



**Investigating the effects of gossypetin on glucose homeostasis and selected complications
of diet-induced prediabetes in male Sprague Dawley rats**

By Karishma Naidoo (217000603)

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Supervisor: Prof. Andile Khathi

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PREFACE

Prediabetes is a globally increasing health concern, primarily attributed to the consumption of high-calorie diets and sedentary lifestyles. It is defined as an intermediate metabolic state between normoglycaemia and type 2 diabetes mellitus (T2DM), and is characterised by impaired glucose regulation. Emerging evidence suggests that many of the complications seen in T2DM, including cardiovascular, hepatic and renal dysfunction, begin during the prediabetic state. The current management strategy of prediabetes involves a combination of dietary and pharmacological interventions. However, poor patient compliance with dietary modifications often reduces the efficacy of pharmacological interventions. Hence, there is a growing need to identify effective therapeutic compounds even in the absence of strict dietary control. Gossypetin (GTIN) is a hexahydroxylated flavonoid that has demonstrated potent antioxidant and anti-inflammatory properties. This study investigated the effects of GTIN on glucose homeostasis and selected markers of cardiovascular, liver and kidney dysfunction in a diet-induced prediabetic rat model. The study also examined the efficacy of GTIN treatment with and without dietary intervention. The experimental work presented in this dissertation was conducted at the University of KwaZulu-Natal, Westville Campus, Durban, South Africa, under the supervision of Prof. Andile Khathi.

DECLARATION

I, **Karishma Naidoo** hereby declare that the dissertation entitled:

“Investigating the effects of gossypetin on glucose homeostasis and selected complications of diet-induced prediabetes in male Sprague Dawley 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. Where use of the work of others was made, it is duly acknowledged in the text.

Student: Karishma Naidoo

Signature _



Date: 07/10/2025

Supervisor: Prof. A. Khathi

Signature _



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DEDICATION

This work is dedicated to God, who has guided me throughout this journey. To my parents and sister, for their unwavering support, love and encouragement every step of the way.

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To my supervisor, **Professor Khathi**: It has been a true privilege to work with such an exceptional academic and mentor. Thank you for your unwavering patience, guidance and encouragement throughout this journey, from my Honours year to the completion of this thesis. Your leadership and mentorship have taught me invaluable skills, both professionally and personally. You consistently pushed me to succeed and become a better version of myself. Your words of motivation, belief in my potential and commitment to excellence have been instrumental in shaping my academic path. Thank you, Prof., for being a great leader, mentor and a source of inspiration.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AGE	Advanced glycated end-product
Akt	Protein kinase B
ALT	Alanine transaminase
AMPK	AMP-activated protein kinase
AST	Aspartate transaminase
AUC	Area under the curve
BMI	Body mass index
ChREBP	Carbohydrate-responsive element-binding protein
CRP	C-reactive protein
CVDs	Cardiovascular diseases
eNOS	Endothelial nitric oxide synthase
FBG	Fasting blood glucose
FFA	Free fatty acids
GFR	Glomerular filtration rate
GLUT-4	Glucose transporter type 4
GPx	Glutathione peroxidase
GTIN	Gossypetin
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein
HFHC	High-fat high-carbohydrate
HOMA-IR	Homeostatic model assessment of insulin resistance
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL-6	Interleukin-6
KIM-1	Kidney injury molecule-1
LDL	Low-density lipoprotein
MAP	Mean arterial pressure
MDA	Malondialdehyde
MET	Metformin
NAFLD	Non-alcoholic fatty liver disease
NPD	Non-prediabetic
PD	Prediabetic

RAAS	Renin–angiotensin–aldosterone system
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SREBP-1c	Sterol regulatory element-binding protein 1c
TAG	Triglycerides
TAS	Total antioxidant status
TC	Total cholesterol
TNF- α	Tumour necrosis factor-alpha
VLDL	Very low-density lipoprotein
WC	Waist circumference
ZDF	Zucker diabetic fatty

PUBLICATIONS

Published Manuscripts

Naidoo K, Khathi A. The Potential Role of Gossypetin in the Treatment of Diabetes Mellitus and Its Associated Complications: A Review. *Int J Mol Sci.* 2023 Dec 18;24(24):17609. doi: 10.3390/ijms242417609. PMID: 38139436; PMCID: PMC10743819.

Naidoo K, Khathi A. Effects of Gossypetin on Glucose Homeostasis in Diet-Induced Pre-Diabetic Rats. *Molecules.* 2024 Sep 17;29(18):4410. doi: 10.3390/molecules29184410. PMID: 39339405; PMCID: PMC11434540.

Naidoo, K.; Khathi, A. Investigating the Effects of Gossypetin on Cardiovascular Function in Diet-Induced Pre-Diabetic Male Sprague Dawley Rats. *Int. J. Mol. Sci.* 2024, 25, 12105. <https://doi.org/10.3390/ijms252212105>

Naidoo K, Khathi A. Investigating the Effects of Gossypetin on Liver Health in Diet-Induced Pre-Diabetic Male Sprague Dawley Rats. *Molecules.* 2025 Apr 19;30(8):1834. doi: 10.3390/molecules30081834. PMID: 40333901; PMCID: PMC12029341.

STUDY OUTLINE

The current thesis is presented and submitted in a manuscript format. The data and findings in this research study are prepared and presented in eight chapters in which four are presented as manuscripts in the following manner:

Chapter 1: This chapter provides an introduction, justification, aim and objectives of the study.

Chapter 2: This chapter provides a comprehensive literature review of prediabetes and related complications affecting the cardiovascular system, liver and kidneys. It discusses conventional and alternative treatments in the management of prediabetes and associated complications. The chapter also includes a review paper on the potential role of gossypetin in the treatment of diabetes, which has been published in *the International Journal of Molecular Sciences*. <https://doi.org/10.3390/ijms242417609>.

Chapter 3: This chapter describes the methodology used in the study, including the experimental design, animal model, dietary interventions, treatment protocols and biochemical analysis.

Chapter 4: This chapter presents the first manuscript, reporting the effects of gossypetin on glucose homeostasis in a diet-induced prediabetic rat model. This manuscript has been published in *Molecules*. <https://doi.org/10.3390/molecules29184410>. See Appendix 3.

Chapter 5: The second manuscript is presented in this chapter, which reports the effects of gossypetin on cardiovascular function in diet-induced prediabetic rats. This manuscript is published in *the International Journal of Molecular Sciences*. <https://doi.org/10.3390/ijms252212105>. See Appendix 4.

Chapter 6: The third manuscript is presented in this chapter, which reports the effects of gossypetin on liver function in diet-induced prediabetic rats. This manuscript is published in *Molecules*. <https://doi.org/10.3390/molecules30081834>. See Appendix 5.

Chapter 7: The fourth manuscript is presented in this chapter, which reports the effects of gossypetin on kidney function in diet-induced prediabetic rats. This manuscript is currently under review in *Pharmaceutics*.

Chapter 8: This final chapter provides an overall synthesis of the study, conclusions, limitations and recommendations for future research.

ABSTRACT

Background

Prediabetes is an intermediate glycaemic state between normoglycaemia and chronic hyperglycaemia. This condition has become increasingly prevalent worldwide due to the consumption of high-calorie diets and a sedentary lifestyle. Prediabetes is characterised by abnormal glucose metabolism and is associated with complications such as non-alcoholic fatty liver disease (NAFLD), cardiovascular and renal dysfunctions, similar to that observed in type 2 diabetes mellitus. The current management strategy for prediabetes involves a combination of dietary and pharmacological interventions. However, patient adherence to dietary intervention is often low, leading to reduced pharmacological effectiveness. Therefore, anti-diabetic agents that are effective even without dietary intervention are needed. Gossypetin (GTIN), a hexahydroxylated flavonoid, has shown potent antioxidant and anti-inflammatory properties. This study sought to investigate the effects of GTIN on glucose homeostasis and selected markers of cardiovascular, hepatic and renal dysfunction in a prediabetic rat model, both with and without dietary intervention.

Materials and Methods

Thirty-six male Sprague Dawley rats (150–180 g) were divided into Group 1 (n=6) and Group 2 (n=30). Prediabetes was induced by feeding Group 2 a high-fat, high-carbohydrate (HFHC) diet for 20 weeks, while Group 1 received a normal diet (ND). Prediabetes was confirmed by fasting blood glucose (FBG) and 2-hour oral glucose tolerance tests (OGTT) according to the American Diabetes Association criteria. The prediabetic rats (n = 30) were subdivided into five groups (n = 6 per group): one group received no therapeutic intervention, while the remaining four groups were treated with either GTIN (15 mg/kg) or metformin (MET, 500 mg/kg), with or without dietary intervention, for 12 weeks. Body mass index (BMI), waist circumference (WC), blood pressure, heart rate, caloric intake, fluid intake and urine output were monitored every four weeks during the treatment period. After treatment, animals were sacrificed; blood and tissues (skeletal muscle, heart, liver, kidney) were collected and stored for biochemical analysis. The thesis comprised four studies evaluating the effects of GTIN on glucose homeostasis, cardiovascular function, hepatic function and renal function in a diet-induced prediabetic rat model.

Results

In the first study, animals in the untreated pre-diabetic (PD) control group exhibited significantly higher fasting and postprandial blood glucose levels, as well as elevated plasma insulin concentrations and increased homeostatic model assessment for insulin resistance (HOMA2-IR) index, relative to the non-prediabetic (NPD) group. Similarly, increased caloric intake, body weight and plasma ghrelin levels were observed in the PD control group. Notably, these parameters were significantly reduced in

the PD animals receiving GTIN treatment. Additionally, glycogen levels in the liver and skeletal muscle, which was disturbed in the PD control group, showed significant improvement following GTIN treatment, both with and without dietary intervention.

In the second study, the PD control group exhibited dyslipidaemia characterized by significantly higher plasma triglyceride, total cholesterol, low-density lipoprotein and very low-density lipoprotein levels, along with decreased high-density lipoprotein (HDL) levels compared with the NPD group. This was accompanied by cardiovascular dysfunction, as evidenced by significantly higher mean arterial pressure (MAP), body mass index (BMI), waist circumference (WC) and plasma endothelial nitric oxide synthase (eNOS) levels in the PD control. Additionally, there were increased heart malondialdehyde levels, reduced heart superoxide dismutase and glutathione peroxidase activity as well as increased plasma interleukin-6, tumour necrosis factor alpha and C-reactive protein levels, indicating enhanced oxidative stress and systemic inflammation. Notably, both GTIN-treated groups showed significantly reduced plasma lipid levels and increased HDL, as well as decreases in MAP, BMI, WC and eNOS levels compared with the PD control. Additionally, GTIN significantly decreased heart lipid peroxidation, enhanced antioxidant activity and decreased plasma inflammation markers.

In the third study, the PD group exhibited hepatic steatosis and liver dysfunction evidenced by significantly higher liver triglyceride (TAG) content, liver weights, sterol regulatory element-binding protein-1c (SREBP-1c), malondialdehyde (MDA) levels, and liver injury enzyme levels, along with decreased liver superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, and plasma bilirubin levels in comparison to NPD. Histologically, there was increased lipid droplet accumulation and structural disarray in the PD group. GTIN treatment significantly reduced liver TAGs, liver weights and plasma SREBP-1c levels, as well as improved liver SOD and GPx activity while decreasing liver MDA levels and liver injury enzymes in comparison to the PD control. Notably, GTIN treatment increased plasma bilirubin levels. Liver histology in the GTIN-treated groups revealed decreased lipid droplet accumulation and improved tissue integrity.

In the fourth study, GTIN significantly reduced fluid intake, urine output and kidney weights in both the presence and absence of dietary intervention compared with the PD group, indicating improved renal function. Additionally, GTIN administration improved glomerular filtration rate, and normalized creatinine, albumin, urea and uric acid levels. It also improved electrolyte homeostasis by restoring renal sodium and potassium handling along with reduced plasma aldosterone levels. GTIN attenuated renal oxidative stress as evidenced by lower kidney MDA, interleukin-6, tumour necrosis factor- α and higher total antioxidant status levels. Additionally, GTIN reduced kidney injury markers including plasma KIM-1 and urinary podocin mRNA expression.

Conclusion

This study demonstrated that chronic consumption of high-calorie diets led to the development of prediabetes. This was associated with impairments in glucose homeostasis as well as cardiovascular, liver and kidney functions. However, treatment with GTIN regardless of dietary intervention may ameliorate disturbed glucose homeostasis as well as cardiovascular, liver and renal dysfunction during prediabetes. These findings suggest that GTIN may represent a promising therapeutic alternative for the management of prediabetes, particularly where adherence to dietary intervention is difficult to maintain. This may help delay the progression from PD to T2DM and reduce the burden of T2DM-associated complications.

CHAPTER 1: INTRODUCTION

1.0 Background

Type 2 diabetes mellitus (T2DM) is a multi-etiological chronic metabolic disorder characterised by hyperglycaemia and insulin resistance in peripheral tissues [1]. Chronic consumption of high-calorie diets combined with a sedentary lifestyle has been shown to promote the development of T2DM [2]. The International Diabetes Federation estimates that around 783 million people worldwide will be affected by T2DM by 2045 [1]. The mortality rate associated with T2DM-related complications including cardiovascular, liver and renal diseases has also gradually increased [3]. The current strategy for the management of T2DM involves the combination of dietary and pharmacological intervention [4, 5].

Metformin is the first-line pharmacotherapy prescribed for the treatment of T2DM [6]. It has been shown to reduce intestinal glucose absorption, hepatic gluconeogenesis and increase glucose uptake in peripheral tissues [6]. Studies have also shown that metformin may exert cardioprotective and hepatoprotective effects beyond its glucose-lowering properties [7, 8]. Dietary modification has been shown to promote weight loss, improve glycaemic control and dyslipidaemia [9]. Notably, studies have shown that many of the complications associated with T2DM, such as cardiovascular diseases, non-alcoholic fatty liver disease (NAFLD) and nephropathy begin during prediabetes [10, 11].

Prediabetes is an intermediate hyperglycaemic state associated with impaired fasting glucose levels and moderate glucose intolerance [12]. The global prevalence of prediabetes in 2017 was 352.1 million which is expected to increase by 8.3% by 2045 [13]. Studies have shown that prediabetes induces oxidative stress and chronic inflammation which contribute to cardiovascular dysfunction, including increased blood pressure, endothelial injury and atherosclerosis [14, 15]. It also leads to liver complications such as NAFLD due to insulin resistance and triglyceride accumulation [16]. Additionally, prediabetes impairs kidney function causing glomerular damage, albuminuria and nephropathy [17]. Studies have shown that prediabetes can be reversed through a combination of dietary and pharmacological intervention [18, 19]. However, there are reports of low patient compliance, as most patients merely use pharmacological intervention without any change in diet [20]. Hence, the efficacy of pharmacological interventions is reduced under these poor compliance situations [20]. Therefore, there is a need to identify compounds to manage prediabetes that are effective in both the presence and absence of dietary intervention.

Plant-derived products have been increasingly recognised for their effectiveness in the treatment of T2DM [21]. Gossypetin (GTIN) is a flavonoid found in various medicinal plants including *Hibiscus* species [22]. Recent studies have shown that GTIN exhibits antioxidant, anti-inflammatory, anti-atherosclerotic, cytoprotective and antimutagenic effects [22, 23], which are bioactivities indispensable in managing diabetes complications. GTIN possesses high structural similarity with

quercetin which is well-known to ameliorate diabetes and its associated complications due to its antioxidant nature and anti-glycation ability [24]. However, the effects of GTIN on glucose homeostasis as well as its effects on cardiovascular, liver and renal complications in the prediabetic state have not been investigated yet.

Animal models have been widely used as a representation of the clinical manifestations of prediabetes and the related complications [25]. Various diet-induced animal models of prediabetes have been established by feeding either a high-fat diet, high-sucrose diet or a combination of both [26]. In our laboratory, we have developed a high-fat high-carbohydrate (HFHC) diet-induced prediabetic rat model to mimic the human condition of prediabetes [27]. Accordingly, this study sought to investigate the effects of GTIN on glucose homeostasis and selected complications of prediabetes using a HFHC-diet induced prediabetic animal model.

2.0 Aim of the study

The aim of the study is to determine the effects of gossypetin (GTIN) on glucose homeostasis as well as its effects on cardiovascular, liver and renal function in diet-induced prediabetic male Sprague Dawley rats.

3.0 Research questions

3.1 What are the effects of GTIN on markers associated with glucose homeostasis in diet-induced prediabetic male Sprague Dawley rats?

3.2 What are the effects of GTIN on markers associated with cardiovascular function in diet-induced prediabetic male Sprague Dawley rats?

3.3 What are the effects of GTIN on markers associated with liver function in diet-induced prediabetic male Sprague Dawley rats?

3.4 What are the effects of GTIN on markers associated with renal function in diet-induced prediabetic male Sprague Dawley rats?

4.0 Aims and Objectives

The objective for each of the respective research questions; 3.1, 3.2, 3.3 and 3.4, are stated below. Research question 3.1 is addressed in Chapter 4 of the dissertation, while research questions 3.2, 3.3 and 3.4 are addressed in Chapter 5, Chapter 6 and Chapter 7, respectively.

Chapter 4 Aim

This chapter seeks to investigate the effects of GTIN on glucose homeostasis in diet-induced prediabetic male Sprague Dawley rats in the presence and absence of dietary intervention. Chapter 4 comprises an original research manuscript and the objectives for the manuscript are described below:

Chapter 4 Objectives

- To determine the effects of GTIN on glucose tolerance by assessing the oral glucose tolerance response (OGT) and calculating the area under the curve (AUC) in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on glycaemic control and insulin resistance by measuring fasting blood glucose and plasma insulin levels as well as calculating the homeostatic model for insulin resistance (HOMA-IR2) index in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on long-term glycaemic control by measuring glycated haemoglobin (HbA1c) levels in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on energy intake and weight regulation by measuring caloric intake and body weight in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on appetite regulation by measuring plasma ghrelin levels in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on glucose storage by measuring liver and skeletal muscle glycogen levels in the presence and absence of dietary intervention in diet-induced prediabetes

Chapter 5 Aim

This chapter seeks to investigate the effects of GTIN on cardiovascular function in diet-induced prediabetic male Sprague Dawley rats in the presence and absence of dietary intervention. Chapter 5 comprises an original research manuscript and the objectives for the manuscript are described below:

Chapter 5 Objectives

- To determine the effects of GTIN on lipid profile by measuring plasma triglyceride (TG), total cholesterol (TC), high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) levels in the presence and absence of dietary intervention in diet-induced prediabetes

- To determine the effects of GTIN on blood pressure by measuring the mean arterial blood pressure (MAP) in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on adiposity by assessing the body mass index (BMI) and waist circumference (WC) in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on endothelial function by measuring endothelial nitric oxide (eNOS) levels in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on heart lipid peroxidation, antioxidant and inflammatory markers by measuring heart malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity and glutathione peroxidase (Gpx) activity as well as plasma interleukin (IL)-6, tumour necrosis factor (TNF)- α and C-reactive protein (CRP) levels in the presence and absence of dietary intervention in diet-induced prediabetes

Chapter 6 Aim

This chapter seeks to investigate the effects of GTIN on liver function in diet-induced prediabetic male Sprague Dawley rats in the presence and absence of dietary intervention. Chapter 6 comprises an original research manuscript and the objectives for the manuscript are described below:

Chapter 6 Objectives

- To determine the effects of GTIN on the extent of lipid accumulation and liver enlargement by measuring liver TAG levels and liver weight in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on lipogenesis by measuring plasma sterol regulatory binding element protein (SREBP)-1c levels in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on liver oxidative status by measuring liver MDA levels, SOD activity and Gpx activity in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on liver injury by measuring plasma aspartate transaminase (AST) and alanine transaminase (ALT) levels in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on liver function and antioxidant status by measuring plasma bilirubin in the presence and absence of dietary intervention in diet-induced prediabetes

- To determine the effects of GTIN on liver histology by assessing lipid droplet accumulation, sinusoid and hepatocyte arrangement in the presence and absence of dietary intervention in diet-induced prediabetes

Chapter 7 Aim

This chapter seeks to investigate the effects of GTIN on renal function in diet-induced prediabetic male Sprague Dawley rats in the presence and absence of dietary intervention. Chapter 7 comprises an original research manuscript and the objectives for the manuscript are described below:

Chapter 7 Objectives

- To determine the effects of GTIN on renal function markers by measuring fluid intake, urine output and calculating the glomerular filtration rate (GFR) in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on renal function markers by measuring plasma and urine creatinine, urea, total protein, albumin, uric acid and electrolyte (Na^+ and K^+) levels in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on kidney injury by measuring plasma kidney injury molecule (KIM)-1 levels and mRNA expression of urinary podocin in the presence and absence of dietary intervention in diet-induced prediabetes
- To determine the effects of GTIN on kidney oxidative status by measuring kidney MDA levels, SOD activity and Gpx activity in the presence and absence of dietary intervention in diet-induced prediabetes

5.0 Hypothesis

The administration of GTIN will improve the biomarkers associated with glucose homeostasis as well as cardiovascular, liver and renal function in diet-induced prediabetic male Sprague Dawley rats.

6.0 Null hypothesis

The administration of GTIN will not improve the biomarkers associated with glucose homeostasis as well as cardiovascular, liver and renal function in diet-induced prediabetic male Sprague Dawley rats.

7.0 References

1. Fisher-Hoch, S. P.; Vatcheva, K. P.; Rahbar, M. H.; McCormick, J. B., Undiagnosed diabetes and pre-diabetes in health disparities. PloS one 2015, 10, (7), e0133135.
2. Bennasar-Veny, M.; Fresneda, S.; López-González, A.; Busquets-Cortés, C.; Aguiló, A.; Yañez, A. M., Lifestyle and progression to type 2 diabetes in a cohort of workers with prediabetes. Nutrients

2020, 12, (5), 1538.

3. Roglic, G., WHO Global report on diabetes: A summary. *International Journal of Noncommunicable Diseases* 2016, 1, (1), 3–8.
4. Borse, S. P.; Chhipa, A. S.; Sharma, V.; Singh, D. P.; Nivsarkar, M., Management of type 2 diabetes: current strategies, unfocussed aspects, challenges, and alternatives. *Medical Principles and Practice* 2021, 30, (2), 109–121.
5. Echouffo-Tcheugui, J. B.; Perreault, L.; Ji, L.; Dagogo-Jack, S., Diagnosis and management of prediabetes: a review. *Jama* 2023, 329, (14), 1206–1216.
6. Shaw, J. E., Prediabetes: lifestyle, pharmacotherapy or regulation? *Therapeutic Advances in Endocrinology and Metabolism* 2019, 10, 2042018819863020.
7. Dutta, S.; Shah, R. B.; Singhal, S.; Dutta, S. B.; Bansal, S.; Sinha, S.; Haque, M., Metformin: a review of potential mechanism and therapeutic utility beyond diabetes. *Drug design, development and therapy* 2023, 1907–1932.
8. Cicero, A. F.; Tartagni, E.; Ertek, S., Metformin and its clinical use: new insights for an old drug in clinical practice. *Archives of medical science: AMS* 2012, 8, (5), 907.
9. Garcia-Molina, L.; Lewis-Mikhael, A.-M.; Riquelme-Gallego, B.; Cano-Ibanez, N.; Oliveras-Lopez, M.-J.; Bueno-Cavanillas, A., Improving type 2 diabetes mellitus glycaemic control through lifestyle modification implementing diet intervention: a systematic review and meta-analysis. *European journal of nutrition* 2020, 59, (4), 1313–1328.
10. Khetan, A. K.; Rajagopalan, S., Prediabetes. *Canadian Journal of Cardiology* 2018, 34, (5), 615–623.
11. Mabuza, L. P.; Gamede, M. W.; Maikoo, S.; Booysen, I. N.; Nguban, P. S.; Khathi, A., Hepatoprotective effects of a ruthenium (II) Schiff base complex in rats with diet-induced prediabetes. *Current Therapeutic Research* 2019, 91, 66–72.
12. Bansal, N., Prediabetes diagnosis and treatment: a review. *World journal of diabetes* 2015, 6, (2), 296.
13. Kabadi, U. M., Major pathophysiology in prediabetes and type 2 diabetes: decreased insulin in lean and insulin resistance in obese. *Journal of the Endocrine Society* 2017, 1, (6), 742–750.
14. Lamprou, S.; Koletsos, N.; Mintziori, G.; Anyfanti, P.; Trakatelli, C.; Kotsis, V.; Gkaliagkousi, E.; Triantafyllou, A., Microvascular and endothelial dysfunction in prediabetes. *Life* 2023, 13, (3), 644.
15. Gumede, N.; Ngubane, P.; Khathi, A., Assessing the risk factors for myocardial infarction in diet-induced prediabetes: myocardial tissue changes. *BMC cardiovascular disorders* 2022, 22, (1), 350.
16. Vesa, C. M.; Behl, T.; Nemeth, S.; Bratu, O. G.; Diaconu, C. C.; Moleriu, R. D.; Negrut, N.; Zaha, D. C.; Bustea, C.; Radu, F. I., Prediction of NAFLD occurrence in prediabetes patients. *Experimental*

and Therapeutic Medicine 2020, 20, (6), 190.

17. Shilpasree, A. S.; Patil, V. S.; Revanasiddappa, M.; Patil, V. P.; Ireshnavar, D., Renal dysfunction in prediabetes: confirmed by glomerular hyperfiltration and albuminuria. *Journal of Laboratory Physicians* 2021, 13, (03), 257–262.

18. Khan, R. M. M.; Chua, Z. J. Y.; Tan, J. C.; Yang, Y.; Liao, Z.; Zhao, Y., From pre-diabetes to diabetes: diagnosis, treatments and translational research. *Medicina* 2019, 55, (9), 546.

19. Galaviz, K. I.; Suvada, K.; Merchant, R.; Dharanendra, S.; Haw, J. S.; Narayan, K. V.; Ali, M. K., Interventions for reversing prediabetes: a systematic review and meta-analysis. *American Journal of Preventive Medicine* 2022, 62, (4), 614–625.

20. Gillies, C. L.; Abrams, K. R.; Lambert, P. C.; Cooper, N. J.; Sutton, A. J.; Hsu, R. T.; Khunti, K., Pharmacological and lifestyle interventions to prevent or delay type 2 diabetes in people with impaired glucose tolerance: systematic review and meta-analysis. *Bmj* 2007, 334, (7588), 299.

21. Panche, A. N.; Diwan, A. D.; Chandra, S. R., Flavonoids: an overview. *J Nutr Sci* 2016, 5, e47.

22. Khan, A.; Manna, K.; Bose, C.; Sinha, M.; Das, D. K.; Kesh, S. B.; Chakrabarty, A.; Banerji, A.; Dey, S., Gossypetin, a naturally occurring hexahydroxy flavone, ameliorates gamma radiation-mediated DNA damage. *International Journal of Radiation Biology* 2013, 89, (11), 965–975.

23. Patel, K.; Patel, D. K., Therapeutic benefit and pharmacological activities of Gossypetin: Biological importance in the medicine through scientific research data analysis. *Metabolism-Clinical and Experimental* 2021, 116.

24. Lin, H.-H.; Hsieh, M.-C.; Wang, C.-P.; Yu, P.-R.; Lee, M.-S.; Chen, J.-H., Anti-atherosclerotic effect of gossypetin on abnormal vascular smooth muscle cell proliferation and migration. *Antioxidants* 2021, 10, (9), 1357.

25. Pandey, S.; Dvorakova, M. C., Future perspective of diabetic animal models. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)* 2020, 20, (1), 25–38.

26. Shakya, A.; Chaudary, S. K.; Garabadu, D.; Bhat, H. R.; Kakoti, B. B.; Ghosh, S. K., A comprehensive review on preclinical diabetic models. *Current diabetes reviews* 2020, 16, (2), 104–116.

27. Luvuno, M.; Mabandla, M.; Khathi, A., Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. *Ponte Int. Sci. Res. J* 2018, 74.

CHAPTER 2: LITERATURE REVIEW

1.0 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder that is increasing in prevalence globally [1]. It is characterised by chronic hyperglycaemia and insulin resistance due to disrupted insulin signalling (see Figure 1) [1]. Under normal physiological conditions, insulin binds to the insulin receptor which triggers autophosphorylation on tyrosine residues, thereby activating the insulin receptor substrates-1 and 2 (IRS-1/2) [2]. This interaction activates phosphoinositide 3-kinase (PI3K), which then activates protein kinase B (Akt) [3]. Protein kinase B promotes glycogen synthesis, inhibits gluconeogenesis and facilitates glucose transporter-4 (GLUT-4) translocation to the cell membrane for glucose uptake [2]. In T2DM, chronic inflammation, elevated free fatty acids and oxidative stress impair the insulin signalling pathway by phosphorylating the serine residues on the insulin receptor [3]. The phosphorylation of serine residues interferes with PI3K-Akt activation [4]. Consequently, glycogen synthesis and glucose uptake in muscle and adipose tissue decrease, contributing to hyperglycaemia [2].

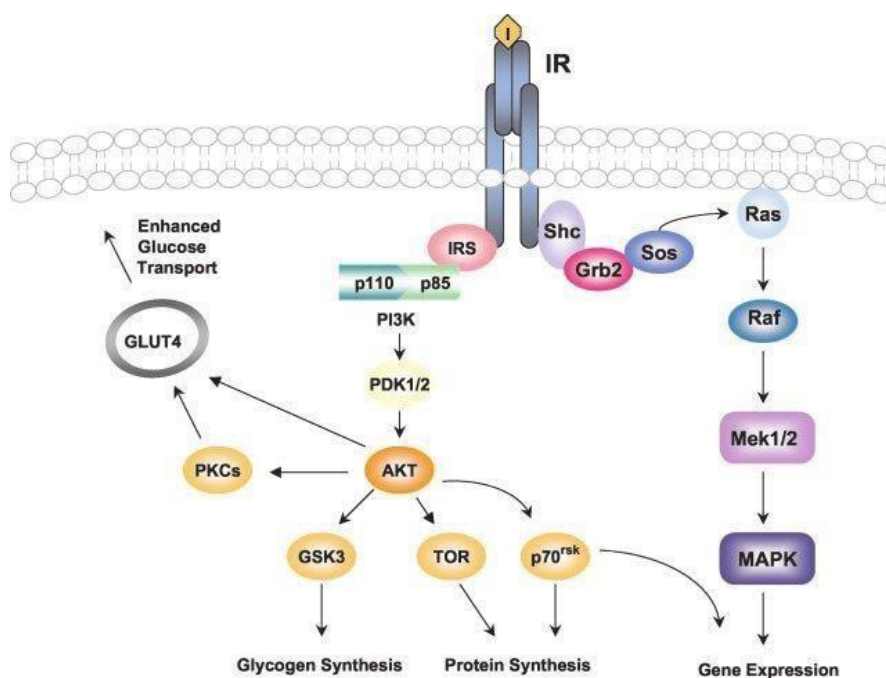


Figure 1: Schematic representation of the insulin signaling pathway and GLUT-4 translocation to the cell membrane [1].

T2DM is diagnosed when fasting blood glucose (FBG) levels exceed 7.0 mmol/L or when postprandial glucose levels are greater than 11.1 mmol/L after a 2-hour oral glucose tolerance test (OGTT) [4]. A glycated haemoglobin (HbA1c) level of 6.5% or greater indicates T2DM [4]. Studies have shown that unhealthy diets and sedentary lifestyles are major risk factors for T2DM [5, 6]. It has been demonstrated that lack of exercise reduces insulin sensitivity and disrupts glucose homeostasis

[6]. Moreover, diets rich in refined sugars and saturated fats were linked with the development of obesity and insulin resistance [7, 8]. According to the International Diabetes Federation (IDF), approximately 536.6 million adults worldwide were diagnosed with T2DM in 2021 [9]. This number is projected to increase to an estimated 783.2 million by 2045 [9]. Among individuals with T2DM, cardiovascular diseases, non-alcoholic fatty liver disease (NAFLD), nephropathy and peripheral neuropathy are notably prevalent [10-12]. It has been estimated that around 50% of T2DM-related mortalities are associated with cardiovascular diseases [13]. Studies suggest that individuals with diabetic nephropathy have a mortality rate that is 2-3 times higher than that of healthy individuals [14, 15]. A study has shown that diabetic peripheral neuropathy is present in up to 35% of individuals at the time of T2DM diagnosis [16].

Notably, studies have shown that many of the complications associated with T2DM, such as cardiovascular diseases, NAFLD and nephropathy, begin during prediabetes [17, 18].

2.0 Prediabetes

Prediabetes is an asymptomatic condition that often remains undetected and precedes the onset of T2DM [19]. It is characterised by elevated blood glucose levels that exceed the normal physiological range but remain below the diagnostic threshold for diabetes [20]. Prediabetes is associated with moderate glucose intolerance, impaired fasting glucose and moderate insulin resistance [21]. In 2017, an estimated 352.1 million individuals worldwide were affected by T2DM [22]. This prevalence is projected to increase to 587 million adults (8.3%) by 2045 [22]. The prevalence of prediabetes in men and women does not differ significantly [23]. Regionally, Africa is projected to experience a rise in prediabetes prevalence by 9.7% due to increasing urbanisation and adoption of westernised lifestyles (see Figure 2) [23]. In South Africa, recent studies have reported prediabetes prevalence rates ranging from 15.6%–70.1% [24, 25]. Notably, a retrospective study conducted in the Durban metropolitan area reported a prediabetes prevalence of 68% among adults aged 25–45 years [26].

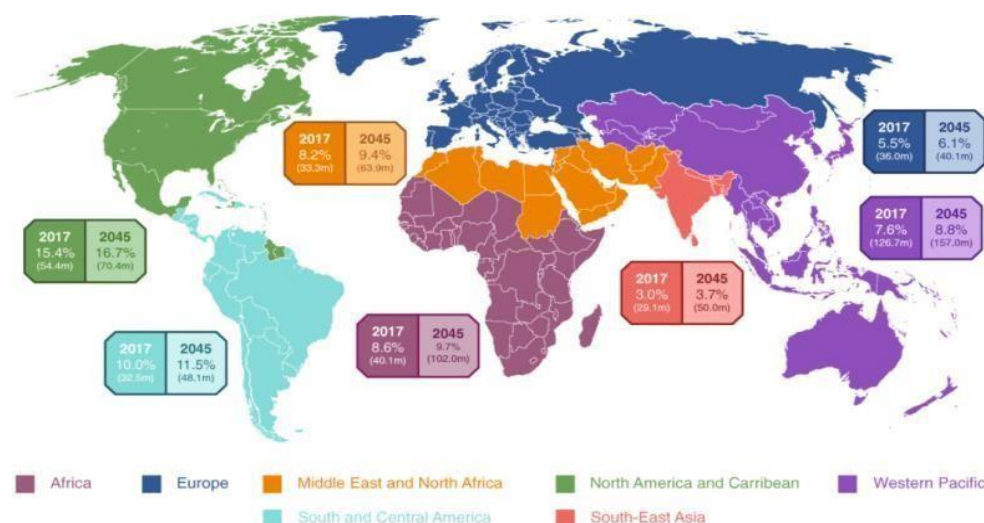


Figure 2: The global estimates of impaired glucose tolerance (IGT) by the International Diabetes

Federation. Numbers in parentheses represent the estimated number of individuals affected by IGT in each region [26].

Several epidemiological studies have demonstrated an association between ethnicity and increased risk of prediabetes [23, 27]. There is a higher prevalence of prediabetes among African Americans, Native Americans, South Asians and Hispanics compared to Caucasians [23]. The factors influencing prediabetes prevalence rates include life expectancy, socioeconomic status, income, access to healthcare and education levels [23]. Consumption of high-calorie diets and lack of exercise have been linked to increased prediabetes prevalence rates [28]. Physical inactivity reduces energy expenditure and impairs glucose metabolism, promoting insulin resistance [29]. Additionally, diets high in refined carbohydrates and saturated fats promote weight gain, insulin resistance and inflammation leading to prediabetes [30, 31].

2.1 Diagnosis of prediabetes

International health organisations utilise various criteria to diagnose prediabetes [20]. The World Health Organization (WHO) defines prediabetes as a state of intermediate hyperglycaemia with impaired fasting glucose (IFG) of 6.1-6.9 mmol/L or impaired glucose tolerance (IGT) of 7.8-11.0 mmol/L [32]. Additionally, the American Diabetes Association (ADA) uses the same threshold for IGT [20]. However, it adopts a lower cut-off value for IFG and includes HbA1c levels between 5.7%–6.4% as an additional diagnostic criterion for prediabetes [20].

Despite the widespread use of these criteria, several studies have reported a poor correlation between HbA1c with IFG and IGT [33, 34]. The use of IFG and IGT as the sole indicators of prediabetes has been challenged [35]. These glycaemic thresholds may not fully reflect the complex pathophysiological mechanism of prediabetes and the probability of developing diabetes in the future [32]. Recently, HbA1c has become more commonly employed in diagnosing prediabetes [32]. Elevated HbA1c levels in prediabetes suggest less efficient blood glucose regulation [33]. Glycated haemoglobin remains in circulation for the lifespan of red blood cells, making it a reliable marker of long-term blood glucose control [36].

