase in glucose levels ($p < 0.05$; Figure 1), and in comparison, with the PD control group, the Re + DI and Re + HFHC groups showed a significant decrease in glucose levels ($p < 0.05$). See Figure 1.

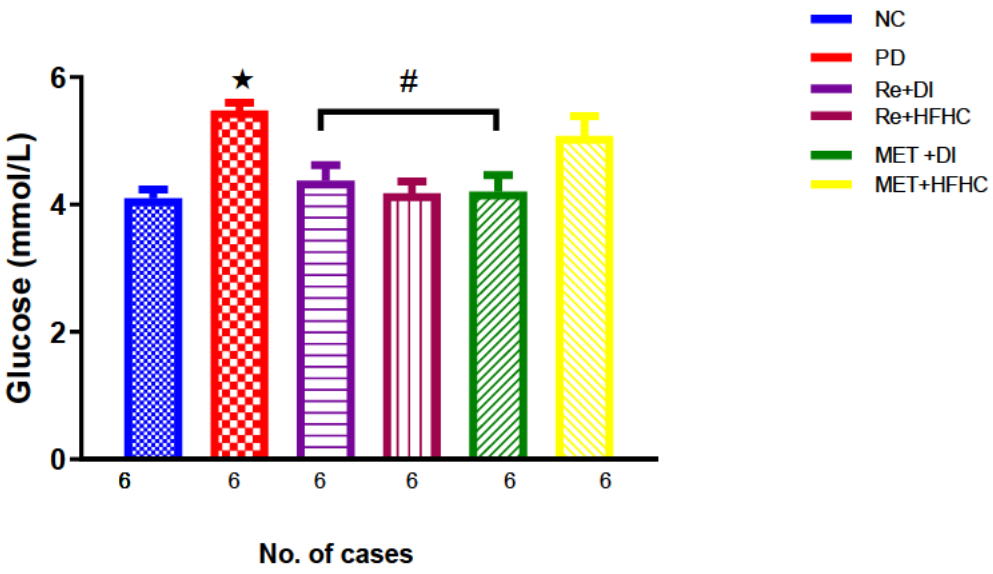

Figure 1. Glucose levels in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 2A, B show the insulin concentration and HOMA2-IR index in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate (Re + HFHC) groups after 12 weeks of treatment. In a comparative evaluation with the NC group, PD showed a significant increase in insulin concentration and HOMA2-IR ($p < 0.05$; Figure 2A, B), and in comparison, with the PD control group, the Re + DI and Re +HFHC groups showed a significant decrease in insulin concentration and the HOMA2-IR index ($p < 0.05$). See Figure 2A, B.

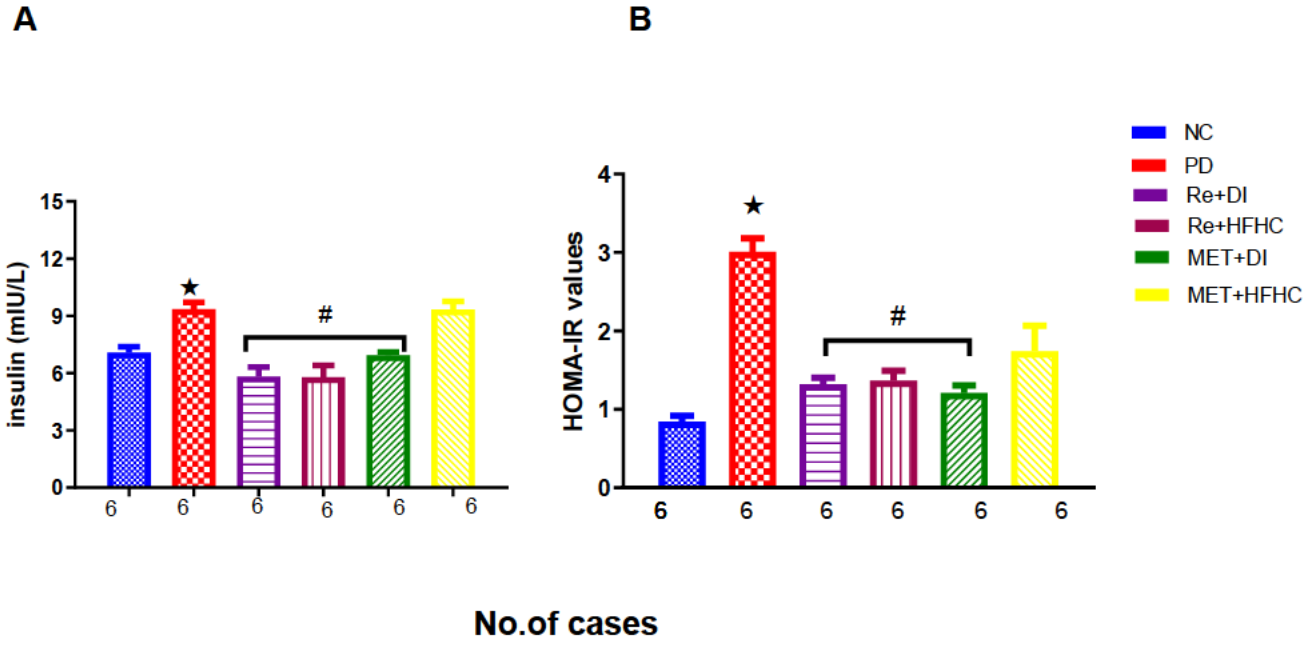

Figure 2. (**A**, **B**) Insulin concentration and HOMA2-IR index in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

2.2. Effects of Rhenium (V) Compound Administration in the Absence and Presence of Diet Intervention on Renal Oxidative Stress and Antioxidant Status

Figures 3–5 show the lipid peroxidation and antioxidant enzyme activities (SOD and GPx) in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. For lipid peroxidation (Figure 3), in comparison with the NC group, PD showed a significant increase in MDA concentration in kidney tissue ($p < 0.05$; Figure 3). In comparison with the PD control group, the Re + DI and Re + HFHC groups showed a significant decrease in MDA concentration ($p < 0.05$). A similar effect was observed in the MET + DI group, with a significantly decreased MDA concentration ($p < 0.05$). For antioxidant activity (Figures 4 and 5), in comparison with the NC group, PD showed a significant decrease in both GPx and SOD activities in kidney tissue ($p < 0.05$; Figures 4 and 5). In comparison with the PD control group, the Re + DI and Re + HFHC groups showed a significant increase in both GPx and SOD activities ($p < 0.05$). A similar effect was observed in the MET + DI group, with a significant increase in antioxidant enzyme activities ($p < 0.05$). See Figures 4 and 5.

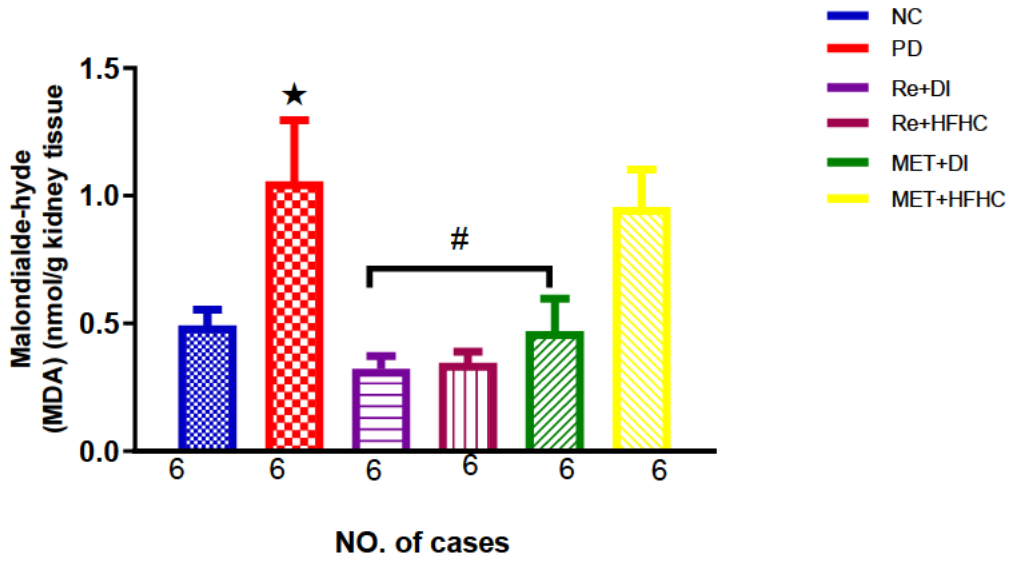

Figure 3. MDA concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI), and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PD, MET +DI, MET + HFHC, Re + DI and Re + HFHC.

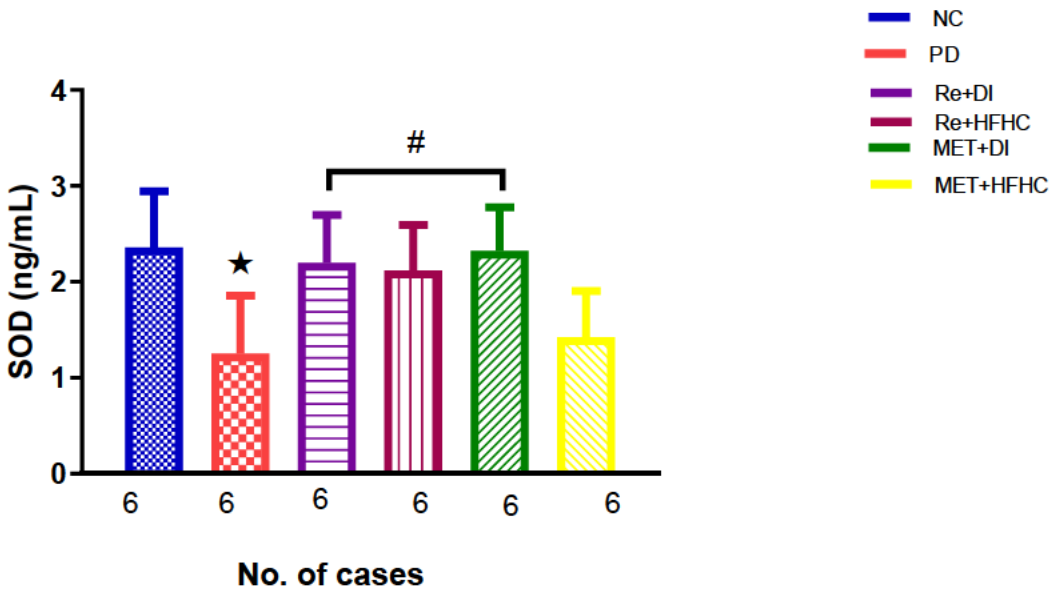

Figure 4. SOD activity in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI), and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PD, MET +DI, MET + HFHC, Re + DI and Re + HFHC.

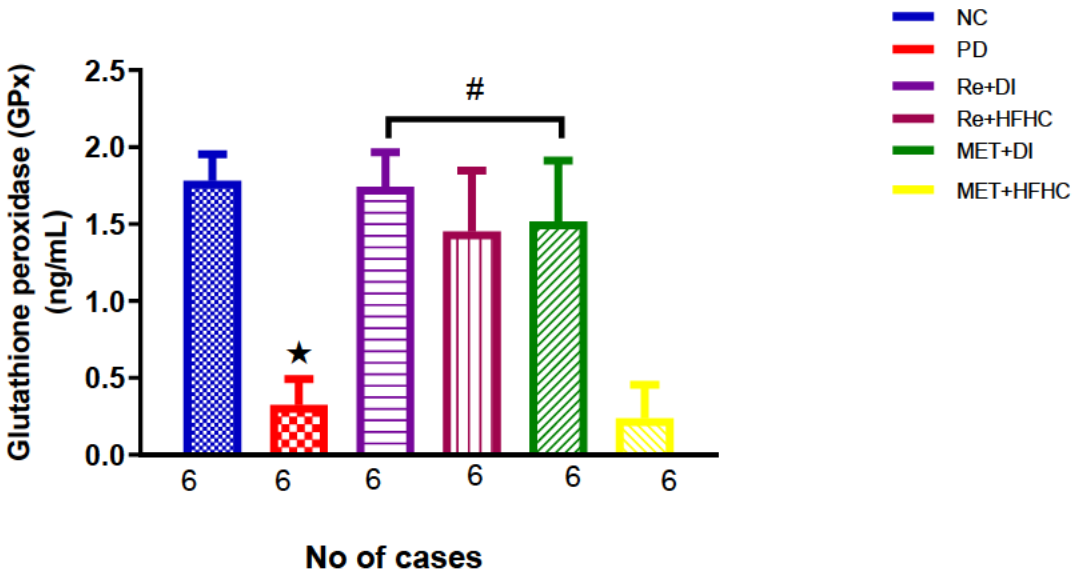

Figure 5. Shows GPx activity in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI), and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PD, MET +DI, MET + HFHC, Re + DI and Re + HFHC.

2.3. Effects of Rhenium (V) Compound Administration in the Absence and Presence of Diet Intervention on Inflammatory Markers: TNF-α and IL-6

Figure 6 shows the TNF-α levels in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In a comparative evaluation with the NC group, PD showed a significant increase in TNF-α levels ($p < 0.05$; Figure 6), and in comparison, with the PD control group, Re + DI and Re + HFHC groups showed a significant decrease in TNF-α levels ($p < 0.05$). See Figure 6.