The HbA1c test is a more convenient screening test as it does not require fasting compared with the fasting blood glucose (FBG) test and the OGTT [20]. It is unclear which diagnostic criteria are best for identifying individuals with prediabetes [20]. However, the lower threshold for IFG in the ADA guidelines compared with the WHO guidelines improves the parity between the number of people identified by IFG and IGT [32]. According to the ADA criteria, an abnormal finding in FBG, OGTT or HbA1c is sufficient to confirm the diagnosis of prediabetes [33]. Moreover, the ADA recommendations are regularly updated with the most recent scientific data and may be considered more globally accepted than other guidelines [32].

2.2 Pathophysiology of prediabetes

The pathophysiological mechanisms of prediabetes involve a complex interplay between impaired insulin action and dysregulated glucose metabolism [37]. In the first stage, moderate insulin resistance develops in peripheral tissues, such as skeletal muscle and adipose tissue [38]. The body responds by stimulating pancreatic β -cells to increase insulin secretion, resulting in compensatory hyperinsulinaemia [39]. This stage is also characterised by β -cell mass expansion [40]. Despite decreased insulin sensitivity, blood glucose levels remain within the normal range during this compensatory phase [39]. The second stage is the stable adaptation period during which β -cells no longer fully compensate for increased insulin resistance [41]. During this phase, fasting and post-load glucose levels begin to rise [41]. These changes are partly driven by glucotoxicity in which elevated glucose concentrations impair β -cell function by promoting oxidative stress and inflammation [42]. The third stage is the unstable early decompensation period in which β -cells cannot compensate for insulin resistance and consequently glucose concentrations start to increase rapidly [41]. It is during this phase that prediabetes typically advances to clinically diagnosable T2DM [42].

The rate of lipolysis in adipocytes of individuals with IGT is significantly higher than that observed with normal glucose tolerance (NGT) [43]. Furthermore, individuals with IGT show resistance to the anti-lipolytic action of insulin, as evidenced by increased free fatty acid (FFA) levels [44]. Increased FFAs contribute to skeletal muscle and adipocyte insulin resistance in prediabetes [42]. Individuals with IGT exhibit a pronounced increase in skeletal muscle insulin resistance with a mild increase in hepatic insulin resistance [45]. In contrast, individuals with IFG exhibit pronounced hepatic insulin resistance with near-normal skeletal muscle insulin sensitivity [46].

2.3 Glucose homeostasis in prediabetes

In the prediabetic state, glucose homeostasis is dysregulated due to moderate insulin resistance and impaired glucose metabolism [47, 48]. Moderate insulin resistance in skeletal muscle reduces glucose uptake due to impaired translocation of GLUT-4 [49]. Consequently, pancreatic β -cells increase insulin secretion as a compensatory mechanism, leading to hyperinsulinaemia [49]. Several studies assess insulin resistance using indices such as the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) [50, 51]. However, HOMA-IR primarily reflects hepatic insulin resistance and is less accurate than the hyperinsulinaemic-euglycaemic clamp, which is considered the gold standard for measuring whole-body insulin sensitivity [52, 53]. Furthermore, ghrelin is released by the oxyntic glands of the stomach in response to reduced glucose uptake in peripheral tissues [54]. Studies have shown elevated plasma ghrelin levels in prediabetic patients [55, 56]. However, other studies have shown decreased or unchanged ghrelin concentrations in obese individuals [57, 58]. These discrepancies may result from differences in body composition, metabolic status and study population [59, 60]. Ghrelin acts centrally on the hypothalamus by activating orexigenic neurons expressing

neuropeptide Y (NPY) and agouti-related peptide (AgRP), thereby promoting hyperphagia [54]. This increase in caloric intake contributes to a positive energy balance, weight gain and further exacerbation of insulin resistance [56].

Glycogen is the main form of glucose storage in the liver and skeletal muscle [61]. It is a readily available source of glucose for energy production [62]. Under normal physiological conditions, elevated plasma glucose stimulates insulin release which activates glycogen synthase [62]. Activated glycogen synthase promotes glycogenesis in the liver and skeletal muscle [62]. Furthermore, impaired insulin signalling in skeletal muscle during prediabetes leads to decreased glucose uptake and glycogen synthesis [55]. The excess plasma glucose is shunted to the liver since it is not reliant on insulin for glucose uptake [63]. The accumulation of glucose in the liver contributes to increased hepatic glycogen accumulation and de novo lipogenesis [63]. Additionally, studies have demonstrated elevated fasting and postprandial plasma glucose concentrations in individuals with prediabetes [64, 65]. HbA1c is a non-enzymatic glycation product of haemoglobin that circulates in the body for approximately 2–3 months [36]. In prediabetic individuals, elevated plasma glucose levels enhance the glycation of haemoglobin, resulting in elevated HbA1c levels [47]. Although FBG and HbA1c are widely used to assess glycaemic control, both have methodological limitations [66]. Fasting plasma glucose reflects glycaemia at a single time point and may fail to identify impaired glucose tolerance, whereas HbA1c may be influenced by conditions affecting erythrocyte lifespan, such as anaemia and chronic kidney disease [67]. Consequently, relying on a single biomarker may lead to the misclassification of some individuals with prediabetes [68, 69]. Furthermore, glucose homeostasis in prediabetes has been widely studied [70, 71]. However, most studies have examined individual metabolic markers rather than the interactions between glucose metabolism, glycogen storage and appetite regulation [72, 73]. Therefore, studies that investigate these pathways together are needed to improve understanding of prediabetes development and identify early therapeutic targets [74].

2.4 Prediabetes-associated complications

Prediabetes is a risk factor for microvascular and macrovascular complications associated with T2DM [75]. Pre-diabetic individuals have been reported to exhibit early stages of cardiovascular diseases, liver dysfunction and nephropathy, which are less severe than those seen in T2DM [41, 76]. It is characterised by damage to peripheral nerves, particularly small unmyelinated sensory fibres, resulting in symptoms such as pain, burning sensations, numbness and impaired temperature sensation [77]. Previous studies have shown that individuals with prediabetes have a higher prevalence of peripheral neuropathy than normoglycaemic individuals, with small-fibre neuropathy representing the earliest manifestation [78, 79]. The development of prediabetic neuropathy is driven by chronic hyperglycaemia, insulin resistance and dyslipidaemia, which promote oxidative stress, endothelial dysfunction and inflammation [80]. These processes induce mitochondrial dysfunction, increase reactive oxygen species production and damage Schwann cells and peripheral neurons, ultimately

impairing nerve function [81]. The early identification and management of prediabetes are therefore important for preventing the development of diabetes-associated complications [82]. The following section describes the effects of prediabetes on the functioning of the cardiovascular, hepatic and renal systems, which are the primary focus of the study.

2.4.1 Effects of prediabetes on cardiovascular function

Previous studies have demonstrated that prediabetes is associated with an increased risk of cardiovascular diseases (CVDs) [83, 84]. The pathophysiological features of cardiovascular dysfunction in prediabetes include vascular insulin resistance, oxidative stress and chronic inflammation [83]. Although these mechanisms are consistently reported, most studies are cross-sectional and therefore cannot establish whether these abnormalities precede cardiovascular dysfunction or develop as a consequence of prediabetes [37, 85]. Studies have shown that insulin resistance contributes to endothelial dysfunction in prediabetes [84]. In insulin-resistant states, insulin-induced nitric oxide (NO)-dependent vasodilation is impaired due to disrupted phosphorylation cascades from insulin receptors to Akt [84]. As a result, endothelial nitric oxide synthase (eNOS) activation is impaired, reducing NO production and leading to increased blood pressure and heart rate [86]. Several studies have reported impaired endothelial function in prediabetes [87, 88]. However, the severity of endothelial dysfunction varies across studies [87, 89]. These differences may reflect variation in study populations, diagnostic criteria for prediabetes and methods used to assess endothelial function [87, 89].

Elevated levels of the lipid peroxidation marker, malondialdehyde (MDA), have been observed in heart tissue during prediabetes [90, 91]. Furthermore, studies have shown reduced heart antioxidant activity including superoxide dismutase (SOD) and glutathione peroxidase (GPx) in prediabetes [37, 92]. Decreased antioxidant enzyme activity contributes to myocardial oxidative damage [92, 93]. Several studies have shown the presence of oxidative stress in heart tissue during prediabetes [94, 95]. However, majority of these studies were derived from experimental animal models [94, 96]. Consequently, the extent to which these findings translate to human prediabetes remains uncertain [82]. In prediabetes, glycated haemoglobin promotes the formation of advanced glycation end-products (AGEs) [33]. AGEs stimulate monocytes to release cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α), which mediate inflammatory reactions associated with cardiovascular diseases [86]. Additionally, C-reactive proteins (CRP) which are sensitive markers for systemic inflammation have been shown to be significantly elevated during prediabetes [93]. However, some clinical studies have reported that the association between inflammatory markers and prediabetes is attenuated after adjustment for adiposity [92, 97]. This suggests that excess adiposity may be an important contributor to inflammation independent of impaired glucose regulation [98].

Elevated FFAs in prediabetes contribute to endothelial dysfunction by impairing insulin signalling

pathways [83]. Elevated plasma FFAs reduce tyrosine phosphorylation of IRS-1/2 and inhibit the PI3K/Akt pathway, leading to decreased glucose uptake and eNOS phosphorylation [86]. Impaired eNOS activity reduces NO bioavailability, which promotes endothelial injury and vascular stiffness [33]. FFAs also activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase via protein kinase C (PKC), which promotes reactive oxygen species (ROS) production [84]. The accumulation of ROS further exacerbates oxidative stress and inflammation by activating nuclear factor kappa B (NF- κ B) [37].

Studies have shown that prediabetes is associated with dyslipidemia as evidenced by elevated triglycerides (TAG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) alongside reduced high-density lipoprotein cholesterol (HDL-C) levels [99, 100]. Dyslipidaemia contributes to atherosclerosis and endothelial dysfunction by promoting lipid accumulation in arterial walls [37]. Studies have also shown that adiposity is a major contributor to endothelial dysfunction in prediabetes [101, 102]. Increased body mass index (BMI) and waist circumference (WC) were associated with elevated heart inflammatory markers and oxidative stress [103, 104].

2.4.2 Effects of prediabetes on liver function

Non-alcoholic fatty liver disease (NAFLD) is characterised by excessive lipid accumulation in hepatocytes and is present in around 60% of pre-diabetic patients [105]. Diets containing high-fat and high-fructose content have been shown to significantly influence the development of NAFLD in prediabetes [106]. Fructose is metabolised exclusively by the liver and converted into triglycerides via *de novo* lipogenesis [105]. Both fructose and glucose upregulate the expression of lipogenic transcription factors such as sterol regulatory element-binding protein (SREBP-1c) and carbohydrate response element binding protein (ChREBP), thereby promoting hepatic TAG synthesis [107]. Additionally, high-fructose diets stimulate the expression of inflammatory mediators which inhibit the phosphorylation of IRS-1, leading to insulin resistance and intrahepatic inflammation [108]. Although studies consistently demonstrate that excessive fat and fructose intake promotes hepatic lipid accumulation, differences in dietary composition, duration of exposure, and experimental models contribute to variation in the severity of NAFLD reported across studies [109, 110].

Moderate insulin resistance in prediabetes impairs glucose uptake in skeletal muscle and adipose tissue [111]. In response to insulin resistance, the pancreas increases insulin production to compensate for the impaired glucose uptake [112]. Elevated insulin levels stimulate adipose tissue lipolysis resulting in an increased release of FFAs into the circulation [113]. Furthermore, insulin resistance disrupts the effects of insulin on hepatic gluconeogenesis and β -oxidation [61]. This disruption enhances hepatic gluconeogenesis and decreases β -oxidation in the liver, leading to intrahepatic TAG accumulation [61]. Hepatic tissue becomes more susceptible to oxidative stress due to aberrant lipid

accumulation in hepatocytes [114]. ROS can initiate lipid peroxidation by targeting polyunsaturated fatty acids leading to the formation of highly reactive aldehyde products such as 4-hydroxy-2-nonenal (4-HNE) and MDA [115]. Moreover, increased oxidative stress triggers inflammatory cascades that contribute to liver injury [105].

Studies have shown increased plasma liver enzyme levels such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and γ -glutamyl transpeptidase (GGT) in prediabetic individuals [116, 117]. These changes suggest hepatocellular stress and impaired liver function [118]. However, findings across studies are inconsistent [119, 120]. Some investigations have reported elevated ALT concentrations without significant changes in AST levels [119, 121]. This may be because ALT is more specific to hepatocellular injury, whereas AST is also expressed in extrahepatic tissues, including skeletal muscle and the heart [122, 123]. In contrast, other studies have observed no significant changes in ALT or AST concentrations in individuals with prediabetes [121, 124]. This may indicate that substantial hepatocellular injury does not occur during the early stages of prediabetes or that conventional liver enzymes may not be sensitive enough to detect early metabolic changes in the liver [121, 124]. Therefore, normal liver enzyme concentrations do not necessarily exclude early liver dysfunction [121, 124].

In addition to liver enzymes, hepatocyte injury can promote the release of damage-associated molecular patterns (DAMPs), which activate innate immune responses and increase the production of pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) [125]. Furthermore, bilirubin concentrations have been reported to be inversely associated with hyperglycaemia and NAFLD, suggesting a potential protective role of bilirubin due to its antioxidant properties [126]. Histological analyses of liver tissue from prediabetic subjects have demonstrated hepatic lipid droplet accumulation, sinusoidal dilation, and disrupted cellular organisation [127, 128]. These structural changes are indicative of hepatic steatosis and early liver injury associated with prediabetic conditions [127, 128]. However, variations in histological findings between studies may be influenced by differences in disease duration, severity of metabolic dysfunction, dietary interventions, and experimental models [129, 130]. Therefore, combining conventional liver enzymes with additional markers of inflammation, oxidative stress, fibrosis, and histology may provide a more comprehensive evaluation of early liver dysfunction in prediabetes [131].

2.4.3 Effects of prediabetes on renal function

In prediabetes, high plasma glucose levels react non-enzymatically with local renal proteins, leading to the formation of AGEs and the production of ROS (see Figure 3) [92, 132]. This induces oxidative stress which disrupts the integrity of the glomerular basement membrane characterised by the upregulation of kidney injury molecule-1 (KIM-1) and loss of podocyte structure [133]. The disruption of the glomerular filtration barrier promotes the loss of proteins such as albumin into the

urine [134]. However, findings regarding albuminuria in prediabetes are inconsistent, with some studies reporting increased urinary albumin excretion, while others have observed no significant changes compared with normoglycaemic individuals [87, 135]. These differences may reflect variation in disease stage, degree of metabolic dysfunction and the methods used to assess urinary albumin excretion [136]. Furthermore, albuminuria may not be sufficiently sensitive to detect subtle renal changes during the early stages of prediabetes, as structural and molecular alterations may occur before measurable increases in urinary albumin [137]. Additionally, reduced renal SOD and GPx activity has been observed in prediabetic animal models [138, 139].

Hyperglycaemia also contributes to renal haemodynamic alterations by increasing sodium reabsorption in the proximal tubules, primarily through upregulation of the sodium-glucose cotransporter 2 (SGLT2) [132]. Increased sodium reabsorption decreases the delivery of sodium to the macula densa within the juxtaglomerular apparatus [140]. The macula densa interprets the reduced tubular sodium levels as a decline in glomerular filtration and subsequently initiates afferent arteriolar vasodilation to increase glomerular filtration [138]. This compensatory response increases glomerular capillary pressure and promotes glomerular hyperfiltration [140]. Persistent elevation of glomerular capillary flow contributes to structural damage and increased glomerular permeability [133]. Consequently, albumin begins to leak into the urine, leading to albuminuria [132].

However, studies investigating changes in glomerular filtration rate (GFR) during prediabetes have reported conflicting findings [141, 142]. Some studies have demonstrated increased GFR, suggesting early glomerular hyperfiltration as an adaptive response to increased glucose and sodium transport [138, 143]. In contrast, other studies have reported reduced GFR, indicating early renal impairment [135, 144]. These differences may be explained by variations in disease progression, with hyperfiltration potentially occurring during the early stages of metabolic dysfunction, followed by a decline in GFR as structural renal damage progresses [145].

Additionally, elevated plasma glucose activates the polyol pathway, hexosamine biosynthetic pathway and renin-angiotensin-aldosterone system (RAAS) which contribute to renal dysfunction [146]. In the polyol pathway, excess intracellular glucose is converted to sorbitol by aldose reductase [147]. Accumulation of sorbitol increases intracellular osmotic stress and depletes nicotinamide adenine dinucleotide phosphate (NADPH), thereby reducing antioxidant defenses and promoting the generation of ROS [147]. In the hexosamine biosynthetic pathway, the increased glycosylation of renal proteins promotes inflammation [148]. RAAS activation increases angiotensin II production and up-regulates angiotensin II type 1 receptors (AT1R) expression in podocytes and mesangial cells [149]. Angiotensin II increases blood pressure, sodium reabsorption and stimulates aldosterone secretion from the adrenal glands, further contributing to renal injury [149]. Albuminuria often reflects established glomerular injury and may not identify subtle tubular damage occurring during the early stages of prediabetes [150]. Therefore, additional biomarkers, including KIM-1, inflammatory

markers, and oxidative stress indicators, may provide a more sensitive assessment of early renal alterations [151].

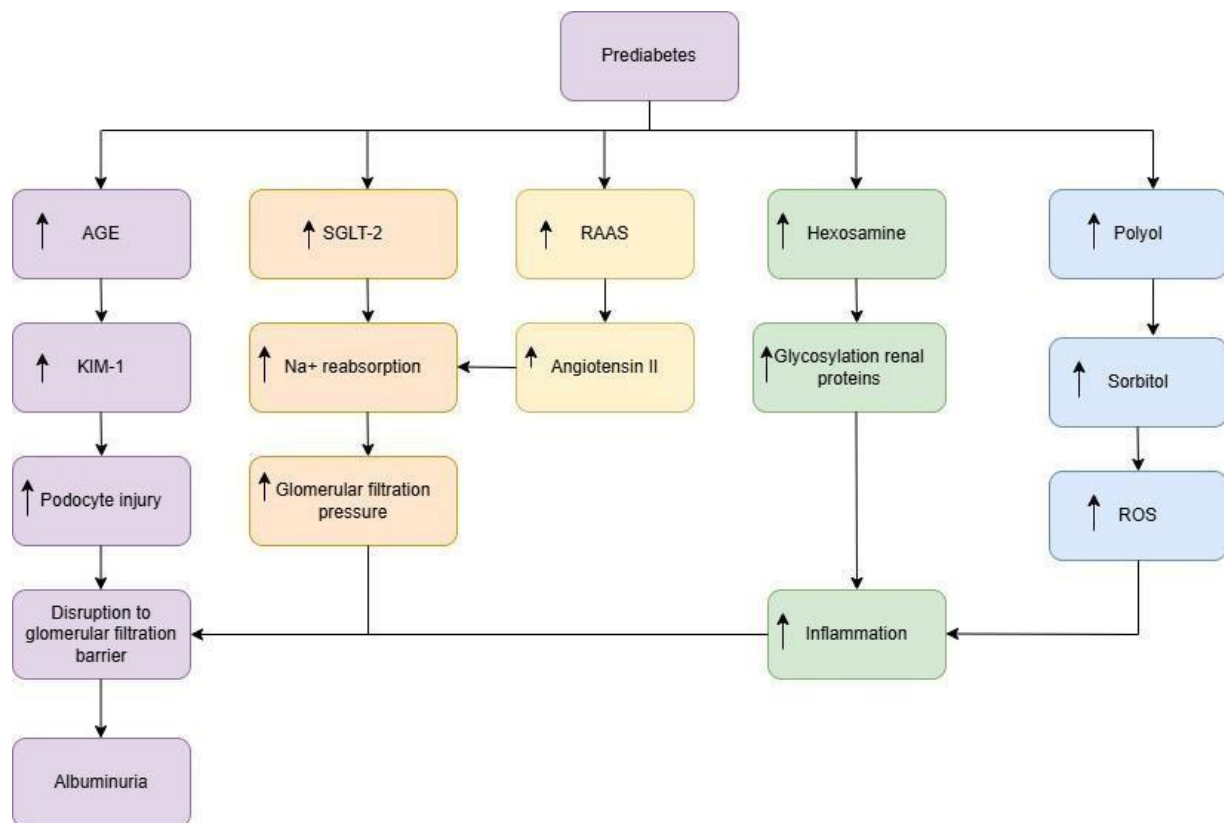


Figure 3: The multifaceted effects of prediabetes on renal function.

3.0 Conventional management of prediabetes

The standard approach for managing prediabetes involves a combination of dietary and pharmacological interventions [3, 18]. In the following sections, the effects of both dietary and pharmacological interventions in the management of prediabetes are discussed in detail.

3.1 Lifestyle intervention

The Diabetes Prevention Program Research Group has published several studies showing that prediabetes and T2DM can be prevented through dietary modifications and physical exercise [33, 152]. Several dietary approaches, such as intermittent fasting regimens, have been shown to improve glycaemic control, insulin sensitivity and promote weight loss [33]. Low-fat diets, low-carbohydrate diets and Mediterranean-style diets have been recommended for the management of prediabetes [45]. Furthermore, studies have shown that consumption of a Mediterranean diet reduces the risk of CVDs in individuals with T2DM [153, 154]. Another study showed that consumption of a low-carbohydrate diet reduced liver fat content and improved liver function markers in individuals with T2DM and NAFLD [155].

Several studies have shown that regular exercise improves insulin sensitivity [45, 156]. A study has shown that engaging in moderate exercise for 150 minutes per week reduces blood glucose levels and improves insulin sensitivity in adults at risk of prediabetes [155]. Additionally, aerobic exercise has been shown to improve cardiac function, endothelial function and reduce blood pressure in T2DM [157]. Studies have also shown that exercise reduces the risk of NAFLD and nephropathy in T2DM [158, 159]. Notably, studies have shown that the implementation of lifestyle interventions can reduce patient reliance on anti-diabetic medications [33, 156]. A study has shown that approximately 49% of individuals participating in lifestyle intervention programs either reduced or discontinued their use of anti-diabetic medications [160].

However, there are limitations associated with lifestyle interventions in the management of prediabetes [161]. One of the major barriers to the implementation of these interventions is the high cost associated with maintaining dietary modifications [162]. Socio-economic disparities also contribute to the variability in adherence, as individuals with lower incomes may face challenges in accessing nutritious foods [163]. Another significant barrier is the low adherence to increased physical activity [164]. Several factors contribute to this low adherence, including a lack of motivation, insufficient time and physical limitations [47, 116]. Despite these challenges, several studies have demonstrated that the benefits of lifestyle interventions extend beyond the intervention period, with lasting effects observed for many years after the completion of the trial [45, 152].

3.2 Pharmacological intervention

Pharmacological interventions play a pivotal role in the management of prediabetes and T2DM [20]. Metformin is a first-line therapy prescribed to delay the onset of diabetes [165]. It is a member of the biguanide class of anti-diabetic medications and works primarily by suppressing hepatic gluconeogenesis and improving insulin sensitivity through AMP-activated protein kinase (AMPK) activation [166]. Clinical trials have demonstrated that metformin reduces the risk of developing T2DM by approximately 45% in individuals with prediabetes [167]. However, adverse gastrointestinal effects such as diarrhoea, bloating and flatulence are frequently reported, which may compromise patient compliance [168].

Furthermore, metformin has been shown to exert cardioprotective effects in T2DM patients [169, 170]. It has been shown to enhance vascular endothelial function and promote angiogenesis [169]. Despite its benefits, metformin is less effective in severe conditions such as myocardial infarction and non-diabetic coronary artery disease, limiting its use as a first-line cardiovascular therapy [171]. Metformin also demonstrates renoprotective effects in diabetic patients, particularly in the early stages of kidney disease and when combined with other therapies [172, 173]. However, its use in advanced stages of kidney disease requires caution due to the potential risk of lactic acidosis, particularly in patients with impaired renal function [174]. Furthermore, studies have shown that metformin

improves liver function in patients with NAFLD [175, 176]. However, it does not significantly improve liver histological features such as steatosis, inflammation or fibrosis [175]. Metformin is administered to humans in tablet form at a dosage starting at 500 mg/day; therefore, we utilised this dosage in our study to replicate similar conditions [177].

Thiazolidinediones are a class of medications used to improve insulin sensitivity in T2DM patients [20]. Troglitazone, pioglitazone and rosiglitazone are the commonly approved drugs belonging to this group [178]. These agents act as agonists of peroxisome proliferator-activated receptor gamma (PPAR- γ) [167]. PPAR- γ is a ligand-activated transcription factors that promotes insulin sensitivity and glycaemic control [178]. However, their clinical use has declined due to safety concerns. Reported adverse effects include weight gain, fluid retention, and an increased risk of heart failure and fractures in certain patient populations [179, 180].

Glucagon-like peptide-1 (GLP-1) agonists represent another class of pharmacological agents used in the management of prediabetes and diabetes [178]. GLP-1 is a gut hormone that slows down diabetes progression by reducing glucagon secretion and suppressing appetite [178]. Recent studies have demonstrated substantial weight loss and cardiovascular benefits with GLP-1 receptor agonists [181, 182]. However, the high cost and limited accessibility remain significant barriers in many healthcare settings [183]. Patients using these drugs may experience nausea, vomiting and gastrointestinal discomfort as side effects [184]. Another class of medications used to treat diabetes are SGLT2 inhibitors [178]. Canagliflozin, dapagliflozin and empagliflozin are commonly approved drugs belonging to the class of SGLT2 inhibitors [184]. SGLT2 inhibitors work by inhibiting glucose reabsorption in the kidneys, leading to increased urinary glucose excretion and reduced plasma glucose levels [184]. However, they are associated with an increased risk of urogenital and genital infections [178].

Studies have shown that prediabetes can be reversed through a combination of dietary and pharmacological interventions [185, 186]. However, there are reports of low patient compliance as most patients merely use pharmacological intervention without any change in diet [167]. Hence, the efficacy of pharmacological interventions is reduced under these conditions [167]. Therefore, there is a need to identify compounds to manage prediabetes that are effective in both the presence and absence of dietary intervention.

4.0 Alternative therapeutic approaches

Plant-derived compounds, particularly phytochemicals, have been extensively utilised in the treatment of various human health conditions, such as diabetes, NAFLD, diabetic nephropathy and cardiovascular diseases [187, 188]. Phytochemicals can be categorised into distinct chemical classes, such as flavonoids, phenolic acids, tannins, alkaloids, coumarins and terpenoids [189]. Flavonoids are polyphenolic compounds abundantly present in plants, particularly in seeds, fruit skin, peels, bark and

flowers [190]. These compounds are well-known for their antidiabetic, antioxidant and anti-inflammatory properties [188, 190]. However, their clinical translation remains limited by poor bioavailability, low absorption, rapid metabolism, and differences in pharmacokinetic properties [191, 192]. Flavonoids share a core structure consisting of two benzene rings (A and B) linked by a three-carbon pyran ring (C) (see Figure 4) [190].

Quercetin is an abundant flavonoid found in fruits such as grapefruit, onion and berries [193]. It has been reported to possess anti-obesity, anti-diabetic, anti-inflammatory and antioxidant properties [193, 194]. Furthermore, it has been shown to ameliorate various diabetes-related complications such as kidney dysfunction, NAFLD and cardiovascular diseases [195, 196]. In vitro studies have demonstrated that quercetin exerts therapeutic effects comparable to those of metformin in cellular models [196, 197]. These pharmacological activities have been associated with quercetin's antioxidant capacity, which is influenced by the type, number and position of sugar moieties attached to its structure [198]. Quercetin functions as a potent antioxidant by directly scavenging ROS, such as superoxide (O_2^-), nitric oxide (NO^-) and peroxynitrite ($ONOO^-$) [92]. It also enhances endogenous antioxidant defence systems by increasing the activity of enzymes, such as SOD, catalase and GPx [92]. The efficacy of quercetin in reducing both metal and non-metal-induced oxidative damage is attributed to the presence of the free 3-OH group (see Figure 4) [198].

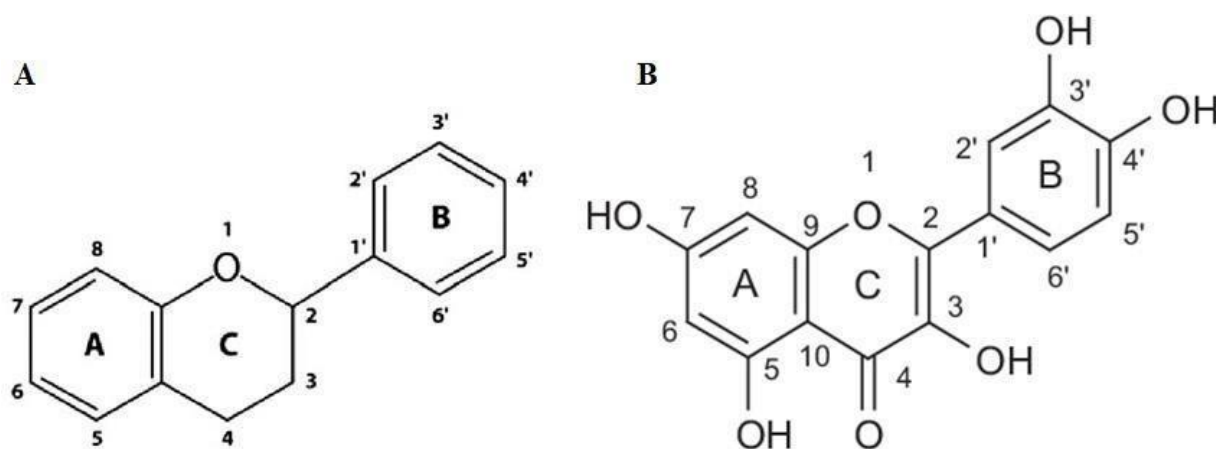


Figure 4: The general structure of A) flavonoids and B) quercetin.

4.1 Gossypetin

Gossypetin (GTIN) is a natural derivative of the well-known flavonoid quercetin [199]. GTIN is a hexahydroxyflavone predominantly found in the calyces of *Hibiscus sabdariffa* [200]. It is also present in other plant species from the Malvaceae family, including *Hibiscus vitifolius*, *Gossypium herbaceum* and *Gossypium arboreum* [201]. These calyces have traditionally been used in tropical regions for their medicinal properties and serve as rich sources of bioactive phytochemicals [202]. Historically, extracts from these plants have been employed in the treatment of conditions such as

inflammation, jaundice and diabetes [203]. Structurally, GTIN shares a catechol moiety on the B-ring similar to that of quercetin (see Figure 5) [204]. However, GTIN has three hydroxyl groups at carbon positions 5, 7 and 8 rather than two as seen in quercetin in the A-ring [205]. Notably, the adjacent hydroxyl groups at carbon positions 7 and 8 in GTIN act as a catechol-like centre [205].

Studies have shown that GTIN exerts potent antioxidant and anti-inflammatory properties (see Table 1) [201, 206]. It has been shown to exhibit therapeutic effects at doses between 10-20 mg/kg in vivo [200, 202]. Hence, in our study we selected an oral dose of 15 mg/kg GTIN for administration to our animals which is within the therapeutic range. A previous study showed that GTIN exhibited the most potent reducing capacity among 15 well-known flavonoids [206]. GTIN has also demonstrated protective effects in various disease models, including improved kidney function following nephrotoxicity [200], enhanced cognition and memory following four weeks of treatment [207], and reduced hepatic steatosis, lobular inflammation and fibrosis in a murine model of non-alcoholic steatohepatitis (NASH) [202].

Although the molecular mechanisms underlying GTIN activity remain incompletely understood, its structural similarity to quercetin suggests that it may influence pathways involved in glucose metabolism, inflammation, oxidative stress and mitochondrial function [208]. In prediabetes, impaired insulin signalling contributes to reduced glucose uptake and altered glucose metabolism [209]. Insulin binding to the insulin receptor activates insulin receptor substrate-1 (IRS-1), which subsequently stimulates phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt) signalling [210]. Activation of Akt promotes glucose transporter-4 (GLUT4) translocation to the skeletal muscle membrane, thereby increasing glucose uptake. Akt also inhibits glycogen synthase kinase-3 (GSK-3), resulting in activation of glycogen synthase and increased glycogen storage [211]. Flavonoids such as quercetin have been reported to improve insulin sensitivity through modulation of this pathway [212]. Therefore, GTIN may potentially improve glucose homeostasis through similar mechanisms. However, the effects of GTIN on IRS-1, PI3K, Akt and GLUT4 signalling have not yet been directly investigated.

Prediabetes is also characterised by increased oxidative stress and chronic low-grade inflammation [213]. Elevated glucose availability and mitochondrial dysfunction increase reactive oxygen species (ROS) generation, which can activate inflammatory pathways such as nuclear factor-kappa B (NF- κ B) [214]. Activation of NF- κ B promotes the transcription of pro-inflammatory cytokines, including TNF- α and IL-6 [214]. These cytokines can impair insulin signalling through inhibitory modifications of IRS proteins, thereby further contributing to insulin resistance [215]. Since GTIN demonstrates antioxidant and anti-inflammatory properties, it may potentially reduce ROS-mediated NF- κ B activation and attenuate inflammatory signalling [199, 200]. However, direct evidence demonstrating GTIN-mediated regulation of NF- κ B activation or inflammatory cytokine production remains limited.

Oxidative stress regulation may also involve activation of endogenous antioxidant defence pathways [216]. Flavonoids such as quercetin have been shown to activate nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates the expression of antioxidant enzymes including haem oxygenase-1 (HO-1) and SOD [217]. Activation of Nrf2 reduces ROS accumulation and lipid peroxidation, thereby limiting oxidative damage [217]. Due to its structural similarity to quercetin, GTIN may potentially exert similar effects through modulation of Nrf2-mediated antioxidant responses [218]. However, the effects of GTIN on Nrf2 signalling and antioxidant enzyme expression have not been directly investigated. These proposed mechanisms are supported by evidence from structurally related flavonoids; however, GTIN-specific molecular mechanisms remain poorly characterised [217].

Mitochondrial dysfunction is an important contributor to impaired glucose metabolism during prediabetes [219]. Reduced mitochondrial oxidative phosphorylation and increased mitochondrial ROS production contribute to insulin resistance and metabolic dysfunction [220]. Quercetin has been reported to improve mitochondrial function through activation of pathways involving peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α) [221]. This promotes mitochondrial biogenesis and enhances oxidative capacity [221]. Therefore, GTIN may potentially preserve mitochondrial function through similar mechanisms due to its structural similarity to quercetin. However, the effects of GTIN on mitochondrial respiration, mitochondrial biogenesis and mitochondrial bioenergetics have not yet been established.

Despite studies demonstrating antioxidant, anti-inflammatory and organ-protective effects of GTIN, its effects on glucose homeostasis and metabolic complications associated with prediabetes remain unknown [200, 201, 222].

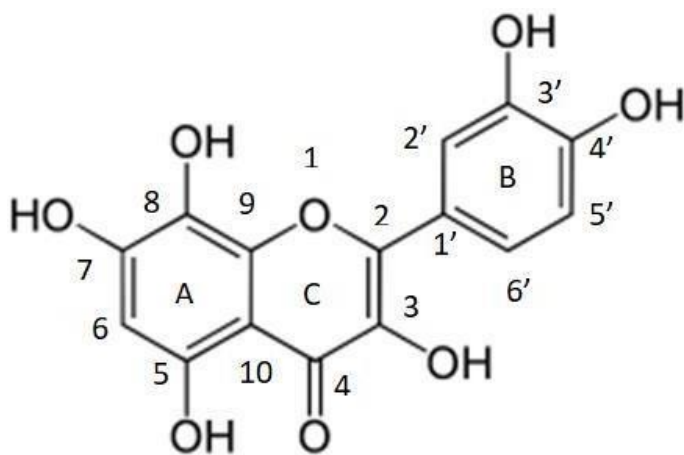


Figure 5: Chemical structure of gossypetin [223].

Table 1: In vitro and In vivo studies on the effects of GTIN in various disease models

Study	Model	Dose	Duration	Key findings
<i>In vitro studies</i>				
Khan et al., 2015 [206]	Hepatocyte cell line	20 mM and 40 mM	1 hour	GTIN at both doses reduced DNA damage and inflammation
Lin et al., 2021 [224]	Aortic cell line	1, 5, 10, 25, 50, 100 and 250 μ M	48 hours	GTIN at all doses reduced cell proliferation, cell cycle progression, migration, matrix degradation and oxidative stress
Lin et al., 2015 [199]	Endothelial cell line	0.1, 0.5, 1.0, 2.0 and 5.0 μ M	24 hours	GTIN at all doses exhibited protective effects against endothelial damage
<i>In vivo studies</i>				
Lin et al., 2015 [199]	New Zealand White rabbits	10 mg/kg oral	10 weeks	GTIN reduced ox-LDL uptake, lipid-laden foam cell formation and promoted cholesterol efflux
Ijaz et al., 2023 [200]	DOX-induced nephrotoxicity in Sprague Dawley rats	30 mg/kg intraperitoneal	30 days	GTIN improved renal function by enhancing antioxidants and reducing urea, creatinine, KIM-1
Samant et al., 2022 [207]	Swiss albino mice exposed to various stressors	5, 10 and 20 mg/kg intraperitoneal	4 weeks	GTIN at all doses improved behaviour, reduced oxidative stress, promoted elevated serotonin, norepinephrine, and BDNF levels
Gulsheen et al., 2024 [225]	Mice	5 and 20 mg/kg oral	1 week	GTIN at both doses showed significant antianxiety and antidepressant effects
Jo et al., 2022 [222]	5xFAD Mice	10 mg/kg oral	13 weeks	GTIN reduced A β plaques and improved cognition

Oh et al., 2023 [202]	C57BL/6 mice	20 mg/kg oral	4 weeks	GTIN reduced hepatic steatosis, lobular inflammation and liver fibrosis
Mustafa et al., 2023 [226]	Sprague-Dawley rats	5 mg/kg and 30 mg/kg oral	56 days	GTIN at both doses reduced oxidative stress, enhanced sperm parameters, restored hormone levels, exhibited androgenic properties and regulated apoptotic marker expression

5.0 Experimental models of prediabetes

In vivo animal models of prediabetes have been widely used to study the pathophysiological mechanisms underlying this condition [227, 228]. While these models do not fully mimic human diabetes mellitus, they remain crucial tools for assessing the efficacy of novel anti-diabetic agents [223]. Animal models allow researchers to control genetic and environmental factors that influence disease onset and progression, thereby yielding useful information for developing effective management strategies [229]. Genetically induced and diet-induced animal models have been used to study prediabetes [230]. Leptin-deficient (ob/ob) mice, insulin receptor substrate (IRS) knockout mice, Goto-Kakizaki (GK) rats and Zucker Diabetic Fatty (ZDF) rats are examples of genetically induced prediabetes models [231]. However, due to their limited availability and the challenge of fully mimicking the human condition of prediabetes, diet-induced animal models are often preferred for studying this condition [229].

Various diet-induced animal models of prediabetes have been established by feeding either a high-fat diet, a high-sucrose diet or a combination of both [232, 233]. A commonly used model involves feeding C57BL/6J mice a high-fat diet for 16 weeks which has been shown to induce prediabetes and prediabetic neuropathy [227]. This model is characterised by the development of obesity, elevated plasma FFA, hyperinsulinemia and IGT [229]. However, this model has not been used to evaluate the efficacy of anti-diabetic and anti-nephropathic agents [229]. Another established model involves feeding adult Wistar rats a 35% sucrose solution ad libitum for 9 weeks [234]. This model is characterised by hyperinsulinemia and hypertriglyceridemia with normoglycaemia during the fed state [229]. Although selected parameters related to prediabetic neuropathy were assessed in this model, the therapeutic effects of anti-diabetic agents have yet to be explored [229]. Additionally, a high-fructose diet-induced prediabetes model has been developed by administering a diet containing 60% fructose to rats for 30 days [235]. This model is characterised by the development of insulin resistance, decreased insulin sensitivity and increased lipid peroxidation [235]. While the suitability of this model has been assessed using the hepatotoxic agent thioacetamide, its potential for evaluating anti-diabetic therapies

has not yet been established [235].

Notably, a combination of high-fat and high-fructose diets has been reported to be more effective in inducing prediabetes than either diet alone [236, 237]. This combined diet model was developed by feeding rodents a diet in which 40%–60% total calories were derived from fat and fructose-enriched drinking water for 6 months [236]. Animals subjected to this regimen exhibited obesity and moderate insulin resistance [236]. The high-fat high-fructose diet has been shown to promote liver fibrosis, inflammation, endoplasmic reticulum stress and lipoapoptosis [238]. However, the macronutrient composition of this diet did not mimic the high-calorie diet that predisposes humans to prediabetes in developed countries [34, 238].

6.0 High-fat high-carbohydrate diet-induced model of prediabetes

In our laboratory, a diet-induced prediabetic animal model was established by feeding male Sprague Dawley rats a high-fat high-carbohydrate (HFHC) diet comprising 55% carbohydrates, 30% fats and 15% protein supplemented with 15% fructose water over 20 weeks [55]. Previous research from our laboratory showed that the HFHC diet induces moderate hyperglycaemia, mild insulin resistance and moderate glucose intolerance, elevated glycated haemoglobin, body weight and ghrelin concentration [34, 116]. It has also been reported that the HFHC diet model of prediabetes exhibits alterations in cardiovascular, hepatic and renal function [90, 116, 149]. Studies have shown that the HFHC diet promotes hepatic oxidative stress and liver damage [239, 240]. Additionally, this diet has been associated with myocardial injury and increased risk of developing cardiovascular diseases [90]. It has also been shown to promote kidney dysfunction, as evidenced by a decreased glomerular filtration rate and increased kidney injury marker levels [149]. Male Sprague Dawley rats are commonly utilised in diabetes research due to their metabolic similarity to humans, ease of handling and docile temperament [55]. Accordingly, this study sought to investigate the effects of GTIN on glucose homeostasis and selected complications of prediabetes using a HFHC diet-induced prediabetic animal model.

7.0 Justification of the study

Prediabetes is an intermediate state of hyperglycaemia which precedes the development of T2DM [20]. The prevalence of prediabetes is increasing rapidly in developed and developing countries such as South Africa due to the consumption of high-calorie diets and sedentary lifestyles [21]. Studies have shown that many of the complications, such as cardiovascular diseases, NAFLD and renal dysfunction, seen in T2DM begin during prediabetes [17, 18]. The combination of pharmacological and dietary interventions is currently the primary therapeutic approach for managing prediabetes [241, 242]. However, patient compliance to these combined approaches is low as patients do not comply with diet modifications due to the high cost, inconvenience of changing eating patterns and lack of nutritional food knowledge [163, 167]. Hence, the efficacy of pharmacological intervention is

reduced [178]. Therefore, there is a need to identify alternative compounds that are effective in managing prediabetes and its associated complications in the presence and absence of dietary intervention. Quercetin is a plant-derived structural analog of gossypetin which has been shown to exhibit high insulin-mimetic properties, antioxidant, cardioprotective, hepatoprotective and renoprotective effects with less toxicity compared with conventional diabetes drugs [198, 243]. Notably, studies have found that gossypetin exhibits potent antioxidant and anti-inflammatory properties [201, 243]. However, no studies have investigated the effects of GTIN on glucose homeostasis as well as its effects on cardiovascular, liver and renal complications in the prediabetic state. Accordingly, this study sought to investigate the effects of GTIN on glucose homeostasis and selected complications of prediabetes using a HFHC-diet induced prediabetic animal model.

8.0 References

1. Galicia-Garcia, U.; Benito-Vicente, A.; Jebari, S.; Larrea-Sebal, A.; Siddiqi, H.; Uribe, K. B.; Ostolaza, H.; Martín, C., Pathophysiology of Type 2 Diabetes Mellitus. In *International Journal of Molecular Sciences*, 2020; Vol. 21.
2. Le, T. K. C.; Dao, X. D.; Nguyen, D. V.; Luu, D. H.; Bui, T. M. H.; Le, T. H.; Nguyen, H. T.; Le, T. N.; Hosaka, T.; Nguyen, T. T. T., Insulin signaling and its application. *Frontiers in endocrinology* 2023, 14, 1226655.
3. Saltiel, A. R., Insulin signaling in health and disease. *The Journal of clinical investigation* 2021, 131, (1).
4. Kubota, T.; Kubota, N.; Kadowaki, T., Imbalanced insulin actions in obesity and type 2 diabetes: key mouse models of insulin signaling pathway. *Cell metabolism* 2017, 25, (4), 797–810.
5. Li, D.-d.; Yang, Y.; Gao, Z.-y.; Zhao, L.-h.; Yang, X.; Xu, F.; Yu, C.; Zhang, X.-l.; Wang, X.-q.; Wang, L.-h., Sedentary lifestyle and body composition in type 2 diabetes. *Diabetology & Metabolic Syndrome* 2022, 14, (1), 8.
6. Eaton, S. B.; Eaton, S. B., Physical inactivity, obesity, and type 2 diabetes: an evolutionary perspective. *Research quarterly for exercise and sport* 2017, 88, (1), 1–8.
7. MacDonald, I. A., A review of recent evidence relating to sugars, insulin resistance and diabetes. *European journal of nutrition* 2016, 55, (Suppl 2), 17–23.
8. von Frankenberg, A. D.; Marina, A.; Song, X.; Callahan, H. S.; Kratz, M.; Utzschneider, K. M., A high-fat, high-saturated fat diet decreases insulin sensitivity without changing intra-abdominal fat in weight-stable overweight and obese adults. *European journal of nutrition* 2017, 56, (1), 431–443.
9. Sun, H.; Saeedi, P.; Karuranga, S.; Pinkepank, M.; Ogurtsova, K.; Duncan, B. B.; Stein, C.; Basit, A.; Chan, J. C.; Mbanya, J. C., IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes research and clinical practice* 2022,

183, 109119.

10. Dai, W.; Ye, L.; Liu, A.; Wen, S. W.; Deng, J.; Wu, X.; Lai, Z., Prevalence of nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus: a meta-analysis. *Medicine* 2017, 96, (39), e8179.
11. Einarson, T. R.; Acs, A.; Ludwig, C.; Panton, U. H., Prevalence of cardiovascular disease in type 2 diabetes: a systematic literature review of scientific evidence from across the world in 2007–2017. *Cardiovascular diabetology* 2018, 17, (1), 83.
12. Low, S.; Sum, C. F.; Yeoh, L. Y.; Tavintharan, S.; Ng, X. W.; Lee, S.; Tang, W. E.; Lim, S. C., Prevalence of chronic kidney disease in adults with type 2 diabetes mellitus. *Ann Acad Med Singapore* 2015, 44, (5), 164–171.
13. Tesfaye, A.; Josef, H.; Wube, T. B.; Girma, Z.; Negasa, B.; Muche, T.; Zewude, B., Magnitude of, and factors associated with cardiovascular disease among type two diabetes mellitus patients. *Diabetes, Metabolic Syndrome and Obesity* 2020, 4123–4129.
14. Selby, N. M.; Taal, M. W., An updated overview of diabetic nephropathy: Diagnosis, prognosis, treatment goals and latest guidelines. *Diabetes, Obesity and Metabolism* 2020, 22, 3–15.
15. Wierzba, W.; Śliwczyński, A.; Karnafel, W.; Gujski, M.; Słodki, M.; Lusawa, A.; Pinkas, J., The association of diabetes with all-cause mortality in patients with end-stage renal disease compared to the general population in Poland—a comparative analysis. *Archives of Medical Science: AMS* 2020, 18, (2), 314.
16. Pfannkuche, A.; Alhajjar, A.; Ming, A.; Walter, I.; Piehler, C.; Mertens, P. R., Prevalence and risk factors of diabetic peripheral neuropathy in a diabetics cohort: Register initiative “diabetes and nerves”. *Endocrine and Metabolic Science* 2020, 1, (1-2), 100053.
17. Khetan, A. K.; Rajagopalan, S., Prediabetes. *Canadian Journal of Cardiology* 2018, 34, (5), 615–623.
18. Mabuza, L. P.; Gamede, M. W.; Maikoo, S.; Booysen, I. N.; Nguban, P. S.; Khathi, A., Hepatoprotective effects of a ruthenium (II) Schiff base complex in rats with diet-induced prediabetes. *Current Therapeutic Research* 2019, 91, 66–72.
19. Control, C. f. D.; Prevention, National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. *Atlanta, GA: US department of health and human services, centers for disease control and prevention* 2011, 201, (1), 2568–2569.
20. Bansal, N., Prediabetes diagnosis and treatment: a review. *World journal of diabetes* 2015, 6, (2), 296.
21. Bhansali, A.; Dutta, P., Pathophysiology of prediabetes. *Journal of the Indian Medical Association* 2005, 103, (11), 594–5, 599.
22. Anjana, R. M.; Shanthi Rani, C. S.; Deepa, M.; Pradeepa, R.; Sudha, V.; Divya Nair, H.; Lakshmi priya, N.; Subhashini, S.; Binu, V. S.; Unnikrishnan, R., Incidence of diabetes and prediabetes

and predictors of progression among Asian Indians: 10-year follow-up of the Chennai Urban Rural Epidemiology Study (CURES). *Diabetes care* 2015, 38, (8), 1441–1448.

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

24. Grundlingh, N.; Zewotir, T. T.; Roberts, D. J.; Manda, S., Assessment of prevalence and risk factors of diabetes and pre-diabetes in South Africa. *Journal of Health, population and Nutrition* 2022, 41, (1), 7.

25. Pheiffer, C., The prevalence of type 2 diabetes in South Africa: A systematic review. 2020.

26. Sosibo, A. M.; Mzimela, N. C.; Ngubane, P. S.; Khathi, A., Prevalence of pre-diabetes in adults aged 25–45 years in a Durban-based clinical setting, South Africa: A retrospective study. *Primary Care Diabetes* 2023, 17, (6), 650–654.

27. Ziegler, D.; Papanas, N.; Vinik, A. I.; Shaw, J. E., Epidemiology of polyneuropathy in diabetes and prediabetes. *Handbook of clinical neurology* 2014, 126, 3–22.

28. Kopp, W., How western diet and lifestyle drive the pandemic of obesity and civilization diseases. *Diabetes, Metabolic Syndrome and Obesity* 2019, 2221–2236.

29. Bird, S. R.; Hawley, J. A., Update on the effects of physical activity on insulin sensitivity in humans. *BMJ open sport & exercise medicine* 2017, 2, (1), e000143.

30. Evert, A. B.; Dennison, M.; Gardner, C. D.; Garvey, W. T.; Lau, K. H. K.; MacLeod, J.; Mitri, J.; Pereira, R. F.; Rawlings, K.; Robinson, S., Nutrition therapy for adults with diabetes or prediabetes: a consensus report. *Diabetes care* 2019, 42, (5), 731.

31. Zhou, Q.; Gao, Y.; Guo, Y.; Zhu, G., Relationship between Dietary Inflammatory Index and Blood Glucose Changes in Patients with Pre-diabetes Mellitus. *Alternative Therapies in Health & Medicine* 2025, 31, (1), 276.

32. Punthakee, Z.; Goldenberg, R.; Katz, P., Definition, classification and diagnosis of diabetes, prediabetes and metabolic syndrome. *Canadian journal of diabetes* 2018, 42, S10–S15.

33. Mainous III, A. G.; Tanner, R. J.; Baker, R., Prediabetes diagnosis and treatment in primary care. *The Journal of the American Board of Family Medicine* 2016, 29, (2), 283–285.

34. Islam, M. S.; Venkatesan, V., Experimentally-induced animal models of prediabetes and insulin resistance: a review. *Acta Pol Pharm* 2016, 73, (4), 827–834.

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

36. Sequeira, I.; Poppitt, S., HbA1c as a marker of prediabetes: A reliable screening tool or not. *Insights Nutr Metabol* 2017; 1 (1): 21-29. 22 *Insights Nutr Metabol* 2017 Volume 1 Issue 2017, 1, 4.

37. Brannick, B.; Dagogo-Jack, S., Prediabetes and cardiovascular disease: pathophysiology and interventions for prevention and risk reduction. *Endocrinology and metabolism clinics of North America* 2018, 47, (1), 33.
38. Ferrannini, E.; Gastaldelli, A.; Iozzo, P., Pathophysiology of prediabetes. *Medical Clinics* 2011, 95, (2), 327–339.
39. Kabadi, U. M., Major pathophysiology in prediabetes and type 2 diabetes: decreased insulin in lean and insulin resistance in obese. *Journal of the Endocrine Society* 2017, 1, (6), 742–750.
40. Lazaridou, S.; Dinas, K.; Tziomalos, K., Prevalence, pathogenesis and management of prediabetes and type 2 diabetes mellitus in patients with polycystic ovary syndrome. *Hormones* 2017, 16, (4), 373–380.
41. Brannick, B.; Dagogo-Jack, S., Prediabetes and Cardiovascular Disease: Pathophysiology and Interventions for Prevention and Risk Reduction. *Endocrinol Metab Clin North Am* 2018, 47, (1), 33–50.
42. Tabák, A. G.; Herder, C.; Rathmann, W.; Brunner, E. J.; Kivimäki, M., Prediabetes: a high-risk state for diabetes development. *The Lancet* 2012, 379, (9833), 2279–2290.
43. Tankova, T.; Chakarova, N.; Dakovska, L.; Atanassova, I., Assessment of HbA1c as a diagnostic tool in diabetes and prediabetes. *Acta diabetologica* 2012, 49, (5), 371–378.
44. Stefanaki, C.; Bacopoulou, F.; Peppas, M., Prediabetes and adolescence—trends, causes, effects, and screening. *US Endocrinol* 2016, 12, (2), 94–8.
45. Kanat, M.; DeFronzo, R. A.; Abdul-Ghani, M. A., Treatment of prediabetes. *World journal of diabetes* 2015, 6, (12), 1207.
46. Jeon, J. Y.; Ko, S.-H.; Kwon, H.-S.; Kim, N. H.; Kim, J. H.; Kim, C. S.; Song, K.-H.; Won, J. C.; Lim, S.; Choi, S. H., Prevalence of diabetes and prediabetes according to fasting plasma glucose and HbA1c. *Diabetes & metabolism journal* 2013, 37, (5), 349.
47. Chakarova, N.; Dimova, R.; Grozeva, G.; Tankova, T., Assessment of glucose variability in subjects with prediabetes. *Diabetes research and clinical practice* 2019, 151, 56–64.
48. Edwards, C. M.; Cusi, K., Prediabetes: a worldwide epidemic. *Endocrinology and Metabolism Clinics* 2016, 45, (4), 751–764.
49. Stringer, D. M.; Zahradka, P.; Taylor, C. G., Glucose transporters: cellular links to hyperglycemia in insulin resistance and diabetes. *Nutrition Reviews* 2015, 73, (3), 140–154.
50. Tahapary, D. L.; Pratisthita, L. B.; Fitri, N. A.; Marcella, C.; Wafa, S.; Kurniawan, F.; Rizka, A.; Tarigan, T. J. E.; Harbuwono, D. S.; Purnamasari, D., Challenges in the diagnosis of insulin resistance: Focusing on the role of HOMA-IR and Tryglyceride/glucose index. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews* 2022, 16, (8), 102581.

51. Horáková, D.; Štěpánek, L.; Janout, V.; Janoutová, J.; Pastucha, D.; Kollárová, H.; Petráková, A.; Štěpánek, L.; Husár, R.; Martiník, K., Optimal homeostasis model assessment of insulin resistance (HOMA-IR) cut-offs: A cross-sectional study in the Czech population. *Medicina* 2019, 55, (5), 158.
52. Gastaldelli, A., Measuring and estimating insulin resistance in clinical and research settings. *Obesity* 2022, 30, (8), 1549–1563.
53. Krentz, A. J.; Weyer, C.; Hompesch, M., Quantification of insulin action in human subjects. In *Translational Research Methods in Diabetes, Obesity, and Nonalcoholic Fatty Liver Disease: A Focus on Early Phase Clinical Drug Development*, Springer: 2019; pp 3–35.
54. Dimitriadis, G. D.; Maratou, E.; Kountouri, A.; Board, M.; Lambadiari, V., Regulation of postabsorptive and postprandial glucose metabolism by insulin-dependent and insulin-independent mechanisms: an integrative approach. *Nutrients* 2021, 13, (1), 159.
55. Khathi, A.; Luvuno, M.; Mabandla, M., Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. *PONTE International Journal of Science and Research* 2018, 74, (5).
56. Chen, S.; Zuo, X.; Li, Y.; Jiang, T.; Zhang, N.; Dai, F.; Chen, Q.; Zhang, Q., Ghrelin is a possible new predictor associated with executive function in patients with type 2 diabetes mellitus. *Journal of diabetes investigation* 2017, 8, (3), 306–313.
57. Mihalache, L.; Gherasim, A.; Niță, O.; Ungureanu, M. C.; Pădureanu, S. S.; Gavril, R. S.; Arhire, L. I., Effects of ghrelin in energy balance and body weight homeostasis. *Hormones* 2016, 15, (2), 186–196.
58. Mitoiu, B. I.; Nartea, R.; Miclaus, R. S., Impact of resistance and endurance training on ghrelin and plasma leptin levels in overweight and obese subjects. *International journal of molecular sciences* 2024, 25, (15), 8067.
59. Wang, Y.; Wu, Q.; Zhou, Q.; Chen, Y.; Lei, X.; Chen, Y.; Chen, Q., Circulating acyl and des-acyl ghrelin levels in obese adults: a systematic review and meta-analysis. *Scientific reports* 2022, 12, (1), 2679.
60. Abassi, W.; Ouerghi, N.; Muscella, A.; Marsigliante, S.; Feki, M.; Bouassida, A., Systematic Review: Does Exercise Training Influence Ghrelin Levels? *International Journal of Molecular Sciences* 2025, 26, (10), 4753.
61. Hatting, M.; Tavares, C. D.; Sharabi, K.; Rines, A. K.; Puigserver, P., Insulin regulation of gluconeogenesis. *Annals of the New York Academy of Sciences* 2018, 1411, (1), 21–35.
62. Jensen, J.; Rustad, P. I.; Kolnes, A. J.; Lai, Y.-C., The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise. *Frontiers in physiology* 2011, 2, 112.
63. Mu, W.; Cheng, X.-f.; Liu, Y.; Lv, Q.-z.; Liu, G.-l.; Zhang, J.-g.; Li, X.-y., Potential nexus of non-alcoholic fatty liver disease and type 2 diabetes mellitus: insulin resistance between hepatic and peripheral tissues. *Frontiers in pharmacology* 2019, 9, 1566.

64. Adin, C. A., Bilirubin as a therapeutic molecule: challenges and opportunities. *Antioxidants* 2021, 10, (10), 1536.
65. Basu, R.; Barosa, C.; Jones, J.; Dube, S.; Carter, R.; Basu, A.; Rizza, R. A., Pathogenesis of prediabetes: role of the liver in isolated fasting hyperglycemia and combined fasting and postprandial hyperglycemia. *The Journal of Clinical Endocrinology & Metabolism* 2013, 98, (3), E409–E417.
66. Yazdanpanah, S.; Rabiee, M.; Tahriri, M.; Abdolrahim, M.; Rajab, A.; Jazayeri, H. E.; Tayebi, L., Evaluation of glycated albumin (GA) and GA/HbA1c ratio for diagnosis of diabetes and glycemic control: A comprehensive review. *Critical reviews in clinical laboratory sciences* 2017, 54, (4), 219–232.
67. Samajdar, S. S.; Joshi, S. R.; Misra, A.; Vikram, N. K., The limitations and fallacies of relying on glycosylated hemoglobin for diagnosing and monitoring diabetes in Indian populations. *Lancet Reg Health Southeast Asia* 2026, 45, 100724.
68. Echouffo-Tcheugui, J. B.; Selvin, E., Prediabetes and What It Means: The Epidemiological Evidence. *Annu Rev Public Health* 2021, 42, 59–77.
69. Dorcely, B.; Katz, K.; Jagannathan, R.; Chiang, S. S.; Oluwadare, B.; Goldberg, I. J.; Bergman, M., Novel biomarkers for prediabetes, diabetes, and associated complications. *Diabetes Metab Syndr Obes* 2017, 10, 345–361.
70. Sallar, A.; Dagogo-Jack, S., Regression from prediabetes to normal glucose regulation: state of the science. *Experimental Biology and Medicine* 2020, 245, (10), 889–896.
71. Sun, C.; Liu, Q.; Ye, X.; Li, R.; Meng, M.; Han, X., The Role of Probiotics in Managing Glucose Homeostasis in Adults with Prediabetes: A Systematic Review and Meta-Analysis. *Journal of Diabetes Research* 2024, 2024, (1), 5996218.
72. Theodorakis, N.; Kreouzi, M.; Pappas, A.; Nikolaou, M., Beyond calories: individual metabolic and hormonal adaptations driving variability in weight management—a state-of-the-art narrative review. *International journal of molecular sciences* 2024, 25, (24), 13438.
73. Canfora, E. E.; Meex, R. C.; Venema, K.; Blaak, E. E., Gut microbial metabolites in obesity, NAFLD and T2DM. *Nature Reviews Endocrinology* 2019, 15, (5), 261–273.
74. Salunkhe, V. A.; Veluthakal, R.; Kahn, S. E.; Thurmond, D. C., Novel approaches to restore beta cell function in prediabetes and type 2 diabetes. *Diabetologia* 2018, 61, (9), 1895–1901.
75. Brannick, B.; Wynn, A.; Dagogo-Jack, S., Prediabetes as a toxic environment for the initiation of microvascular and macrovascular complications. *Experimental biology and medicine* 2016, 241, (12), 1323–1331.
76. Sosibo, A. M.; Mzimela, N. C.; Ngubane, P. S.; Khathi, A., Prevalence of pre-diabetes in adults aged 25 – 45 years in a Durban-based clinical setting, South Africa: A retrospective study. *Primary Care Diabetes* 2023, 17, (6), 650–654.

77. Mauermann, M. L.; Staff, N. P., Peripheral neuropathy: a review. *Jama* 2026, 335, (3), 255–266.
78. Kirthi, V.; Perumbalath, A.; Brown, E.; Nevitt, S.; Petropoulos, I. N.; Burgess, J.; Roylance, R.; Cuthbertson, D. J.; Jackson, T. L.; Malik, R. A., Prevalence of peripheral neuropathy in pre-diabetes: a systematic review. *BMJ Open Diabetes Research & Care* 2021, 9, (1), e002040.
79. Stino, A. M.; Smith, A. G., Peripheral neuropathy in prediabetes and the metabolic syndrome. *Journal of diabetes investigation* 2017, 8, (5), 646–655.
80. Méndez-Morales, S.; Pérez-De Marcos, J.; Rodríguez-Cortés, O.; Flores-Mejía, R.; Martínez-Venegas, M.; Sánchez-Vera, Y.; Tamay-Cach, F.; Lomeli-González, J.; Reyes, A. E.; Lehman-Mendoza, R., Diabetic neuropathy: molecular approach a treatment opportunity. *Vascular Pharmacology* 2022, 143, 106954.
81. Wu, L.; Wang, X. J.; Luo, X.; Zhang, J.; Zhao, X.; Chen, Q., Diabetic peripheral neuropathy based on Schwann cell injury: Mechanisms of cell death regulation and therapeutic perspectives. *Frontiers in endocrinology* 2024, 15, 1427679.
82. Unnikrishnan, R.; Shaw, J. E.; Chan, J. C.; Wild, S. H.; Peters, A. L.; Orrange, S.; Roden, M.; Mohan, V., Prediabetes. *Nature Reviews Disease Primers* 2025, 11, (1), 49.
83. Cosentino, F.; Grant, P. J.; Aboyans, V.; Bailey, C. J.; Ceriello, A.; Delgado, V.; Federici, M.; Filippatos, G.; Grobbee, D. E.; Hansen, T. B., 2019 ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD: The Task Force for diabetes, pre-diabetes, and cardiovascular diseases of the European Society of Cardiology (ESC) and the European Association for the Study of Diabetes (EASD). *European heart journal* 2020, 41, (2), 255–323.
84. Færch, K.; Vistisen, D.; Johansen, N. B.; Jørgensen, M. E., Cardiovascular risk stratification and management in pre-diabetes. *Current diabetes reports* 2014, 14, (6), 493.
85. Naicker, Y.; Khathi, A., Pathophysiological Links Between Stroke and Prediabetes: A Systematic Review. *Current Issues in Molecular Biology* 2025, 47, (10), 854.
86. Grundy, S. M., Pre-diabetes, metabolic syndrome, and cardiovascular risk. *Journal of the American College of Cardiology* 2012, 59, (7), 635–643.
87. Lamprou, S.; Koletsos, N.; Mintziori, G.; Anyfanti, P.; Trakatelli, C.; Kotsis, V.; Gkaliagkousi, E.; Triantafyllou, A., Microvascular and endothelial dysfunction in prediabetes. *Life* 2023, 13, (3), 644.
88. Holm, H.; Kennbäck, C.; Laucyte-Cibulskiene, A.; Nilsson, P. M.; Jujic, A., The impact of prediabetes and diabetes on endothelial function in a large population-based cohort. *Blood Pressure* 2024, 33, (1), 2298309.
89. Sörensen, B. M.; Houben, A. J.; Berendschot, T. T.; Schouten, J. S.; Kroon, A. A.; van der Kallen, C. J.; Henry, R. M.; Koster, A.; Sep, S. J.; Dagnelie, P. C., Prediabetes and type 2 diabetes are associated with generalized microvascular dysfunction: the Maastricht Study. *Circulation* 2016, 134, (18), 1339–1352.

90. Gumedde, N.; Ngubane, P.; Khathi, A., Assessing the risk factors for myocardial infarction in diet-induced prediabetes: myocardial tissue changes. *BMC cardiovascular disorders* 2022, 22, (1), 350.
91. Mahat, R. K.; Singh, N.; Rathore, V.; Arora, M.; Yadav, T., Cross-sectional correlates of oxidative stress and inflammation with glucose intolerance in prediabetes. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews* 2019, 13, (1), 616–621.
92. Luc, K.; Schramm-Luc, A.; Guzik, T.; Mikolajczyk, T., Oxidative stress and inflammatory markers in prediabetes and diabetes. *Journal of Physiology & Pharmacology* 2019, 70, (6), 1.
93. Kato, K.; Otsuka, T.; Saiki, Y.; Kobayashi, N.; Nakamura, T.; Kon, Y.; Kawada, T., Association between elevated C-reactive protein levels and prediabetes in adults, particularly impaired glucose tolerance. *Canadian journal of diabetes* 2019, 43, (1), 40–45. e2.
94. Koncsos, G.; Varga, Z. V.; Baranyai, T.; Boengler, K.; Rohrbach, S.; Li, L.; Schlüter, K.-D.; Schreckenber, R.; Radovits, T.; Oláh, A., Diastolic dysfunction in prediabetic male rats: Role of mitochondrial oxidative stress. *American Journal of Physiology-Heart and Circulatory Physiology* 2016, 311, (4), H927–H943.
95. Dubois-Deruy, E.; Peugnet, V.; Turkieh, A.; Pinet, F., Oxidative stress in cardiovascular diseases. *Antioxidants* 2020, 9, (9), 864.
96. Faria, A.; Persaud, S. J., Cardiac oxidative stress in diabetes: mechanisms and therapeutic potential. *Pharmacology & therapeutics* 2017, 172, 50–62.
97. Dorcelly, B.; Katz, K.; Jagannathan, R.; Chiang, S. S.; Oluwadare, B.; Goldberg, I. J.; Bergman, M., Novel biomarkers for prediabetes, diabetes, and associated complications. *Diabetes, metabolic syndrome and obesity: targets and therapy* 2017, 345–361.
98. Kawai, T.; Autieri, M. V.; Scalia, R., Adipose tissue inflammation and metabolic dysfunction in obesity. *American journal of physiology-cell physiology* 2021, 320, (3), C375–C391.
99. Al Amri, T.; Bahijri, S.; Al-Raddadi, R.; Ajabnoor, G.; Al Ahmadi, J.; Jambi, H.; Borai, A.; Tuomilehto, J., The association between prediabetes and dyslipidemia among attendants of primary care health centers in Jeddah, Saudi Arabia. *Diabetes, Metabolic Syndrome and Obesity* 2019, 2735–2743.
100. Neves, J. S.; Newman, C.; Bostrom, J. A.; Buysschaert, M.; Newman, J. D.; Medina, J. L.; Goldberg, I. J.; Bergman, M., Management of dyslipidemia and atherosclerotic cardiovascular risk in prediabetes. *Diabetes research and clinical practice* 2022, 190, 109980.
101. Wasserman, D. H.; Wang, T. J.; Brown, N. J., The vasculature in prediabetes. *Circulation research* 2018, 122, (8), 1135–1150.
102. Anastasiou, E., Endothelial dysfunction in pre-diabetes. *ENDOCRINOLOGIA Y NUTRICION* 1999, 46, (9), 279–281.
103. Powell-Wiley, T. M.; Poirier, P.; Burke, L. E.; Després, J.-P.; Gordon-Larsen, P.; Lavie, C. J.;

- Lear, S. A.; Ndumele, C. E.; Neeland, I. J.; Sanders, P., Obesity and cardiovascular disease: a scientific statement from the American Heart Association. *Circulation* 2021, 143, (21), e984–e1010.
104. Bibi, S.; Naeem, M.; Mousa, M. F. M.; Bahls, M.; Dörr, M.; Friedrich, N.; Nauck, M.; Bülow, R.; Völzke, H.; Markus, M. R., Body composition markers are associated with changes in inflammatory markers but not vice versa: A bi-directional longitudinal analysis in a population-based sample. *Nutrition, Metabolism and Cardiovascular Diseases* 2024, 34, (5), 1166–1174.
105. Ortiz-Lopez, C.; Lomonaco, R.; Orsak, B.; Finch, J.; Chang, Z.; Kochunov, V. G.; Hardies, J.; Cusi, K., Prevalence of prediabetes and diabetes and metabolic profile of patients with nonalcoholic fatty liver disease (NAFLD). *Diabetes care* 2012, 35, (4), 873–878.
106. Jensen, T.; Abdelmalek, M. F.; Sullivan, S.; Nadeau, K. J.; Green, M.; Roncal, C.; Nakagawa, T.; Kuwabara, M.; Sato, Y.; Kang, D.-H., Fructose and sugar: A major mediator of non-alcoholic fatty liver disease. *Journal of hepatology* 2018, 68, (5), 1063–1075.
107. Linden, A. G.; Li, S.; Choi, H. Y.; Fang, F.; Fukasawa, M.; Uyeda, K.; Hammer, R. E.; Horton, J. D.; Engelking, L. J.; Liang, G., Interplay between ChREBP and SREBP-1c coordinates postprandial glycolysis and lipogenesis in livers of mice [S]. *Journal of lipid research* 2018, 59, (3), 475–487.
108. Hossain, I. A.; Akter, S.; Bhuiyan, F. R.; Shah, M. R.; Rahman, M. K.; Ali, L., Subclinical inflammation in relation to insulin resistance in prediabetic subjects with nonalcoholic fatty liver disease. *BMC Research Notes* 2016, 9, (1), 266.
109. Eng, J. M.; Estall, J. L., Diet-induced models of non-alcoholic fatty liver disease: food for thought on sugar, fat, and cholesterol. *Cells* 2021, 10, (7), 1805.
110. Hydes, T.; Alam, U.; Cuthbertson, D. J., The impact of macronutrient intake on non-alcoholic fatty liver disease (NAFLD): too much fat, too much carbohydrate, or just too many calories? *Frontiers in nutrition* 2021, 8, 640557.
111. Mayans, L., Metabolic syndrome: insulin resistance and prediabetes. *FP essentials* 2015, 435, 11–16.
112. Cavaghan, M. K.; Ehrmann, D. A.; Polonsky, K. S., Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. *The Journal of clinical investigation* 2000, 106, (3), 329–333.
113. Boden, G., 45Obesity, insulin resistance and free fatty acids. *Current opinion in endocrinology, diabetes, and obesity* 2011, 18, (2), 139.
114. TAHARBOUCHT, S.; GUERMAZ, R.; BROURI, M.; CHIBANE, A., Pre-diabetes and NAFLD; a study of an Algerian population sample. *Endocrine and Metabolic Science* 2020, 1, (3-4), 100060.
115. Petersen, M. C.; Vatner, D. F.; Shulman, G. I., Regulation of hepatic glucose metabolism in health and disease. *Nature reviews endocrinology* 2017, 13, (10), 572–587.
116. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Plant-derived oleanolic acid ameliorates

- markers associated with non-alcoholic fatty liver disease in a diet-induced pre-diabetes rat model. *Diabetes, Metabolic Syndrome and Obesity* 2019, 1953–1962.
117. Nguyen, Q. M.; Srinivasan, S. R.; Xu, J.-H.; Chen, W.; Hassig, S.; Rice, J.; Berenson, G. S., Elevated liver function enzymes are related to the development of prediabetes and type 2 diabetes in younger adults: the Bogalusa Heart Study. *Diabetes care* 2011, 34, (12), 2603–2607.
 118. Krisnamurti, D.; Purwaningsih, E.; Tarigan, T.; Nugroho, C.; Soetikno, V.; Louisa, M., Alterations of Liver Functions and Morphology in a Rat Model of Prediabetes After a Short-term Treatment of a High-fat High-glucose and Low-dose Streptozotocin. *Open Access Maced J Med Sci. Scientific Foundation SPIROSKI*; 2022; 10 (A): 668–74. In 2022.
 119. Faramarzi, E.; Mehrtabar, S.; Molani-Gol, R.; Dastgiri, S., The relationship between hepatic enzymes, prediabetes, and diabetes in the Azar cohort population. *BMC Endocrine Disorders* 2025, 25, (1), 41.
 120. Bril, F.; Portillo Sanchez, P.; Lomonaco, R.; Orsak, B.; Hecht, J.; Tio, F.; Cusi, K., Liver safety of statins in prediabetes or T2DM and nonalcoholic steatohepatitis: post hoc analysis of a randomized trial. *The Journal of Clinical Endocrinology & Metabolism* 2017, 102, (8), 2950–2961.
 121. Dinarvand, N.; Cheraghian, B.; Rahimi, Z.; Salehipour Bavarsad, S.; Bavarsad, A.; Mohammadtaghvaei, N., Examining dyslipidaemia, metabolic syndrome and liver enzyme levels in patients with prediabetes and type 2 diabetes in population from Hoveyzeh cohort study: a case–control study in Iran. *Endocrinology, Diabetes & Metabolism* 2023, 6, (2), e401.
 122. Xu, X.; Poulsen, K. L.; Wu, L.; Liu, S.; Miyata, T.; Song, Q.; Wei, Q.; Zhao, C.; Lin, C.; Yang, J., Targeted therapeutics and novel signaling pathways in non-alcohol-associated fatty liver/steatohepatitis (NAFL/NASH). *Signal transduction and targeted therapy* 2022, 7, (1), 287.
 123. Samy, A. M.; Kandeil, M. A.; Sabry, D.; Abdel-Ghany, A.; Mahmoud, M. O., From NAFLD to NASH: Understanding the spectrum of non-alcoholic liver diseases and their consequences. *Heliyon* 2024, 10, (9).
 124. Kushkestanti, M.; Parvani, M.; Nosrani, S. E.; Rezaei, S.; Karimi, M., Lipid Profile and hepatic enzymes differences between pre-diabetes and normal staff. *Journal of Sports Science* 2020, 8, 67–75.
 125. Chen, L.; Deng, H.; Cui, H.; Fang, J.; Zuo, Z.; Deng, J.; Li, Y.; Wang, X.; Zhao, L., Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget* 2017, 9, (6), 7204.
 126. Inoguchi, T.; Sonoda, N.; Maeda, Y., Bilirubin as an important physiological modulator of oxidative stress and chronic inflammation in metabolic syndrome and diabetes: a new aspect on old molecule. *Diabetology international* 2016, 7, (4), 338–341.
 127. Koenig, A. B.; Tan, A.; Abdelaal, H.; Monge, F.; Younossi, Z. M.; Goodman, Z. D., Hepatic steatosis and its associations with acute and chronic liver diseases. *Alimentary pharmacology &*

therapeutics 2024, 60, (2), 167–200.