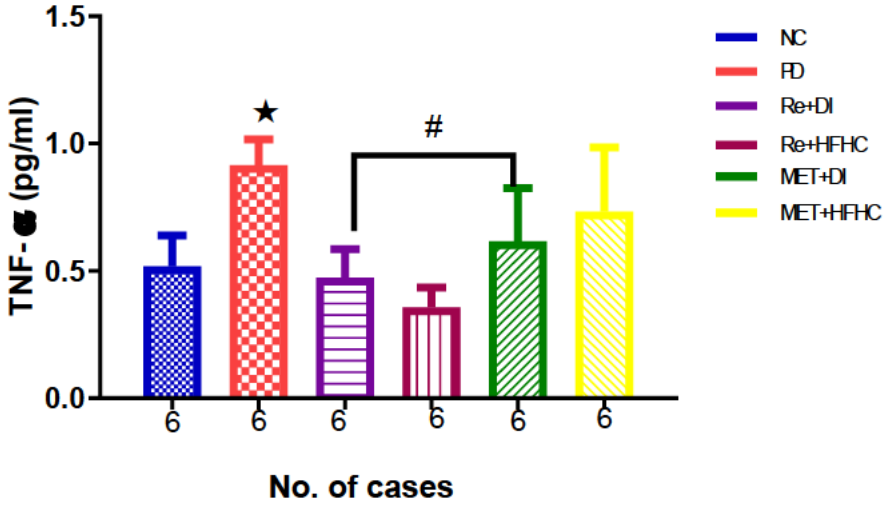

Figure 6. TNF α in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) com/pound and diet intervention (Re + DI), and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p <$ 0.05 in comparison with NC; # $p < 0.05$ in comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 7 shows the IL-6 levels in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In a comparative evaluation with the NC group, PD showed a significant increase in IL-6 levels ($p < 0.05$; Figure 6), and in comparison, with the PD control group, Re + DI and Re +HFHC groups showed a significant decrease in IL-6 ($p < 0.05$). See Figure 7.

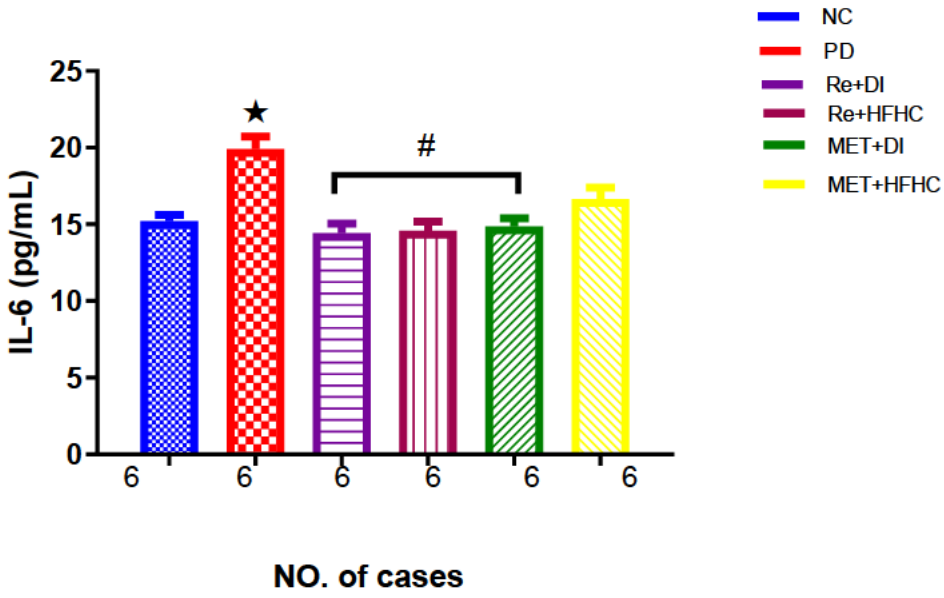

Figure 7. IL-6 concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI), and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

2.4. Effects of Rhenium (V) Compound Administration in the Absence and Presence of Diet Intervention on KIM 1 and GFR

Figure 8 shows the KIM-1 concentration in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant increase in KIM-1 concentration ($p < 0.05$) (Figure 8). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant decrease in KIM-1 concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 8.

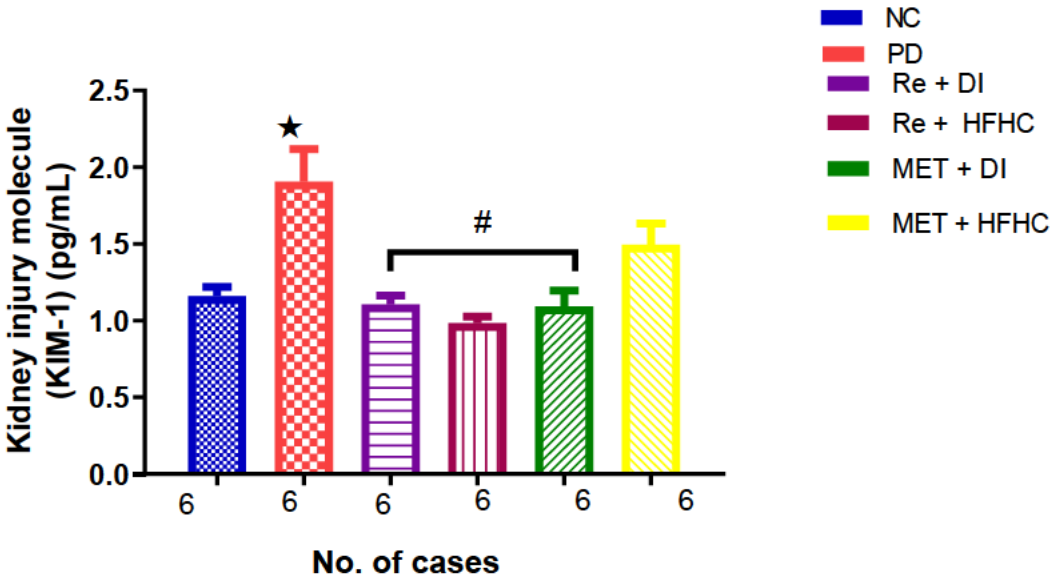

Figure 8. Plasma KIM-1 concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; α $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC. ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PD, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 9 shows the GFR in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant decrease in GFR ($p < 0.05$) (Figure 2). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant increase in GFR when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 9.

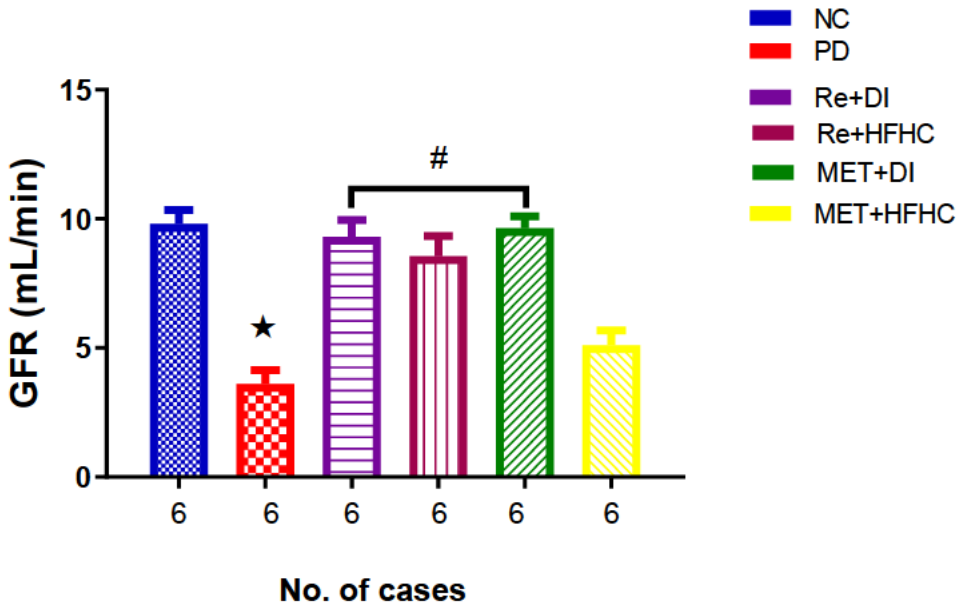

Figure 9. GFR in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

2.5. Effects of Rhenium (V) Compound Administration in the Absence and Presence of Diet Intervention on Plasma and Urinary Sodium and Potassium (Electrolytes Na^{+} and K^{+}), Fluid Intake and Urine Output

Figure 10 shows the fluid intake in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI), and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. There was a significant increase in fluid intake in PD when compared to NC ($p < 0.05$). After treatment for 12 weeks with the rhenium (V) compound, there was a noticeable significant decrease in fluid intake and urine output in treated groups when compared to PD ($p < 0.05$ Figure 10). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group. See Figure 10

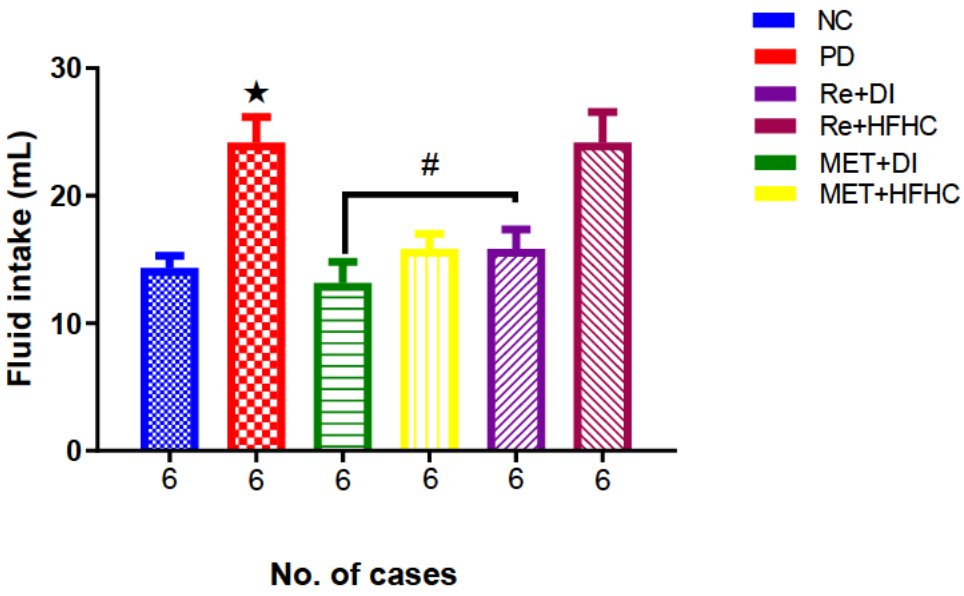

Figure 10. Fluid intake volume in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 11 shows the urine output in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. There was a significant increase in urine output in PD when compared to NC ($p < 0.05$). After treatment for 12 weeks with the rhenium (V) compound, there was a noticeable significant decrease in fluid intake and urine output in treated groups when compared to PD ($p < 0.05$ Figure 11). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 11.

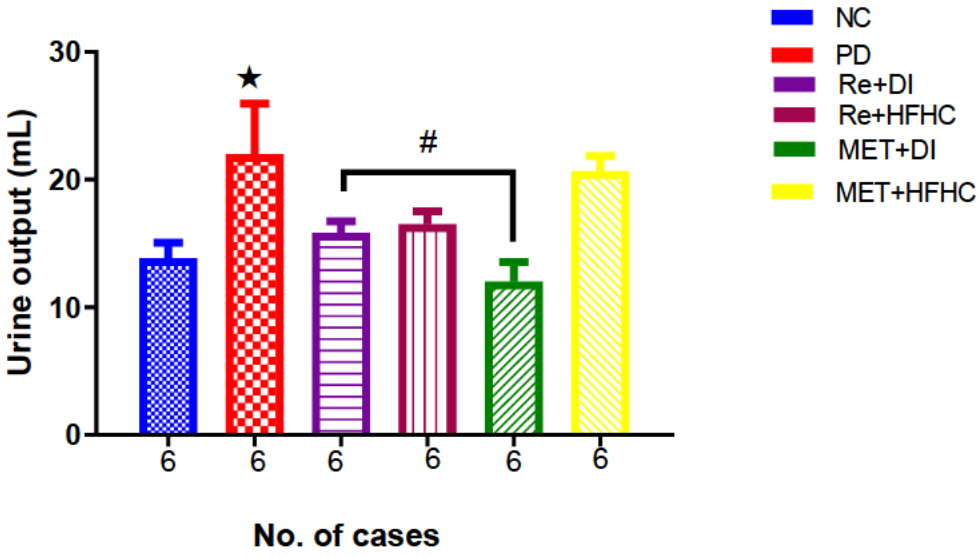

Figure 11. Urinary output volume in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 12A, B show plasma and urinary potassium (K^+) in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In Figure 12 A, there is a significant increase in urinary k^+ concentrations in the PD group when compared to the NC group ($p < 0.05$). In contrast, after 12 weeks of treatment with the rhenium (V) compound, there was a significant decrease in urinary K^+ concentration when compared with the PD group. In Figure 12 B, there was a significant increase in plasma K^+ concentration in the PD group when compared to the NC group ($p < 0.05$ Figure 12B). In contrast, after 12 weeks of treatment with the rhenium (V) compound, there was a significant increase in plasma K^+ concentration in the rhenium (V) compound-treated groups when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group. See Figure 12A, B.