128. Burgeiro, A.; Cerqueira, M. G.; Varela-Rodríguez, B. M.; Nunes, S.; Neto, P.; Pereira, F. C.; Reis, F.; Carvalho, E., Glucose and lipid dysmetabolism in a rat model of prediabetes induced by a high-sucrose diet. *Nutrients* 2017, 9, (6), 638.
129. Van Herck, M. A.; Vonghia, L.; Francque, S. M., Animal models of nonalcoholic fatty liver disease—a starter’s guide. *Nutrients* 2017, 9, (10), 1072.
130. Flessa, C.-M.; Nasiri-Ansari, N.; Kyrou, I.; Leca, B. M.; Lianou, M.; Chatzigeorgiou, A.; Kaltsas, G.; Kassi, E.; Randeva, H. S., Genetic and diet-induced animal models for non-alcoholic fatty liver disease (NAFLD) research. *International Journal of Molecular Sciences* 2022, 23, (24), 15791.
131. Ahmadizar, F.; Younossi, Z. M., Exploring biomarkers in nonalcoholic fatty liver disease among individuals with type 2 diabetes mellitus. *Journal of clinical gastroenterology* 2024, 59, (1), 36.
132. Okada, R.; Yasuda, Y.; Tsushita, K.; Wakai, K.; Hamajima, N.; Matsuo, S., Glomerular hyperfiltration in prediabetes and prehypertension. *Nephrology Dialysis Transplantation* 2012, 27, (5), 1821–1825.
133. Palatini, P., Glomerular hyperfiltration: a marker of early renal damage in pre-diabetes and pre-hypertension. In Oxford University Press: 2012; Vol. 27, pp 1708–1714.
134. Barutta, F.; Bellini, S.; Gruden, G., Mechanisms of podocyte injury and implications for diabetic nephropathy. *Clinical Science* 2022, 136, (7), 493–520.
135. Melsom, T.; Schei, J.; Stefansson, V. T. N.; Solbu, M. D.; Jenssen, T. G.; Mathisen, U. D.; Wilsgaard, T.; Eriksen, B. O., Prediabetes and risk of glomerular hyperfiltration and albuminuria in the general nondiabetic population: a prospective cohort study. *American Journal of Kidney Diseases* 2016, 67, (6), 841–850.
136. Claudel, S. E.; Verma, A., Albuminuria in cardiovascular, kidney, and metabolic disorders: a state-of-the-art review. *Circulation* 2025, 151, (10), 716–732.
137. Shilpasree, A. S.; Patil, V. S.; Revanasiddappa, M.; Patil, V. P.; Ireshnavar, D., Renal Dysfunction in Prediabetes: Confirmed by Glomerular Hyperfiltration and Albuminuria. *J Lab Physicians* 2021, 13, (3), 257–262.
138. Narongkiatikhun, P.; Bjornstad, P., Unmasking the hidden burden of prediabetes on kidney function. *Kidney International* 2025, 108, (5), 751–753.
139. Noeman, S. A.; Hamooda, H. E.; Baalash, A. A., Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats. *Diabetology & metabolic syndrome* 2011, 3, (1), 17.
140. Echouffo-Tcheugui, J.; Narayan, K.; Weisman, D.; Golden, S.; Jaar, B., Association between prediabetes and risk of chronic kidney disease: a systematic review and meta-analysis. *Diabetic Medicine* 2016, 33, (12), 1615–1624.

141. Tonneijck, L.; Muskiet, M. H.; Smits, M. M.; van Bommel, E. J.; Heerspink, H. J.; van Raalte, D. H.; Joles, J. A., Glomerular Hyperfiltration in Diabetes: Mechanisms, Clinical Significance, and Treatment. *J Am Soc Nephrol* 2017, 28, (4), 1023–1039.
142. Melsom, T.; Brobak, K. M.; Norvik, J. V.; Enoksen, I. T.; Rinde, L.; Jenssen, T. G.; Eriksen, B. O., Iohexol clearance, but not estimated GFR, reveals a steeper GFR decline in patients with prediabetes. *Kidney International* 2025, 108, (5), 938–947.
143. Di Pino, A.; Scicali, R.; Marchisello, S.; Zanolì, L.; Ferrara, V.; Urbano, F.; Filippello, A.; Di Mauro, S.; Scamporrino, A.; Piro, S., High glomerular filtration rate is associated with impaired arterial stiffness and subendocardial viability ratio in prediabetic subjects. *Nutrition, Metabolism and Cardiovascular Diseases* 2021, 31, (12), 3393–3400.
144. Wang, X.; Huang, C.; Liu, Y.; Han, Y.; Hu, H., Association of estimated glomerular filtration rate and incident pre-diabetes: a secondary 5-year longitudinal cohort study in Chinese people. *Frontiers in Endocrinology* 2022, 13, 965545.
145. Premaratne, E.; Verma, S.; Ekinçi, E. I.; Theverkalam, G.; Jerums, G.; MacIsaac, R. J., The impact of hyperfiltration on the diabetic kidney. *Diabetes & metabolism* 2015, 41, (1), 5–17.
146. Wu, T.; Ding, L.; Andoh, V.; Zhang, J.; Chen, L., The mechanism of hyperglycemia-induced renal cell injury in diabetic nephropathy disease: an update. *Life* 2023, 13, (2), 539.
147. Dănilă, A.-I.; Ghenciu, L. A.; Stoicescu, E. R.; Bolintineanu, S. L.; Iacob, R.; Săndesc, M.-A.; Faur, A. C., Aldose reductase as a key target in the prevention and treatment of diabetic retinopathy: a comprehensive review. *Biomedicines* 2024, 12, (4), 747.
148. Paneque, A.; Fortus, H.; Zheng, J.; Werlen, G.; Jacinto, E., The Hexosamine biosynthesis pathway: regulation and function. *Genes (Basel)* 14. In 2023.
149. Mkhize, B. C.; Mosili, P.; Ngubane, P. S.; Sibiya, N. H.; Khathi, A., Diet-induced prediabetes: Effects on the activity of the renin–angiotensin–aldosterone system in selected organs. *Journal of Diabetes Investigation* 2022, 13, (5), 768–780.
150. Heyman, S. N.; Raz, I.; Dwyer, J. P.; Weinberg Sibony, R.; Lewis, J. B.; Abassi, Z., Diabetic proteinuria revisited: updated physiologic perspectives. *Cells* 2022, 11, (18), 2917.
151. Yang, H.; Chen, Y.; He, J.; Li, Y.; Feng, Y., Advances in the diagnosis of early biomarkers for acute kidney injury: a literature review. *BMC nephrology* 2025, 26, (1), 115.
152. Mainous III, A. G.; Tanner, R. J.; Scuderi, C. B.; Porter, M.; Carek, P. J., Prediabetes screening and treatment in diabetes prevention: the impact of physician attitudes. *The Journal of the American Board of Family Medicine* 2016, 29, (6), 663–671.
153. Martín-Peláez, S.; Fito, M.; Castaner, O., Mediterranean diet effects on type 2 diabetes prevention, disease progression, and related mechanisms. A review. *Nutrients* 2020, 12, (8), 2236.
154. Scaglione, S.; Di Chiara, T.; Daidone, M.; Tuttolomondo, A., Effects of the Mediterranean diet

on the components of metabolic syndrome concerning the cardiometabolic risk. *Nutrients* 2025, 17, (2), 358.

155. Torres-Peña, J. D.; Arenas-de Larriva, A. P.; Alcala-Diaz, J. F.; Lopez-Miranda, J.; Delgado-Lista, J., Different dietary approaches, non-alcoholic fatty liver disease and cardiovascular disease: a literature review. *Nutrients* 2023, 15, (6), 1483.

156. Tuso, P., Prediabetes and lifestyle modification: time to prevent a preventable disease. *The Permanente Journal* 2014, 18, (3), 88.

157. Kourek, C.; Karatzanos, E.; Raidou, V.; Papazachou, O.; Philippou, A.; Nanas, S.; Dimopoulos, S., Effectiveness of high intensity interval training on cardiorespiratory fitness and endothelial function in type 2 diabetes: a systematic review. *World Journal of Cardiology* 2023, 15, (4), 184.

158. Li, R.-Y.; Guo, L., Exercise in diabetic nephropathy: protective effects and molecular mechanism. *International Journal of Molecular Sciences* 2024, 25, (7), 3605.

159. Xue, Y.; Peng, Y.; Zhang, L.; Ba, Y.; Jin, G.; Liu, G., Effect of different exercise modalities on nonalcoholic fatty liver disease: a systematic review and network meta-analysis. *Scientific reports* 2024, 14, (1), 6212.

160. Bertram, M.; Lim, S.; Barendregt, J.; Vos, T., Assessing the cost-effectiveness of drug and lifestyle intervention following opportunistic screening for pre-diabetes in primary care. *Diabetologia* 2010, 53, (5), 875–881.

161. Glechner, A.; Keuchel, L.; Affengruber, L.; Titscher, V.; Sommer, I.; Matyas, N.; Wagner, G.; Kien, C.; Klerings, I.; Gartlehner, G., Effects of lifestyle changes on adults with prediabetes: A systematic review and meta-analysis. *Primary care diabetes* 2018, 12, (5), 393–408.

162. Bennasar-Veny, M.; Fresneda, S.; López-González, A.; Busquets-Cortés, C.; Aguiló, A.; Yañez, A. M., Lifestyle and progression to type 2 diabetes in a cohort of workers with prediabetes. *Nutrients* 2020, 12, (5), 1538.

163. Rezaei, M.; Valiee, S.; Tahan, M.; Ebtekar, F.; Ghanei Gheshlagh, R., Barriers of medication adherence in patients with type-2 diabetes: a pilot qualitative study. *Diabetes, Metabolic Syndrome and Obesity* 2019, 589–599.

164. Shibayama, K.; Furushima, C.; Saka, M.; Sakamoto, T.; Takahashi, H., Barriers to lifestyle modification in patients with non-alcoholic fatty liver disease: a scoping review. *Journal of Rural Medicine* 2024, 19, (1), 1–9.

165. Herman, R.; Kravos, N. A.; Jensterle, M.; Janež, A.; Dolžan, V., Metformin and insulin resistance: a review of the underlying mechanisms behind changes in GLUT4-mediated glucose transport. *International journal of molecular sciences* 2022, 23, (3), 1264.

166. Li, M.; Hu, X.; Xu, Y.; Hu, X.; Zhang, C.; Pang, S., A possible mechanism of metformin in improving insulin resistance in diabetic rat models. *International journal of endocrinology* 2019,

2019, (1), 3248527.

167. Gillies, C. L.; Abrams, K. R.; Lambert, P. C.; Cooper, N. J.; Sutton, A. J.; Hsu, R. T.; Khunti, K., Pharmacological and lifestyle interventions to prevent or delay type 2 diabetes in people with impaired glucose tolerance: systematic review and meta-analysis. *Bmj* 2007, 334, (7588), 299.

168. Zhou, G.; Myers, R.; Li, Y.; Chen, Y.; Shen, X.; Fenyk-Melody, J.; Wu, M.; Ventre, J.; Doebber, T.; Fujii, N., Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of clinical investigation* 2001, 108, (8), 1167–1174.

169. Dutta, S.; Shah, R. B.; Singhal, S.; Dutta, S. B.; Bansal, S.; Sinha, S.; Haque, M., Metformin: a review of potential mechanism and therapeutic utility beyond diabetes. *Drug design, development and therapy* 2023, 1907–1932.

170. Cicero, A. F.; Tartagni, E.; Ertek, S., Metformin and its clinical use: new insights for an old drug in clinical practice. *Archives of medical science: AMS* 2012, 8, (5), 907.

171. Bu, Y.; Peng, M.; Tang, X.; Xu, X.; Wu, Y.; Chen, A. F.; Yang, X., Protective effects of metformin in various cardiovascular diseases: clinical evidence and AMPK-dependent mechanisms. *Journal of Cellular and Molecular Medicine* 2022, 26, (19), 4886–4903.

172. Hsu, W.-H.; Hsiao, P.-J.; Lin, P.-C.; Chen, S.-C.; Lee, M.-Y.; Shin, S.-J., Effect of metformin on kidney function in patients with type 2 diabetes mellitus and moderate chronic kidney disease. *Oncotarget* 2017, 9, (4), 5416.

173. Siboto, A.; Akinnuga, A. M.; Khumalo, B.; Ismail, M. B.; Booysen, I. N.; Sibiya, N. H.; Ngubane, P.; Khathi, A., Ameliorative Effects of a Rhenium (V) Compound with Uracil-Derived Ligand Markers Associated with Hyperglycaemia-Induced Renal Dysfunction in Diet-Induced Prediabetic Rats. *International Journal of Molecular Sciences* 2022, 23, (23), 15400.

174. Inzucchi, S. E.; Lipska, K. J.; Mayo, H.; Bailey, C. J.; McGuire, D. K., Metformin in patients with type 2 diabetes and kidney disease: a systematic review. *Jama* 2014, 312, (24), 2668–2675.

175. Pinyopornpanish, K.; Leerapun, A.; Pinyopornpanish, K.; Chattipakorn, N., Effects of metformin on hepatic steatosis in adults with nonalcoholic fatty liver disease and diabetes: insights from the cellular to patient levels. *Gut and liver* 2021, 15, (6), 827.

176. Fujita, Y.; Hosokawa, M.; Fujimoto, S.; Mukai, E.; Abudukadier, A.; Obara, A.; Ogura, M.; Nakamura, Y.; Toyoda, K.; Nagashima, K., Metformin suppresses hepatic gluconeogenesis and lowers fasting blood glucose levels through reactive nitrogen species in mice. *Diabetologia* 2010, 53, (7), 1472–1481.

177. Bosi, E., Metformin—the gold standard in type 2 diabetes: what does the evidence tell us? *Diabetes, Obesity and Metabolism* 2009, 11, 3–8.

178. Shaw, J. E., Prediabetes: lifestyle, pharmacotherapy or regulation? *Therapeutic Advances in Endocrinology and Metabolism* 2019, 10, 2042018819863020.

179. Lebovitz, H. E., Thiazolidinediones: the forgotten diabetes medications. *Current diabetes reports* 2019, 19, (12), 151.
180. Raveendran, A. V.; Fernandez, C. J.; Jacob, K., Efficacy and cardiovascular safety of thiazolidinediones. *Current Drug Safety* 2021, 16, (2), 233–249.
181. Le, R.; Nguyen, M. T.; Allahwala, M. A.; Psaltis, J. P.; Marathe, C. S.; Marathe, J. A.; Psaltis, P. J., Cardiovascular protective properties of GLP-1 receptor agonists: more than just diabetic and weight loss drugs. *Journal of clinical medicine* 2024, 13, (16), 4674.
182. Raza, F. A.; Altaf, R.; Bashir, T.; Asghar, F.; Altaf, R.; Tousif, S.; Goyal, A.; Mohammed, A.; Mohammad, M. F.; Anan, M., Effect of GLP-1 receptor agonists on weight and cardiovascular outcomes: A review. *Medicine* 2024, 103, (44), e40364.
183. Hamed, K.; Alosaimi, M. N.; Ali, B. A.; Alghamdi, A.; Alkhashi, T.; Alkhaldi, S. S.; Altowarqi, N. A.; Alzahrani, H.; Alshehri, A. M.; Alkhaldi, R. K., Glucagon-like peptide-1 (GLP-1) receptor agonists: exploring their impact on diabetes, obesity, and cardiovascular health through a comprehensive literature review. *Cureus* 2024, 16, (9).
184. Dandona, P.; Chaudhuri, A., Sodium-glucose co-transporter 2 inhibitors for type 2 diabetes mellitus: An overview for the primary care physician. *International journal of clinical practice* 2017, 71, (5), e12937.
185. Khan, R. M. M.; Chua, Z. J. Y.; Tan, J. C.; Yang, Y.; Liao, Z.; Zhao, Y., From pre-diabetes to diabetes: diagnosis, treatments and translational research. *Medicina* 2019, 55, (9), 546.
186. Galaviz, K. I.; Suvada, K.; Merchant, R.; Dharanendra, S.; Haw, J. S.; Narayan, K. V.; Ali, M. K., Interventions for reversing prediabetes: a systematic review and meta-analysis. *American Journal of Preventive Medicine* 2022, 62, (4), 614–625.
187. Panche, A. N.; Diwan, A. D.; Chandra, S. R., Flavonoids: an overview. *Journal of nutritional science* 2016, 5, e47.
188. Dias, M. C.; Pinto, D. C.; Silva, A. M., Plant flavonoids: Chemical characteristics and biological activity. *Molecules* 2021, 26, (17), 5377.
189. Kumar, A.; P, N.; Kumar, M.; Jose, A.; Tomer, V.; Oz, E.; Proestos, C.; Zeng, M.; Elobeid, T.; K, S., Major phytochemicals: recent advances in health benefits and extraction method. *Molecules* 2023, 28, (2), 887.
190. Nicolle, E.; Souard, F.; Faure, P.; Boumendjel, A., Flavonoids as promising lead compounds in type 2 diabetes mellitus: molecules of interest and structure-activity relationship. *Current medicinal chemistry* 2011, 18, (17), 2661–2672.
191. Chen, L.; Cao, H.; Huang, Q.; Xiao, J.; Teng, H., Absorption, metabolism and bioavailability of flavonoids: A review. *Critical reviews in food science and nutrition* 2022, 62, (28), 7730–7742.
192. Hu, L.; Luo, Y.; Yang, J.; Cheng, C., Botanical flavonoids: efficacy, absorption, metabolism and

- advanced pharmaceutical technology for improving bioavailability. *Molecules* 2025, 30, (5), 1184.
193. Aguirre, L.; Arias, N.; Macarulla, M. T.; Gracia, A.; Portillo, M. P., Beneficial effects of quercetin on obesity and diabetes. *Open Nutraceuticals J* 2011, 4, (1), 189–198.
 194. Michala, A.-S.; Pritsa, A., Quercetin: a molecule of great biochemical and clinical value and its beneficial effect on diabetes and cancer. *Diseases* 2022, 10, (3), 37.
 195. Miltonprabu, S.; Tomczyk, M.; Skalicka-Woźniak, K.; Rastrelli, L.; Daglia, M.; Nabavi, S. F.; Alavian, S. M.; Nabavi, S. M., Hepatoprotective effect of quercetin: From chemistry to medicine. *Food and Chemical Toxicology* 2017, 108, 365–374.
 196. Gnoni, A.; Di Chiara Stanca, B.; Giannotti, L.; Gnoni, G. V.; Siculella, L.; Damiano, F., Quercetin reduces lipid accumulation in a cell model of NAFLD by inhibiting de novo fatty acid synthesis through the acetyl-CoA carboxylase 1/AMPK/PP2A axis. *International Journal of Molecular Sciences* 2022, 23, (3), 1044.
 197. Ansari, P.; Choudhury, S. T.; Seidel, V.; Rahman, A. B.; Aziz, M. A.; Richi, A. E.; Rahman, A.; Jafrin, U. H.; Hannan, J.; Abdel-Wahab, Y. H., Therapeutic potential of quercetin in the management of type-2 diabetes mellitus. *Life* 2022, 12, (8), 1146.
 198. Ozgen, S.; Kilinc, O. K.; Selamoğlu, Z., Antioxidant activity of quercetin: a mechanistic review. *Turkish Journal of Agriculture-Food Science and Technology* 2016, 4, (12), 1134–1138.
 199. Lin, H.-H., In vitro and in vivo atheroprotective effects of gossypetin against endothelial cell injury by induction of autophagy. *Chemical Research in Toxicology* 2015, 28, (2), 202–215.
 200. Ijaz, M. U.; Alvi, K.; Khan, H. A.; Imran, M.; Afsar, T.; Almajwal, A.; Amor, H.; Razak, S., Gossypetin mitigates doxorubicin-induced nephrotoxicity: A histopathological and biochemical evaluation. *Journal of King Saud University-Science* 2023, 35, (7), 102830.
 201. Proença, C.; Rufino, A. T.; Santos, I.; Albuquerque, H. M.; Silva, A. M.; Fernandes, E.; Ferreira de Oliveira, J. M. P., Gossypetin is a novel modulator of inflammatory cytokine production and a suppressor of osteosarcoma cell growth. *Antioxidants* 2023, 12, (9), 1744.
 202. Oh, E.; Lee, J.; Cho, S.; Kim, S. W.; Jo, K. W.; Shin, W. S.; Gwak, S. H.; Ha, J.; Jeon, S. Y.; Park, J.-H., Gossypetin prevents the progression of nonalcoholic steatohepatitis by regulating oxidative stress and AMP-activated protein kinase. *Molecular Pharmacology* 2023, 104, (5), 214–229.
 203. Montalvo-González, E.; Villagrán, Z.; González-Torres, S.; Iñiguez-Muñoz, L. E.; Isiordia-Espinoza, M. A.; Ruvalcaba-Gómez, J. M.; Arteaga-Garibay, R. I.; Acosta, J. L.; González-Silva, N.; Anaya-Esparza, L. M., Physiological effects and human health benefits of Hibiscus sabdariffa: a review of clinical trials. *Pharmaceuticals* 2022, 15, (4), 464.
 204. Gao, S.-T.; Xin, X.; Wang, Z.-y.; Hu, Y.-y.; Feng, Q., USP5: Comprehensive insights into structure, function, biological and disease-related implications, and emerging therapeutic opportunities. *Molecular and Cellular Probes* 2024, 73, 101944.

205. Bachar, S. C.; Bachar, R.; Jannat, K.; Jahan, R.; Rahmatullah, M., Hepatoprotective natural products. *Annual reports in medicinal chemistry* 2020, 55, 207–249.
206. Khan, A.; Manna, K.; Das, D. K.; Kesh, S. B.; Sinha, M.; Das, U.; Biswas, S.; Sengupta, A.; Sikder, K.; Datta, S., Gossypetin ameliorates ionizing radiation-induced oxidative stress in mice liver—a molecular approach. *Free Radical Research* 2015, 49, (10), 1173–1186.
207. Samant, N. P.; Gupta, G. L., Gossypetin-based therapeutics for cognitive dysfunction in chronic unpredictable stress-exposed mice. *Metabolic Brain Disease* 2022, 37, (5), 1527–1539.
208. Dubey, R.; Prabhakar, P. K.; Gupta, J., Identification of structurally similar phytochemicals to quercetin with high sirt1 binding affinity and improving diabetic wound healing by using in silico approaches. *Biointerface Res. Appl. Chem* 2021, 12, 7621–7632.
209. Samuel, V. T.; Shulman, G. I., The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. *The Journal of clinical investigation* 2016, 126, (1), 12–22.
210. Tang, W.; Liu, H.; Li, X.; Deng, S.; Gao, C., Influence and treatment of insulin receptor substrate/PI3K/Akt-mediated insulin resistance in diabetes mellitus. *Molecular medicine reports* 2025, 33, (2), 63.
211. Huang, X.; Liu, G.; Guo, J.; Su, Z., The PI3K/AKT pathway in obesity and type 2 diabetes. *International journal of biological sciences* 2018, 14, (11), 1483.
212. Russo, B.; Picconi, F.; Malandrucchio, I.; Frontoni, S., Flavonoids and insulin-resistance: from molecular evidences to clinical trials. *International journal of molecular sciences* 2019, 20, (9), 2061.
213. Naseri, K.; Saadati, S.; Ghaemi, F.; Ashtary-Larky, D.; Asbaghi, O.; Sadeghi, A.; Afrisham, R.; de Courten, B., The effects of probiotic and synbiotic supplementation on inflammation, oxidative stress, and circulating adiponectin and leptin concentration in subjects with prediabetes and type 2 diabetes mellitus: A GRADE-assessed systematic review, meta-analysis, and meta-regression of randomized clinical trials. *European journal of nutrition* 2023, 62, (2), 543–561.
214. Rauf, A.; Khalil, A. A.; Awadallah, S.; Khan, S. A.; Abu-Izneid, T.; Kamran, M.; Hemeg, H. A.; Mubarak, M. S.; Khalid, A.; Wilairatana, P., Reactive oxygen species in biological systems: Pathways, associated diseases, and potential inhibitors—A review. *Food science & nutrition* 2024, 12, (2), 675–693.
215. Zhao, X.; An, X.; Yang, C.; Sun, W.; Ji, H.; Lian, F., The crucial role and mechanism of insulin resistance in metabolic disease. *Frontiers in endocrinology* 2023, 14, 1149239.
216. Liu, S.; Liu, J.; Wang, Y.; Deng, F.; Deng, Z., Oxidative stress: signaling pathways, biological functions, and disease. *MedComm* 2025, 6, (7), e70268.
217. Moratilla-Rivera, I.; Sánchez, M.; Valdés-González, J. A.; Gómez-Serranillos, M. P., Natural products as modulators of Nrf2 signaling pathway in neuroprotection. *International Journal of Molecular Sciences* 2023, 24, (4), 3748.

218. L. Suraweera, T.; Rupasinghe, H. V.; Dellaire, G.; Xu, Z., Regulation of Nrf2/ARE pathway by dietary flavonoids: a friend or foe for cancer management? *Antioxidants* 2020, 9, (10), 973.
219. Veluthakal, R.; Esparza, D.; Hoolachan, J. M.; Balakrishnan, R.; Ahn, M.; Oh, E.; Jayasena, C. S.; Thurmond, D. C., Mitochondrial dysfunction, oxidative stress, and inter-organ miscommunications in T2D progression. *International journal of molecular sciences* 2024, 25, (3), 1504.
220. Prasun, P., Mitochondrial dysfunction in metabolic syndrome. *Biochimica et biophysica acta (BBA)-Molecular basis of disease* 2020, 1866, (10), 165838.
221. Ungurianu, A.; Zanfirescu, A.; Margină, D., Exploring the therapeutic potential of quercetin: A focus on its sirtuin-mediated benefits. *Phytotherapy Research* 2024, 38, (5), 2361–2387.
222. Jo, K. W.; Lee, D.; Cha, D. G.; Oh, E.; Choi, Y. H.; Kim, S.; Park, E. S.; Kim, J. K.; Kim, K.-T., Gossypetin ameliorates 5xFAD spatial learning and memory through enhanced phagocytosis against A β . *Alzheimer's research & therapy* 2022, 14, (1), 158.
223. Huang, K.; Liu, Z.; Kim, M.-O.; Kim, K.-R., Anticancer effects of gossypetin from Hibiscus sabdariffa in oral squamous cell carcinoma. *Journal of Applied Oral Science* 2023, 31, e20230243.
224. Lin, H.-H.; Hsieh, M.-C.; Wang, C.-P.; Yu, P.-R.; Lee, M.-S.; Chen, J.-H., Anti-atherosclerotic effect of gossypetin on abnormal vascular smooth muscle cell proliferation and migration. *Antioxidants* 2021, 10, (9), 1357.
225. Gulsheen; Kumar, A.; Sharma, A., Antianxiety and antidepressant activity guided isolation and characterization of gossypetin from Hibiscus sabdariffa Linn. calyces. *Journal of Biologically Active Products from Nature* 2019, 9, (3), 205–214.
226. Mustafa, S.; Anwar, H.; Ain, Q. U.; Ahmed, H.; Iqbal, S.; Ijaz, M. U., Therapeutic effect of gossypetin against paraquat-induced testicular damage in male rats: a histological and biochemical study. *Environmental Science and Pollution Research* 2023, 30, (22), 62237–62248.
227. Leal, J.; Morrow, L. M.; Khurshid, W.; Pagano, E.; Feenstra, T., Decision models of prediabetes populations: a systematic review. *Diabetes, Obesity and Metabolism* 2019, 21, (7), 1558–1569.
228. Tripathi, V.; Verma, J., Different models used to induce diabetes: a comprehensive review. *Int J Pharm Pharm Sci* 2014, 6, (6), 29–32.
229. Wilson, R. D.; Islam, M. S., Fructose-fed streptozotocin-injected rat: an alternative model for type 2 diabetes. *Pharmacological reports* 2012, 64, (1), 129–139.
230. Ble-Castillo, J. L.; Aparicio-Trapala, M. A.; Juárez-Rojop, I. E.; Torres-Lopez, J. E.; Mendez, J. D.; Aguilar-Mariscal, H.; Olvera-Hernández, V.; Palma-Cordova, L. C.; Diaz-Zagoya, J. C., Differential effects of high-carbohydrate and high-fat diet composition on metabolic control and insulin resistance in normal rats. *International journal of environmental research and public health* 2012, 9, (5), 1663–1676.
231. Panchal, S. K.; Brown, L., Rodent models for metabolic syndrome research. *BioMed Research*

International 2011, 2011, (1), 351982.

232. Pinyo, J.; Hira, T.; Hara, H., Continuous feeding of a combined high-fat and high-sucrose diet, rather than an individual high-fat or high-sucrose diet, rapidly enhances the glucagon-like peptide-1 secretory response to meal ingestion in diet-induced obese rats. *Nutrition* 2019, 62, 122–130.

233. Shakya, A.; Chaudary, S. K.; Garabadu, D.; Bhat, H. R.; Kakoti, B. B.; Ghosh, S. K., A comprehensive review on preclinical diabetic models. *Current diabetes reviews* 2020, 16, (2), 104–116.

234. Lemos, C.; Rial, D.; Gonçalves, F.; Pires, J.; Silva, H.; Matheus, F.; Da Silva, A.; Marques, J.; Rodrigues, R.; Jarak, I., High sucrose consumption induces memory impairment in rats associated with electrophysiological modifications but not with metabolic changes in the hippocampus. *Neuroscience* 2016, 315, 196–205.

235. Melo, B. P.; Zacarias, A. C.; Oliveira, J. C. C.; de Souza, L. M. C.; Sabino, J.; Ferreira, A. V. M.; Tonoli, C.; Dos Santos, M. L.; de Avelar, G. F.; Meeusen, R., Thirty days of combined consumption of a high-fat diet and fructose-rich beverages promotes insulin resistance and modulates inflammatory response and histomorphometry parameters of liver, pancreas, and adipose tissue in Wistar rats. *Nutrition* 2021, 91, 111403.

236. Messier, C.; Whately, K.; Liang, J.; Du, L.; Puissant, D., The effects of a high-fat, high-fructose, and combination diet on learning, weight, and glucose regulation in C57BL/6 mice. *Behavioural brain research* 2007, 178, (1), 139–145.

237. Coate, K. C.; Kraft, G.; Lautz, M.; Smith, M.; Neal, D. W.; Cherrington, A. D., A high-fat, high-fructose diet accelerates nutrient absorption and impairs net hepatic glucose uptake in response to a mixed meal in partially pancreatectomized dogs. *The Journal of nutrition* 2011, 141, (9), 1643–1651.

238. Balakumar, M.; Raji, L.; Prabhu, D.; Sathishkumar, C.; Prabu, P.; Mohan, V.; Balasubramanyam, M., High-fructose diet is as detrimental as high-fat diet in the induction of insulin resistance and diabetes mediated by hepatic/pancreatic endoplasmic reticulum (ER) stress. *Molecular and cellular biochemistry* 2016, 423, (1), 93–104.

239. Wei, X.; Li, X.; Hu, S.; Cheng, J.; Cai, R., Regulation of ferroptosis in lung adenocarcinoma. *International journal of molecular sciences* 2023, 24, (19), 14614.

240. Shah, M.; Garg, A., High-fat and high-carbohydrate diets and energy balance. *Diabetes Care* 1996, 19, (10), 1142–1152.

241. Borse, S. P.; Chhipa, A. S.; Sharma, V.; Singh, D. P.; Nivsarkar, M., Management of type 2 diabetes: current strategies, unfocussed aspects, challenges, and alternatives. *Medical Principles and Practice* 2021, 30, (2), 109–121.

242. Echouffo-Tcheugui, J. B.; Perreault, L.; Ji, L.; Dagogo-Jack, S., Diagnosis and management of prediabetes: a review. *Jama* 2023, 329, (14), 1206–1216.

243. Cruz, K. J. C.; de Oliveira, A. R. S.; do Nascimento Marreiro, D., Antioxidant role of zinc in diabetes mellitus. *World journal of diabetes* 2015, 6, (2), 333.



Review

The Potential Role of Gossypetin in the Treatment of Diabetes Mellitus and Its Associated Complications: A Review

Karishma Naidoo and Andile Khathi*

Department of Human Physiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, 4000, South Africa; 217000603@stu.ukzn.ac.za

* Correspondence: khathia@ukzn.ac.za Tel.: +31-260-7132

Abstract: Type 2 diabetes mellitus (T2DM) is a metabolic disorder caused by *insulin resistance* and dysfunctional beta (β)-cells in the pancreas. Hyperglycaemia is a characteristic of uncontrolled diabetes which eventually leads to fatal organ system damage. In T2DM, free radicals are continuously produced, causing extensive tissue damage and subsequent macro- and microvascular complications. The standard approach to managing T2DM is pharmacological treatment with anti-diabetic medications. However, patients' adherence to treatment is frequently decreased by the side effects and expense of medications, which has a detrimental impact on their health outcomes. Quercetin, a flavonoid, is one of the most potent anti-oxidants which ameliorates T2DM. Thus, there is an increased demand to investigate quercetin and its derivatives, as it is hypothesised that similar structured compounds may exhibit similar biological activity. *Gossypetin* is a hexahydroxylated flavonoid found in the calyx of *Hibiscus sabdariffa*. Gossypetin has a similar chemical structure to quercetin with an extra hydroxyl group. Furthermore, previous literature has elucidated that gossypetin exhibits neuroprotective, hepatoprotective, reprotoective and nephroprotective properties. The mechanisms underlying gossypetin's therapeutic potential have been linked to its anti-oxidant, anti-inflammatory and immunomodulatory properties. Hence, this review highlights the potential role of gossypetin in the treatment of diabetes and its associated complications.

Keywords: gossypetin; diabetes mellitus; flavonoid

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1. Introduction

Approximately 90% of all cases of diabetes are type 2 diabetes mellitus (T2DM) [1]. The global prevalence of T2DM in adults was 536.6 million in 2021, and by 2045, 783.2 million people are expected to have

the condition, according to the International Diabetes Federation (IDF) (Figure 1) [2, 3]. The response to insulin is diminished in T2DM, which is characterised as insulin resistance [4]. It has been demonstrated that macro- and microvascular complications such as atherosclerosis, stroke, kidney failure and non-alcoholic fatty liver disease (NAFLD) are associated with diabetes mellitus (DM) [5, 6]. Oxidative stress has been demonstrated to be an essential component in the pathophysiology of these complications [7]. Unfortunately, conventional medical treatments for T2DM exhibit unfavourable side effects, such as adverse reactions in the gastrointestinal tract and damage to the kidneys and liver [8].

There has been increased global interest currently to identify anti-oxidant compounds that are pharmacologically potent with little or no side effects [9]. As the primary source of anti-oxidants, flavonoids have emerged as an effective tool to combat against oxidative stress [10]. According to previous studies, one of the most prevalent flavonols derived from plants is quercetin, which has demonstrated various anti-diabetic effects, along with reduced side effects [11, 12]. Thus, due to the notable therapeutic activities of quercetin, it has gained considerable interest [11]. Moreover, there exists an extensive reserve of phytochemicals that remain unexplored but may possess similar therapeutic properties to quercetin [13, 14]. It has been demonstrated that similarly structured compounds exhibit similar biological activity [13]. Moreover, research has demonstrated that the number and location of hydroxyl groups in flavonoids affect their anti-oxidant activity [15]. Gossypetin is a flavonoid that is found in the calyx of *Hibiscus sabdariffa* [16]. Interestingly, gossypetin has been shown to exhibit a similar structure to quercetin with an extra hydroxyl group, which may suggest more potent anti-oxidant activity [17]. Relevant to this review, studies have demonstrated the anti-oxidant, anti-inflammatory, nephroprotective, neuroprotective and hepatoprotective properties of gossypetin, all without any discernible toxicity [18-20]. Gossypetin has been demonstrated to be an effective dual-targeting agent that activates AMP-activated protein kinase (AMPK) and reduces oxidative stress, in contrast to conventional anti-diabetic medications that target one pathological mechanism [21]. In addition, the *Hibiscus* species is easily accessible and offers an inexpensive source of gossypetin [22]. The aim of the present review is to elucidate the potential role of gossypetin in the treatment of diabetes and its associated complications.

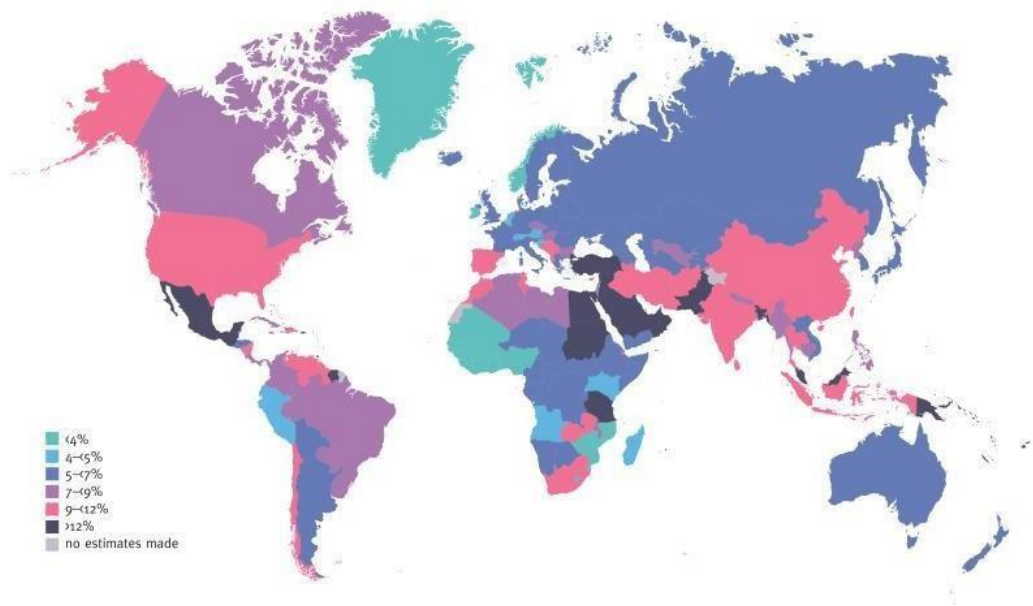


Figure 1. Estimated age-adjusted comparative prevalence of diabetes in adults (20–79 years) in 2021 Adapted with permission from International Diabetes Federation, 2013 [3].

1.1. Type 2 Diabetes Mellitus Complications

Type 2 diabetes (T2DM) is a chronic metabolic disease that is rapidly becoming more prevalent across the world [23]. It is characterised by insulin resistance and dysfunction in the β -cells in the pancreas [24]. The pathophysiology and progression of this metabolic disorder is associated with hyperglycaemia-induced reactive oxygen species (ROS) production and oxidative stress [7]. Oxidative stress under hyperglycaemic conditions is caused by an imbalance between the production of ROS and the cellular anti-oxidant system [7]. This leads to the development of diabetes [7]. Peroxisomes, phagocytic cells and the endoplasmic reticulum all produce reactive oxygen species (ROS), with the mitochondrial electron transport chain (ETC) playing a major role [25]. Structural and functional changes in proteins, lipids and nucleic acids have been shown to be induced by the increased production of ROS [26]. Moreover, ROS alters multiple intracellular signaling pathways, which results in insulin resistance and decreased β -cell performance [7]. In addition, the generation of ROS induced by hyperglycaemia also plays a role in the development of the macro- and microvascular complications associated with diabetes (Figure 2)[5, 6, 27]. Furthermore, it has been suggested that the generation of ROS through hyperglycaemia triggers several stress-sensitive signaling pathways, such as p-38 mitogen-activated protein kinases (MAPK), nuclear factor kappa B (NF- κ B) and Jun amino-terminal kinases/stress-activated protein kinases (JNK/SAPK), which in turn accelerates the development of complications related to T2DM [27]. Furthermore, research has demonstrated that the development of microvascular diabetic complications is significantly influenced by pro-inflammatory cytokines [28, 29]. Thus, clinical treatments may be developed from therapeutic approaches based on anti-oxidant and anti-inflammatory properties that have beneficial action against diabetic complications [30].

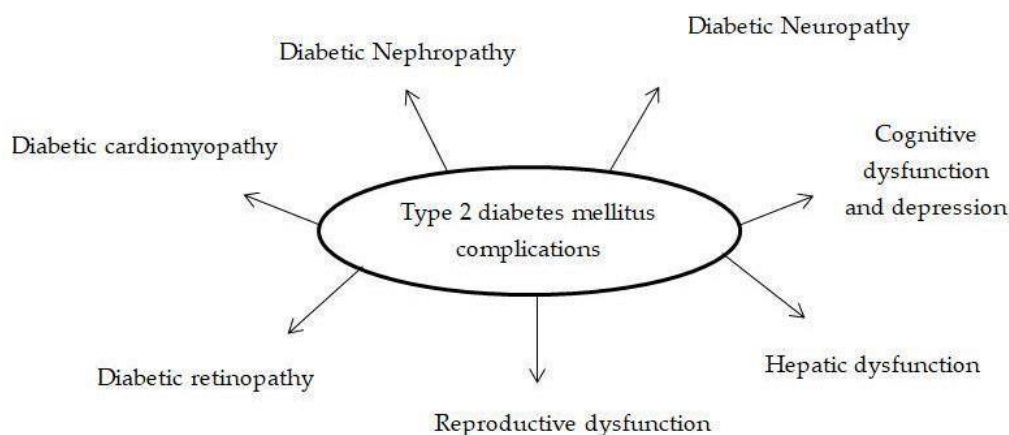


Figure 2. The complications associated with type 2 diabetes mellitus [5, 6, 27].

1.2. Flavonoids

The majority of phytochemicals with anti-oxidant activity in plants are phenolics, which make up the largest class of phytochemicals [31]. Of all the phenolic compounds that occur naturally, flavonoids make up the largest group [32]. They are responsible for the different colours seen in leaves, seeds, bark, flowers and fruits [10]. The fundamental flavone skeleton of flavonoids is composed of 15 carbons and includes two benzene rings (A and B) connected by a three-carbon pyran ring (C) (Figure 3) [33, 34]. The distinct subclasses of flavonoidal compounds are derived from variations in this fundamental structure (Figure 3) [10, 34]. These include chalcones, anthocyanins, flavanones, isoflavones, flavones and flavanols [10]. These secondary metabolites have been the subject of recent research due to their potential to prevent metabolic disorders such as obesity and diabetes mellitus [35]. The research findings indicate that dietary flavonoids have the potential to enhance insulin sensitivity, improve dysregulated lipid metabolism and reduce oxidative stress in metabolic disorders [36]. The research has shown that quercetin, one of the most abundant flavonols found in plants, has potent anti-diabetic properties [37, 38].

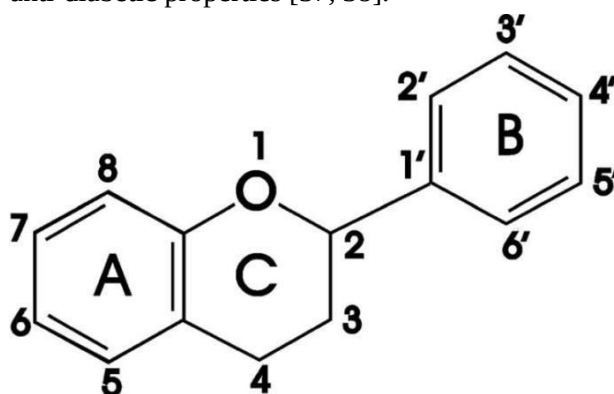


Figure 3. General structure of flavonoids. Adapted with permission from Ekalu and Habila, 2020 [34].

1.2.1. Quercetin

Quercetin is a flavonoid that is mainly found in citrus fruits, grapes, berries and broccoli [11]. It exhibits potent anti-oxidant properties [39].

Studies have indicated that quercetin is a beneficial natural substance that regulates important signaling pathways and acts on various diabetes targets [40]. Unlike the currently used therapeutics, quercetin improves both hyperglycaemia and its associated macrovascular and microvascular complications [12, 37]. Quercetin increases the production of insulin, protects pancreatic beta-cells from oxidative stress and improves the cells' ability to defend against reactive oxygen species [37, 41]. Diabetic complications including retinopathy, nephropathy and neuropathy have been shown to be induced by inflammation and oxidative stress [42, 43]. Quercetin has been shown to prevent diabetic complications by blocking NF- κ B cells, monocyte chemoattractant protein 1 (MCP-1) and intercellular adhesion molecule 1 (ICAM1) in T2DM patients (Figure 4)[44-48]. Moreover, quercetin has been shown to decrease nephropathy biomarker levels, such as creatinine, blood urea nitrogen (BUN) and 8-hydroxydeoxyguanosin [49, 50]. Furthermore, the research has demonstrated that quercetin has a safer profile than conventional anti-diabetic medications that are marketed [37, 51]. Additionally, a novel fermentation-based glycosylation technique using inexpensive substrates such as underutilised food waste can be used to produce quercetin on a large scale [37]. Furthermore, a study showed that similar structured compounds may exhibit similar biological activities [13]. There exists a large pool of unexplored compounds with similar therapeutic activities to quercetin [52].

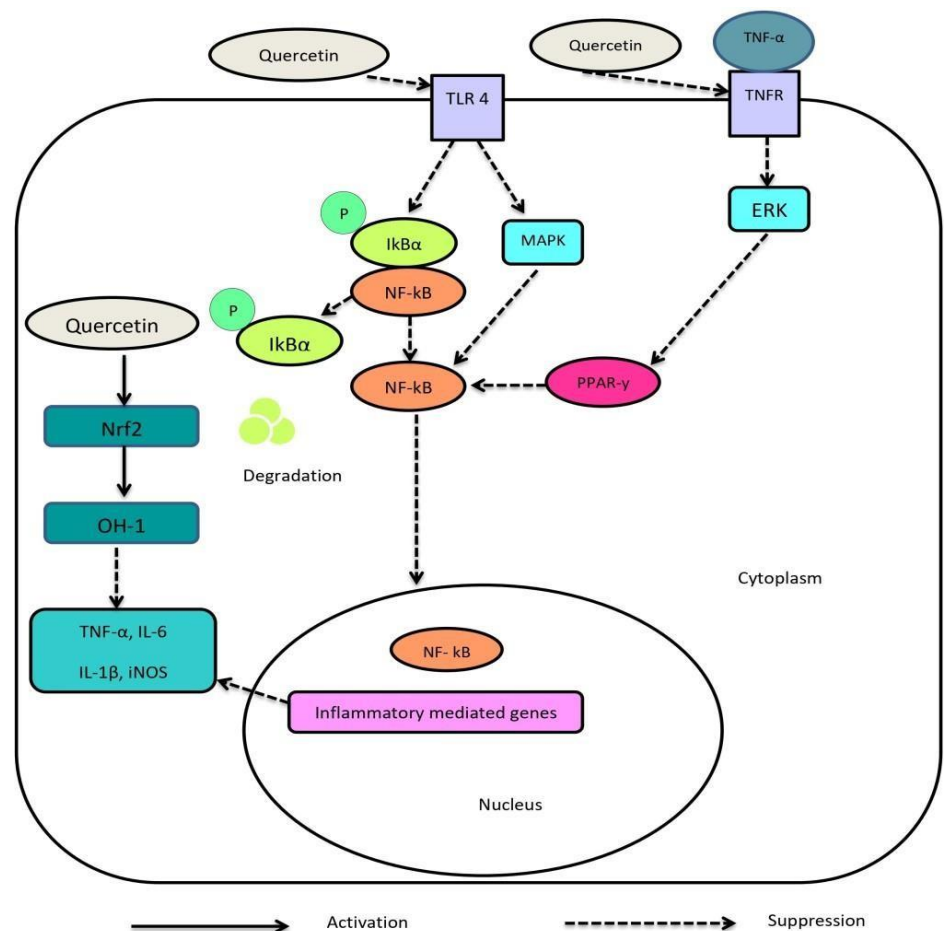


Figure 4. The potential molecular mechanism of quercetin on the NF- κ B inflammatory pathway [44, 45]. The activation of toll-like receptor (TLR) pathways encourages the production of pro-inflammatory

cytokines through the upregulation of transcription factors such as NF- κ B [44, 45]. Quercetin suppresses inflammatory cytokines production such as interleukin (IL)-6, tumour necrosis factor (TNF)- α and IL-1 β via downregulating TLR4 [46, 53]. Subsequently, repressed receptors yield targeted suppression of I κ B α phosphorylation and mitogen-activated protein kinase (MAPK) expression and thereby inhibit nuclear transfer of the NF- κ B [46, 47]. Phosphorylation is represented as p in the above figure. In addition, quercetin may indirectly prevent inflammation by increasing peroxisome proliferator-activated receptor γ (PPAR γ) activity, thereby antagonising NF- κ B [46, 48]. In addition, TNF- α binds to TNF receptors, which causes the activation of extracellular signal-regulated kinase (ERK) [46]. Activated ERK inactivates PPAR γ , therefore preventing nuclear translocation and the DNA binding of NF- κ B [46, 54]. This prevents the transcription of inflammatory mediators and triggers oxidative stress [46]. Quercetin activates nuclear factor erythroid 2-related factor 2 (Nrf2) and thereby contributes to increased heme oxygenase (HO)-1 levels, which are responsible for the downregulation of TNF- α [46]. Together, these block NF- κ B-mediated induction of inflammatory cascades [46].

1.2.2. Gossypetin

Gossypetin (GTIN) is a natural derivative of well-known quercetin [55]. It has previously been noted that quercetin is beneficial against diabetes mellitus and its associated complications [21, 37]. Gossypetin is hexahydroxyflavone and is found in the calyx of *Hibiscus sabdariffa* [16]. Tropical regions have made extensive use of these calyces as potential sources of medicinal phytochemicals [56]. Traditionally, these plant extracts were used to treat inflammation, jaundice and diabetes [57, 58]. GTIN is a yellow pigment, with a chemical formula of C₁₅H₁₀O₈, a molecular weight of 318.24 g/mol and a boiling point temperature of 679.30 °C @ 760.00 mmHg [59]. Since reducing agents exhibit anti-oxidant properties, an organic molecule's reducing power may serve as an indicator to evaluate its anti-oxidant activity [60]. The anti-oxidant actions of flavonoids are mediated by their functional hydroxyl groups, which chelate metal ions and scavenge free radicals [61]. The number and arrangement of hydroxyl groups in flavonoids also significantly influence their anti-oxidant properties [15]. The high anti-oxidant activity which gossypetin exhibits may be attributed to the presence of a carbonyl group at position C4, a double bond between C2 and C3 and the 3-OH and 4-OH groups in the B ring (Figure 5) [55, 62, 63]. Interestingly, gossypetin has a catechol moiety as a B-ring, similarly to quercetin [17]. In addition, gossypetin has three hydroxyl groups rather than two at positions 5, 7 and 8 in the A-ring (Figure 5) [17, 55, 63]. In gossypetin, the two subsequent hydroxyl groups at positions 7 and 8 function as another catechol-like center [17]. This suggests that gossypetin has three oxidisable redox active centers while quercetin only has two [17]. In addition, previous research showed that among 15 well-known flavonoids, including quercetin, gossypetin possessed the most potent reducing capacity [17]. This may suggest that gossypetin exhibits more potent anti-oxidant activity than quercetin. In addition, gossypetin may exhibit similar pharmacological properties to quercetin due to the structural similarity. Studies have shown that gossypetin exerts various pharmacological properties, including anti-oxidant, anti-atherosclerotic, anti-nephrotoxic, as well as reprotective and hepatoprotective effects [18, 21, 55]. Gossypetin has been shown to exhibit therapeutic effects at doses as low as 10 mg/kg and 20 mg/kg in

vivo [20, 64]. Therefore, gossypetin may serve as a potential candidate in the treatment of diabetes and its associated complications.

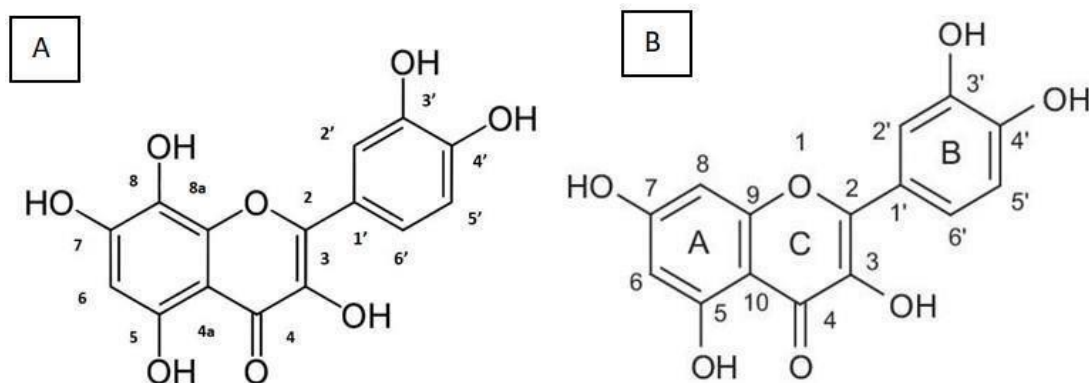


Figure 5. Structure of (A) gossypetin (GTIN) and (B) quercetin. Adapted with permission from Khan et al., 2013 and Zheng and Chow, 2009 [55, 63].

1.3. Pharmacological Action of Gossypetin

As mentioned above, diabetes mellitus causes numerous micro- and macrovascular complications in affected patients [5, 65]. Gossypetin may serve as an appropriate candidate for the prevention and treatment of diabetes mellitus complications. In the below section, we discuss the therapeutic potential of gossypetin for the treatment of inflammation, oxidative stress, kidney dysfunction, liver dysfunction, cognitive dysfunction, atherosclerosis and reproductive dysfunction (Figure 6)[18, 19, 62, 66, 67].

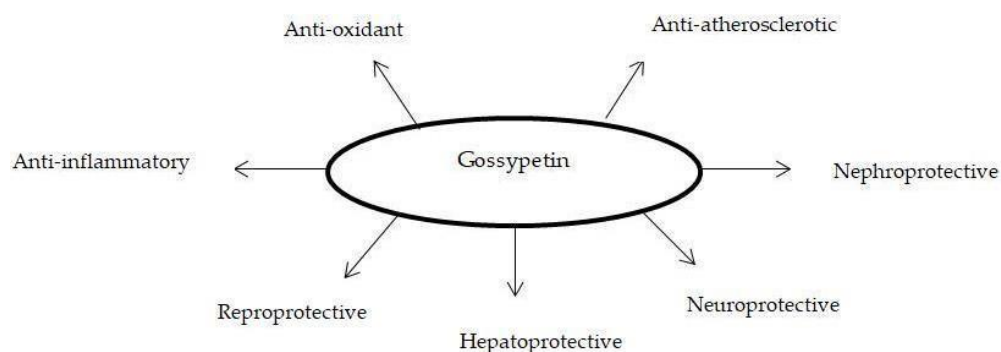


Figure 6. The reported pharmacological importance of gossypetin [18, 19, 62, 66, 67].

1.3.1. Anti-Oxidant and Anti-Inflammatory Effects of Gossypetin

The oxidative stress caused by ROS and free radicals has been shown to be linked with numerous complications in T2DM [68]. The cellular alterations that result in diabetic complications have been demonstrated to be caused by increased oxidative stress and inflammation, which are both a cause and an effect of diabetes [68]. Radiation promotes the production of hydroxyl radicals, which leads to

lipid peroxidation and significant biological damage [69]. It is responsible for the development of inflammation through nitric acid (NO) production [69]. In a previous study, hepatocytes were treated with 20 mM and 40 mM GTIN for one hour before exposure to 5 Gy radiation [55]. The treatment with 20 mM of GTIN offered more effective protection against damage to supercoiled DNA than 40 mM of quercetin after exposure to 5 Gy radiation [70]. According to the study, GTIN significantly protected against radiation-induced DNA damage and inflammation directly and indirectly by scavenging ROS and NO [55]. The study demonstrated the anti-oxidant potential of GTIN, evidenced by the high ferric-reducing anti-oxidant power (FRAP) value [55]. FRAP denotes the total anti-oxidant pool [71]. GTIN contains a pair of parahydroxyls at positions 5 and 8 and two pairs of orthohydroxyls [55]. It is therefore oxidisable because it can form two o-quinone intermediates and one p-quinone intermediate, which aids in scavenging free radicals [55]. Moreover, it was found that GTIN inhibited free-radical-mediated DNA strand breakage and decreased radiation-mediated oxidative stress in the study's ex vivo model [55]. According to the study, GTIN terminates a radical chain reaction by reacting with radicals, transforming them into more stable compounds [55]. In addition, in the study's ex vivo model, it was made evident that gossypetin exhibited the most potent anti-oxidant activity at a dosage of 50 mM [70]. It was also suggested that exposure to 50 mM gossypetin exhibits therapeutic effects against DNA damage at even a higher radiation dose exposure between 5 Gy and 20 Gy [55]. The aforementioned studies have demonstrated the anti-inflammatory and anti-oxidant properties of gossypetin in the management of radiation-induced oxidative stress (Figure 7) [55, 70, 72, 73]. Taken together, gossypetin may alleviate oxidative-stress-induced diabetic complications due to its potent anti-oxidant and anti-inflammatory potential.

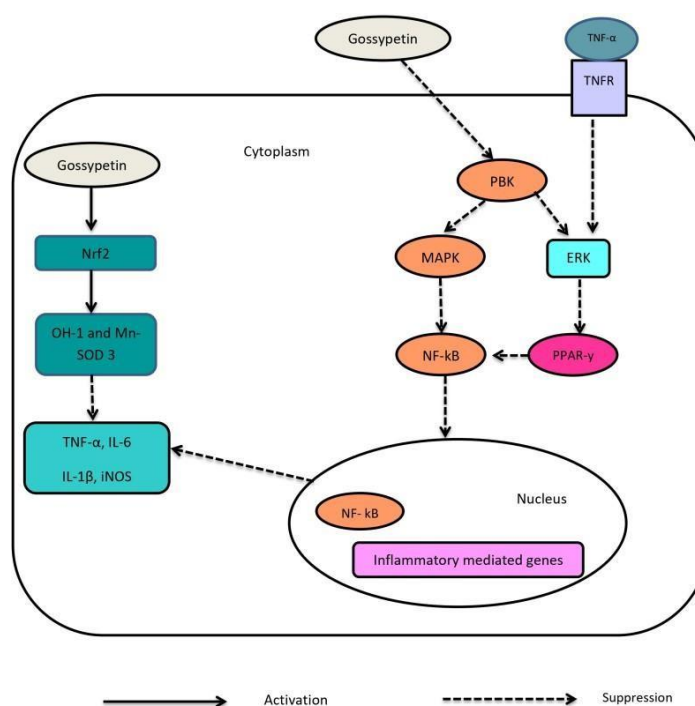


Figure 7. The potential molecular mechanism of gossypetin on the NF-κB inflammatory pathway [55, 73]. It is shown that gossypetin inhibits PDZ-binding kinase (PBK) phosphorylation, which is involved in the regulation of p38 MAPK and ERK1/2 kinases [73]. Consequently,

MAPK and ERK are inhibited by gossypetin [55, 73]. The downregulation of MAPK inhibits NF- κ B activation and translocation into the nucleus [55, 73]. As a result, there is a downregulation in the transcription of inflammatory mediators. In addition, the downregulation of ERK inhibits PPAR- γ , thereby antagonising NF- κ B [55, 73]. Furthermore, gossypetin has been shown to stimulate Nrf2, thereby activating OH-1 and superoxide dismutase (SOD)-3 [73]. This promotes anti-oxidant and anti-inflammatory properties.

1.3.2. Anti-Atherosclerotic Effects of Gossypetin

One of the main complications of DM is atherosclerosis, which is caused by endothelial dysfunction, inflammation and oxidative stress [74]. According to previous studies, increased low-density lipoprotein (LDL) oxidation has been linked to hyperinsulinaemia and impaired glucose tolerance in T2DM [75, 76]. Oxidised low-density lipoprotein (ox-LDL) induces damage to the vascular endothelial cells, which is a major factor in the development of atherosclerosis [77]. In T2DM, oxidative stress promotes the onset of vascular smooth muscle (VSMC) dysfunction [78]. Plaque formation results from VSMCs' progressive proliferation and migration from the vascular media to the neointima during the pathogenesis of atherosclerosis [78]. According to a previous study, starved rat aortic VSMCs were treated with 0, 1, 5, 10, 25, 50, 100 and 250 μ M GTIN for 48 h [66]. The research findings indicated that GTIN inhibits the dysfunction of VSMCs through various mechanisms, including the suppression of cell proliferation, cell cycle progression, migration, matrix degradation and oxidative stress [66]. It was concluded that GTIN may demonstrate protective effects against VSMC dysfunction, which may delay atherosclerotic pathogenesis [20, 66]. According to another study, gossypetin demonstrated protective effects against endothelial damage both in vitro and in vivo [20]. In the in vitro model, cells were treated with 0.1, 0.5, 1.0, 2.0 and 5.0 μ M for 24 h [20]. In the study's in vivo model, New Zealand white rabbits were fed on a high-fat diet for 10 weeks to induce atherosclerosis and treated with 10 mg/kg gossypetin [20]. The results showed that in the in vitro model, gossypetin inhibited ox-LDL uptake, lipid-laden foam cell formation and promoted cholesterol efflux in a dose-dependent manner [20]. Furthermore, treatment with gossypetin has been shown to decrease liver injury markers and improve the lipid profile [20]. It was stated that numerous signals may have been involved in this process and the mechanisms are likely to be complex [20]. Gossypetin may have a major role in atheroprotection through upregulating autophagy [20]. Gossypetin inhibits oxidised low-density lipoprotein (ox-LDL) by activating peroxisome proliferator-activated receptor (PPAR)- α and inhibiting PPAR- γ , which promotes macrophage cholesterol clearance and delays atherosclerosis [20]. These studies have highlighted the anti-atherosclerotic property of gossypetin in the treatment of endothelial dysfunction [20, 66]. Taken together, gossypetin may alleviate atherosclerosis in T2DM due to its anti-oxidant potential and ability to target endothelial dysfunction.

1.3.3. Nephroprotective Effects of Gossypetin

Oxidative stress and inflammation play a major role in the development of renal dysfunction in T2DM [43, 79]. A previous study has demonstrated that in a nephrotoxicity model, kidney function was significantly improved in Sprague-Dawley rats administered 30 mg/Kg GTIN intraperitoneally for 30 days [18]. Anti-oxidant enzyme activities were significantly increased by GTIN supplementation, which was also

associated with a reduction in ROS and malondialdehyde (MDA) levels [18]. Higher creatinine clearance was linked to an improved glomerular filtration rate, which was reflected by a reduction in urea, creatinine, kidney injury molecule-1 (KIM-1) and neutrophil-gelatinase-associated lipocalin (NGAL) levels after GTIN supplementation [18]. Urea and creatinine are used to monitor renal function [80]. The breakdown of phosphocreatine, which is catalysed by creatine kinase, produces creatinine, which is then eliminated from the kidney via glomerular filtration [81]. Any damage to the renal tissues caused by oxidation increases the levels of urea and creatinine while decreasing creatinine clearance [81]. Additionally, urine normally does not contain the transmembrane protein KIM-1, but its presence suggests damage to the proximal tubules [81]. The results of the study showed that GTIN treatment reduced the levels of inflammatory markers such as interleukin-10 and tumour necrosis factor- α (TNF- α) [18]. The results were corroborated by previous studies which suggested that flavonoids may be responsible for suppressing inflammation [82, 83]. It was suggested that the free radical scavenging and anti-inflammatory properties of GTIN may be directly related to its renoprotective effects [18]. Taken together, gossypetin may serve as a novel compound in the treatment of diabetic nephropathy due to its ability to decrease the levels of renal injury biomarkers, oxidative markers, apoptotic markers and inflammatory markers and alleviate damage to the architecture of renal tissue. In addition, the anti-oxidant and anti-inflammatory properties of gossypetin may ameliorate diabetic nephropathy by targeting oxidative stress and inflammation.

1.3.4. Neuroprotective Effects of Gossypetin

Cognitive function is compromised by the oxidative stress and inflammation caused by the high blood glucose concentrations in the brain as a result of T2DM [84, 85]. In a previous study, stress was used to induce memory and spatial learning deficits in mice [64]. Furthermore, long-term unpredictable stress leads to adverse consequences, such as neuropsychiatric conditions including dementia, depression and anxiety [86]. In this study, five weeks of exposure to different stressors were followed by four weeks of intraperitoneal administration of gossypetin at doses of 5, 10 and 20 mg/kg [64]. The behaviour pattern significantly improved following gossypetin administration, according to the results [64]. It has been found that mice exposed to 10 and 20 mg/kg of gossypetin had significantly lower levels of corticosterone and oxidative stress [64]. The main cause of synaptic loss and cognitive deficits is oxidative stress, which is accelerated by high cortisol levels [87]. Moreover, gossypetin administration was found to increase the levels of serotonin, norepinephrine and brain-derived neurotrophic factor (BDNF) [64]. A crucial molecule in the plastic changes linked to memory and learning is known as BDNF [88]. In patients with T2DM, lower levels of BDNF have been linked to cognitive impairment [89, 90]. Another study found that at doses of 5 and 20 mg/kg po, respectively, gossypetin demonstrates significant anti-anxiety and anti-depressant activity in mice [91]. Diabetes promotes an increase in the renin–angiotensin–aldosterone system (RAAS) both locally and systemically in the brain [92]. Pro-inflammatory processes are induced in the brain by elevated RAAS activation [93]. One factor influencing the decreased synthesis and release of BDNF is neuroinflammation [94]. Reduced BDNF levels have been linked to the development of depression by promoting neuronal damage [95]. Furthermore, a previous study showed that gossypetin administration in

vitro and in vivo decreased several of the hallmarks associated with Alzheimer's disease (AD), including microgliosis and astrogliosis [67]. In the study's in vitro model, the primary microglial cells were treated with 25 μ M gossypetin for 24 h [67], and mice were intragastrically administered 10 mg/kg gossypetin daily for 13 weeks [67]. The results demonstrated that gossypetin reduced the formation of various types of beta-amyloid (A β) protein plaques, which delayed the progression of AD [67]. Interestingly, cognition and memory function was reversed to the same level as the control group after treatment with gossypetin [67]. Insulin resistance contributes to the development of Alzheimer's disease by promoting the build-up of A β proteins [96, 97]. Taken together, gossypetin may serve as a novel compound in the treatment of cognition impairments in T2DM due to its ability to reduce oxidative stress and inflammation, decrease cortisol levels and increase BDNF levels.

1.3.5. Hepatoprotective Effects of Gossypetin

Systemic lipid and glucose homeostasis is maintained largely in part by the liver [98]. Studies have demonstrated that non-alcoholic fatty liver disease (NAFLD), fibrosis, cirrhosis and aberrant glycogen deposition are all associated with T2DM [99, 100]. It has been demonstrated that oxidative stress and inflammation play a significant role in the development of liver dysfunction in T2DM patients [100, 101]. Previous studies on drug development for nonalcoholic steatohepatitis (NASH) were based on the one-hit hypothesis [21, 102]. However, the development of therapeutic agents that target one specific mechanism to improve NASH has failed [102, 103]. As a result, the "multiple-hit hypothesis," which takes into account focusing on multiple factors concurrently to improve the effectiveness of therapeutic agents, has become the new paradigm in NASH drug development [21, 104]. It has been suggested that targeting oxidative stress and metabolic dysfunction may be an approach to prevent NASH since these factors promote the development of fibrosis and inflammation [105]. According to a previous study, gossypetin reduced hepatic steatosis, lobular inflammation and liver fibrosis in mice induced with NASH for four weeks at a dose of 20 mg/kg/day [21]. According to the study, gossypetin directly acted as an anti-oxidant in both the in vitro and in vivo models, reducing the oxidative stress caused by hydrogen peroxide and palmitate [21]. It supports previous studies which have shown that anti-oxidants have the ability to alleviate NASH [21, 106]. Furthermore, the study revealed that gossypetin induced higher AMPK phosphorylation compared to the other flavonoids, including quercetin [21]. Acetyl-CoA carboxylase 1 (ACC), an essential enzyme for fatty acid synthesis that controls hepatic insulin resistance, is phosphorylated by AMPK [21]. According to the study, mice treated with gossypetin exhibited increased AMPK activation, which reduced liver steatosis [21]. Taken together, gossypetin may serve as a novel therapeutic compound in the treatment of NASH and NAFLD due to its double targeting action against oxidative stress and AMPK phosphorylation.

1.3.6. Reproprotective Effects of Gossypetin

Male reproductive dysfunction in T2DM has been demonstrated to be exacerbated by oxidative stress, which promotes changes in hormone levels, apoptosis in germ cells and a decline in semen quality [107, 108]. An organic substance known as paraquat (PQ) has been shown to promote major damage to the male reproductive system [19, 109]. In a previous study, male reproductive dysfunction was induced by PQ exposure through the production of free radicals [19]. In the study, adult

male Sprague-Dawley rats were administered with gossypetin at doses of 5 mg/kg and 30 mg/kg for 56 days [19]. Gossypetin was found to improve PQ-induced reproductive dysfunctions in the study [19]. Gossypetin reduced the levels of reactive oxygen species (ROS) and malondialdehyde (MDA) while increasing the activities of glutathione reductase (GSR), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) [19]. Gossypetin also enhanced sperm viability, motility, as well as the quantity of hypo-osmotic tail-swelled spermatozoa and epididymal sperms [19]. In addition, it decreased sperm morphological abnormalities [19]. Gossypetin alleviated the luteinising hormone (LH) and follicle-stimulating hormone (FSH) reductions induced by PQ [19]. Additionally, gossypetin significantly increased the level of testosterone by upregulating the expression of steroidogenic enzymes, indicating that it has androgenic properties [19]. Gossypetin downregulated the gene expression of apoptotic markers such as caspase-3 and upregulated the gene expression of anti-apoptotic markers [19]. Taken together, gossypetin demonstrated reproprotective effects against paraquat-induced male reproductive dysfunction through its antioxidant, androgenic and anti-apoptotic properties. Since oxidative stress is a major contributor underlying T2DM-induced male reproductive dysfunction, gossypetin may have the potential to confer similar protective effects during T2DM. However, further studies are required to evaluate the therapeutic efficacy of GTIN using type 2 diabetic animals.