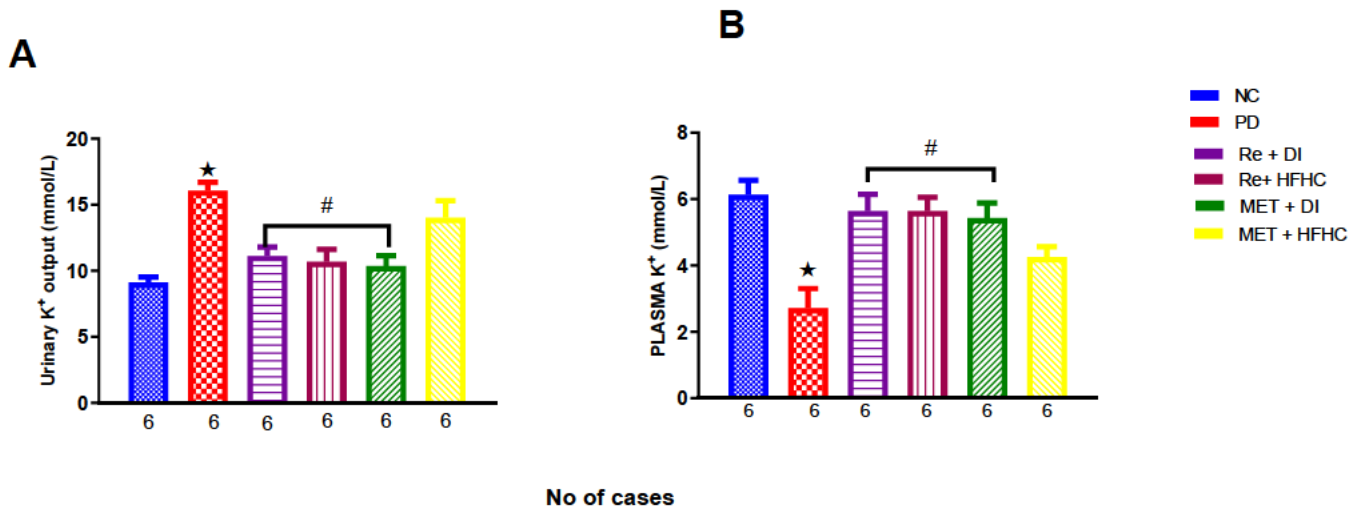

Figure 12. (**A**,**B**) Urinary and plasma K^+ concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 13A, B show plasma and urinary sodium in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI), and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In Figure 13A, there is a significant decrease in urinary Na^+ concentrations in the PD group when compared to the NC group ($p < 0.05$). In contrast, after 12 weeks of treatment with the rhenium (V) compound, there was a significant increase in urinary Na+ concentration when compared with the PD group. In Figure 13B, there is a significant increase in plasma Na^+ concentration in the PD group when compared to the NC group ($p < 0.05$ Figure 13B). In contrast, after 12 weeks of treatment with the rhenium (V) compound, there was a significant decrease in plasma Na^+ concentration in the rhenium (V) compound-treated groups when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group. See Figure 13A, B.

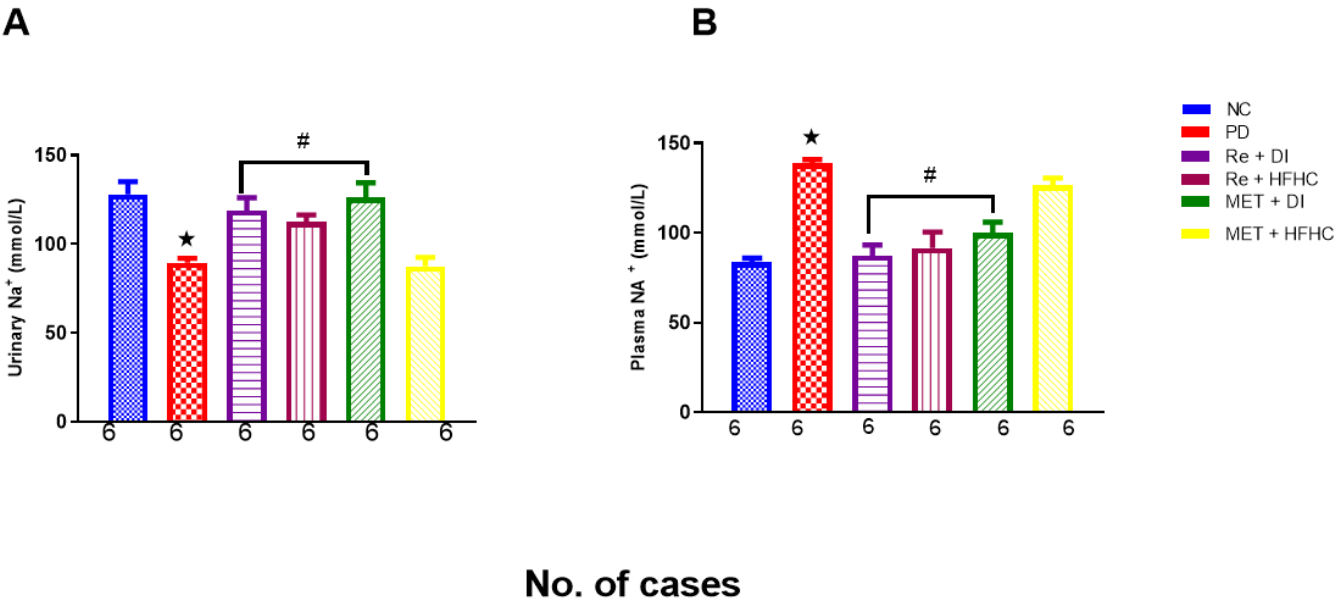

Figure 13. (**A**, **B**) Urinary and plasma NA^+ concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

2.6. Effects of Rhenium (V) Compound Administration in the Absence and Presence of Diet Intervention on Albumin Uric Acid, Urea and Creatinine (Both Plasma and Urine)

Figure 14A, B show plasma and urinary albumin concentrations in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. For plasma albumin, in comparison with the NC group, the PD group showed a significant increase in plasma albumin concentration ($p < 0.05$) (Figure 14A). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant decrease in plasma albumin concentration when compared with the PD group ($p < 0.05$). For urinary albumin, in comparison with the NC group, the PD group showed a significant decrease in urinary albumin concentration ($p < 0.05$) (Figure 14B). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant increase in urinary albumin concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 14A, B.

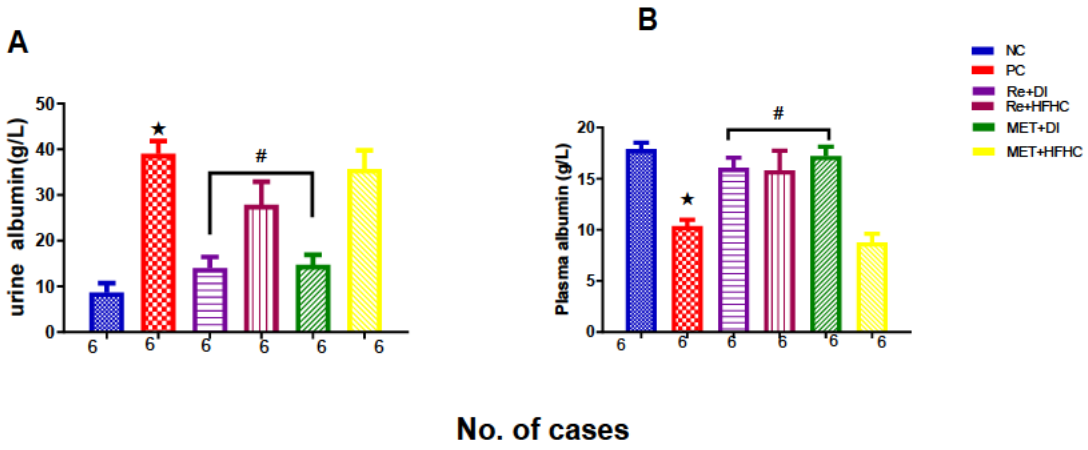

Figure 14. (**A**, **B**) Plasma and urinary albumin concentrations in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 15A, B show plasma and urinary uric acid concentrations in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. For both plasma and urinary uric acid, in comparison with the NC group, the PD group showed a significant increase in plasma and urinary uric acid concentrations ($p < 0.05$) (Figure 15A, B). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant decrease in plasma and urinary uric acid concentrations when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 15A, B.

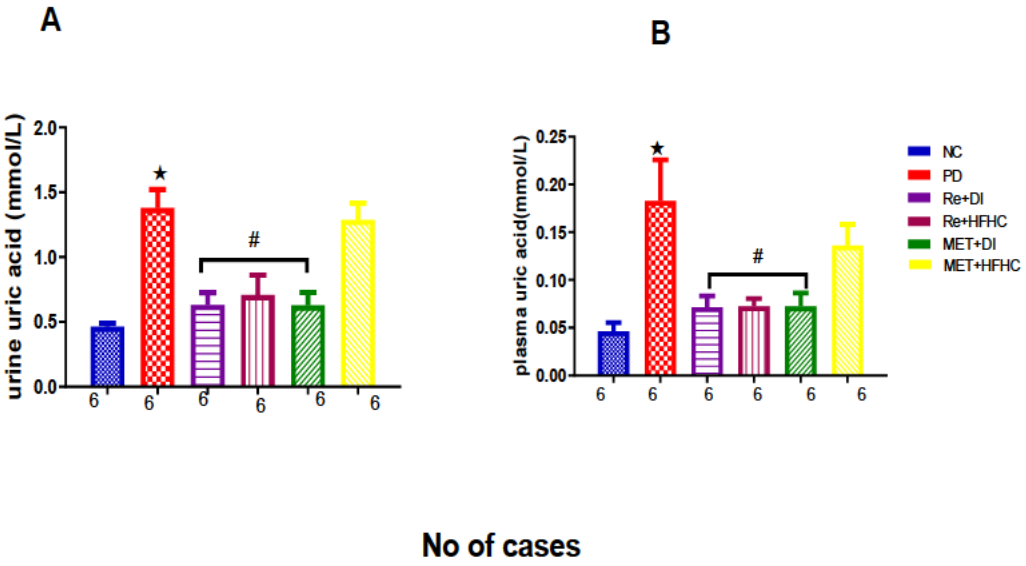

Figure 15. (**A**, **B**) Plasma and urinary uric acid concentrations in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 16A, B show urinary and plasma urea concentrations in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. For plasma urea, in comparison with the NC group, the PD group showed a significant increase in plasma urea concentration ($p < 0.05$) (Figure 16A). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant decrease in plasma urea concentration when compared with the PD group ($p < 0.05$). For urinary urea, in comparison with the NC group, the PD group showed a significant decrease in urinary urea concentration ($p < 0.05$) (Figure 16B). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant increase in urinary urea concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 16A, B.

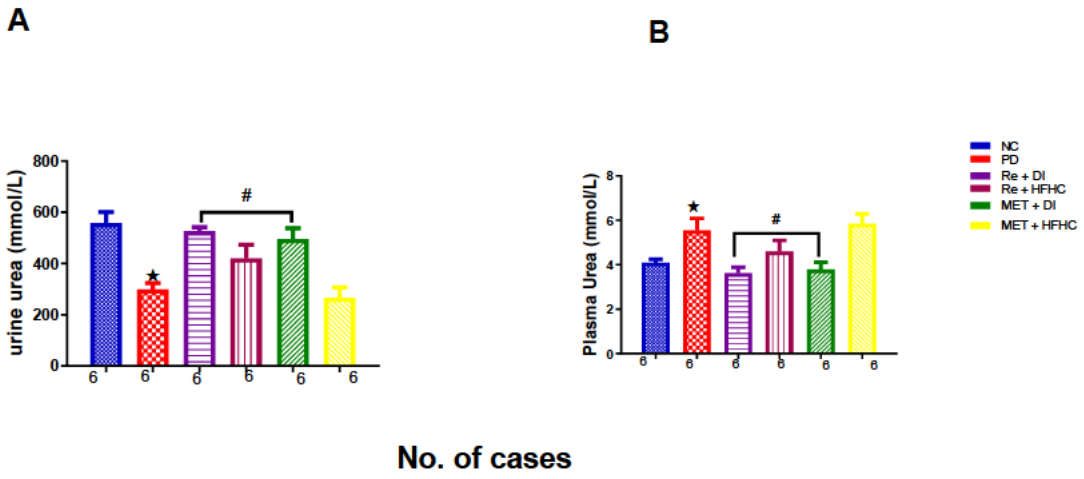

Figure 16. (**A**, **B**) Urinary and plasma urea concentrations in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 17A, B show urinary and plasma creatinine concentrations in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. For plasma creatinine, in comparison with the NC group, the PD group showed a significant increase in plasma creatinine concentration ($p < 0.05$) (Figure 17A). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant decrease in plasma creatinine concentration when compared with the PD group ($p < 0.05$). For urinary creatinine, in comparison with the NC group, the PD group showed a significant decrease in urinary albumin concentration ($p < 0.05$) (Figure 17B). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant increase in urinary creatinine concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 17A, B.