1.4. Potential Use of Gossypetin in Diabetes Mellitus

The above section highlights the reported findings of gossypetin in the treatment of dysfunctions in the renal, cardiovascular, hepatic, reproductive and cognition systems [20, 21, 67]. Many of the complications that occur in diabetes are associated with oxidative stress [110, 111]. An imbalance between the generation of reactive oxygen species (ROS) and the anti-oxidant system's capacity to neutralise these molecules results in oxidative stress [112]. Diabetes promotes oxidative stress due to a variety of mechanisms, such as increased oxygen radical production from glucose auto-oxidation, the production of glycated proteins and the glycation of anti-oxidant enzymes, which limits the capacity of anti-oxidants to neutralise free radicals [7, 113]. Gossypetin may prevent diabetic complications due to its anti-oxidant, anti-apoptotic and anti-inflammatory actions [21, 73]. Elevated blood glucose levels pose a challenge in the treatment of diabetes mellitus [114]. Consequently, therapies that lower blood glucose levels may help treat diabetes mellitus [114]. Previous studies have shown that diabetes is associated with the impairment of AMPK activity [115, 116]. AMPK stimulates β -oxidation and fatty acid uptake while suppressing the synthesis of triglycerides, cholesterol and fatty acids [117]. Moreover, AMPK signaling reduces oxidative stress, inflammation and apoptosis [118]. Insulin resistance is inhibited and beta (β)-cell survival is promoted by AMPK activation [119]. AMPK activation has been shown in numerous studies to improve glucose uptake into the cells and reduce intracellular glucose production in diabetics [116, 117]. In addition, metformin and rosiglitazone, the two main conventional medications used in diabetes, demonstrate their metabolic effects partially by activating AMPK [120]. This makes the pharmacological activation of AMPK a viable target for DM drug development and discovery [121]. Unfortunately, there are negative side effects associated with these conventional anti-diabetic drugs [122, 123]. Consequently, it is imperative to investigate natural medications derived from plants that

treat diabetes and lack adverse side effects [124]. There is significant potential for managing diabetes mellitus and its complications with fewer side effects when natural products such as quercetin are used to activate and regulate the AMPK pathway [116, 125]. It is well known that gossypetin is structurally similar to quercetin with an extra hydroxyl [20, 126]. Therefore, gossypetin may exhibit similar anti-diabetic [20, 126]. Interestingly, gossypetin has been shown to possess higher AMPK activity than quercetin [21]. For this reason, gossypetin may serve as a promising treatment for diabetes mellitus and its associated complications.

1.5. Conclusions

In this review, we highlighted the pharmacological properties of gossypetin and its potential in the treatment of diabetes and the associated complications. Our review of the literature demonstrated that gossypetin exhibited anti-oxidant, anti-inflammatory, nephroprotective, neuroprotective and hepatoprotective properties [21, 67, 126]. Gossypetin ameliorates many complications by targeting oxidative stress and inflammation [17, 21]. Oxidative stress and inflammation play a critical role in the development of T2DM [68]. It is worthy to note that gossypetin offered better protection against DNA damage from oxidative stress than quercetin [70]. In addition, gossypetin possessed the highest AMPK activity in comparison to other flavonoids, including quercetin [21]. Consequently, gossypetin may serve as a potential candidate in the treatment of diabetes and its associated complications. Future studies should investigate the therapeutic potential of gossypetin in diabetes mellitus. Moreover, further studies are also required to establish the pharmacokinetic and safety profiles of gossypetin.

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References

1. Erzse, A., et al., *The direct medical cost of type 2 diabetes mellitus in South Africa: a cost of illness study*. Global Health Action, 2019. **12**(1): p. 1636611.
2. Yan, Y., et al., *Prevalence, awareness and control of type 2 diabetes mellitus and risk factors in Chinese elderly population*. BMC Public Health, 2022. **22**(1): p. 1382.
3. Federation, I.D. *Idf Diabetes Atlas*; International Diabetes Federation: Brussels, Belgium, 2013. <https://doi.org/10.1289/image.ehp.v119.i03>.
4. Galicia-Garcia, U., et al. *Pathophysiology of Type 2 Diabetes Mellitus*. International Journal of Molecular Sciences, 2020. **21**, DOI: 10.3390/ijms21176275.
5. Rangel, E.B.; Rodrigues, C.O.; De Sa, J.R. *Micro- and Macrovascular Complications in Diabetes Mellitus: Preclinical and Clinical Studies*; Hindawi: London, UK, 2019.
6. Chawla, A., R. Chawla, and S. Jaggi, *Microvascular and macrovascular complications in diabetes mellitus: Distinct or continuum?* Indian J Endocrinol Metab, 2016. **20**(4): p. 546-51.
7. Bhatti, J.S., et al., *Oxidative stress in the pathophysiology of type 2 diabetes and related complications: Current therapeutics strategies and future perspectives*. Free Radical Biology and Medicine, 2022. **184**: p. 114-134.
8. Marín-Peñalver, J.J., et al., *Update on the treatment of type 2 diabetes mellitus*. World J Diabetes, 2016. **7**(17): p. 354-95.
9. Unuofin, J.O. and S.L. Lebelo, *Antioxidant Effects and Mechanisms of Medicinal Plants and Their Bioactive Compounds for the Prevention and Treatment of Type 2 Diabetes: An Updated Review*. Oxid Med Cell Longev, 2020. **2020**: p. 1356893.
10. Panche, A.N., A.D. Diwan, and S.R. Chandra, *Flavonoids: an overview*. J Nutr Sci, 2016. **5**: p. e47.
11. David, A.V.A., R. Arulmoli, and S. Parasuraman, *Overviews of biological importance of quercetin: A bioactive flavonoid*. Pharmacognosy reviews, 2016. **10**(20): p. 84.
12. Ansari, P., et al., *Therapeutic potential of quercetin in the management of type-2 diabetes mellitus*. Life, 2022. **12**(8): p. 1146.
13. Dubey, R., D.P. Prabhakar, and J. Gupta, *Identification of Structurally Similar Phytochemicals to Quercetin with High SIRT1 Binding Affinity and Improving Diabetic Wound Healing by Using In silico Approaches*. Biointerface Research in Applied Chemistry, 2021. **12**: p. 7621-7632.
14. Rubin Thapa, M., et al., *A Review on Structure, Modifications and Structure-Activity Relation of Quercetin and Its Derivatives*. Journal of Microbiology and Biotechnology, 2020. **30**(1): p. 11-20.
15. Banjarnahor, S.D. and N. Artanti, *Antioxidant properties of flavonoids*. Medical Journal of Indonesia, 2014. **23**(4): p. 239-44.
16. Huang, K., et al., *Anticancer effects of gossypetin from Hibiscus sabdariffa in oral squamous cell carcinoma*. Journal of Applied Oral Science, 2023. **31**: p. e20230243.
17. Dutta, M.S., et al., *Estimation of the reducing power and electrochemical behavior of few flavonoids and polyhydroxybenzophenones substantiated by bond dissociation energy: A comparative analysis*. Molecular Diversity, 2022: p. 1-13.
18. Ijaz, M.U., et al., *Gossypetin mitigates doxorubicin-induced nephrotoxicity: A histopathological and biochemical evaluation*. Journal of King Saud University-Science, 2023. **35**(7): p. 102830.
19. Mustafa, S., et al., *Therapeutic effect of gossypetin against paraquat-induced testicular damage in male rats: a histological and biochemical study*. Environmental Science and Pollution Research, 2023. **30**(22): p. 62237-62248.
20. Lin, H.-H., *In vitro and in vivo atheroprotective effects of gossypetin against endothelial cell injury by induction of autophagy*. Chemical Research in Toxicology, 2015. **28**(2): p. 202-215.
21. Oh, E.; Lee, J.; Cho, S.; Kim, S.W.; Jo, K.W.; Shin, W.S.; Gwak, S.H.; Ha, J.; Jeon, S.Y.; Park, J.-H.; et al. Gossypetin prevents the progression of nonalcoholic steatohepatitis by regulating oxidative stress and AMP-activated protein kinase. *Mol. Pharmacol.* **2023**, *104*, 214–229.
22. Jejurkar, G. and M. Chavan, *Therapeutic benefits of gossypin as an emerging phytoconstituents of Hibiscus spp.: a critical review*. Future Journal of Pharmaceutical Sciences, 2023. **9**(1): p. 95.
23. Khan, M.A.B., et al., *Epidemiology of Type 2 Diabetes - Global Burden of Disease and Forecasted Trends*. J Epidemiol Glob Health, 2020. **10**(1): p. 107-111.

24. Saisho, Y., *β -cell dysfunction: Its critical role in prevention and management of type 2 diabetes*. World J Diabetes, 2015. **6**(1): p. 109-24.
25. Phaniendra, A., D.B. Jestadi, and L. Periyasamy, *Free radicals: properties, sources, targets, and their implication in various diseases*. Indian J Clin Biochem, 2015. **30**(1): p. 11-26.
26. Redza-Dutordoir, M. and D.A. Averill-Bates, *Activation of apoptosis signalling pathways by reactive oxygen species*. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2016. **1863**(12): p. 2977-2992.
27. Yaribeygi, H., et al., *Molecular Mechanisms Linking Oxidative Stress and Diabetes Mellitus*. Oxid Med Cell Longev, 2020. **2020**: p. 8609213.
28. Nguyen, D.V., L.C. Shaw, and M.B. Grant, *Inflammation in the pathogenesis of microvascular complications in diabetes*. Front Endocrinol (Lausanne), 2012. **3**: p. 170.
29. Devaraj, S., M.R. Dasu, and I. Jialal, *Diabetes is a proinflammatory state: a translational perspective*. Expert Rev Endocrinol Metab, 2010. **5**(1): p. 19-28.
30. Kong, M., et al., *Anti-inflammatory phytochemicals for the treatment of diabetes and its complications: Lessons learned and future promise*. Biomedicine & Pharmacotherapy, 2021. **133**: p. 110975.
31. Tungmunnithum, D., et al., *Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview*. Medicines (Basel), 2018. **5**(3).
32. Sun, W. and M.H. Shahrajabian, *Therapeutic Potential of Phenolic Compounds in Medicinal Plants—Natural Health Products for Human Health*. Molecules, 2023. **28**(4): p. 1845.
33. Dias, M.C., D. Pinto, and A.M.S. Silva, *Plant Flavonoids: Chemical Characteristics and Biological Activity*. Molecules, 2021. **26**(17).
34. Ekalu, A. and J.D. Habila, *Flavonoids: isolation, characterization, and health benefits*. Beni-Suef University Journal of Basic and Applied Sciences, 2020. **9**(1): p. 1-14.
35. Shehadeh, M.B., G. Suaifan, and A.M. Abu-Odeh, *Plants Secondary Metabolites as Blood Glucose-Lowering Molecules*. Molecules, 2021. **26**(14).
36. Yi, X., et al., *Flavonoids improve type 2 diabetes mellitus and its complications: a review*. Front Nutr, 2023. **10**: p. 1192131.
37. Dhanya, R., *Quercetin for managing type 2 diabetes and its complications, an insight into multitarget therapy*. Biomedicine & Pharmacotherapy, 2022. **146**: p. 112560.
38. Aghababaei, F. and M. Hadidi *Recent Advances in Potential Health Benefits of Quercetin*. Pharmaceuticals, 2023. **16**, DOI: 10.3390/ph16071020.
39. Ozgen, S., O.K. Kilinc, and Z. Selamoğlu, *Antioxidant activity of quercetin: a mechanistic review*. Turkish Journal of Agriculture-Food Science and Technology, 2016. **4**(12): p. 1134-1138.
40. Ghafouri-Fard, S., et al., *The impact of the phytotherapeutic agent quercetin on expression of genes and activity of signaling pathways*. Biomedicine & Pharmacotherapy, 2021. **141**: p. 111847.
41. Youl, E., et al., *Quercetin potentiates insulin secretion and protects INS-1 pancreatic β -cells against oxidative damage via the ERK1/2 pathway*. Br J Pharmacol, 2010. **161**(4): p. 799-814.
42. Hammes, H.-P., *Diabetic retinopathy: hyperglycaemia, oxidative stress and beyond*. Diabetologia, 2018. **61**(1): p. 29-38.
43. Charlton, A., et al., *Oxidative stress and inflammation in renal and cardiovascular complications of diabetes*. Biology, 2020. **10**(1): p. 18.
44. De Nardo, D., *Toll-like receptors: Activation, signalling and transcriptional modulation*. Cytokine, 2015. **74**(2): p. 181-189.
45. Roy, A., et al., *Potential therapeutic targets for inflammation in toll-like receptor 4 (TLR4)-mediated signaling pathways*. International immunopharmacology, 2016. **40**: p. 79-89.
46. Wang, Y., et al., *Drug delivery based pharmacological enhancement and current insights of quercetin with therapeutic potential against oral diseases*. Biomedicine & Pharmacotherapy, 2020. **128**: p. 110372.
47. Cheng, S.-C., et al., *Quercetin inhibits the production of IL-1 β -induced inflammatory cytokines and chemokines in ARPE-19 cells via the MAPK and NF- κ B signaling pathways*. International journal of molecular sciences, 2019. **20**(12): p. 2957.
48. Chuang, C.-C., et al., *Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor- α -mediated inflammation and insulin resistance in primary human adipocytes*. The American journal of clinical nutrition, 2010. **92**(6): p. 1511-1521.
49. Lai, P.B., L. Zhang, and L.Y. Yang, *Quercetin ameliorates diabetic nephropathy by reducing*

the expressions of transforming growth factor- β 1 and connective tissue growth factor in streptozotocin-induced diabetic rats. *Ren Fail*, 2012. **34**(1): p. 83-7.

50. Yang, H., et al., *Quercetin Treatment Improves Renal Function and Protects the Kidney in a Rat Model of Adenine-Induced Chronic Kidney Disease*. *Med Sci Monit*, 2018. **24**: p. 4760-4766.

51. Yi, H., et al., *The Therapeutic Effects and Mechanisms of Quercetin on Metabolic Diseases: Pharmacological Data and Clinical Evidence*. *Oxid Med Cell Longev*, 2021. **2021**: p. 6678662.

52. Salehi, B., et al., *Therapeutic Potential of Quercetin: New Insights and Perspectives for Human Health*. *ACS Omega*, 2020. **5**(20): p. 11849-11872.

53. Xiong, G., et al., *Quercetin inhibits inflammatory response induced by LPS from Porphyromonas gingivalis in human gingival fibroblasts via suppressing NF- κ B signaling pathway*. *BioMed research international*, 2019. **2019**.

54. Krajka-Kuźniak, V. and W. Baer-Dubowska, *Modulation of Nrf2 and NF- κ B signaling pathways by naturally occurring compounds in relation to cancer prevention and therapy. Are combinations better than single compounds?* *International Journal of Molecular Sciences*, 2021. **22**(15): p. 8223.

55. Khan, A., et al., *Gossypetin, a naturally occurring hexahydroxy flavone, ameliorates gamma radiation-mediated DNA damage*. *International Journal of Radiation Biology*, 2013. **89**(11): p. 965-975.

56. Riaz, G. and R. Chopra, *A review on phytochemistry and therapeutic uses of Hibiscus sabdariffa L.* *Biomedicine & Pharmacotherapy*, 2018. **102**: p. 575-586.

57. Da-Costa-Rocha, I., et al., *Hibiscus sabdariffa L.—A phytochemical and pharmacological review*. *Food chemistry*, 2014. **165**: p. 424-443.

58. González-Stuart, A. Multifaceted therapeutic value of roselle (*Hibiscus sabdariffa* L.—Malvaceae). *Nutr. Diet. Suppl. Nutr. Cost Anal. Versus Clin. Benefits* **2011**, 2011, 215–226.

59. Har Bhajan, S. and K.A. Bharati, 6 - Enumeration of dyes, in *Handbook of Natural Dyes and Pigments*, S. Har Bhajan and K.A. Bharati, Editors. 2014, Woodhead Publishing India. p. 33-260.

60. Shahidi, F. and Y. Zhong, *Measurement of antioxidant activity*. *Journal of Functional Foods*, 2015. **18**: p. 757-781.

61. Kumar, S. and A.K. Pandey, *Chemistry and biological activities of flavonoids: an overview*. *ScientificWorldJournal*, 2013. **2013**: p. 162750.

62. Puthanveedu, V. and K. Muraleedharan, *Study on structural detailing of gossypetin and its medicinal application in UV filtering, radical scavenging, and metal chelation open up through NCI, TD-DFT, QTAIM, ELF, and LOL analysis*. *Computational and Theoretical Chemistry*, 2023. **1225**: p. 114126.

63. Zheng, Y.; Chow, A. Production and characterization of a spray-dried hydroxypropyl- β -cyclodextrin/quercetin complex. *Drug Dev. Ind. Pharm.* **2009**, 35, 727–734.

64. Samant, N.P. and G.L. Gupta, *Gossypetin-based therapeutics for cognitive dysfunction in chronic unpredictable stress-exposed mice*. *Metabolic Brain Disease*, 2022. **37**(5): p. 1527-1539.

65. Folli, F., et al., *The role of oxidative stress in the pathogenesis of type 2 diabetes mellitus micro-and macrovascular complications: avenues for a mechanistic-based therapeutic approach*. *Current diabetes reviews*, 2011. **7**(5): p. 313-324.

66. Lin, H.-H., et al., *Anti-atherosclerotic effect of gossypetin on abnormal vascular smooth muscle cell proliferation and migration*. *Antioxidants*, 2021. **10**(9): p. 1357.

67. Jo, K.W., et al., *Gossypetin ameliorates 5xFAD spatial learning and memory through enhanced phagocytosis against A β* . *Alzheimer's Research & Therapy*, 2022. **14**(1): p. 1-18.

68. Oguntibeju, O.O., *Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links*. *International journal of physiology, pathophysiology and pharmacology*, 2019. **11**(3): p. 45.

69. Fischer, N., E.-J. Seo, and T. Efferth, *Prevention from radiation damage by natural products*. *Phytomedicine*, 2018. **47**: p. 192-200.

70. Devipriya, N., et al., *Quercetin ameliorates gamma radiation-induced DNA damage and biochemical changes in human peripheral blood lymphocytes*. *Mutat Res*, 2008. **654**(1): p. 1-7.

71. Benzie, I.F.; Devaki, M. The ferric reducing/antioxidant power (FRAP) assay for non-enzymatic antioxidant capacity: Concepts, procedures, limitations and applications. *Meas. Antioxid. Act. Capacit. Recent Trends Appl.* **2018**, 2018, 77–106.

72. Mounnissamy, V., et al., *Antiinflammatory activity of gossypetin isolated from Hibiscus sabdariffa*. *Indian Journal of Heterocyclic Chemistry*, 2002. **12**(1): p. 85-86.

73. Proença, C.; Rufino, A.T.; Santos, I.; Albuquerque, H.M.T.; Silva, A.M.S.; Fernandes, E.; Ferreira de Oliveira, J.M.P. Gossypetin Is a Novel Modulator of Inflammatory Cytokine Production and a Suppressor of Osteosarcoma Cell Growth. *Antioxidants* **2023**, *12*, 1744.
74. Marchio, P., et al., *Targeting early atherosclerosis: a focus on oxidative stress and inflammation*. *Oxidative medicine and cellular longevity*, 2019. **2019**.
75. Tangvarasittichai, S., *Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus*. *World journal of diabetes*, 2015. **6**(3): p. 456.
76. Hirano, T., *Pathophysiology of diabetic dyslipidemia*. *Journal of atherosclerosis and thrombosis*, 2018. **25**(9): p. 771-782.
77. Khatana, C., et al., *Mechanistic insights into the oxidized low-density lipoprotein-induced atherosclerosis*. *Oxidative medicine and cellular longevity*, 2020. **2020**.
78. Casella, S., et al., *Molecular pathways regulating macrovascular pathology and vascular smooth muscle cells phenotype in type 2 diabetes*. *International journal of molecular sciences*, 2015. **16**(10): p. 24353-24368.
79. Jha, J.C., et al., *A causal link between oxidative stress and inflammation in cardiovascular and renal complications of diabetes*. *Clinical Science*, 2018. **132**(16): p. 1811-1836.
80. Bamanikar, S., A.A. Bamanikar, and A. Arora, *Study of Serum urea and Creatinine in Diabetic and nondiabetic patients in a tertiary teaching hospital*. *The Journal of Medical Research*, 2016. **2**(1): p. 12-15.
81. Luft, F.C., *Biomarkers and predicting acute kidney injury*. *Acta Physiologica*, 2021. **231**(1): p. e13479.
82. Leyva-López, N., et al., *Flavonoids as cytokine modulators: a possible therapy for inflammation-related diseases*. *International journal of molecular sciences*, 2016. **17**(6): p. 921.
83. Al-Khayri, J.M., et al., *Flavonoids as potential anti-inflammatory molecules: A review*. *Molecules*, 2022. **27**(9): p. 2901.
84. Barone, E., et al., *The interplay among oxidative stress, brain insulin resistance and AMPK dysfunction contribute to neurodegeneration in type 2 diabetes and Alzheimer disease*. *Free Radical Biology and Medicine*, 2021. **176**: p. 16-33.
85. Rojas-Gutierrez, E., et al., *Alzheimer's disease and metabolic syndrome: A link from oxidative stress and inflammation to neurodegeneration*. *Synapse*, 2017. **71**(10): p. e21990.
86. Antonelli, M.C., et al., *Long-term consequences of prenatal stress and neurotoxicants exposure on neurodevelopment*. *Progress in neurobiology*, 2017. **155**: p. 21-35.
87. Bisht, K., K. Sharma, and M.-È. Tremblay, *Chronic stress as a risk factor for Alzheimer's disease: roles of microglia-mediated synaptic remodeling, inflammation, and oxidative stress*. *Neurobiology of stress*, 2018. **9**: p. 9-21.
88. Miranda, M., et al., *Brain-derived neurotrophic factor: a key molecule for memory in the healthy and the pathological brain*. *Frontiers in cellular neuroscience*, 2019: p. 363.
89. Ortiz, B.M., et al., *Brain-derived neurotrophic factor plasma levels and premature cognitive impairment/dementia in type 2 diabetes*. *World Journal of Diabetes*, 2016. **7**(20): p. 615.
90. Gatkikh, I.V., *Association of Serum BDNF with Severity of Cognitive Disorders in Patients with Type 2 Diabetes*. *Personalized Psychiatry and Neurology*, 2022. **2**(2): p. 67-77.
91. Gulsheen, A. Kumar, and A. Sharma, *Antianxiety and antidepressant activity guided isolation and characterization of gossypetin from Hibiscus sabdariffa Linn. calyces*. *Journal of Biologically Active Products from Nature*, 2019. **9**(3): p. 205-214.
92. Balogh, D.B., et al., *Antidepressant effect in diabetes-associated depression: A novel potential of RAAS inhibition*. *Psychoneuroendocrinology*, 2020. **118**: p. 104705.
93. Gimenez, V.M., et al., *Vitamin D-RAAS connection: an integrative standpoint into cardiovascular and neuroinflammatory disorders*. *Current Protein and Peptide Science*, 2020. **21**(10): p. 948-954.
94. Lima Giacobbo, B., et al., *Brain-derived neurotrophic factor in brain disorders: focus on neuroinflammation*. *Molecular neurobiology*, 2019. **56**: p. 3295-3312.
95. Colucci-D'Amato, L., L. Speranza, and F. Volpicelli, *Neurotrophic factor BDNF, physiological functions and therapeutic potential in depression, neurodegeneration and brain cancer*. *International journal of molecular sciences*, 2020. **21**(20): p. 7777.
96. Nguyen, T.T., et al., *Role of insulin resistance in the Alzheimer's disease progression*. *Neurochemical research*, 2020. **45**: p. 1481-1491.

97. Sharma, V.K. and T.G. Singh, *Insulin resistance and bioenergetic manifestations: Targets and approaches in Alzheimer's disease*. Life Sciences, 2020. **262**: p. 118401.
98. Jones, J.G., *Hepatic glucose and lipid metabolism*. Diabetologia, 2016. **59**(6): p. 1098-1103.
99. Mu, W., et al., *Potential nexus of non-alcoholic fatty liver disease and type 2 diabetes mellitus: insulin resistance between hepatic and peripheral tissues*. Frontiers in pharmacology, 2019. **9**: p. 1566.
100. Mohamed, J., et al., *Mechanisms of diabetes-induced liver damage: the role of oxidative stress and inflammation*. Sultan qaboos university medical journal, 2016. **16**(2): p. e132.
101. Masarone, M., et al., *Role of oxidative stress in pathophysiology of nonalcoholic fatty liver disease*. Oxidative medicine and cellular longevity, 2018. **2018**.
102. Ballestri, S., et al., *The independent predictors of non-alcoholic steatohepatitis and its individual histological features. Insulin resistance, serum uric acid, metabolic syndrome, alanine aminotransferase and serum total cholesterol are a clue to pathogenesis and candidate targets for treatment*. Hepatology Research, 2016. **46**(11): p. 1074-1087.
103. Yang, K., et al., *Efficacy and safety of dietary polyphenol supplementation in the treatment of non-alcoholic fatty liver disease: a systematic review and meta-analysis*. Frontiers in Immunology, 2022. **13**: p. 949746.
104. Myint, M., et al., *Inflammatory signaling in NASH driven by hepatocyte mitochondrial dysfunctions*. Journal of Translational Medicine, 2023. **21**(1): p. 757.
105. Luangmonkong, T., et al., *Targeting oxidative stress for the treatment of liver fibrosis*. Reviews of Physiology, Biochemistry and Pharmacology, Vol. 175, 2018: p. 71-102.
106. Eslamparast, T., et al., *Recent advances in dietary supplementation, in treating non-alcoholic fatty liver disease*. World journal of hepatology, 2015. **7**(2): p. 204.
107. He, Z., et al., *Diabetes mellitus causes male reproductive dysfunction: a review of the evidence and mechanisms*. in vivo, 2021. **35**(5): p. 2503-2511.
108. Rato, L.; Oliveira, P.F.; Sousa, M.; Silva, B.M.; Alves, M.G. Role of reactive oxygen species in diabetes-induced male reproductive dysfunction. In *Oxidants, Antioxidants and Impact of the Oxidative Status in Male Reproduction*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 135–147.
109. Chen, J., et al., *Effect of paraquat on cytotoxicity involved in oxidative stress and inflammatory reaction: A review of mechanisms and ecological implications*. Ecotoxicology and Environmental Safety, 2021. **224**: p. 112711.
110. Asmat, U., K. Abad, and K. Ismail, *Diabetes mellitus and oxidative stress—A concise review*. Saudi pharmaceutical journal, 2016. **24**(5): p. 547-553.
111. Pickering, R.J., et al., *Recent novel approaches to limit oxidative stress and inflammation in diabetic complications*. Clinical & translational immunology, 2018. **7**(4): p. e1016.
112. Pisoschi, A.M. and A. Pop, *The role of antioxidants in the chemistry of oxidative stress: A review*. European journal of medicinal chemistry, 2015. **97**: p. 55-74.
113. Thakur, P., A. Kumar, and A. Kumar, *Targeting oxidative stress through antioxidants in diabetes mellitus*. Journal of drug targeting, 2018. **26**(9): p. 766-776.
114. Wang, P., et al., *Diabetes mellitus—advances and challenges in human β -cell proliferation*. Nature Reviews Endocrinology, 2015. **11**(4): p. 201-212.
115. Entezari, M., et al., *AMPK signaling in diabetes mellitus, insulin resistance and diabetic complications: A pre-clinical and clinical investigation*. Biomedicine & Pharmacotherapy, 2022. **146**: p. 112563.
116. Joshi, T., et al., *Targeting AMPK signaling pathway by natural products for treatment of diabetes mellitus and its complications*. Journal of Cellular Physiology, 2019. **234**(10): p. 17212-17231.
117. Wang, Q., et al., *AMPK-mediated regulation of lipid metabolism by phosphorylation*. Biological and Pharmaceutical Bulletin, 2018. **41**(7): p. 985-993.
118. Marino, A., et al., *AMP-activated protein kinase: A remarkable contributor to preserve a healthy heart against ROS injury*. Free Radical Biology and Medicine, 2021. **166**: p. 238-254.
119. Coughlan, K.A., et al., *AMPK activation: a therapeutic target for type 2 diabetes?* Diabetes Metab Syndr Obes, 2014. **7**: p. 241-53.
120. Misra, P. and R. Chakrabarti, *The role of AMP kinase in diabetes*. Indian J Med Res, 2007. **125**(3): p. 389-98.
121. Madhavi, Y., et al., *Targeting AMPK in diabetes and diabetic complications: energy*

- homeostasis, autophagy and mitochondrial health*. Current medicinal chemistry, 2019. **26**(27): p. 5207-5229.
122. Babiker, A. and M. Al Dubayee, *Anti-diabetic medications: How to make a choice?* Sudanese journal of paediatrics, 2017. **17**(2): p. 11.
123. Kumar, R., D. Kerins, and T. Walther, *Cardiovascular safety of anti-diabetic drugs*. European Heart Journal-Cardiovascular Pharmacotherapy, 2016. **2**(1): p. 32-43.
124. Blahova, J., et al., *Pharmaceutical drugs and natural therapeutic products for the treatment of type 2 diabetes mellitus*. Pharmaceuticals, 2021. **14**(8): p. 806.
125. Francini, F., G.R. Schinella, and J.-L. Ríos, *Activation of AMPK by medicinal plants and natural products: its role in type 2 diabetes mellitus*. Mini reviews in medicinal chemistry, 2019. **19**(11): p. 880-901.
126. Hurtová, M., et al., *Preparation of Synthetic and Natural Derivatives of Flavonoids Using Suzuki–Miyaura Cross-Coupling Reaction*. Molecules, 2022. **27**(3): p. 967.

Bridge

The comprehensive review of existing literature in Chapter 2 highlighted key gaps in understanding the metabolic and physiological effects of gossypetin (GTIN) in the prediabetic state. In particular, limited evidence exists on the potential of GTIN to modulate glucose homeostasis and mitigate associated complications, such as cardiovascular dysfunction, hepatic impairment and renal disturbances. In order to address these gaps in the literature, Chapter 3 outlines the detailed methodology employed to investigate the effects of GTIN on glucose homeostasis, cardiovascular function, liver health and kidney function in diet-induced prediabetes.

CHAPTER 3: METHODOLOGY

1.0 Chemicals and drugs

The chemicals employed were of analytical grade and sourced from standard commercial suppliers (Merck, Darmstadt, Germany). Gossypetin was purchased from INDOFINE Chemical Company (Hillsborough, NJ, USA). Metformin was purchased from Sigma-Aldrich (St. Louis, MI, USA).

2.0 Ethical considerations

The ethical clearance of the study was approved by the Animal Research Ethics Committee (AREC) of the College of Health Sciences, the University of KwaZulu Natal (AREC NO.: AREC/00004965/2022).

3.0 Sample size calculation

In this study, we have utilised G Power software (version 3.1) to calculate the sample size. The calculation was based on fasting blood glucose values obtained from a previously published study conducted in our laboratory using the same diet-induced prediabetic male Sprague Dawley rat model [1]. Using an estimated effect size (f) of 0.68, a significance level (α) of 0.05, a statistical power ($1 - \beta$) of 0.80, and six experimental groups, G Power analysis indicated that a minimum total sample size of 36 animals ($n = 6$ per group) was required, achieving an actual statistical power of 0.84 (Table 1). Furthermore, previous studies conducted in our laboratory using the same experimental model have successfully employed six animals per group to detect significant differences in metabolic, biochemical and histological outcomes [1]. Therefore, six animals per group were considered sufficient to achieve adequate statistical power while adhering to the principles of Reduction under the 3Rs framework for the ethical use of animals in research.

Table 1: Sample size calculation on G power software

F tests—ANOVA: Fixed effects, omnibus, one-way	
Analysis: A priori: Compute required sample size	
Input	
Effect size f	0.68
α err probability	0.05
Power ($1 - \beta$ err prob)	0.80
Number of groups	6
Output	
Non-centrality parameter λ	16.65
Critical F	2.53

Numerator <i>df</i>	5
Denominator <i>df</i>	30
Total sample size	36
Actual power	0.84

4.0 Animals and housing

The study involved 36 male Sprague Dawley rats (150–180 g), which were bred and kept in the Biomedical Research Unit (BRU) at the University of KwaZulu-Natal (UKZN), Westville campus. Under standard laboratory conditions, the animals were housed at a constant temperature of 22 ± 2 °C, a carbon dioxide (CO₂) content of <5000 p.m, relative humidity of $55 \pm 5\%$ and a 12 h light/dark cycle (lights on at 07:00). Noise levels were kept under 65 decibels. Food and water were provided *ad libitum* to all animals. All animal experiments and handling were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (ethics number: AREC/00004965/2022). Before the experimental diet (high fat high carbohydrate) was introduced, the rats were allowed to acclimatize to their new environment while consuming a standard rat diet (Meadow Feeds, Lanseria, South Africa) and tap water for 1 week.

5.0 Induction of prediabetes

The animals were randomly distributed into two groups based on diet: group 1 ($n = 6$) and group 2 ($n = 30$). The induction of experimental prediabetes in the animals was carried out using the protocol outlined by Luvuno et al. [1]. Group 1 animals were given a standard rat diet and tap water, while group 2 animals received a high-fat, high-carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, Johannesburg, South Africa) supplemented with 15% fructose-enriched water to induce prediabetes over a period of 20 weeks. Following 20 weeks, the animals were assessed for prediabetes using the American Diabetes Association criteria [2]. Animals with a fasting blood glucose concentration of 5.6 to 6.9 mmol/L and a 2 h oral glucose tolerance test glucose concentration of 7.8 to 11.0 mmol/L were classified as the prediabetic group. The animals on the standard diet were also assessed at week 20 to confirm normoglycaemia and were categorised as the non-prediabetic group.

6.0 Experimental design and treatment

Following the induction of prediabetes, the prediabetic group ($n = 30$) was further divided into five sub-groups (Group 2-6), consisting of six animals each (see Figure 1). The prediabetic animals either continued the HFHC diet or changed to a normal standard diet (ND) while receiving either 15 mg/kg gossypetin (GTIN) or 500 mg/kg metformin (MET) orally once every third day for 12 weeks. We selected an oral dose of 15 mg/kg GTIN based on previous studies showing its effectiveness and safety within the 10–20 mg/kg range [3-5]. Similarly, 500 mg/kg MET was chosen due to its proven therapeutic effects at this dose and minimal side effects [6, 7]. Group 1 were the non-prediabetic

control (NPD) animals that continued the ND while group 2 were the prediabetic control animals (PD) that remained on the HFHC diet and received as the vehicle 3 mL/kg of diluted dimethyl sulphoxide (2 mL DMSO: 19 mL normal saline, p.o.)(Merck, Darmstadt, Germany). Group 3 (GTIN + ND) animals received GTIN and were changed to the ND diet while Group 4 (GTIN + HFHC) received GTIN and continued the HFHC diet. Group 5 (MET + ND) animals received MET and were changed to the ND diet while Group 6 animals (MET + HFHC) received MET and continued the HFHC diet.

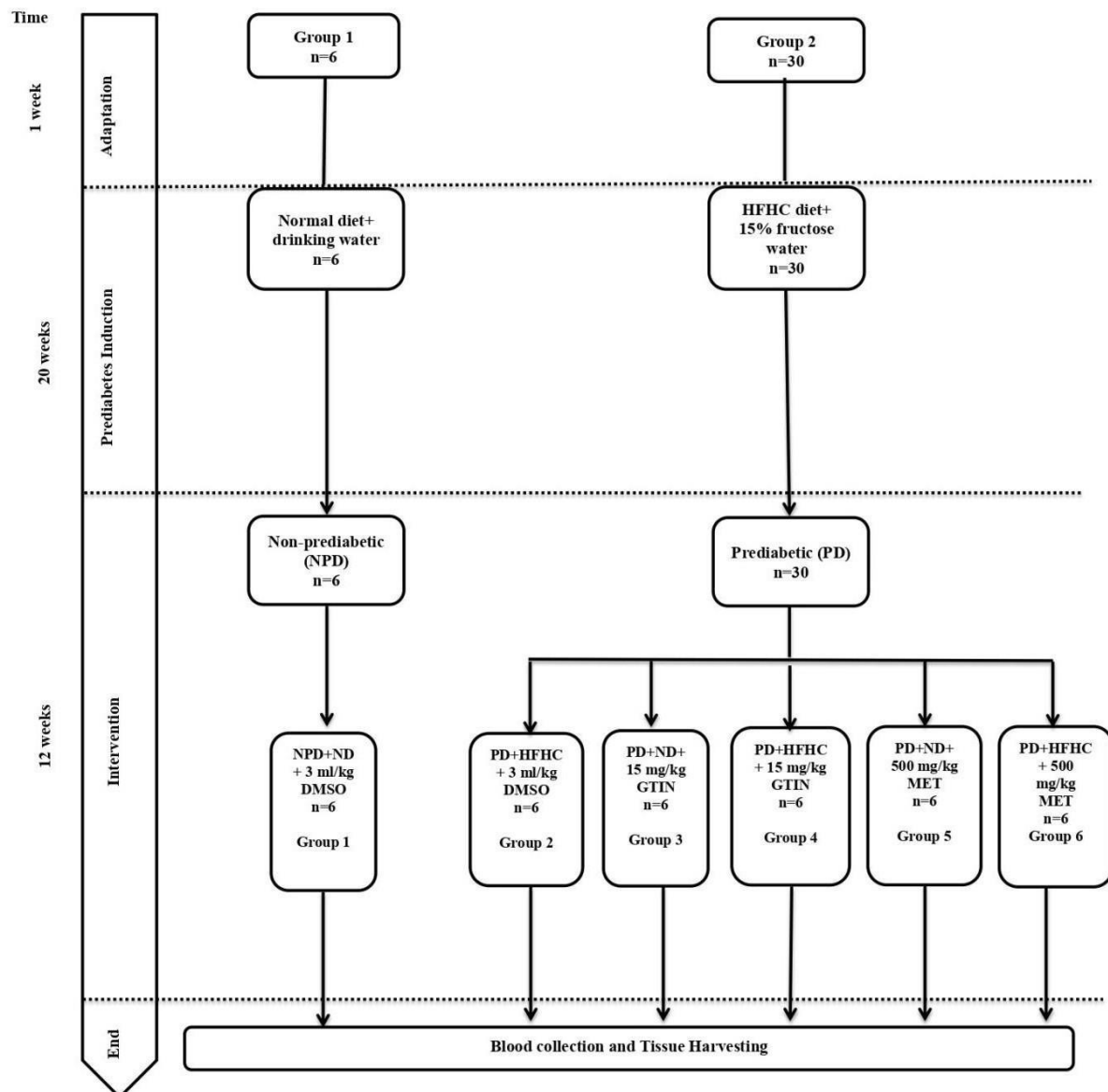


Figure 1: Experimental layout and design

7.0 Blood collection and tissue harvesting

At the end of week 32, all animals were anaesthetised with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd., Roodeport, South Africa) for 3 min using a gas anaesthetic chamber

(BRU, UKZN, Durban, South Africa). Once the animals were unconscious, blood samples were obtained from the animals through cardiac puncture and transferred into individual pre-cooled heparinised containers. The blood samples were centrifuged (Eppendorf AG, Eppendorf centrifuge 5403, Hamburg, Germany) at 4 °C, 503× *g* for 15 min to obtain plasma. Each plasma sample was aspirated into plain sample bottles and stored at –80 °C in a Bio Ultra freezer (Snijers Scientific, Tilburg, The Netherlands) until ready for biochemical analysis. Skeletal muscle, heart, liver and kidneys were removed and rinsed in normal saline. The tissues were snap-frozen in liquid nitrogen and stored at –80 °C in a BioUltra freezer for biochemical analysis. The right liver lobe was preserved in 10% formalin buffer for histopathological examination.

8.0 Methodology for study 1

Parameters such as body weight, percentage body weight change, caloric intake, percentage caloric intake change and fasting blood glucose (FBG) levels were monitored at weeks 20, 24, 28, and 32. The oral glucose tolerance test (OGTT) was conducted at week 32 following the protocol established in prior studies [6, 7].

8.1 Biochemical analysis

At the end of week 32, plasma insulin levels were measured using sandwich ELISA while ghrelin levels were measured using competitive ELISA as per the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). Glycated haemoglobin (HbA1c) levels were measured using competitive ELISA (Cloud-Clone Corp, Wuhan, China). In sandwich ELISA, the analyte is captured between two antibodies, whereas in competitive ELISA the sample antigen competes with a labelled antigen for binding to a limited amount of antibody.

The homeostatic model assessment for insulin resistance (HOMA2-IR) index was calculated from the insulin and FBG concentrations (<https://www.dtu.ox.ac.uk/homacalculator/>) [8]. Glycogen levels in liver and skeletal muscle tissues were assessed following an established protocol [6, 7]. The absorbance was read using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Württemberg, Germany) at 620 nm. The glycogen concentrations were calculated from a glycogen standard curve.

More information on the methodology for study 1 is given in Chapter 4.

9.0 Methodology for study 2

Parameters such as body mass index (BMI), waist circumference (WC) and blood pressure were monitored at weeks 20, 24, 28 and 32 as described in the established protocol [9, 10]. The systolic and diastolic pressure of all experimental animals was measured using a non-invasive tail-cuff method and the mean arterial pressure (MAP) was calculated using the formula below [10, 11]:

$$\text{MAP (mmHg)} = \frac{(2 \times \text{diastolic pressure}) + \text{systolic pressure}}{3}$$

9.1 Biochemical analysis

At the end of week 32, the plasma lipid profile was analysed by measuring triglyceride (TAG), total cholesterol (TC) and high density lipoprotein (HDL) using the respective assay kits according to the manufacturer instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). These assays were based on the principle of enzymatic reactions that produce a colour change proportional to the concentration of the analyte. VLDL and LDL cholesterol levels were calculated using Friedewald's formula as seen below [12]:

$$\text{LDL (mmol/L)} = \text{TC} - \text{HDL} - (\text{TG}/2.2) \text{ and } \text{VLDL (mmol/L)} = \text{TG}/2.2$$

Lipid peroxidation was determined by measuring heart malondialdehyde (MDA) levels using a previously published protocol [9, 11]. The assay is based on the principle that MDA reacts with thiobarbituric acid (TBA) under high temperature and acidic conditions to form a pink MDA-TBA adduct, which can be quantified spectrophotometrically at 532 nm. The absorbance from these wavelengths was used to calculate the concentration of MDA using Beer's Law as follows [9, 11]:

$$\text{MDA concentration (nmol/g)} = \frac{\text{Average Absorbance}}{\text{Absorption coefficient (156 nm)}}$$

Heart antioxidant enzyme activity including superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured according to manufacturer instruction (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). Endothelial function and inflammatory marker levels were determined by measuring plasma endothelial nitric oxide (eNOS), tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6) and C-reactive protein (CRP) using sandwich ELISA according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

More information on the methodology for study 2 is given in Chapter 5.

10.0 Methodology for study 3

The liver weights of all the animals in each experimental group were measured using an analytical balance at week 32.

10.1 Biochemical analysis

At the end of week 32, plasma aspartate transaminase (AST), alanine transaminase (ALT) and bilirubin levels were analysed with IDEXX Catalyst One Chemistry Analyzer (IDEXX Laboratories Inc. Westbrook, ME, USA). Plasma sterol regulatory element binding protein 1c (SREBP-1c) levels

were measured using a competitive ELISA kit (Biomatik USA, LLC, Wilmington, DE, USA).

Liver tissue samples and the homogenate medium used for determining liver TAG levels, SOD activity and Gpx activity were prepared according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). Liver triglyceride levels were measured according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). Liver antioxidant enzyme activity including SOD and GPx was determined using the respective assay kits according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). The liver MDA concentration was quantified following a previously established laboratory protocol [9, 11].

10.2 Histology

At week 32, histological examination of the right liver lobe was performed following a previously documented protocol [13]. The slides were stained with hematoxylin and eosin (H&E). The processed tissue sections were visualized and captured with a Leica SCN400 scanner and analysed using Aperio ImageScope software (version 12.4.6; Leica Biosystems, Buffalo Grove, IL, USA). The liver tissue slides were assessed for the following: Lipid droplet accumulation, sinusoid disruption and hepatocyte plate arrangement.

More information on the methodology for study 3 is given in Chapter 6.

11.0 Methodology for study 4

Fluid intake and urine output were measured in all the animals by placing them in individual metabolic cages for 24 hours during weeks 20, 24, 28 and 32. The urine samples were centrifuged at 13000 rpm for 5 minutes at 4°C and the supernatants were stored at -80°C in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) until ready for biochemical analysis.

11.1 Biochemical analysis

At week 32, kidney function parameters (such as creatinine, urea, uric acid, albumin) were determined in the plasma and urine samples by using their respective assay kits according to the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). Urine albumin and electrolytes (Na⁺ and K⁺) levels were measured by Global Clinical and Viral Laboratories (Amanzimtoti, Durban, South Africa). Plasma kidney injury molecule (KIM-1) and aldosterone concentrations were determined from their specific ELISA kits as instructed by the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

The glomerular filtration rate (GFR) of all the animals was determined from the estimation of

creatinine in the plasma and urine (creatinine clearance) as follows [14]:

$$\text{GFR [mL/min]} = \frac{\text{Urine creatinine } \left(\frac{\text{mg}}{\text{dl}}\right) \times 24 \text{ hrs urine volume (mL)}}{\text{Plasma creatinine } \left(\frac{\text{mg}}{\text{dl}}\right) \times 60 \text{ min} \times 24 \text{ hours}}$$

Kidney total antioxidant status (TAS), IL-6 and TNF- α levels were measured using their respective ELISA kits (Elabsience Biotechnology Co., Ltd., Houston, TX, USA).

11.2 PCR

Ribonucleic acid (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. The purity of the RNA was confirmed by the relative absorbance ratio of 260/280 nm via a Nanodrop 1000 spectrophotometer (Thermo Scientific, Santa Clara, CA, USA).

A cDNA synthesis kit was used to produce reverse transcription reactions using 2 μ g of total RNA as described by the manufacturer (New England Biolabs, Ipswich, MA, USA). The Luna Universal qPCR Master Mix was utilized for amplification (New England Biolabs, Ipswich, MA, USA). All samples were run in duplicate and β -actin mRNA was used as a housekeeping gene (β -actin: forward primer 5'-AGTGCCAGCCTCGTCTCATA-3'; reverse primer 5'-GATGGTGATGGGTTTCCCGT-3' to normalise the podocin mRNA level. The sequences of the used oligonucleotide primers were as follows: podocin forward 5'-TGGAAGCTGAGGCACAAAGA-3' and podocin reverse 5'-AGAATCTCAGCCGCCATCCT-3' (Inqaba Biotec, Pretoria, South Africa). The $2^{-\Delta\Delta C_t}$ relative quantification method was used to represent gene expression values.

More information on the methodology for study 4 is given in Chapter 7.

12.0 Statistical analysis

GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA, USA) was used to analyse data for all groups in all studies (Study 1-4). Prior to statistical analysis, the assumptions of normality and homogeneity of variance were assessed to ensure the suitability of the data for parametric analyses. Data collected repeatedly from the same animals over time, including fasting blood glucose, caloric intake, mean arterial pressure, BMI, WC, fluid intake and urine output, were analysed using two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons post hoc test. Variables measured at a single time point were analysed by one-way analysis of variance followed by Tukey–Kramer via GraphPad Prism 5. The statistical data were presented as mean $\pm$ SEM. Statistical significance was determined at $p < 0.05$.

13.0 References

1. Khathi, A.; Luvuno, M.; Mabandla, M., Voluntary ingestion of a high-fat high-carbohydrate diet:

- a model for prediabetes. *PONTE International Journal of Science and Research* 2018, 74, (5).
2. Hostalek, U., Global epidemiology of prediabetes-present and future perspectives. *Clinical diabetes and endocrinology* 2019, 5, (1), 5.
 3. Ijaz, M. U.; Alvi, K.; Khan, H. A.; Imran, M.; Afsar, T.; Almajwal, A.; Amor, H.; Razak, S., Gossypetin mitigates doxorubicin-induced nephrotoxicity: A histopathological and biochemical evaluation. *Journal of King Saud University-Science* 2023, 35, (7), 102830.
 4. Lin, H.-H., In vitro and in vivo atheroprotective effects of gossypetin against endothelial cell injury by induction of autophagy. *Chemical Research in Toxicology* 2015, 28, (2), 202–215.
 5. Proença, C.; Rufino, A. T.; Santos, I.; Albuquerque, H. M.; Silva, A. M.; Fernandes, E.; Ferreira de Oliveira, J. M. P., Gossypetin is a novel modulator of inflammatory cytokine production and a suppressor of osteosarcoma cell growth. *Antioxidants* 2023, 12, (9), 1744.
 6. Khumalo, B.; Siboto, A.; Akinnuga, A. M.; Sibiya, N.; Khathi, A.; Ngubane, P. S., Momordica balsamina improves glucose handling in a diet-induced prediabetic rat model. *PLoS One* 2023, 18, (12), e0295498.
 7. Khathi, A.; Serumula, M. R.; Myburg, R. B.; Van Heerden, F. R.; Musabayane, C. T., Effects of Syzygium aromaticum-derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge. *PloS one* 2013, 8, (11), e81632.
 8. Delanghe, J. R.; Verlinde, E.; Speeckaert, M. M.; Maenhout, T., HOMA-IR and HOMA2-IR estimation based on glycated hemoglobin as an alternative for fasting glucose. *Acta Clinica Belgica* 2023, 78, (4), 308–312.
 9. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Plant-derived oleanolic acid (OA) ameliorates risk factors of cardiovascular diseases in a diet-induced pre-diabetic rat model: effects on selected cardiovascular risk factors. *Molecules* 2019, 24, (2), 340.
 10. Mbatha, B.; Khathi, A.; Sibiya, N.; Booysen, I.; Mangundu, P.; Ngubane, P., Cardio-protective effects of a dioxidovanadium (V) complex in male sprague–dawley rats with streptozotocin-induced diabetes. *BioMetals* 2021, 34, (1), 161–173.
 11. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Plant-derived oleanolic acid ameliorates markers associated with non-alcoholic fatty liver disease in a diet-induced pre-diabetes rat model. *Diabetes, Metabolic Syndrome and Obesity* 2019, 1953–1962.
 12. Knopfholz, J.; Disserol, C. C. D.; Pierin, A. J.; Schirr, F. L.; Streisky, L.; Takito, L. L.; Massucheto Ledesma, P.; Faria-Neto, J. R.; Olandoski, M.; Da Cunha, C. L. P., Validation of the friedewald formula in patients with metabolic syndrome. *Cholesterol* 2014, 2014, (1), 261878.
 13. Mabuza, L. P.; Gamede, M. W.; Maikoo, S.; Booysen, I. N.; Nguban, P. S.; Khathi, A., Hepatoprotective effects of a ruthenium (II) Schiff base complex in rats with diet-induced prediabetes. *Current Therapeutic Research* 2019, 91, 66–72.

14. Siboto, A.; Sibiya, N.; Khathi, A.; Ngubane, P., The Effects of Momordica balsamina Methanolic Extract on Kidney Function in STZ-Induced Diabetic Rats: Effects on Selected Metabolic Markers. *Journal of diabetes research* 2018, 2018, (1), 7341242.

Bridge

The methodology presented in Chapter 3 included the experimental design, animal model, treatment interventions and biochemical analysis. Chapter 4 consists of an original research manuscript that presents the findings of manuscript 1, which investigates the effects of gossypetin (GTIN) on glucose homeostasis in both the presence and absence of dietary intervention in diet-induced prediabetic rats. The study also compares the effects of GTIN with those of metformin, a standard anti-diabetic agent.

CHAPTER 4: MANUSCRIPT 1

PROLOGUE

The habitual consumption of high-calorie diets has been shown to promote the development of prediabetes and type 2 diabetes mellitus (T2DM). Prediabetes is an intermediate hyperglycaemic state which often precedes the development of T2DM. In prediabetes, glucose homeostasis becomes progressively impaired due to the early onset of insulin resistance and compensatory hyperinsulinaemia. The current management strategies for prediabetes involve a combination of dietary and pharmacological interventions. However, there still remains a low level of patient compliance with this approach as most patients merely use pharmacological intervention without any change in diet. Hence, the efficacy of pharmacological interventions is reduced. This limitation highlights a significant gap in the literature regarding the identification of compounds that remain effective even in the absence of dietary intervention. Flavonoids have garnered attention for their potent antihyperglycaemic and insulin-sensitising properties. Gossypetin (GTIN) is a hexahydroxylated flavonoid which has recently demonstrated potent antioxidant and anti-inflammatory effects. However, no studies have shown the effects of GTIN on glucose homeostasis during the prediabetic state. Hence, in manuscript 1, we investigated the effects of GTIN on glucose homeostasis in both the presence and absence of dietary intervention in a diet-induced prediabetic rat model.

The manuscript in Chapter 4 is titled “**Effects of gossypetin on glucose homeostasis in diet-induced prediabetic rats**” and is authored by Naidoo K and Khathi A.

The manuscript has been published in the journal “**Molecules**” and formatted according to the journal’s guidelines for authors. See Appendix 3.

Candidate Contribution: Karishma Naidoo was responsible for study conceptualisation, study design, all experimental work, statistical analysis, first draft writing and manuscript editing.

Investigating the effects of gossypetin on glucose homeostasis in diet-induced pre-diabetic male Sprague Dawley rats

Karishma Naidoo^{1,2}, Andile Khathi¹

1. University of KwaZulu-Natal, Westville, KwaZulu-Natal South Africa
2. Corresponding Author: Karishma Naidoo

School of Laboratory Medicine & Medical Sciences

University of KwaZulu-Natal

Private Bag X54001

Durban,

SOUTH AFRICA

Phone: 0711607469 /27 (0) 31 260 7585, Fax: 27 (0) 31 260 7132

Email: karishma280199@gmail.com /khathia@ukzn.ac.za

Keywords: Gossypetin, high fat high carbohydrate, flavonoid, pre-diabetes

Abstract:

Flavonoid compounds have gained considerable interest due to their diverse biological activities, including their potential role in improving metabolic dysfunction associated with impaired glucose regulation. However, the effects of gossypetin (GTIN) on glucose homeostasis during the pre-diabetic state remain unexplored. This study examined the effects of GTIN on key markers of glucose homeostasis in a diet-induced pre-diabetic rat model. Pre-diabetes was induced by allowing the animals to feed on a high-fat high-carbohydrate (HFHC) diet supplemented with 15% fructose water for 20 weeks. Following pre-diabetes induction, the pre-diabetic animals were sub-divided into five groups (n = 6), where they were either orally treated with GTIN (15 mg/kg) or metformin (MET) (500 mg/kg), both with and without dietary intervention, over a 12-week period. The results demonstrated that animals in the untreated pre-diabetic (PD) control group exhibited significantly higher fasting and postprandial blood glucose levels, as well as elevated plasma insulin concentrations and increased homeostatic model assessment for insulin resistance (HOMA2-IR) index, relative to the non-pre-diabetic (NPD) group. Similarly, increased caloric intake, body weight and plasma ghrelin levels were observed in the PD control group. Notably, these parameters were significantly reduced in the PD animals receiving GTIN treatment. Additionally, glycogen levels in the liver and skeletal muscle, which were disturbed in the PD control group, showed significant improvement in both GTIN-treated groups. These findings may suggest that GTIN administration, with or without dietary modifications, may offer therapeutic benefits in ameliorating glucose homeostasis disturbances associated with the PD state.

1.0 Introduction

Pre-diabetes is a metabolic state characterised by blood glucose concentrations above normal physiological levels but below the diagnostic threshold for type 2 diabetes mellitus (T2DM) [1]. This condition is associated with early insulin resistance and hyperinsulinaemia [2, 3]. The prevalence of pre-diabetes has been linked to dietary patterns, particularly those high in fats and carbohydrates, commonly observed in “Westernized” diets [4]. As of 2017, the global prevalence of impaired glucose tolerance (IGT) was estimated at 7.3% among adults, affecting approximately 352.1 million individuals, with projections indicating an increase to 8.3% by 2045 [1, 4]. Untreated pre-diabetes is recognised as a precursor to T2DM development and its associated complications [4, 5].

The development of pre-diabetes involves progressive disruption of glucose regulation through impaired peripheral glucose utilisation and altered hepatic glucose metabolism [5]. Additionally, decreased cellular glucose uptake stimulates increased food intake, mediated by elevated ghrelin levels [6]. The progression from pre-diabetes to T2DM is significantly influenced by lifestyle factors, particularly dietary habits and adherence to prescribed interventions. Despite the proven efficacy of dietary modifications and pharmacological treatments in mitigating the risk of T2DM, adherence to these lifestyle changes remains a challenge [7]. Thus, there is a need for alternative treatments that remain effective even in the absence of dietary modifications [6].

Flavonoids, a diverse group of polyphenolic compounds found abundantly in plants, have demonstrated significant antioxidant properties, capable of scavenging various free radicals, including hydroxyl radicals, superoxide anions and lipid peroxy radicals [8, 9]. The antioxidant efficacy of flavonoids is influenced by their structural configuration and the number of hydroxyl groups present [10, 11]. Quercetin, a well-known flavonoid, has been reported to improve glucose homeostasis in both pre-diabetic and diabetic states due to its antioxidant activity [12, 13]. GTIN, a hexahydroxylated flavonoid extracted from the calyx of *Hibiscus sabdariffa*, possesses a chemical structure similar to that of quercetin. It has been reported to exhibit more potent antioxidant effects compared to quercetin [14, 15]. Previous studies have reported that GTIN exhibits several biological effects, including anti-inflammatory, neuroprotective, hepatoprotective, and renoprotective activities [14, 15]. However, despite these promising pharmacological properties, the influence of GTIN on glucose homeostasis during the pre-diabetic state remains unexplored. Therefore, this study aims to evaluate the effects of GTIN on key markers associated with glucose homeostasis in a diet-induced PD rat model, both with and without dietary modifications.

2.0 Results

2.1 Oral glucose tolerance (OGT) test

The OGTT was measured at the end of week 12 of the treatment period ($n = 6$, per group). At 0 min (Figure 1a), the PD control group showed significantly ($p = < 0.0001$) higher fasting blood glucose (FBG) levels in comparison to the NPD group. However, PD animals receiving GTIN with either a normal diet (GTIN + ND) ($p = 0.0017$) or a high-fat high-carbohydrate diet (GTIN + HFHC) ($p = 0.0146$) showed significantly lower plasma glucose levels in comparison to the PD control group. Similarly, FBG levels were significantly ($p = 0.0005$) lower in PD animals receiving MET with a normal diet (MET + ND) in comparison to the PD control group. At 120 min post-glucose load, the PD control group showed significantly ($p = 0.0015$) higher plasma glucose levels in comparison to the NPD group. The GTIN + ND and GTIN + HFHC groups had significantly lower blood glucose levels by 37.72% and 27.09%, while the MET + ND and MET + HFHC groups showed reductions of 30.10% and 23.93% in comparison to the PD control group. Additionally, the PD control group exhibited a significantly higher area under the curve (AUC) at all intervals (Figure 1b) in comparison to the NPD group. Both GTIN-treated groups (31.16% and 19.32%) and MET-treated groups (25.30% and 3.45%) exhibited significantly lower AUC values at 60–120 min in comparison to the PD control group.

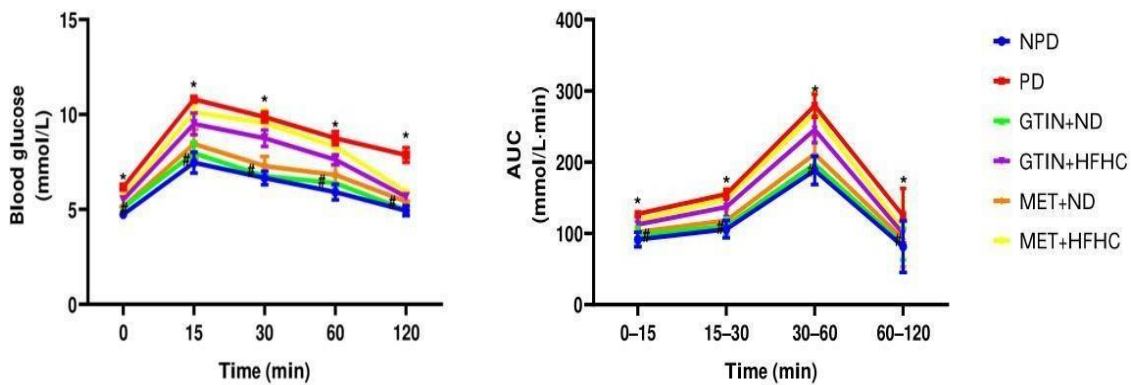


Figure 1. The effects of GTIN on (a) blood glucose concentrations during the 2-h OGTT and (b) AUC after 12 weeks of treatment. Data are presented as mean $\pm$ SEM ($n = 6$ per group). * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

2.2. HOMA2-IR Index

The HOMA2-IR values were calculated at week 12 of the treatment period ($n = 6$, per group). The results (Table 1) showed that the FBG ($p = < 0.0001$) and insulin ($p = < 0.0001$) levels were significantly higher in the PD control group in comparison to the NPD group. The HOMA2-IR value for the NPD group was within the insulin-sensitive range (< 1.0), while the PD control group had a

significantly ($p = < 0.0001$) higher HOMA2-IR value compared to the NPD, which was in the range of early insulin resistance (>1.9). Both GTIN + ND and GTIN + HFHC groups showed significantly lower FBG (17.44% and 10.76%), insulin levels (57.88% and 53.05%) and HOMA2-IR index values (70.37% and 62.0%) in comparison to the PD control group. The MET + ND group also displayed significantly lower FBG (16.80%) ($p = < 0.0001$), insulin (57.20%) ($p = < 0.0001$) and HOMA2-IR values (60.07%) ($p = < 0.0001$) in comparison to the PD control group.

Table 1. The effects of GTIN on FBG, plasma insulin and HOMA2-IR index in rats with or without dietary modification at week 12 of the treatment period (n = 6, per group). Values are presented as mean $\pm$ SEM.

Experimental Group	Plasma Fasting Glucose (mmol/L)	Plasma Insulin (pmol/L)	HOMA2-IR Index
NPD	4.73 $\pm$ 0.12	28.03 $\pm$ 0.79	0.85 $\pm$ 0.038
PD	6.17 $\pm$ 0.08 *	68.36 $\pm$ 4.09 *	2.74 $\pm$ 0.19 *
GTIN + ND	5.08 $\pm$ 0.14 # ^	28.22 $\pm$ 0.83 #	0.92 $\pm$ 0.036 #
GTIN + HFHC	5.50 $\pm$ 0.073 #	31.57 $\pm$ 0.43 #	1.11 $\pm$ 0.022 #
MET + ND	5.13 $\pm$ 0.12 #	28.70 $\pm$ 0.66 #	0.94 $\pm$ 0.034 #
MET + HFHC	5.70 $\pm$ 0.073 *	34.51 $\pm$ 1.30 #	1.26 $\pm$ 0.063 *

* = $p < 0.05$ denotes comparison with NPD; # = $p < 0.05$ denotes comparison with PD; ^ = $p < 0.05$ denotes comparison with MET + HFHC.

2.3. Glycated Haemoglobin

Glycated haemoglobin (HbA1c) concentrations were measured in all experimental groups at week 12 of the treatment period (n = 6, per group). The results (Figure 2) showed that the HbA1c levels were significantly ($p = < 0.0001$) higher in the PD control group in comparison to the NPD group. However, it was observed that the HbA1c levels were significantly lower in both the GTIN + ND ($p = < 0.0001$) and GTIN + HFHC ($p = < 0.0001$) groups by 54.24% and 50.22% in comparison to the PD control group. Similarly, both the MET + ND ($p = < 0.0001$) and MET + HFHC ($p = < 0.0001$) groups showed significantly lower HbA1c levels by 53.66% and 30.70% in comparison to the PD control group.

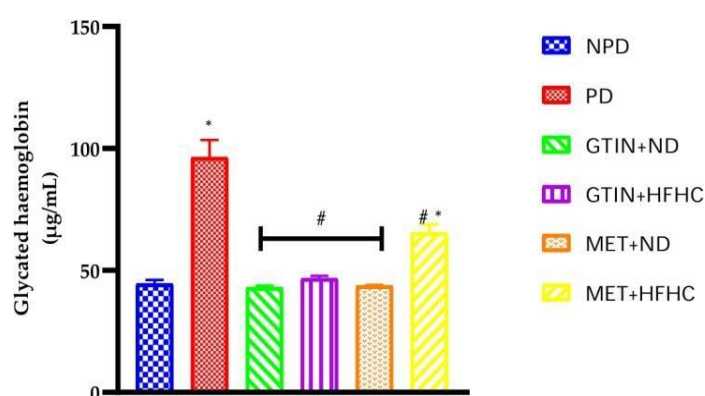


Figure 2. Effects of GTIN on HbA1c levels after 12 weeks of treatment. Data are presented as mean $\pm$ SEM (n = 6 per group). * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

2.4. Caloric Intake

The caloric intake and percentage increase in caloric intake of the animals were determined every fourth week of the 12-week treatment period (n = 6, per group). The results (Table 2) showed that the PD control group had significantly ($p = < 0.0001$) higher caloric intake in comparison to the NPD group throughout the treatment period. At week 12, both the GTIN + ND ($p = < 0.0001$) and GTIN + HFHC ($p = < 0.0001$) groups showed significantly reduced caloric intake (17.26% and 11.86%) and percentage increase in caloric intake (19.18% and 6.76%) in comparison to the PD control group. The MET + ND group also exhibited significantly ($p = < 0.0001$) lower caloric intake (15.46%) and percentage increase (14.53%) in comparison to the PD control group.

Table 2. The effects of GTIN on caloric intake and percentage increase in caloric intake from week 0 to week 12 of the treatment phase in rats with or without dietary modification (n = 6, per group). Values are presented as mean $\pm$ SEM and in percentage increase ($\uparrow$ = increase and $\downarrow$ = decrease).

Caloric Intake (Kcal/g)				
Experimental Group	Week 0	Week 4	Week 8	Week 12
NPD	139.92 $\pm$ 2.25	142.90 $\pm$ 2.44	144.34 $\pm$ 2.23	144.16 $\pm$ 2.12
	(100%)	($\uparrow$ 2.15%)	($\uparrow$ 3.14%)	($\uparrow$ 3.02%)
PD	162.14 $\pm$ 2.64 *	171.16 $\pm$ 2.39 *	169.43 $\pm$ 2.74 *	170.93 $\pm$ 2.99 *
	(100%)	($\uparrow$ 5.28%) *	($\uparrow$ 4.30%)	($\uparrow$ 5.13%)
GTIN + ND	168.13 $\pm$ 2.84 *	160.47 $\pm$ 2.79 *	150.87 $\pm$ 1.98	141.10 $\pm$ 2.62 #^
	(100%)	($\downarrow$ 4.77%) *#^	($\downarrow$ 11.42%) *#^	($\downarrow$ 19.18%) *#^
GTIN + HFHC	163.31 $\pm$ 4.37 *	165.08 $\pm$ 4.51 *	166.06 $\pm$ 4.78 *	150.65 $\pm$ 3.64 #
	(100%)	($\uparrow$ 2.98%) #	($\uparrow$ 3.09%)	($\downarrow$ 6.76%) *#
MET + ND	165.12 $\pm$ 1.66 *	162.09 $\pm$ 1.67 *	159.02 $\pm$ 1.89 *	144.18 $\pm$ 1.56 #^
	(100%)	($\downarrow$ 1.87%) *#^	($\downarrow$ 2.12%) *#^	($\downarrow$ 14.53%) *#^
MET + HFHC	162.79 $\pm$ 2.65 *	168.86 $\pm$ 2.92 *	168.45 $\pm$ 2.04 *	158.17 $\pm$ 2.55 *
	(100%)	($\uparrow$ 3.58%)	($\uparrow$ 3.39%)	($\downarrow$ 2.92%) *#

* = $p < 0.05$ denotes comparison with NPD, # = $p < 0.05$ denotes comparison with PD, ^ = $p < 0.05$ denotes comparison with MET + HFHC.

2.5. Body Weight

The body weight and percentage increase body weight of the animals were determined every fourth week of the 12-week treatment period ($n = 6$, per group). The results (Table 3) showed that the body weight of the PD control group was significantly ($p = < 0.0001$) higher throughout the experimental period in comparison to the NPD group. At week 8, the GTIN + ND group demonstrated a significantly ($p = < 0.0001$) lower body weight and percentage increase (11.35% and 11.34%) in comparison to the PD control group. By week 12, reductions were 12.28% in body weight and 4.92% in percentage increase. The GTIN + HFHC group showed a significant ($p = < 0.0001$) reduction in percentage body weight increase by 4.59% at week 12 in comparison to the PD control group. The MET + ND group also exhibited a significantly ($p = < 0.0001$) lower percentage increase in body weight by 3.08% in comparison to the PD control group.

Table 3. The effects of GTIN on body weight and percentage increase in body weight from week 0 to week 12 of the treatment phase in rats with or without dietary modification ($n = 6$, per group). Values are presented as mean $\pm$ SEM and in percentage increase ($\uparrow$ = increase and $\downarrow$ = decrease).

Body Weight (g)				
Experimental Group	Week 0	Week 4	Week 8	Week 12
NPD	528.2 $\pm$ 18.21 (100%)	540.7 $\pm$ 18.53 ($\uparrow$ 2.30%)	554.0 $\pm$ 17.87 ($\uparrow$ 4.65%)	554.8 $\pm$ 17.51 ($\uparrow$ 4.77%)
PD	650.2 $\pm$ 9.46 * (100%)	673.7 $\pm$ 11.46 * ($\uparrow$ 3.47%)	692.7 $\pm$ 10.17 * ($\uparrow$ 6.13%)	708.0 $\pm$ 15.71 * ($\uparrow$ 8.09%)
GTIN + ND	665.3 $\pm$ 16.86 * (100%)	634.0 $\pm$ 15.08 * ($\downarrow$ 8.63%) *#^	612.7 $\pm$ 17.04 # ($\downarrow$ 11.34%) *#^	597.7 $\pm$ 15.74 #^ ($\downarrow$ 4.92%) ^#*
GTIN + HFHC	633.0 $\pm$ 13.09 (100%)	637.0 $\pm$ 12.96 #^ ($\uparrow$ 0.67%)	651.5 $\pm$ 12.21 # ($\uparrow$ 2.83%)	663.0 $\pm$ 11.88 ($\uparrow$ 4.59%) #^
MET + ND	638.5 $\pm$ 18.71 * (100%)	612.7 $\pm$ 18.78 #* ($\downarrow$ 4.24%)	618.8 $\pm$ 18.19 #^* ($\downarrow$ 3.18%)	619.5 $\pm$ 18.73 ($\downarrow$ 3.08%) *#
MET + HFHC	651.3 $\pm$ 23.82 * (100%)	671.3 $\pm$ 24.47 ($\uparrow$ 2.98%)	690.7 $\pm$ 22.40 * ($\uparrow$ 5.75%)	700.5 $\pm$ 23.49 * ($\uparrow$ 7.06%) *

* = $p < 0.05$ denotes comparison with NPD; # = $p < 0.05$ denotes comparison with PD; ^ = $p < 0.05$ denotes comparison with MET + HFHC.

2.6. Plasma Ghrelin Concentrations

Plasma ghrelin concentrations were measured in all experimental groups at week 12 of the treatment

period ($n = 6$, per group). The results (Figure 3) showed that the plasma ghrelin levels were significantly ($p = < 0.0001$) higher in the PD control group in comparison to the NPD group. Furthermore, both the GTIN + ND ($p = < 0.0001$) and GTIN + HFHC ($p = < 0.0001$) groups had significantly lower plasma ghrelin levels by 41.53% and 33.79% in comparison to the PD control group. Interestingly, there was no significant difference ($p = 0.4213$) in the ghrelin concentrations between the NPD and GTIN + ND groups.

Similarly, both the MET + ND ($p = < 0.0001$) and MET + HFHC ($p = 0.0005$) groups had significantly lower plasma ghrelin by 40.29% and 13.51% in comparison to the PD control group.

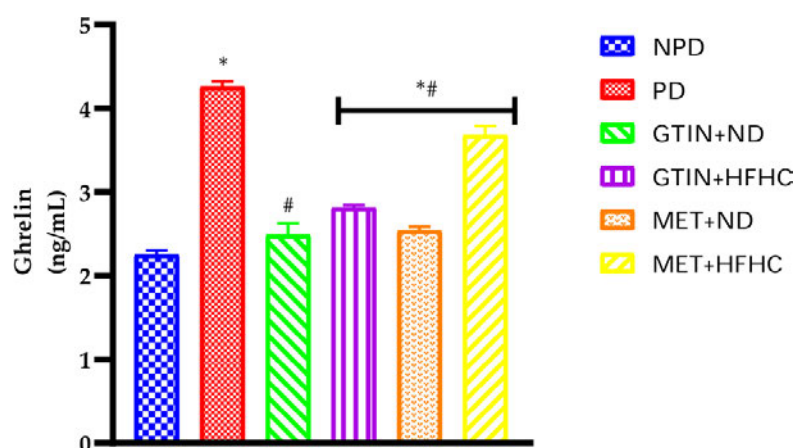


Figure 3. Effects of GTIN on plasma ghrelin concentration after 12 weeks of treatment. Data are presented as mean $\pm$ SEM ($n = 6$ per group). * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

2.7. Liver and Skeletal Muscle Glycogen Concentrations

Liver and skeletal muscle glycogen concentrations were measured in all experimental groups at week 12 of the treatment period ($n = 6$, per group). The results showed (Figure 4) that the glycogen concentrations in the PD control group were significantly ($p = < 0.0001$) higher in the liver, while significantly ($p = < 0.0001$) lower in the skeletal muscle in comparison to the NPD group. Both the GTIN + ND and GTIN + HFHC groups showed significantly lower liver glycogen levels ($p = < 0.0001$, $p = < 0.0001$) by 54.26% and 52.86% and significantly higher skeletal muscle glycogen ($p = < 0.0001$, $p = < 0.0001$) levels by 60.40% and 47.66% in comparison to the PD control group. Similarly, the MET + ND and MET + HFHC groups exhibited significantly lower liver glycogen levels ($p = < 0.0001$, $p = < 0.0001$) by 51.70% and 45.27% and higher skeletal muscle glycogen ($p = < 0.0001$, $p = < 0.0001$) levels by 53.08% and 27.51% in comparison to the PD control group.