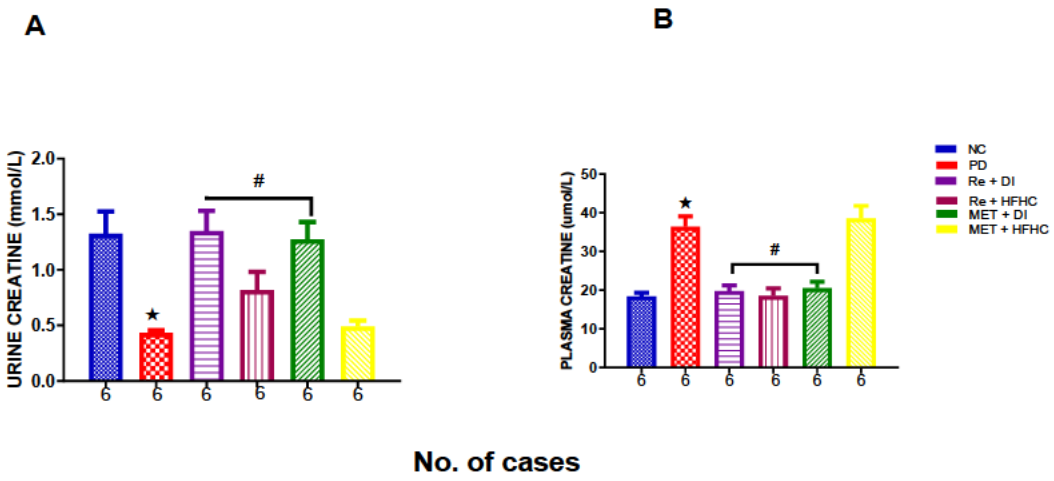

Figure 17. (**A**, **B**) Urinary and plasma creatinine concentrations in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

2.7. Effects of Rhenium (V) Compound Administration in the Absence and Presence of Diet Intervention on Aldosterone and Levels of mRNA Expression of Urinary Podocin

Figure 18 shows aldosterone concentration in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group showed a significant increase in aldosterone concentration ($p < 0.05$) (Figure 18). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in a significant decrease in aldosterone concentration when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 18.

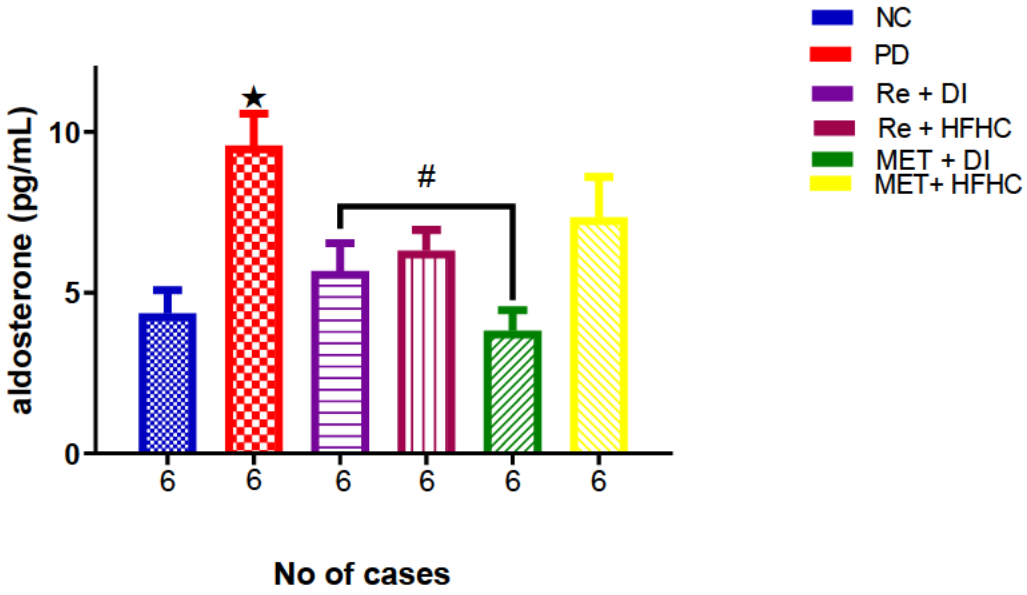

Figure 18. Plasma aldosterone concentration in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

Figure 19 shows the levels of the mRNA expression of urinary podocin in the normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate diet (Re + HFHC) groups after 12 weeks of treatment. In comparison with the NC group, the PD group was shown to have high levels of podocin mRNA expression ($p < 0.05$) (Figure 19). The administration of the rhenium (V) compound in both the diet intervention and high-fat/high-carbohydrate treatments resulted in significantly reduced levels of podocin mRNA when compared with the PD group ($p < 0.05$). A similar effect was observed with the MET + DI-treated group when compared with the PD group ($p < 0.05$). See Figure 19.

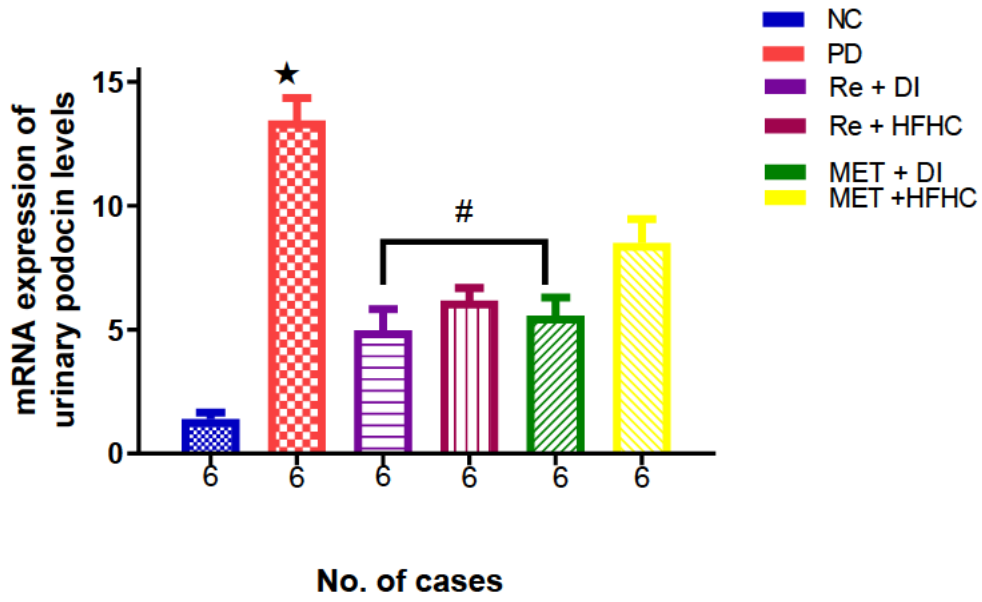

Figure 19. Levels of mRNA expression of urinary podocin in normal control (NC), prediabetic control (PD), metformin and diet intervention (MET + DI), metformin and high-fat/high-carbohydrate diet (MET + HFHC), rhenium (V) compound and diet intervention (Re + DI) and rhenium (V) compound and high-fat/high-carbohydrate (Re + HFHC) groups after 12 weeks of treatment. Values are presented as means ± SEM (n = 6). ★ $p < 0.05$ in comparison with NC; # $p < 0.05$ in comparison with PC, MET + DI, MET + HFHC, Re + DI and Re + HFHC.

3. Discussion

There has been growing interest in the use of metal complexes to treat prediabetes in the pharmacotherapy industry (32, 33). The focus of the current study is on a [3+1] oxo-free rhenium (V) compound with uracil-derived ligands in the treatment of prediabetes in diet-induced prediabetic rats.

Type 2 diabetes (T2DM) typically starts as prediabetes and progresses as

a chronic condition marked by persistent high blood glucose concentration due to the body's resistance to insulin (169). Insulin resistance is a key feature of T2DM and is associated with the cardiorenal metabolic syndrome, leading to the initial stages of cardiovascular and renal diseases in T2DM patients (166, 170). Untreated prediabetic rats had hyperglycaemia and insulin resistance, as shown by the HOMA2-IR index, and also abnormalities in electrolytes and signs of kidney damage, as determined by KIM 1 and low GFR. The groups treated with the rhenium (V) compound in both the presence and absence of a diet intervention had improved glycaemic control, as evidenced by lower glucose levels, low plasma insulin and regulated HOMA-IR values. Only the metformin-treated group with the diet intervention showed a reduction in glucose, insulin and the HOMA-IR value, while the metformin-treated group that consumed an HFHC diet did not show changes in these parameters. This agrees with the literature trend that suggests that metformin is effective with dietary intervention. However, uncontrolled hyperglycaemia and hyperinsulinaemia affect kidney cells during the prediabetic state.

In this study, we looked at hyperglycaemia-induced oxidative stress as a cause of kidney damage. Hyperglycaemia causes the auto-oxidation of glucose, the glycation of proteins and the activation of the polyol mechanism (164, 171). The polyol pathway is activated by hyperglycaemia, especially in non-insulin target tissues, including the kidneys. In this pathway, the conversion of glucose to sorbitol via aldose reduction is at the expense of the overconsumption of NADPH, which is essential for glutathione synthesis, a major antioxidant (172). The over-production of intracellular reactive oxygen species and antioxidant deficiency contribute to several microvascular and macrovascular complications of the kidney (77, 171). Oxidative stress stimulates the generation of inflammatory mediators and inflammation, which in turn enhance the production of reactive oxygen species (166). The untreated prediabetic group showed increased levels of the lipid peroxidation marker (MDA) and increased KIM 1 urinary and podocin levels, accompanied by a decline in GFR. Studies on prediabetes and T2DM have shown evidence that the consumption of a high-fat/high-carbohydrate diet has a positive correlation with systemic oxidative stress and renal disease (86, 135). Studies have also reported on the downregulation of the Nrf2/ARE pathway, which is responsible for the expression of antioxidants and anti-inflammatory proteins (173). Emerging studies have demonstrated that chronic oxidative stress, as occurs in diabetes, leads to the irregular inhibition of the Nrf2/ARE pathway by Keap1 (174). Recent reports have also suggested that the activation of the Nrf2/ARE pathway can prevent kidney disease progression (175). Various molecules, such as resveratrol, have been shown to stimulate this pathway, thus ameliorating oxidative stress (Eun Nim Kim). The antioxidative effects observed in this study could partly be attributed to the interaction of the investigated compound with kinases upstream of the NrF2/ARE pathway; however, confirmatory studies are necessary. Furthermore, it has been reported that the upregulation of NAD(P)H oxidase 4 (NOX4) plays an important role in causing renal oxidative stress and kidney injury in animal models of chronic kidney disease (CKD) and diabetic nephropathy (DN) (25, 176). Oxidative stress also plays an important role in podocyte injury and the downregulation of glomerular filtration barrier proteins, nephron and podocin (177, 178).

Damage to podocytes results in proteinuria and, eventually, renal failure, which is due to a decrease in glomerular permeability due to mesangial expansion (178, 179). Apart from oxidative stress and associated inflammation, podocyte injury or death could be attributed to insulin resistance (180). These cells, which are crucial for the integrity of the glomerular basement membrane, have been reported to be insulin-responsive, and hence, their survival is insulin-dependent. Amongst other renal cells, podocytes highly express insulin receptors and Akt2 and GLUT 4 (55, 181). Insulin signalling has been reported to be key in podocyte functions, including the maintenance of glomerulus integrity.

Upon treatment with the rhenium (V) compound, there was improved kidney function. This is observed through the improved GFR, as there is less damage to the kidney, which is evidenced by reduced KIM 1 and podocin levels in rhenium-treated groups. We speculate that the rhenium (V) compound facilitates kidney recovery by first preventing hyperglycaemia-induced oxidative stress by normalizing oxidative stress and antioxidant defence enzymes, as the results show improved antioxidant enzyme activities of SOD and GPx and the suppression of lipid peroxidation in treated prediabetic rats. The ability of the rhenium (V) compound to attenuate hyperglycaemia could also be beneficial in improving kidney function. Many reports agree that adequate glycaemic control can prevent kidney dysfunction or injury. Additionally, the renoprotective effect could also be attributed to the ability of rhenium compounds to enhance the insulin sensitivity of podocytes and, ultimately, their function and survival. A previous study has shown that the administration of this compound led to lower levels of glycated haemoglobin (HbA1c) and increased insulin sensitivity in prediabetic rats (34, 99). The prevention of renal damage in rhenium (V) compound-treated rats in both the presence and absence of a dietary intervention could then explain the significantly lower levels of urine albumin in comparison with untreated prediabetic rats. It is noteworthy that while there are significant improvements in the rhenium (V) compound-treated groups without the dietary intervention, these improvements are not as pronounced as those observed in the groups treated with the dietary intervention. This may be attributable to the continued assault on the kidneys as a result of the continued ingestion of the HFHC diet. In a previous paper, it was shown that even though there is an amelioration of hyperinsulinaemia in the absence of the dietary intervention, the decrease in insulin levels is not as pronounced in comparison with the groups with the dietary intervention (34). Continued ingestion may still lead to pronounced insulin resistance, which could mask the effect of the rhenium compound. As there are no statistically significant differences between the two groups, this may indicate that there may be improved efficacy with the dietary intervention.

Inflammatory cytokines, including transforming growth factor, are involved in the development and progression of diabetic nephropathy by promoting mesangium expansion and cellular matrix thickening (166, 182). In this study, we looked at IL-6 and TNF-α to check the effects of the rhenium (V) compound on preventing inflammation. Increased levels of IL-6 and TNF-α in the untreated prediabetic group indicated glomerular basement membrane thickening and high levels of IL-6, which are associated with an elevation in its urinary excretion (182). TNF-α is cytotoxic to renal cells and is able to induce direct renal injury (182, 183). TNF-α also directly induces reactive oxygen species (ROS) in diverse

cells, including mesangial cells (166, 183). However, the prediabetic group treated with the rhenium (V) compound had reduced levels of inflammatory markers, and this may be due to the beneficial effects mentioned above, which resulted in the prevention of immune system activation; therefore, the rhenium (V) compound may have anti-inflammatory properties.

Both the inflammation and oxidative stress mentioned above can cause the activation of various pathways, including NF-kB, which further activate apoptosis and cell proliferation and differentiation and lead to the loss of renal function (184, 185). However, NF-Kb can be downregulated by pharmaceutical therapies that have anti-inflammatory and antioxidant properties via targeting the Nrf2 pathway, which has a central role in encoding antioxidants (175, 185). Nrf2 inhibits reactive oxygen species (ROS) and inflammatory pathways that lead to kidney dysfunction (175, 186). We speculate that the rhenium (V) compound targets Keap1, which results in the release of Nrf2 into the nucleus. Once Nrf2 is activated and in the nucleus, it binds to the gene regulator antioxidant response element (ARE) region and mediates the transcription of antioxidant genes. Therefore, antioxidant activity is increased via this pathway, which is supported by the increased activity of SOD and GPx in the rhenium-treated groups.

Prediabetes has been shown to be an intermediate-stage complication associated with T2DM; for example, this is when renal dysfunction often begins (23, 187). Kidney dysfunction is known to be caused by a combination of fat accumulation as a result of insulin resistance in peripheral tissues and primary hypertension (71, 84). In an insulin-resistant state, Na+/K+-ATPase activity is decreased, and insulin resistance induces hyperinsulinaemia (63, 85). Sodium reabsorption from renal tubules is increased and leads to high blood pressure, and lastly, the circulatory fluid volume can also increase relative to hyperglycaemia-induced hyperosmolarity (84, 85). Indeed, rats treated with the rhenium (V) compound in both the presence and absence of the dietary intervention had significantly lower blood pressure as compared to untreated prediabetic rats. We speculate that this could be due to the glucose-metabolism-ameliorating effects of the rhenium (V) compound, as previously shown by Siboto and colleagues (34). In that study, the rhenium (V) compound reduced hyperinsulinaemia, and this is a positive effect because, when insulin is regulated, Na+/K+-ATPase activity will increase. This leads to sodium not being reabsorbed at a high rate, which results in reduced blood pressure. In metformin-treated rats, the group that received diet modification sustained glomerular capillary hypertension and subsequent glomerular barrier injury and microalbumin leakage; metformin exerts pleiotropic actions on the kidney, beyond its effects as a glucose-lowering agent by attenuating DN, associated with its ability to improve insulin resistance, lipid metabolism, and antioxidative and anti-inflammatory functions (29, 188).

Blood pressure is not the only marker that the rhenium (V) compound ameliorated in the treated prediabetic rats; the GFR function was also improved, as we observed that the treated group had improved electrolyte reabsorption and excretion. Studies have shown that diabetic nephropathy is associated with a decline in renal function, including the reduced function of the glomerular filtration rate (GFR), which is associated with electrolyte abnormalities, water imbalance and hypernatremia (83, 84). Elevated plasma uric acid is associated with a

higher risk of insulin resistance (189, 190). Uric acid is a weak acid generated by purine metabolism. It has been recognised as the cause of gout since the early 1800s (189, 190). High circulating uric acid levels might increase the risk of T2DM and metabolic syndrome, thereby contributing to a higher risk of diabetic complications among T2DM patients (169). Experiments in rats showed an association between lower serum uric acid levels and improved insulin sensitivity (99).

Diet-induced prediabetic rats had high uric acid levels in comparison with non-prediabetic rats. However, rats treated with the rhenium (V) compound in both the presence and absence of the diet intervention had reduced uric acid, suggesting the amelioration of uric acid. Lastly, the significantly increased urea, creatinine and uric acid levels in untreated prediabetic rats demonstrated renal damage and metabolic alterations resulting from insulin resistance and hyperglycaemia. However, treatment with the rhenium (V) compound in both the presence and absence of the dietary intervention resulted in a significant decrease in creatinine, plasma urea and uric acid levels, signifying its nephroprotective potential.

Prediabetes is associated with the increased activity of the renin–angiotensin–aldosterone system (RAAS), resulting in the retention of sodium and water and a rise in blood pressure (85, 165). The upregulation of RAAS contributes to the impairment of the renal system, which was evidenced by the significantly increased concentration of aldosterone in the untreated prediabetic group (82, 86). High levels of aldosterone have negative effects on the system, such as inflammation, oxidative stress and even insulin resistance (86, 191). Arterial hypertension plays an important role in the development of kidney dysfunction by causing glomerular hyperfiltration and glomerular hypertrophy, followed by the expansion of the mesangium and the accumulation of the extracellular matrix (37, 83). Prediabetic rats treated with the rhenium (V) compound in both the presence and absence of the dietary intervention had lower levels of aldosterone in comparison with untreated prediabetic rats. Metal complexes including transition metals such as vanadium have been shown to be useful in treating prediabetic hypertension by targeting the RAAS by decreasing angiotensin concentrations (95). We speculate that the rhenium (V) compound also uses the same mechanism to reduce aldosterone and high blood pressure, but further studies will be conducted on the effects of the rhenium (V) compound on regulating blood pressure, including mean arterial pressure (95).

4. Materials and Methods

4.1. Animals.

Thirty-six [36] male Sprague-Dawley rats (150–180 g) obtained from Biomedical Research Unit, University of KwaZulu-Natal (UKZN), were kept under standard environmental conditions, i.e., constant humidity (55 ± 5%), temperature (22 ± 2 °C) and 12 h day/12 h night cycle. The rats were acclimatised for 2 weeks and consumed standard rat chow (Meadow Feeds, South Africa) and water ad libitum before being fed on the experimental high-fat/high-carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, South Africa) to induce prediabetes. The HFHC diet consists of carbohydrates (55% kcal/g), fats (30% kcal/g) and proteins (15% kcal/g), as described in our previous studies (133, 135). All

experimental designs and procedures were carried out according to the ethics and guidelines of the Animal Research Ethics Committee (AREC, ethical clearance code: AREC/00003221/2021) of UKZN, Durban, South Africa.

4.2. Induction of Prediabetes.

Sprague-Dawley rats were randomly assigned to the following diet groups (n = 6 per group): a standard rat chow with normal drinking water (ND + H_2O) or a high-fat/high-carbohydrate diet with drinking water supplemented with fructose (HFHC + Fructose). The experimental prediabetes induction period was 20 weeks (135, 136). Rats with fasting blood glucose of more than 5.6 mmol/L were considered prediabetic and further grouped for pharmacological studies (137, 138). The treatment started on the subsequent day, and this was considered the first day of treatment.

4.3. *Experimental Design.*

The animals were randomly divided into 6 groups of 6 animals in each (30 with persisting prediabetes; 6 normal). Group 1: Normal healthy control rats received vehicle (NC); Group 2: Prediabetic control rats continued with the HFHC diet and received vehicle (PD); Group 3: Treated prediabetic rats continued with the STD diet and received metformin (MET + DI); Group 4: Treated prediabetic rats continued with the HFHC diet and received metformin (MET + HFHC); Group 5: Prediabetic rats continued with the STD diet and received the rhenium (V) compound (Re + DI); Group 6: Treated prediabetic rats continued with the HFHC diet and received the rhenium (V) compound (Re + HFHC).

4.4. Treatment of Prediabetic Animals.

The treatment period started after 20 weeks of prediabetes induction and lasted an additional 12 weeks. The animals were treated once every third day at 9:00 a.m., where the MET + HFHC and MET + DI groups received an oral dose of metformin (500 mg/kg), while the Re + HFHC and Re + DI groups received a subcutaneous injection of the rhenium (V) compound (15 mg/kg). The concentration of the rhenium (V) compound was chosen from a previous study conducted in our laboratory (34). Parameters including blood glucose, blood pressure, fluid intake and urine output were monitored every 4 weeks during the treatment period.

4.4.1. *Determination of Fluid Intake and Urine Output.*

At the beginning of the treatment period and every 4 weeks thereafter, all of the animals in each group were placed in different metabolic cages for 24 h to measure fluid intake and urine output. The urine samples were measured and centrifuged at 13,000 rpm for 5 min at 4 °C, and the supernatants were stored at −80 °C in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) until ready for kidney function parameter analysis.

4.4.2. *Blood collection and tissue harvesting.*

For blood collection, all rats were anaesthetised with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd., Roodeport, South Africa), 4–5% for the induction and 1–2% for maintenance, as is recommended by the anaesthesia guideline, and Isofor showed no negative health effects

on the rats when administered via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for 3 min. While rats were unconscious, blood was collected by cardiac puncture into individual pre-cooled heparinisedblood collection tubes. The blood was then centrifuged (Eppendorf centrifuge 5403, Germany) at 4 °C and 503 g for 15 min. Plasma was collected and stored at −80 °C in a Bio Ultra freezer (name of the manufacturer: Snijers Scientific labs;CityHolland, country: The Netherlands) pending biochemical analysis. Following blood collection, kidney was removed, weighed and rinsed with cold normal saline solution and snap frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at −80 °C until biochemical analysis.

4.5. *Biochemical Analysis*

The biochemical analysis of kidney function parameters (such as creatinine, urea, uric acid, albumin and electrolytes (Na^+ and K^+)) was performed on plasma and urine samples in the 32nd week by using their respective assay kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) as instructed by the manufacturer. However, the kidney injury molecule (KIM-1) and aldosterone plasma concentrations were determined using their specific ELISA kits (KIM 1: E-EL-R3019; aldosterone: E-EL-0070) as instructed by the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) via a microplate reader (SPECTROstar Nano spectrophotometer; BMG LABTECH, Ortenburg, LGBW, Germany).

4.5.1. Determination of GFR

The GFRs of all animals were determined in the 32nd week of the experiment from the estimation of creatinine in the plasma and urine (creatinine clearance) as follows:

GFR mL/min = Urine creatinine mg/dL × 24 h urine volume mL
Plasma creatinine mg/dL × 60 min × 24 h

4.5.2. Lipid Peroxidation and Antioxidant Status**.**

Lipid peroxidation was assessed by determining the concentration of malondialdehyde (MDA) in homogenised kidney tissues according to a previously established protocol (141). However, the antioxidant status of the kidney homogenate was assessed by determining the concentration of superoxide dismutase (SOD) and glutathione peroxidase (GPx) by using their specific ELISA kits (SOD: E-EL-R1324; GPx: E-EL-R2491) according to the instructions of the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). The inflammatory markers TNF-α and IL-6 were measured using their specific ELISA kits (Rat TNF-α: E-EL-R2856; Rat IL-6: E-EL-R0015).

4.5.2. Urine RNA Isolation

The kit was purchased from Inqaba Biotechnical Industries (pty) Ltd. RNA was isolated from urine (4 mL) by using the ZR Urine RNA Isolation Kit™ (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocol. RNA was treated with DNAse before reverse transcription. The purity of the RNA was confirmed by the relative

absorbance ratio of 260/280 nm via a Nanodrop 1000 spectrophotometer (name of the manufacturer: Thermo Scientific, city: CA ; country :USA).

4.5.3. Urine Complementary DNA (cDNA) Synthesis

Urine RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) by using the iScript™ cDNA Synthesis Kit (name of the manufacturer: Bio-Rad, city: CA, country USA) through incubation in a thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems, Life Technologies). For cDNA synthesis, urine RNA (2 μL) was mixed with 5× iScript reaction (4 μL), iScript reverse transcriptase enzyme (1 μL) (name of the manufacturer: Bio-Rad, city: CA; country 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 min, 42 °C for 30 min and, finally, 85 °C for 5 min. Thereafter, the synthesised cDNA was stored at −80 °C until its use for real-time PCR (polymerase chain reaction).

4.5.4. Real-Time PCR

The urinary mRNA level of podocin was quantified by a real-time PCR LightCycler (name of the manufacturer: Roche LightCycler 96, city: CA; country: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 s, 60 °C for 20 s, and 72 °C for 20 s in the LightCycler (Roche LightCycler 96, USA). All samples were run in duplicate, and β-actin mRNA was used as a housekeeping gene (β-actin primers: forward primer GCA CCA CAC CTT CTA CAA TG; reverse primer TGC TTG CTG ATC CAC ATC TG) to normalise the podocin mRNA level. The sequences of the used oligonucleotide primers (Metabion International AG, Planegg, Germany) were as follows: podocin forward 5′-TGG AAG CTG AGG CAC AAA GA-3′ and podocin reverse 5′-AGA ATC TCA GCC GCC ATC CT-3′.

4.6. Statistical Analysis

All data are expressed as means ± SEM. Statistical comparisons were performed with Graph Pad In Stat Software (version 5.00, Graph Pad Software, Inc., San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test. A value of $p < 0.05$ was considered statistically significant

5. Conclusions

The treatment of diet-induced prediabetic rats with the rhenium (V) compound with uracil-derived ligands in both the presence and absence of a diet intervention did not only markedly improve insulin sensitivity but also effectively decreased metabolic disturbances, thereby preventing hyperglycaemia-induced oxidative stress in the kidney. We believe that these findings warrant more studies into the biological activity of this compound, as it may have the potential to not only restore glucose homeostasis in the prediabetic state but also prevent prediabetes-associated complications.

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Chapter 5

Synthesis

Prediabetes is an asymptomatic state of moderate dysglycaemia that often precedes the onset of T2DM (9, 11). It is diagnosed using the following parameters, glycated haemoglobin (HbA1c) of a level of 5.7% to 6.4%, impaired glucose tolerance (IGT) defined as 2 h plasma glucose of 7.8-11.0 mmol/L after ingestion of 75 g of oral glucose load based on a 2 h oral glucose tolerance test (OGTT) and lastly impaired fasting glucose (IFG) defined as fasting plasma glucose of 6.1-6.9 mmol/L (4, 106). Studies have reported an increase in the prevalence of both T2DM and prediabetes in developing countries (1, 4, 24). This increase may be attributed to increased urbanization which results in decreased physical activity and chronic ingestion of high caloric foods such as refined carbohydrates and diets rich in saturated fatty acids (4, 192). The uncontrolled dysglycaemia seen in prediabetes has been shown to lead to complications often associated with T2DM are NAFLD, CVD and renal dysfunction (9, 106).

Studies have however shown that prediabetes is reversible (106, 192). This knowledge has been used to form a therapeutic strategy to try and reduce the prevalence of T2DM (192). The management of prediabetes requires a combination of anti-hyperglycaemic pharmacological agents like metformin and lifestyle modification that is consumption of low-calorie diets (28, 91). Metformin has been shown to be effective in managing prediabetes (24, 91, 107). However, patients tend to become overly-dependent on the pharmacological treatment and neglect the dietary modifications thus reducing the efficacy of the pharmacological agents (28, 107). Prolonged use of these pharmacological agents can also lead to toxicity and cause organ damage (24, 107). Therefore, there is a need for novel anti-diabetic pharmacological agents that can remain therapeutic in low concentrations even in the absence of dietary modification.

Metal complexes that have organic ligands have been used experimentally as anti-hyperglycaemic pharmacological agents to try and find an alternative to metformin (33, 98). These complexes have shown promise in improving insulin sensitivity and ameliorating some complications associated with T2DM (97). However, their application has been limited by different reports of toxicity (193, 194). The metal of interest in this study, [3+1] oxo- free rhenium (V) compound was combined with an uracil-derived ligand in an attempt to minimize its toxicity. This ligand has been shown to improve rhenium stability as well as allow rhenium to mimic the biodistribution patterns of liposomal and liposomal pharmacological agents (100,

101). This was found to increase its permeability and promote the physiological compatibility of the compound (100-102). A study conducted in our laboratory showed that the [3+1] oxo-free rhenium (V) compound with a uracil-derived ligand stimulated glucose uptake in insulin resistant skeletal muscle cell lines *in-vitro* (101). In our laboratory, a high fat high carbohydrate diet-induced prediabetic rat model was established that mimics the human prediabetes condition (5). Previously, we investigated the effect of this compound in markers of glucose homeostasis in diet-induced prediabetic rats and demonstrated that it improved glucose tolerance, reduced plasma ghrelin concentration and ameliorated blood HbA1c levels in both the presence and absence of dietary intervention (34). These promising observations prompted us to investigate the effects of the rhenium (V) compound on NAFLD, CVD and renal dysfunction all which are complications of prediabetes using a rodent model.

Prediabetes is associated with NAFLD which is characterized by IFG, abnormal plasma liver enzymes, AST and ALT, indicating liver damage and accumulation of hepatic triglyceride (78). In manuscript 1, Investigated the effects of the rhenium (V) compound with uracil-derived ligands on selected markers of liver function in diet-induced prediabetic rats. During the prediabetic phase, moderate insulin resistance in tissues like skeletal muscle causes high blood glucose to redirect to the liver, using methods independent of insulin for glucose processing (40, 46). This surplus glucose triggers heightened processes like lipogenesis and glycogenesis, leading to irregular lipid levels and excessive glycogen storage in the liver. Physical changes like enlarged liver (hepatomegaly) and increased enzyme levels such as AST and ALT indicate tissue damage. The moderate increase in blood glucose during prediabetes also prompts inflammation, exacerbating insulin resistance, as seen through elevated markers like CRP, IL-6, and TNF α. In this study, our rodent model displayed all these traits, affirming its suitability as a model for prediabetes-induced NAFLD.

The use of rhenium (V) compound alongside uracil-based ligands, regardless of dietary changes, demonstrated a positive impact on indicators linked to NAFLD. This was evident through reduced hepatic triglycerides and glycogen storage in the liver. Additionally, lower plasma AST and ALT levels indicated an improvement in liver damage caused by prediabetes. Moreover, there was a decrease in inflammatory markers associated with enhanced control of blood glucose. These findings aligned with an earlier study where the rhenium (V) compound was found to improve glucose regulation irrespective of dietary changes (34). When comparing with our standard drug, metformin, similar observations were noted. However, these outcomes were apparent only when metformin was used alongside dietary intervention. Although there

was some progress in NAFLD-related markers when metformin was administered without dietary changes, it did not reach statistical significance. This mirrored a previous study where administering metformin without dietary intervention showed improvements in glucose regulation that were not statistically significant (34).

The improvement of NAFLD indicators in the prediabetic state appears to hinge on the enhancement of glucose regulation. This study marks the initial evidence showcasing that administering the rhenium (V) compound improves markers linked to NAFLD in prediabetes, regardless of dietary changes. These findings indicate the potential hepato-protective qualities of our compound, likely achieved through enhanced control of blood glucose. Consequently, this compound might not only benefit glucose regulation but also mitigate the advancement of NAFLD.

Moreover, the liver plays a pivotal role in the normal functioning of other organs. In the prediabetic state, heightened levels of insulin and blood glucose trigger a process called *de novo* lipogenesis in the liver through SREBP-1c, leading to extensive storage of triglycerides (TGs) and other fatty substances like LDL. As these substances are released into the bloodstream, they instigate damage to the arterial walls, causing constriction due to the accumulation of fat (152). Uncontrolled accumulation of fat can create arterial blockages, inducing shear stress and resulting in elevated cholesterol levels. This process contributes to endothelial damage and high blood pressure, ultimately leading to the onset of cardiovascular complications. Hence, in manuscript 2, explored the impact of the innovative rhenium (V) compound on specific indicators of cardiovascular function in rats with diet-induced prediabetes, considering both scenarios with and without dietary intervention. The onset of cardiovascular complications is linked to various risk factors like dyslipidemia, high BMI, and hypertension (59, 69). Individuals with excess body weight or obesity face heightened cardiac risks due to elevated levels of TGs, TC, and LDL, which foster the development of atherosclerosis (12, 69). Reduced availability of nitric oxide in the bloodstream triggers vasoconstriction, leading to increased blood pressure (151, 195). Prediabetic patients experience persistent low-level inflammation, evident through elevated cytokines such as TNF-α, alongside inflammatory markers like CRP and IL-6 (61, 77). Elevated levels of these inflammatory markers signify a higher risk of heart attack and stroke (12, 71). The moderate increase in blood glucose observed in prediabetes can also enhance markers associated with chronic inflammation and contribute to increased generation of reactive oxygen species (ROS), indicating reduced levels of antioxidants in the system (61). Normalizing the lipid profile has

been identified as a preventive measure against cardiovascular risks (45, 80). The onset of heart failure triggers the activation of two systems: the renin-angiotensin-aldosterone system (RAAS), causing sodium and water retention, and the sympathetic nervous system, leading to lipolysis (37, 78). The RAAS plays a pivotal role in the development and progression of diabetic renal disease (196). In prediabetic patients, uncontrolled activation of this system leads to fluid retention and hypertension. It also triggers inflammatory, thrombotic, and atherogenic effects that might contribute to long-term damage in end organs (77, 81).

In this research, the untreated prediabetic rats exhibited these traits, reinforcing the credibility of using the diet-induced prediabetes rodent model. Administration of the rhenium (V) compound not only reduced triglyceride (TG) production but also lowered other indicators like plasma LDL and total cholesterol. This compound, based on a metallic element, additionally promoted the generation of HDL, which could be beneficial for cardiomyocytes, possibly through enhanced apoA-I activity. An earlier study indicated that this metallic compound contributed to weight loss by suppressing ghrelin secretion in treated prediabetic rats, regardless of dietary changes. (34). In this study, prediabetic rats treated with the rhenium (V) compound exhibited increased nitric oxide (NO) availability, regardless of dietary changes. This elevation could facilitate vasodilation, potentially explaining the observed decrease in blood pressure. It implies that the compound's ability to reduce blood pressure might be linked to its capacity to normalize the lipid profile, which is associated with its effects on both glucose and liver regulation. Furthermore, the prediabetic rats treated with the rhenium (V) compound showcased reduced levels of IL-6, TNF-α, and CRP compared to untreated prediabetic rats. These findings suggest that the compound's beneficial effects might, in part, stem from its anti-inflammatory properties, likely influenced by its impact on glucose regulation.

Similar to manuscript 1, the administration of metformin, our standard drug, resulted in a similar trend only where metformin was coupled with dietary intervention. Therefore, the mechanism of preventing development of CVD in the prediabetic state seems to depend on amelioration of both glucose and liver homeostasis. This study, for the first time, shows that the administration of the rhenium (V) compound could prevent the development of CVD in the prediabetic state in both the presence and absence of dietary intervention.

Prediabetes is linked to a moderate increase in the risk of chronic kidney disease (197). IFG, IGT and elevated blood pressure are identified as risk factors that potentially contribute to the

onset of kidney disease (197). In a prior study conducted within our laboratory, the rhenium (V) compound was demonstrated to restore normal glucose tolerance specifically in skeletal muscle tissues (34). Building on the discoveries from manuscripts 1 and 2, the subsequent phase aimed to assess metabolic factors associated with the progression of kidney disease. Therefore, in manuscript 3 we investigated the effects of the rhenium (V) compound on prediabetes-induced renal dysfunction in both the presence and absence of dietary modification.

Arterial hypertension is closely linked to diabetic nephropathy and significantly contributes to the progression of kidney dysfunction. It triggers glomerular hyperfiltration, glomerular hypertrophy, subsequently leading to mesangial expansion, and the accumulation of extracellular matrix within the kidneys (37, 83). In diabetic nephropathy, there's a decline in kidney function, notably affecting the glomerular filtration rate (GFR). This decline is linked to electrolyte imbalances, disrupted water regulation, and an increased presence of hypernatremia (83, 84). Hyperglycaemia-induced oxidative stress as a cause of kidney damage is seen by increased urinary KIM 1 and podocin levels as well as increased concentrations of plasma aldosterone. In this study, the untreated prediabetic rats displayed these characteristics, further validating the use of the diet-induced prediabetes animal model in the management of prediabetes-induced renal damage.

Treatment with the rhenium (V) compound notably enhanced insulin sensitivity, evident through lower HOMA IR index values. This improvement suggests a prevention of hyperglycemia-induced oxidative stress. Kidney function showed improvement in rats treated with the rhenium (V) compound, regardless of dietary changes. This was indicated by increased glomerular filtration rate (GFR) and reduced urinary levels of KIM 1 and podocin, markers of kidney injury. Moreover, there was a decrease in plasma aldosterone levels, accompanied by a drop in blood pressure. Beyond regulating blood glucose, the rhenium (V) compound also boosted the antioxidant activity of both SOD and GPx. Additionally, there was a noteworthy reduction in plasma urea and uric acid levels, highlighting the potential of the compound in safeguarding kidney function. Similar outcomes were observed with the administration of metformin, but notably when coupled with dietary intervention.

The data seems to suggest that the improvement of renal function in the prediabetic state relies primarily on the amelioration of glucose homeostasis with secondary contributions from the improvement of hepatic and cardiovascular homeostasis. This study, for the first time, shows

that the administration of the rhenium (V) compound improved renal function in the prediabetic state in both the presence and absence of dietary intervention.

Taken together, the study showed that diet-induced prediabetes results in moderate hyperglycaemia. This hyperglycaemia then becomes the key driver in the dysregulation of hepatic, cardiovascular and renal homeostasis. The administration of the standard drug was shown to be beneficial in managing these derangements, albeit in the presence of dietary intervention. The administration of the rhenium (V) compound, however, was able to ameliorate the moderate hyperglycaemia in both the presence and absence of dietary intervention. This could therefore explain how this compound was able to ameliorate prediabetes-associated NAFLD, CVD and renal dysfunction in both the presence and absence of dietary intervention. See Figure 1

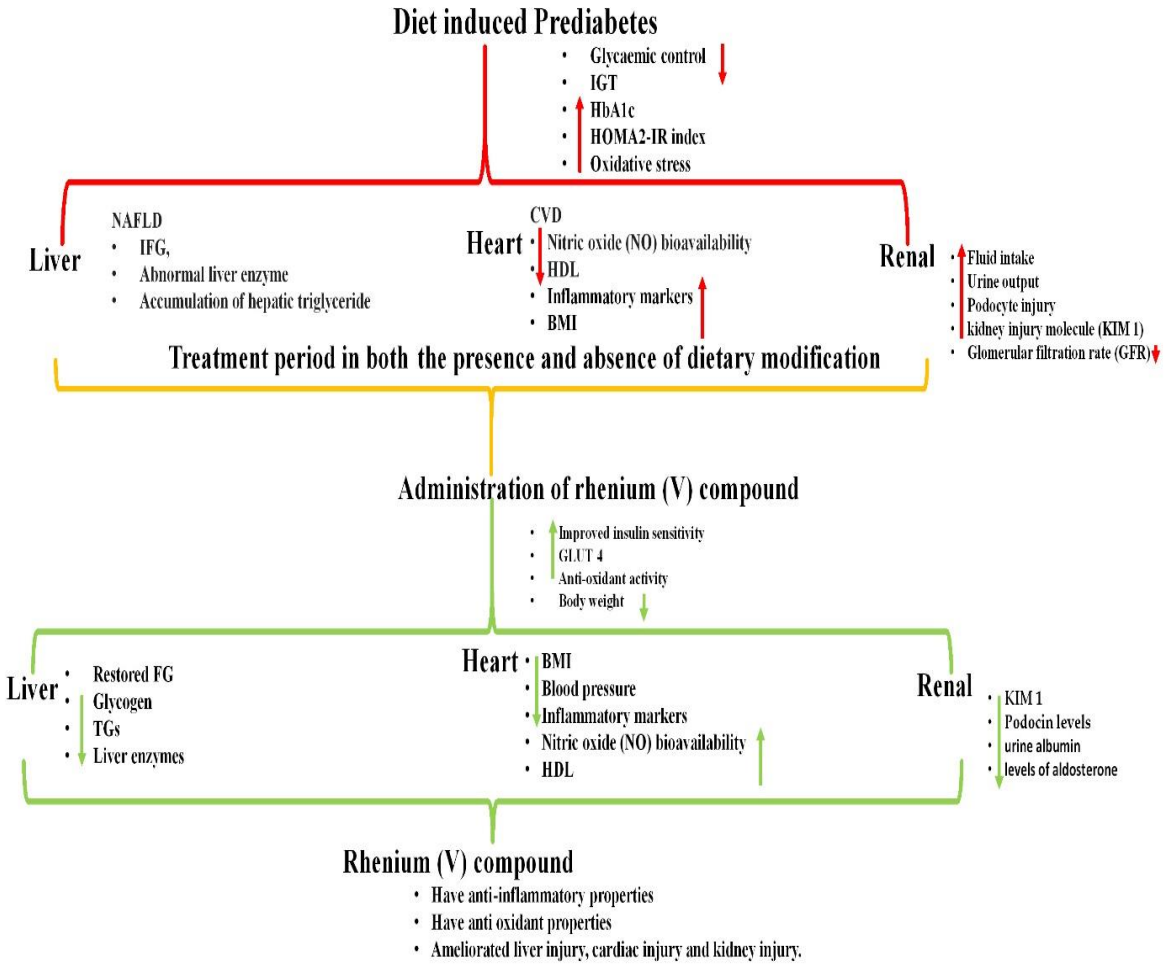

Figure 1: Summary of findings from the study

Conclusion

The results of this study show that the rhenium (V) compound with uracil-derived ligands have ameliorative effects on NAFLD, CVD and renal dysfunction which are metabolic complications associated with prediabetes. These effects were observed in both the presence and absence of dietary intervention. The findings of this study further suggest that the rhenium (V) compound's health benefits stem from its ability to mediate enhanced insulin sensitivity which translates to improved glycaemic control . Potentially, the the rhenium (V) compound with uracil-derived ligands can offer a new patient-friendly alternative in the treatment of prediabetes and thus assist prevent its (prediabetes) progression to type 2 diabetes.

Study limitations and recommendations

Due to limited funding, key markers of glycaemic GLUT2 and SREBP1c were not measured in the current study. GLUT2 expression could have given insights on how the excess glucose was utilised in the hepatocytes and the regulation of SREBP1c. Liver histology was also not done which could have provided an overview of fat accumulation in liver and possible development of steatosis. The study could have further further evaluated the effects of the rhenium (V) compound on hepatic synthetic function and renal excretory function by determining effects on plasma albumin and bilirubin, respectively. Thus future studies should interrogate further on the liver function function including doing assays that help identify mechanisms at play . The current study could also have evaluated the compound's effects on endothelial nitric oxide synthase (eNOS) and endothelin1 (ET1) which are key markers of endothelial health, but this was not done due to limited funding. Furthermore, an in-depth investigation could be done to investigate the effects of rhenium (V) compound on the regulation of the RAAS. Furthermore, histological assays on the heart microarchitecture following treatment were not done neither were the downstream effects of the rhenium (V) compound on kidney function, for example, red blood cell production. Thus, future research should tackle such gaps which the current research did not address.

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Appendices

Appendix 1: AREC Ethics Approval Letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

23 June 2022

Ms Angezwa Siboto (212518628)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Ms Siboto,

Protocol reference number: AREC/00003221/2021 (previous AREC reference number: AREC/039/018M)
Project title: Evaluating the protective effects of rhenium (V) compound with uracil derived ligands in cardiovascular, hepatic and renal function in a high-fat high-carbohydrate diet-induced prediabetes rat model.

Full Approval – Research Application

With regard to your revised application received on 09 June 2022, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

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 22 June 2023.

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

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Dr Andile Khathi; Dr Phikelelani Ngubane
cc BRU Manager: Dr Jaca

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix 2: Manuscript 1 Journal guide: diabetology Journal

Diabetology MDPI Instructions for Authors

Manuscript Submission Overview

Types of Publications

Diabetology has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. Diabetology requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on Supplementary Materials and references to unpublished data).

Manuscripts submitted to Diabetology should neither be published previously nor be under consideration for publication in another journal. The main article types are as follows:

Articles: Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. Authors should not unnecessarily divide their work into several related manuscripts, although short Communications of preliminary, but significant, results will be considered. The quality and impact of the study will be considered during peer review.

Reviews: These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA guidelines.

Case reports: Case reports present detailed information on the symptoms, signs, diagnosis, treatment (including all types of interventions), and outcomes of an individual patient. Case reports usually describe new or uncommon conditions that serve to enhance medical care or highlight diagnostic approaches.

Submission Process

Manuscripts for Diabetology should be submitted online at susy.mdpi.com. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the submission website. Once you have registered, click here to go to the submission form for Diabetology. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors are encouraged to use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office diabetology@mdpi.com. Accepted file formats are:

Microsoft Word: Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, we encourage you to use the Diabetology Microsoft Word template file. Please insert your graphics (schemes, figures, etc.) in the main text after the paragraph of its first citation.

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Supplementary files: May be any format, but it is recommended that you use common, non-proprietary formats where possible (see below for further details).

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Free Format Submission

Diabetology now accepts free format submission:

We do not have strict formatting requirements, but all manuscripts must contain the required sections: Author Information, Abstract, Keywords, Introduction, Materials & Methods, Results, Conclusions, Figures and Tables with Captions, Funding Information, Author Contributions, Conflict of Interest and other Ethics Statements. Check the Journal Instructions for Authors for more details.

Your references may be in any style, provided that you use the consistent formatting throughout. It is essential to include author(s) name(s), journal or book title, article or chapter title (where required), year of publication, volume and issue (where appropriate) and pagination. DOI numbers (Digital Object Identifier) are not mandatory but highly encouraged. The bibliography software package EndNote, Zotero, Mendeley, Reference Manager are recommended.

When your manuscript reaches the revision stage, you will be requested to format the manuscript according to the journal guidelines.

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work. It should explain why the manuscript fits the scope of the journal.

Any prior submissions of the manuscript to MDPI journals must be acknowledged. If this is the case, it is strongly recommended that the previous manuscript ID is provided in the submission system, which will ease your current submission process. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

All cover letters are required to include the statements:

We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.

All authors have approved the manuscript and agree with its submission to (journal name).

Author Biography

Authors are encouraged to add a biography (maximum 150 words) to the submission and post it to SciProfiles. This should be a single paragraph and should contain the following points:

Authors' full names followed by current positions;

Education background including institution information and year of graduation (type and level of degree received);

Work experience;

Current and previous research interests;

Memberships of professional societies and awards received.

Note for Authors Funded by the National Institutes of Health (NIH)

The editors of this journal are able to deposit papers to the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/) on your behalf. If you are funded by NIH, please request this service from our editors after acceptance of your paper.

[Return to top]

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:

A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, TIFF, or SVG. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.

Acronyms/Abbreviations/Initialisms should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.

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 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.

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Front Matter

These sections should appear in all manuscript types

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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 work and 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.

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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.

Funding: 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.

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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.

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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.

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Preparing Figures, Schemes and Tables

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All Figures, Schemes and Tables should have a short explanatory title and caption.

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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.

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For the main text, please ensure that:

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Supplementary Materials, Data Deposit and Software Source Code

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MDPI is committed to supporting open scientific exchange and enabling our authors to achieve best practices in sharing and archiving research data. We encourage all authors of articles published in MDPI journals to share their research data. Individual journal guidelines can be found at the journal 'Instructions for Authors' page. Data sharing policies concern the minimal dataset that supports the central findings of a published study. Generated data should be publicly available and cited in accordance with journal guidelines.

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Where ethical, legal or privacy issues are present, data should not be shared. The authors should make any limitations clear in the Data Availability Statement upon submission. Authors should ensure that data shared are in accordance with consent provided by participants on the use of confidential data.

Data Availability Statements provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study.

Below are suggested Data Availability Statements:

Data available in a publicly accessible repository
The data presented in this study are openly available in [repository name e.g., FigShare] at [doi], reference number [reference number].

Data available in a publicly accessible repository that does not issue DOIs
Publicly available datasets were analyzed in this study. This data can be found here: [link/accession number]

Data available on request due to restrictions eg privacy or ethical
The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [insert reason here]

3rd Party Data
Restrictions apply to the availability of these data. Data was obtained from [third party] and are available [from the authors / at URL] with the permission of [third party].

Data sharing not applicable
No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Data is contained within the article or supplementary material
The data presented in this study are available in [insert article or supplementary material here]

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Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to https://www.mdpi.com/authors/layout#_bookmark83.

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Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

New nucleic acid sequences must be deposited into an acceptable repository such as GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.

New high throughput sequencing (HTS) datasets (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive (SRA).

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New protein sequences obtained by protein sequencing must be submitted to UniProt (submission tool SPIN). Annotated protein structure and its reference sequence must be submitted to RCSB of Protein Data Bank.

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Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "Minimum Information About a Proteomics Experiment". All generated mass spectrometry raw data must be deposited in the appropriate public database such as ProteomeXchange, PRIDE or jPOST. At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/), revised in 2013. According to point 23 of this declaration, an approval from the local institutional review board (IRB) or other appropriate ethics committee must be obtained before

undertaking the research to confirm the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in Section 'Institutional Review Board Statement' of the article.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

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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

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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.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

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NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: https://www.animalethics.org.au/three-rs

Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf

American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use

European Animal Research Association. EU regulations on animal research. Available online: https://www.eara.eu/animal-research-law

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished de novo cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The MLH1+ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

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Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

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Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from ‘Crown Mix’ (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) guidelines which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

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Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include clinicaltrials.gov, the EU Clinical Trials Register and those listed by the World Health Organisation International Clinical Trials Registry Platform.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

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MDPI requires a completed CONSORT 2010 checklist and flow diagram as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (http://www.consort-statement.org) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

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Sex and Gender in Research

We encourage our authors to follow the 'Sex and Gender Equity in Research – SAGER – guidelines' and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full guidelines before submission.

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Appendix 4: Manuscript 2 Journal guide: Diabetes and Vascular Disease Research Journal.

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Group as author:

Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension*. 2002;40(5):679–686.

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Ahrar K, Madoff DC, Gupta S, Wallace MJ, Price RE, Wright KC. Development of a large animal model for lung tumors. *J Vasc Interv Radiol*. 2002;13(9 Pt 1):923–928.

Issue with no volume:

Banit DM, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop*. 2002;(401):230–238.

No volume or issue:

Outreach: bringing HIV-positive individuals into care. *HRSA Careaction*. 2002 Jun:1–6.

Type of article indicated as needed:

Lofwall MR, Strain EC, Brooner RK, Kindbom KA, Bigelow GE. Characteristics of older methadone maintenance (MM) patients [abstract]. *Drug Alcohol Depend*. 2002;66 Suppl 1:S105.

Article published electronically ahead of the print:

Yu WM, Hawley TS, Hawley RG, Qu CK. Immortalization of yolk sac-derived precursor cells. *Blood*. Epub 2002 Jul 5.

Foreign language:

Virchow R. Aetiologie der neoplastischen Geschwulst/Pathogenie der neoplastischen Geschwulste [Etiology and pathology of cancerous tumors]. Die Krankhaften Geschwulste. Berlin: Verlag von August Hirschwald; 1865:57–101. German.

Books and other monographs

Personal author(s):

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. *Medical Microbiology*. 4th ed. St Louis: Mosby; 2002.

Editor(s), compiler(s) as author:

Gilstrap LC 3rd, Cunningham FG, VanDorsten JP, eds. *Operative Obstetrics*. 2nd ed. New York: McGraw-Hill; 2002.

Author(s) and editor(s):

Breedlove GK, Schorfheide AM. *Adolescent Pregnancy*. 2nd ed. Wieczorek RR, ed. White Plains (NY): March of Dimes Education Services; 2001.

Appendix 5: Manuscript 3 Journal guide: International Journal of molecular science

International Journal of molecular science

Instructions for Authors

Shortcuts

- Manuscript Submission Overview
- Manuscript Preparation
- Preparing Figures, Schemes and Tables
- Original Images for Blots and Gels Requirements
- Supplementary Materials, Data Deposit and Software Source Code
- Research and Publication Ethics
- Reviewer Suggestions
- English Corrections
- Preprints and Conference Papers
- Authorship
- Editorial Independence
- Conflict of Interests
- Editorial Procedures and Peer-Review
- Promoting Equity, Diversity and Inclusiveness Within MDPI Journals
- Resource Identification Initiative

Submission Checklist

Please:

1. Read the Aims & Scope to gain an overview and assess if your manuscript is suitable for this journal;
2. Use the Microsoft Word template or LaTeX template to prepare your manuscript;
3. Make sure that issues about publication ethics, research ethics, copyright, authorship, figure formats, data and references format have been appropriately considered;
4. Ensure that all authors have approved the content of the submitted manuscript.
5. Authors are encouraged to add a biography (optional) to the submission and post it to SciProfiles.

Manuscript Submission Overview

Types of Publications

IJMS has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *IJMS* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on **Supplementary Materials** and references to unpublished data).

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A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work. It should explain why the manuscript fits the scope of the journal.

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- We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.
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Authors are encouraged to add a biography (maximum 150 words) to the submission and post it to **SciProfiles**. This should be a single paragraph and should contain the following points:

1. Authors' full names followed by current positions;

2. Education background including institution information and year of graduation (type and level of degree received);
3. Work experience;
4. Current and previous research interests;
5. Memberships of professional societies and awards received.

Note for Authors Funded by the National Institutes of Health (NIH)

This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, **http://nihms.nih.gov/**).

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Manuscript Preparation

General Considerations

- **Important note:**
    - Substances without clear ingredients, such as complex prescriptions, crude extracts, and herbal mixtures, are not considered;
    - Papers that only contain clinical trials/data are not acceptable for *IJMS*.
- **Research manuscripts** should comprise:
    - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords
    - **Research manuscript sections**: Introduction, Results, Discussion, Materials and Methods, Conclusions (optional).
    - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.
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- **Graphical Abstract:**

A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple

superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, TIFF, or SVG. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.

- **Acronyms/Abbreviations/Initialisms** should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.
- **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 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.
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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. Please do not include abbreviated or short forms of the title, such as a running title or head. These will be removed by our Editorial Office.
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- **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.

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- **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.

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- **Funding:** 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.
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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.

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- 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.
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- 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.
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For the main text, please ensure that:

- All experimental samples and controls used for one comparative analysis are run on the same blot/gel.
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- You have checked figures for duplications and ensured the figure legends are clear and accurate. Please include all relevant information in the figure legends and clearly indicate any re-arrangement of lanes.

In order to ensure the integrity and scientific validity of blots (including, but not limited to, Western blots) and the reporting of gel data, original, uncropped and unadjusted images should be uploaded as Supporting Information files at the time of initial submission.

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Supplementary Materials, Data Deposit and Software Source Code

MDPI Research Data Policies

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Where ethical, legal or privacy issues are present, data should not be shared. The authors should make any limitations clear in the Data Availability Statement upon submission. Authors should ensure that data shared are in accordance with consent provided by participants on the use of confidential data.

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  The data presented in this study are openly available in [repository name e.g., FigShare] at [doi], reference number [reference number].
- Data available in a publicly accessible repository that does not issue DOIs
  Publicly available datasets were analyzed in this study. This data can be found here: [link/accession number]
- Data available on request due to restrictions eg privacy or ethical
  The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [insert reason here]
- 3rd Party Data
  Restrictions apply to the availability of these data. Data was obtained from [third party] and are available [from the authors / at URL] with the permission of [third party].
- Data sharing not applicable
  No new data were created or analyzed in this study. Data sharing is not applicable to this article.
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  The data presented in this study are available in [insert article or supplementary material here]

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Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to https://www.mdpi.com/authors/layout#_bookmark83.

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Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

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Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult **databib.org** or **re3data.org**. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal **Data** also accepts submissions of data set papers.

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New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited into an acceptable repository such as **GenBank**, **EMBL**, or **DDBJ**. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the **GEO database** or in the NCBI's **Sequence Read Archive (SRA)**.
- *New microarray data* must be deposited either in the **GEO** or the **ArrayExpress** databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
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Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "**Minimum Information About a Proteomics Experiment**". All generated mass spectrometry raw data must be deposited in the appropriate public database such as **ProteomeXchange**, **PRIDE** or **jPOST**. At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (**https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/**), revised in 2013. According to point 23 of this declaration, an approval from the local institutional review board (IRB) or other appropriate ethics committee must be obtained before undertaking the research to confirm the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in Section 'Institutional Review Board Statement' of the article.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

A written informed consent for publication must be obtained from participating patients. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent for publication from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A **template permission form** is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission. Editors reserve the right to reject any submission that does not meet these requirements.

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If the study reports research involving vulnerable groups, an additional check may be performed. The submitted manuscript will be scrutinized by the editorial office and upon request, documentary evidence (blank consent forms and any related discussion documents from the ethics board) must be supplied. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., explanation regarding why such categorization was needed must be clearly stated in the article.

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 [1]':

- 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.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

MDPI endorses the ARRIVE guidelines (**arriveguidelines.org/**) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found

at https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20Compliance%20Questionnaire.pdf. Editors reserve the right to ask for the checklist and to reject submissions that do not adhere to these guidelines, to reject submissions based on ethical or animal welfare concerns or if the procedure described does not appear to be justified by the value of the work presented.

1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: https://www.animalethics.org.au/three-rs
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf
3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use
4. European Animal Research Association. EU regulations on animal research. Available online: https://www.eara.eu/animal-research-law

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

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Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) **guidelines** which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation **International Clinical Trials Registry Platform**.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 **checklist** and **flow diagram** as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (**http://www.consort-statement.org**) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

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Sex and Gender in Research

We encourage our authors to follow the **'Sex and Gender Equity in Research – SAGER – guidelines'** and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full **guidelines** before submission.

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Potential disputes over borders and territories may have particular relevance for authors in describing their research or in an author or editor correspondence address, and should be respected. Content decisions are an editorial matter and where there is a potential or perceived dispute or complaint, the editorial team will attempt to find a resolution that satisfies parties involved.

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- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- The journal accepts exact translations of previously published work. All submissions of translations must conform with our **policies on transla**

Appendix 6: Certificate of acceptance for the manuscript (ijms-2009699) titled: Rhenium (V) compound with uracil derived ligands ameliorates renal dysfunction by suppressing hyperglycaemia mediated renal oxidative stress and inflammation in diet induced prediabetic rats.

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CERTIFICATE OF ACCEPTANCE

Certificate of acceptance for the manuscript (**ijms-2009699**) titled:

Rhenium (V) compound with uracil derived ligands ameliorates renal dysfunction by suppressing hyperglycaemia mediated renal oxidative stress and inflammation in diet induced prediabetic rats.

Authored by:

Angezwa Siboto; Akinjide Moses Akinnuga; Bongiwe Khumalo; Muhammed Bilaal Ismail; Irvin Noel Booysen; Ntethelelo Hopewell Sibiya; Phikelelani Ngubane; Andile Khathi

has been accepted in *Int. J. Mol. Sci.* (ISSN 1422-0067) on 22 November 2022

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Appendix 7: Abstract of CHS symposium 2022 oral presentation

SCHOOL OF LABORATORY MEDICINE AND MEDICAL SCIENCES RESEARCH DAY

30 NOVEMBER 2022

SENATE CHMABER, WESTVILLE CAMPUS

INSTRUCTIONS:

- Please forward your abstracts electronically to dudhrajhp@ukzn.ac.za by **11 Nov 2022**.
- Participants should indicate their preference of presentation type (oral or poster).

CRITERIA FOR THE ACCEPTANCE OF ABSTRACTS:

- Abstracts are open to all postgraduate and post-doc students, developmental lecturers and credentialing staff in the School of Laboratory Medicine and Medical Sciences.
- Abstracts must adhere to the format indicated on the template provided.
- It is the author's responsibility to submit an abstract that is free of spelling and grammatical errors.
- All submissions must have ethical clearance.
- Abstracts will be reviewed by the scientific committee based on the following:
    (1) Clear background/statement of the problem.
    (2) Appropriateness of the methodology/study design to achieve the objectives.
    (3) Clear presentation/significance of the results
    (4) Relevance of the research findings; are the conclusions supported by the results.
    (5) Novelty of the study.

GUIDELINES:

Use Arial 11-point font throughout.

The abstract must have a title: In bold type and upper case (capitals).

All authors must be listed: Surname followed by initials; do not include titles. (bold and underline the presenter's name).

Author affiliations must be shown: Department, Centre or Unit; Use * and # symbols to match affiliation with author.

Example: Taylor, M.*, Suleman, F..#

*Department of Public Health; # Discipline of Pharmaceutical Sciences

- Abstract Layout: Please fit into the frame. **250 words max**.
  - **Background/Aim(s):** Clearly state the purpose(s) of the study.
  - **Methods:** Clearly state how your study was conducted, sample selection, tools and instruments used
  - **Results:** Present your results in a logical sequence
  - **Discussion/Conclusion:** Emphasize new and important aspects of the study and conclusions that are drawn from them.

PLEASE FILL IN ALL PRESENTER DETAILS

Presenter Information

Surname: Siboto	First Name: Angezwa
Research Theme: Endocrinology	Tel: 0632028630
E-Mail: 212518628@stu.ukzn.ac.za	Date: 02/11/2022

ABSTRACT TEMPLATE

Only abstracts that have strictly adhered to this template will be considered.

250 words max.

TITLE, AUTHOR AND AFFILIATION

Investigating the Protective Effects of a Rhenium (V) Compound with Uracil-Derived Ligands on Liver Damage Associated with Prediabetes in Diet-Induced Prediabetic Rats

Angezwa Siboto [1],* , Akinjide Moses Akinnuga [1] , Muhammed Bilaal Ismail [2] , Irvin Noel Booysen [2],

Ntethelelo Hopewell Sibiya [3], Phikelelani 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

2 School of Chemistry and Physics, College of Agriculture, Engineering Sciences, University of KwaZulu-Natal, Pietermaritzburg 3209, South Africa

3 Pharmacology Division, Rhodes University, Grahamstown 6140, South Africa

***** Correspondence: 212518628@stu.ukzn.ac.za

ABSTRACT

BODY:

Background/Aim(s): Non-alcoholic fatty liver disease (NAFLD) is associated with prediabetes and can be treated by using a combination of metformin and dietary modification. However, people often fail to adhere to dietary modifications and become more dependent on pharmaceutical intervention, and this affects the effectiveness of the drug. In this study, we investigated the effects of rhenium (V) compound with uracil-derived ligands on liver health in diet-induced prediabetic rats in both the presence and absence of dietary modification.

Methods: Prediabetic male Sprague Dawley rats were treated with the rhenium (V) compound for 12 weeks in both the presence and absence of dietary modification while monitoring fasting blood glucose levels. Antioxidant enzyme activity, inflammation markers and liver enzymes were measured together with liver glycogen and plasma triglycerides after sacrificing.

Results: The administration of rhenium (V) compound to prediabetic rats in both the presence and absence of dietary modification resulted in reduced concentrations of fasting blood glucose and triglycerides. There was also reduced liver glycogen, oxidative stress and liver enzymes while increasing antioxidant enzymes.

Discussion/Conclusion: Rhenium (V) compound ameliorated liver injury and prevented hepatotoxicity.

Research Theme: ENDOCRIOLOGY_____

Ethics Number: AREC/00003221/2021 _____

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	Poster Presentation
X	Oral Presentation

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