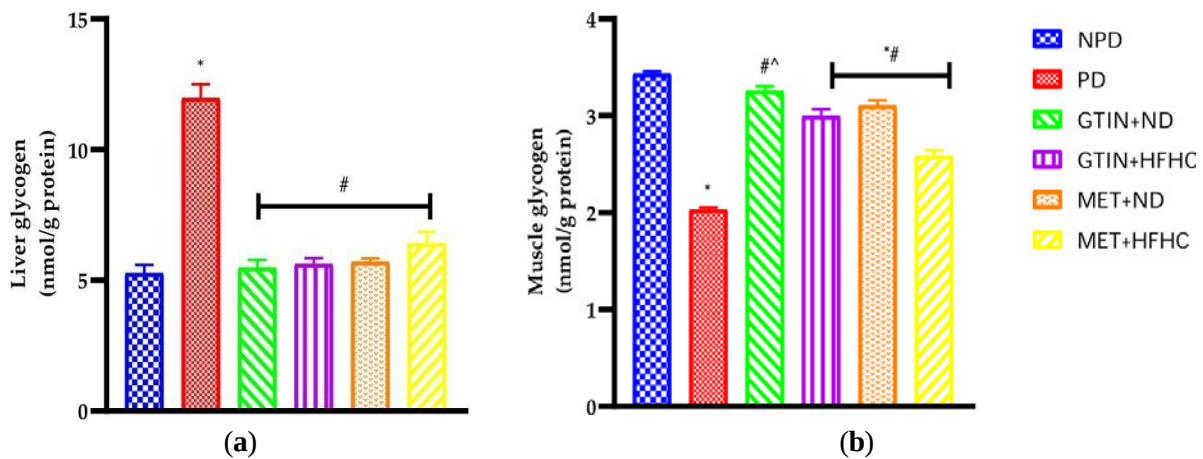


Figure 4. Effects of GTIN on (a) liver and (b) skeletal muscle glycogen concentration after 12 weeks of treatment. Data are presented as mean $\pm$ SEM ($n = 6$ per group). * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD; ^ $p < 0.05$ vs. MET + HFHC.

3.0 Discussion

Flavonoids are phenolic compounds found in fruits and vegetables as secondary metabolites [16]. These naturally occurring compounds possess anti-diabetic effects [10, 17]. Quercetin, a well-known flavonoid, has been shown to significantly improve insulin sensitivity, reduce caloric intake, HbA1c levels and FBG in the PD state [10, 18, 19]. Thus, there is an increased demand to investigate quercetin and its derivatives, as it is hypothesised that similarly structured compounds may exhibit similar biological activity [20, 21]. GTIN ameliorates many complications by targeting oxidative stress and inflammation [22, 23]. However, the effects of this compound on glucose homeostasis in the PD state have not been explored. Hence, this study sought to investigate the effects of GTIN on selected glucose homeostasis-associated markers in PD rats, both with and without dietary modification.

Pre-diabetes is a state of intermediate hyperglycaemia associated with IGT, impaired FBG and early insulin resistance [1]. In normal glucose tolerant (NGT) individuals, blood glucose levels temporarily increase following glucose ingestion but return to fasting levels within two hours [24]. Insulin resistance impairs glucose disposal in skeletal muscle due to disrupted insulin signalling, glucose transport and glycogen synthesis, resulting in elevated blood glucose levels [25, 26]. This impairment is also associated with reduced GLUT4 translocation to the skeletal muscle membrane, limiting peripheral glucose uptake [27]. Furthermore, HFHC-induced metabolic stress may promote inflammatory activation, including NF- κ B signalling, which can further disrupt insulin signalling and contribute to insulin resistance [28]. Consistent with previous studies, chronic consumption of a HFHC diet induced pre-diabetes, as evidenced by the impaired FBG, IGT and HOMA2-IR index in the PD control group in comparison to the NPD group [29-31]. However, both the GTIN + ND and GTIN + HFHC diet groups showed significantly lower HOMA2-IR index, fasting and postprandial

glucose levels compared to the PD control group. This may suggest that GTIN has a beneficial effect on FBG levels and improves glucose tolerance even when combined with the HFHC diet, though its effects are more pronounced with a normal diet. The AUC was significantly lower in the GTIN + ND in comparison to the PD control group. This may suggest that GTIN improves glucose handling and reduces glucose exposure. Previous studies have shown that quercetin may stimulate glucose uptake, glucose transporter (GLUT)-4 translocation and insulin signalling pathways [32, 33]. It is speculated that GTIN may improve glucose parameters by regulating key signalling pathways involved in glucose metabolism [34]. One possible mechanism may involve activation of AMP-activated protein kinase (AMPK), a key regulator of glucose homeostasis that promotes glucose uptake, improves insulin sensitivity and suppresses hepatic glucose production [35]. Additionally, the MET + HFHC and MET + ND groups exhibited similar therapeutic effects as the GTIN-treated groups. Metformin improves glycaemic control primarily through suppression of hepatic gluconeogenesis and activation of AMPK, which enhances insulin sensitivity and peripheral glucose uptake [36]. Therefore, the comparable effects observed between GTIN and MET may reflect overlapping metabolic actions, although GTIN may additionally influence oxidative stress and inflammatory pathways [37, 38]. Both the MET- and GTIN-treated groups were effective in improving glucose tolerance in PD animals, with GTIN exhibiting more potent anti-hyperglycaemic effects.

HbA1c is a non-enzymatic glycation product of haemoglobin that persists in the body for 2–3 months [39]. Therefore, the HbA1c test is an effective means to screen for pre-diabetes and T2DM [39]. In the PD condition, plasma insulin does not effectively stimulate insulin-responsive tissues such as skeletal muscle [40, 41]. This results in increased glycation of haemoglobin due to elevated plasma glucose levels [42]. In this study, at week 12 of the treatment period there were significantly higher HbA1c levels in the PD control group in comparison to the NPD group. The HFHC diet may have induced insulin resistance resulting in elevated HbA1c levels, consistent with previous studies [43, 44]. However, both GTIN-treated groups showed significant reductions in HbA1c levels in comparison to the PD control group, with no change in comparison to the NPD group. GTIN was more effective at lowering HbA1c levels when combined with a normal diet compared to the HFHC diet, suggesting that a healthier diet enhances the efficacy of this compound. Previous studies have shown that flavonoids such as quercetin significantly improve HbA1c levels by increasing glucose uptake and improving insulin sensitivity [45, 46]. This improvement may be associated with enhanced peripheral glucose disposal through improved insulin signalling and GLUT4-mediated glucose transport [47]. Additionally, the antioxidant and anti-inflammatory properties of flavonoids may reduce metabolic stress that contributes to impaired glucose regulation [48, 49]. Additionally, MET has been shown to decrease HbA1c by decreasing hepatic glucose production and enhancing peripheral glucose uptake, as evidenced by the decreased HbA1c levels in the MET-treated groups [50, 51]. However, GTIN appears slightly more effective than MET, particularly in the GTIN + ND group. The effect of GTIN was comparable to MET, indicating its potential as a promising alternative treatment in the

management of diabetes.

Energy homeostasis is the state of equilibrium between energy intake and expenditure, which is impacted by caloric intake and energy storage [52]. High-fat diets (HFD) in rodents increase caloric intake, correlating with weight gain [53, 54]. High-fructose diets promote rapid intestinal glucose absorption, resulting in repeated postprandial hyperglycaemia and compensatory hyperinsulinaemia, which contribute to the development of insulin resistance [55, 56]. This impaired glucose utilisation stimulates the secretion of ghrelin, an orexigenic hormone that regulates appetite and energy homeostasis [57]. Elevated ghrelin levels increase food intake, thereby promoting body weight gain [58]. In this study, at week 12 of the treatment period, the PD control group had significantly higher caloric intake, percentage body weight increase and plasma ghrelin levels in comparison to the NPD group. The results corroborated with previous findings which have associated elevated ghrelin with increased caloric intake and weight gain during pre-diabetes [59, 60].

However, caloric intake, percentage body weight gain and ghrelin levels were significantly lower in both GTIN-treated groups at week 12 of the treatment period in comparison to the PD control group. These results may suggest that GTIN administration under both dietary regimens, significantly improves satiety and reduces body weight gain. Previous studies have shown that flavonoids reduce body weight gain by decreasing caloric intake and increasing satiety [61, 62]. These effects may be partly attributed to the inhibition of amylolytic enzymes, including α -amylase and α -glucosidase, which delays carbohydrate digestion and reduces intestinal glucose absorption [63]. Consequently, postprandial glucose excursions are attenuated, resulting in improved glycaemic control [64]. Improved glycaemic control may enhance satiety and reduce caloric intake, thereby limiting body weight gain [65]. Similarly, GTIN may have reduced body weight gain through mechanisms similar to those reported for flavonoids. These mechanisms may include reduced intestinal glucose absorption, resulting in enhanced satiety and lower caloric intake. Additionally, the MET + ND group exhibited similar anti-obesity effects as the GTIN-treated groups. MET has been shown to reduce caloric intake and body weight through multiple intestinal mechanisms [66, 67]. It decreases intestinal glucose absorption and enhances the secretion of glucagon-like peptide-1 (GLP-1), which promotes satiety [66]. MET also increases circulating growth differentiation factor-15 (GDF-15), which suppresses appetite and promotes weight loss [68]. These mechanisms may have contributed to the reductions in caloric intake and body weight observed in the MET+ND group. However, reductions in caloric intake and body weight were not significant in the MET + HFHC group. This may suggest that GTIN exhibits more potent effects on controlling obesity even in the absence of dietary intervention, as compared to MET administration. The results of this study suggest that GTIN may have potential metabolic benefits in controlling weight gain; however, further studies are required to determine its suitability as a therapeutic intervention for obesity.

Glycogen is the main form of glucose storage in the liver and skeletal muscles [69]. It is a readily available source of glucose for energy production [69]. Under normal physiological conditions, elevated plasma glucose stimulates insulin release, activating glycogen synthase and increasing glycogen synthesis in the liver and muscles [70, 71]. In the PD state, insulin resistance impairs insulin signalling in skeletal muscle, reducing the translocation of GLUT-4 to the cell membrane and limiting peripheral glucose uptake [72]. Consequently, less glucose is available for skeletal muscle glycogen synthesis, while excess circulating glucose is diverted to the liver for increased glycogen synthesis [73, 74]. In this study, skeletal muscle glycogen levels were significantly lower while liver glycogen levels were significantly higher in the PD control group in comparison to the NPD group. During the PD state, the excess plasma glucose in the presence of skeletal muscle insulin resistance may have been diverted to the liver for storage [75]. However, both GTIN-treated groups had significantly higher skeletal muscle glycogen and lower liver glycogen levels in comparison to the PD control group. Glycogen distribution is more favourable in the GTIN + ND group in comparison to the GTIN + HFHC group. Previous studies have shown that quercetin enhances insulin signalling in skeletal muscle by promoting GLUT-4 translocation to the cell membrane, thereby increasing glucose uptake [76, 77]. Similarly, GTIN may have improved insulin signalling and glucose uptake in skeletal muscle, reducing glucose shunting to the liver. Additionally, the MET-treated groups showed similar effects on glycogen levels in the liver and muscle as the GTIN-treated groups. MET has been shown to improve insulin sensitivity by promoting GLUT-4 translocation to the plasma membrane of skeletal muscle and adipose tissue, thereby increasing peripheral glucose uptake without directly stimulating pancreatic insulin secretion [78]. These mechanisms may account for the improved glycogen distribution observed in the MET-treated groups. The two treatments were comparable in this regard. However, the effects of GTIN on GLUT4 expression and translocation were not investigated in the present study. Therefore, future studies should investigate whether GTIN enhances peripheral glucose uptake by modulating GLUT4 translocation and insulin signalling pathways.

The HFHC diet effectively induced pre-diabetes, as evidenced by impaired FBG, IGT, increased HbA1c, hyperinsulinaemia and early insulin resistance in comparison to the NPD group. However, the administration of GTIN, both in the presence and absence of dietary modification, significantly decreased FBG and HbA1c levels as well as improved glucose tolerance and insulin sensitivity in comparison to the PD control group. Furthermore, both GTIN-treated groups showed significantly lower caloric intake, body weight gain and plasma ghrelin levels in comparison to the PD control group. GTIN improved disturbed glycogen levels in the liver and skeletal muscle. Additionally, the effects of GTIN were comparable to the conventional anti-diabetic drug MET. This may suggest that GTIN, with or without dietary modification, ameliorates disturbed glucose homeostasis in the PD state. Although these findings demonstrate promising metabolic effects, translation to humans requires further investigation. Important considerations include GTIN pharmacokinetics, oral bioavailability, metabolism, tissue distribution, toxicity and determination of an appropriate therapeutic dose. Many

flavonoids exhibit limited bioavailability due to rapid metabolism; therefore, future studies should establish whether biologically active concentrations of GTIN can be achieved safely in humans [79].

4.0 Materials and Methods

4.1. Chemicals and Drugs

All chemicals and drugs used were of analytical grade and obtained from reputable suppliers (Merck, Darmstadt, Germany).

4.2. Animals and Housing

This study involved the use of 36 male Sprague-Dawley rats (150–180 g) which were bred and housed in the Biomedical Research Unit (BRU) at the University of KwaZulu-Natal (UKZN), Westville campus. The animals were maintained under controlled laboratory conditions: a constant temperature of 22 ± 2 °C, carbon dioxide (CO₂) levels below 5000 ppm, relative humidity of $55 \pm 5\%$, and a 12-h light/dark cycle with lights on at 07:00. Noise levels were kept below 65 decibels, and the rats had ad libitum access to food and water. All protocols and animal care procedures adhered to the guidelines of the Animal Research Ethics Committee of the University of KwaZulu-Natal (ETHICS: AREC/00004965/2022). Prior to the study, the rats were acclimatized to their environment for one week with standard rat chow and tap water before exposure to the HFHC diet (AVI Products (Pty) Ltd., Waterfall, South Africa) [60].

4.3. Induction of Pre-Diabetes

The rats were randomly assigned to two diet groups: Group 1 (n = 6) and Group 2 (n = 30) (Figure 5). Pre-diabetes was induced in Group 2 according to the protocol described by Luvuno et al. [60]. Group 1 rats were maintained on a standard rat diet with tap water, while Group 2 rats were given a HFHC diet supplemented with 15% fructose-enriched water. After 20 weeks, pre-diabetes was assessed using the American Diabetes Association (ADA) criteria [80]. Rats with FBG levels between 5.6 to 6.9 mmol/L, an OGTT 2-h glucose concentration between 7.8 to 11.0 mmol/L, and HbA1c levels between 5.7 to 6.4% were classified as pre-diabetic. Rats fed the standard diet were also evaluated at week 20 to confirm normoglycemia and were classified as the non-pre-diabetic group. This period marked the beginning of the treatment phase (Week 0).

4.4. Experimental Design and Treatment

Following pre-diabetes induction, the rats were categorized into two primary groups: non-pre-diabetic (n = 6) and pre-diabetic (n = 30). The treatment phase commenced the day after pre-diabetes diagnosis and continued for 12 weeks. The pre-diabetic rats (n = 30) were subdivided into five experimental groups (Groups 2 to 6), each comprising six rats. These groups were as follows: Group 2 (pre-diabetic control) continued on the HFHC diet and received as the vehicle, 3 mL/kg of diluted dimethyl

sulfoxide (DMSO) (2 mL DMSO: 19 mL normal saline, p.o.); Group 3 (GTIN + ND) received GTIN while on the normal diet; Group 4 (GTIN + HFHC) received GTIN while continuing on the HFHC diet; Group 5 (MET + ND) received MET (MET) while on the normal diet; and Group 6 (MET + HFHC) received MET while on the HFHC diet. GTIN and MET were administered orally at dosages of 15 mg/kg and 500 mg/kg, respectively, every third day at 09:00 am. These dosages were selected based on previous studies [13, 59, 81]. Parameters such as body weight, caloric intake, and FBG levels were monitored at week 0, 4, 8, and 12. The OGTT was conducted at week 12 following the protocol established in prior studies [59, 60].

4.5. Blood Collection and Tissue Harvesting

After 12 weeks of treatment, all animals were anaesthetised with Isoflurane (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd., Roodeport, South Africa) for 3 min using a gas anaesthetic chamber (BRU, UKZN, Durban, SA). Following the onset of anaesthesia, blood samples were collected via cardiac puncture and transferred into pre-cooled heparinized tubes. The samples were then centrifuged (Eppendorf centrifuge 5403, Hamburg, Germany) at 4 °C and 503 g for 15 min to separate the plasma, which was subsequently stored at –80 °C in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) until ready for biochemical analysis. The liver and skeletal muscle tissues were excised after the animals were sacrificed, snap-frozen in liquid nitrogen, and stored at –80 °C in a BioUltra freezer until ready for glycogen assay.

4.6. Biochemical Analysis

Plasma insulin, HbA1c and ghrelin levels were measured using ELISA kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) as per the manufacturer's instructions. The HOMA2-IR index was calculated from the insulin and FBG concentrations <https://www.dtu.ox.ac.uk/homacalculator/> (accessed on 8 July 2024).

4.7. Glycogen Assay

Glycogen levels in liver and skeletal muscle tissues were assessed following established protocols [81-83]. Tissue samples (50 mg) were homogenized with 2 mL of 30% potassium hydroxide (KOH) and heated at 100 °C for 30 min. The reaction was terminated by the addition of 0.194 mL of 10% of sodium tetraoxosulphate VI (Na₂SO₄). After cooling, the glycogen precipitate was collected, washed with ethanol (95%), and resolubilized in 1 mL of H₂O. The glycogen content was then quantified by mixing 200 µL of the resolubilized sample with 4 mL of anthrone reagent (0.5 g anthrone in 250 mL of 95% sulfuric acid) and boiling for 10 min. Absorbance was measured at 620 nm using a Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Germany).

4.8. Statistical Analysis

Data are expressed as mean ± SEM. Prior to statistical analysis, normality was assessed using the

Shapiro–Wilk test, and homogeneity of variance was assessed using Bartlett's test. Statistical analyses were performed using two-way analysis of variance (ANOVA) for OGTT, caloric intake and body weight data, while all other data were analysed using one-way ANOVA, followed by Tukey–Kramer post hoc tests. Area under the curve (AUC) was calculated using GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was determined at $p < 0.05$.

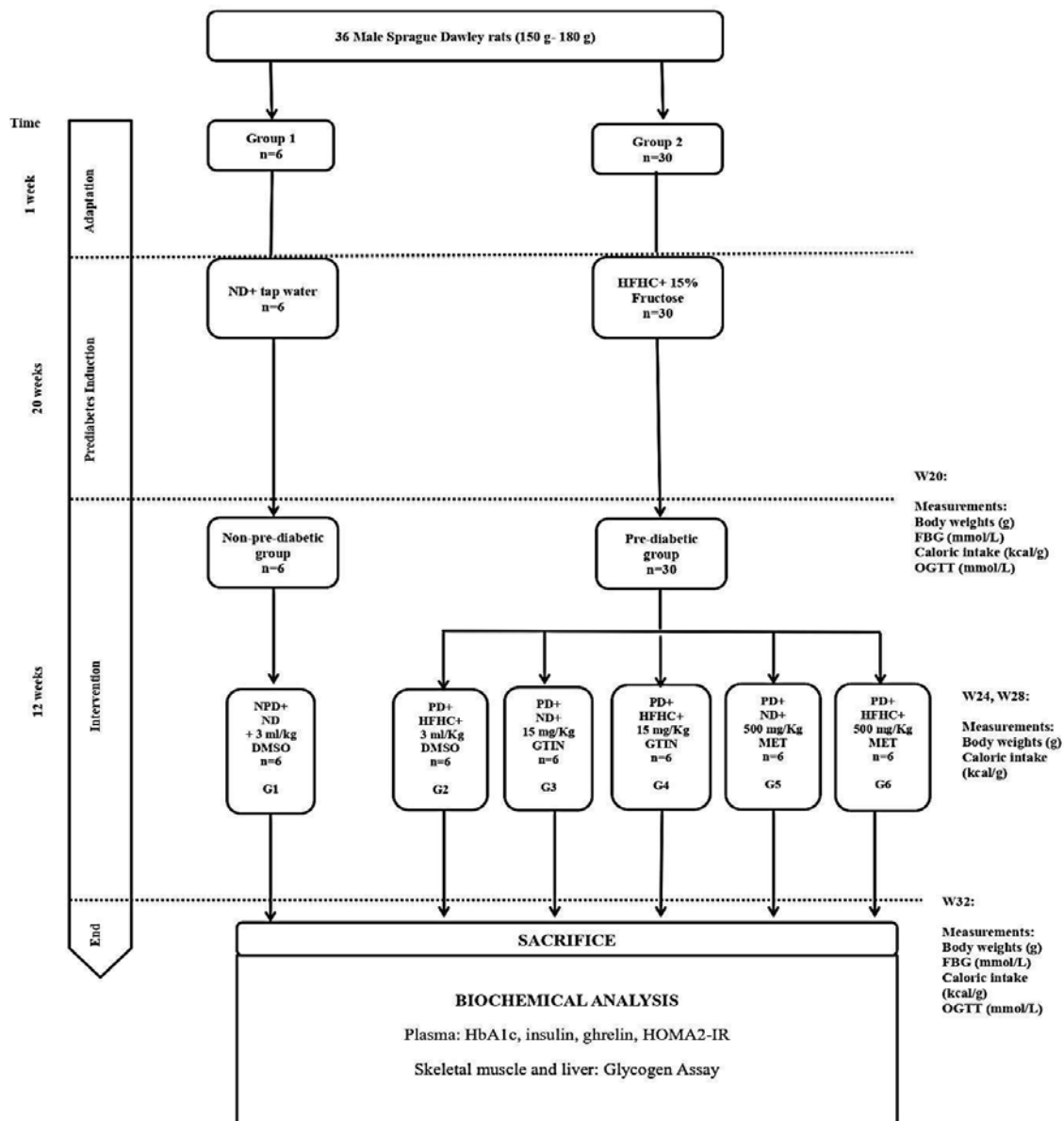


Figure 5. Layout of experimental design and grouping

5. Conclusions

The findings of this study demonstrate that GTIN supplementation attenuates disturbed glucose homeostasis associated with diet-induced pre-diabetes. GTIN improved glycaemic regulation, as evidenced by reduced FBG, enhanced glucose tolerance and lowered HbA1c levels. It also improved metabolic parameters by reducing caloric intake, body weight gain and plasma ghrelin levels,

suggesting its benefits in appetite control and weight management. Additionally, GTIN positively influenced glycogen distribution, enhancing glucose uptake in muscles and reducing liver glycogen. These beneficial effects were evident regardless of dietary intervention, although greater improvements were observed in animals receiving dietary modification. The efficacy of GTIN in glucose management was comparable to that of MET, indicating its potential as an alternative therapeutic agent in diabetes management.

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

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References

1. Bansal, N., Prediabetes diagnosis and treatment: A review. *World journal of diabetes* 2015, 6, (2), 296.
2. Tabák, A. G.; Herder, C.; Rathmann, W.; Brunner, E. J.; Kivimäki, M., Prediabetes: a high-risk state for diabetes development. *The Lancet* 2012, 379, (9833), 2279–2290.
3. Ligthart, S.; van Herpt, T. T.; Leening, M. J.; Kavousi, M.; Hofman, A.; Stricker, B. H.; van Hoek, M.; Sijbrands, E. J.; Franco, O. H.; Dehghan, A., Lifetime risk of developing impaired glucose metabolism and eventual progression from prediabetes to type 2 diabetes: a prospective cohort study. *The lancet Diabetes & endocrinology* 2016, 4, (1), 44–51.
4. Buysschaert, M.; Bergman, M., Definition of prediabetes. *Medical Clinics* 2011, 95, (2), 289–297.

5. Khetan, A. K.; Rajagopalan, S., Prediabetes. *Canadian Journal of Cardiology* 2018, 34, (5), 615–623.
6. Derrick, S. A.; Kristo, A. S.; Reaves, S. K.; Sikalidis, A. K., Effects of dietary red raspberry consumption on pre-diabetes and type 2 diabetes mellitus parameters. *International Journal of Environmental Research and Public Health* 2021, 18, (17), 9364.
7. Wadden, T. A.; Berkowitz, R. I.; Sarwer, D. B.; Prus-Wisniewski, R.; Steinberg, C., Benefits of lifestyle modification in the pharmacologic treatment of obesity: a randomized trial. *Archives of internal medicine* 2001, 161, (2), 218–227.
8. Choudhary, R.; Swarnkar, P., Antioxidant activity of phenolic and flavonoid compounds in some medicinal plants of India. *Natural product research* 2011, 25, (11), 1101–1109.
9. Batiha, G. E.-S.; Beshbishy, A. M.; Ikram, M.; Mulla, Z. S.; El-Hack, M. E. A.; Taha, A. E.; Algammal, A. M.; Elewa, Y. H. A., The pharmacological activity, biochemical properties, and pharmacokinetics of the major natural polyphenolic flavonoid: quercetin. *Foods* 2020, 9, (3), 374.
10. Kumar, S.; Pandey, A. K., Chemistry and biological activities of flavonoids: an overview. *The scientific world journal* 2013, 2013, (1), 162750.
11. Mondal, S.; Rahaman, S., Flavonoids: A vital resource in healthcare and medicine. *Pharm. Pharmacol. Int. J* 2020, 8, (2), 91–104.
12. R. Dias, T.; G. Alves, M.; Casal, S.; F. Oliveira, P.; M. Silva, B., Promising potential of dietary (poly) phenolic compounds in the prevention and treatment of diabetes mellitus. *Current Medicinal Chemistry* 2017, 24, (4), 334–354.
13. Abdelmoaty, M. A.; Ibrahim, M.; Ahmed, N.; Abdelaziz, M., Confirmatory studies on the antioxidant and antidiabetic effect of quercetin in rats. *Indian Journal of Clinical Biochemistry* 2010, 25, (2), 188–192.
14. Khan, A.; Manna, K.; Bose, C.; Sinha, M.; Das, D. K.; Kesh, S. B.; Chakrabarty, A.; Banerji, A.; Dey, S., Gossypetin, a naturally occurring hexahydroxy flavone, ameliorates gamma radiation-mediated DNA damage. *International journal of radiation biology* 2013, 89, (11), 965–975.
15. Ijaz, M. U.; Alvi, K.; Khan, H. A.; Imran, M.; Afsar, T.; Almajwal, A.; Amor, H.; Razak, S., Gossypetin mitigates doxorubicin-induced nephrotoxicity: A histopathological and biochemical evaluation. *Journal of King Saud University-Science* 2023, 35, (7), 102830.
16. De La Rosa, L. A.; Moreno-Escamilla, J. O.; Rodrigo-García, J.; Alvarez-Parrilla, E., Phenolic compounds. In *Postharvest physiology and biochemistry of fruits and vegetables*, Elsevier: 2019; pp 253–271.
17. Hossain, M. K.; Dayem, A. A.; Han, J.; Yin, Y.; Kim, K.; Saha, S. K.; Yang, G.-M.; Choi, H. Y.; Cho, S.-G., Molecular mechanisms of the anti-obesity and anti-diabetic properties of flavonoids. *International journal of molecular sciences* 2016, 17, (4), 569.

18. Chen, J.; Jin, L.; Chen, M.; Xu, K.; Huang, Q.; He, B., Application of natural compounds in the treatment and prevention of prediabetes. *Frontiers in Nutrition* 2023, 10, 1301129.
19. Bule, M.; Abdurahman, A.; Nikfar, S.; Abdollahi, M.; Amini, M., Antidiabetic effect of quercetin: A systematic review and meta-analysis of animal studies. *Food and chemical toxicology* 2019, 125, 494–502.
20. Singh, P.; Arif, Y.; Bajguz, A.; Hayat, S., The role of quercetin in plants. *Plant Physiology and Biochemistry* 2021, 166, 10–19.
21. Michala, A.-S.; Pritsa, A., Quercetin: a molecule of great biochemical and clinical value and its beneficial effect on diabetes and cancer. *Diseases* 2022, 10, (3), 37.
22. Mounnissamy, V.; Gopal, V.; Gunasegaran, R.; Saraswathy, A., Antiinflammatory activity of gossypetin isolated from Hibiscus sabdariffa. *Indian Journal of Heterocyclic Chemistry* 2002, 12, (1), 85–86.
23. Oh, E.; Lee, J.; Cho, S.; Kim, S. W.; Jo, K. W.; Shin, W. S.; Gwak, S. H.; Ha, J.; Jeon, S. Y.; Park, J.-H., Gossypetin prevents the progression of nonalcoholic steatohepatitis by regulating oxidative stress and AMP-activated protein kinase. *Molecular Pharmacology* 2023, 104, (5), 214–229.
24. Nakrani, M. N.; Wineland, R. H.; Anjum, F., Physiology, glucose metabolism. 2020.
25. Deshmukh, A. S., Insulin-stimulated glucose uptake in healthy and insulin-resistant skeletal muscle. *Hormone Molecular Biology and Clinical Investigation* 2016, 26, (1), 13–24.
26. Emanuel, A. L.; Meijer, R. I.; Muskiet, M. H.; Van Raalte, D. H.; Eringa, E. C.; Serné, E. H., Role of insulin-stimulated adipose tissue perfusion in the development of whole-body insulin resistance. *Arteriosclerosis, thrombosis, and vascular biology* 2017, 37, (3), 411–418.
27. Whytock, K. L.; Goodpaster, B. H., Unraveling skeletal muscle insulin resistance: molecular mechanisms and the restorative role of exercise. *Circulation research* 2025, 137, (2), 184–204.
28. Gao, Q.; Li, Y.; Wang, L., Dietary Carbohydrate modulation in metabolic syndrome: Mechanistic insights, gut microbiota interactions, and precision nutrition approaches. *Food Reviews International* 2026, 1–29.
29. Beulens, J.; Rutters, F.; Ryden, L.; Schnell, O.; Mellbin, L.; Hart, H.; Vos, R., Risk and management of pre-diabetes. *European journal of preventive cardiology* 2019, 26, (2_suppl), 47–54.
30. Burgeiro, A.; Cerqueira, M. G.; Varela-Rodríguez, B. M.; Nunes, S.; Neto, P.; Pereira, F. C.; Reis, F.; Carvalho, E., Glucose and lipid dysmetabolism in a rat model of prediabetes induced by a high-sucrose diet. *Nutrients* 2017, 9, (6), 638.
31. Miao, L.; Zhang, X.; Zhang, H.; Cheong, M. S.; Chen, X.; Farag, M. A.; San Cheang, W.; Xiao, J., Baicalin ameliorates insulin resistance and regulates hepatic glucose metabolism via activating insulin signaling pathway in obese pre-diabetic mice. *Phytomedicine* 2024, 124, 155296.

32. Eldamarawi, M.; Abdelazeem, M., Effect of quercetin and metformin on glucose transporter-4 expression, oxidative stress, inflammation markers and insulin resistance in type 2 diabetes mellitus. *Bulletin of Egyptian Society for Physiological Sciences* 2020, 40, (2), 70–85.
33. Eid, H. M.; Nachar, A.; Thong, F.; Sweeney, G.; Haddad, P. S., The molecular basis of the antidiabetic action of quercetin in cultured skeletal muscle cells and hepatocytes. *Pharmacognosy magazine* 2015, 11, (41), 74.
34. Moore, W. T.; Bowser, S. M.; Fausnacht, D. W.; Staley, L. L.; Suh, K.-S.; Liu, D., Beta cell function and the nutritional state: dietary factors that influence insulin secretion. *Current Diabetes Reports* 2015, 15, (10), 76.
35. Naidoo, K.; Khathi, A., The potential role of gossypetin in the treatment of diabetes mellitus and its associated complications: a review. *International Journal of Molecular Sciences* 2023, 24, (24), 17609.
36. Bailey, C. J., Metformin: Therapeutic profile in the treatment of type 2 diabetes. *Diabetes, Obesity and Metabolism* 2024, 26, 3–19.
37. Lin, H.-H., In vitro and in vivo atheroprotective effects of gossypetin against endothelial cell injury by induction of autophagy. *Chemical Research in Toxicology* 2015, 28, (2), 202–215.
38. Khan, A.; Manna, K.; Das, D. K.; Kesh, S. B.; Sinha, M.; Das, U.; Biswas, S.; Sengupta, A.; Sikder, K.; Datta, S., Gossypetin ameliorates ionizing radiation-induced oxidative stress in mice liver—a molecular approach. *Free Radical Research* 2015, 49, (10), 1173–1186.
39. Chen, Z.; Shao, L.; Jiang, M.; Ba, X.; Ma, B.; Zhou, T., Interpretation of HbA1c lies at the intersection of analytical methodology, clinical biochemistry and hematology. *Experimental and therapeutic medicine* 2022, 24, (6), 707.
40. Zhang, Z.; Liu, H.; Liu, J., Akt activation: A potential strategy to ameliorate insulin resistance. *Diabetes research and clinical practice* 2019, 156, 107092.
41. Janssen, J. A., Overnutrition, hyperinsulinemia and ectopic fat: it is time for a paradigm shift in the management of type 2 diabetes. *International Journal of Molecular Sciences* 2024, 25, (10), 5488.
42. Sherwani, S. I.; Khan, H. A.; Ekhzaimy, A.; Masood, A.; Sakharkar, M. K., Significance of HbA1c test in diagnosis and prognosis of diabetic patients. *Biomarker insights* 2016, 11, BMI. S38440.
43. Guo, F.; Moellering, D. R.; Garvey, W. T., Use of HbA1c for diagnoses of diabetes and prediabetes: comparison with diagnoses based on fasting and 2-hr glucose values and effects of gender, race, and age. *Metabolic syndrome and related disorders* 2014, 12, (5), 258–268.
44. Tankova, T.; Chakarova, N.; Dakovska, L.; Atanassova, I., Assessment of HbA1c as a diagnostic tool in diabetes and prediabetes. *Acta diabetologica* 2012, 49, (5), 371–378.
45. Ansari, P.; Choudhury, S. T.; Seidel, V.; Rahman, A. B.; Aziz, M. A.; Richi, A. E.; Rahman, A.;

- Jafrin, U. H.; Hannan, J.; Abdel-Wahab, Y. H., Therapeutic potential of quercetin in the management of type-2 diabetes mellitus. *Life* 2022, 12, (8), 1146.
46. Hosseini, A.; Razavi, B. M.; Banach, M.; Hosseinzadeh, H., Quercetin and metabolic syndrome: A review. *Phytotherapy Research* 2021, 35, (10), 5352–5364.
47. Popescu, M.; Radivojevic, K.; Trasca, D.-M.; Varut, R. M.; Enache, I.; Osman, A., Natural antidiabetic agents: Insights into Ericaceae-derived phenolics and their role in metabolic and oxidative modulation in diabetes. *Pharmaceuticals* 2025, 18, (5), 682.
48. Krawczyk, M.; Burzynska-Pedziwiatr, I.; Wozniak, L. A.; Bukowiecka-Matusiak, M., Impact of polyphenols on inflammatory and oxidative stress factors in diabetes mellitus: nutritional antioxidants and their application in improving antidiabetic therapy. *Biomolecules* 2023, 13, (9), 1402.
49. Yi, X.; Dong, M.; Guo, N.; Tian, J.; Lei, P.; Wang, S.; Yang, Y.; Shi, Y., Flavonoids improve type 2 diabetes mellitus and its complications: A review. *Frontiers in Nutrition* 2023, 10, 1192131.
50. Wu, T.; Horowitz, M.; Rayner, C. K., New insights into the anti-diabetic actions of metformin: from the liver to the gut. *Expert review of gastroenterology & hepatology* 2017, 11, (2), 157–166.
51. Sansome, D. J.; Xie, C.; Veedfald, S.; Horowitz, M.; Rayner, C. K.; Wu, T., Mechanism of glucose-lowering by metformin in type 2 diabetes: role of bile acids. *Diabetes, Obesity and Metabolism* 2020, 22, (2), 141–148.
52. Sovetkina, A.; Nadir, R.; Fung, J. N. M.; Nadjarpour, A.; Beddoe, B., The physiological role of ghrelin in the regulation of energy and glucose homeostasis. *Cureus* 2020, 12, (5).
53. Licholai, J. A.; Nguyen, K. P.; Fobbs, W. C.; Schuster, C. J.; Ali, M. A.; Kravitz, A. V., Why do mice overeat high-fat diets? how high-fat diet alters the regulation of daily caloric intake in mice. *Obesity* 2018, 26, (6), 1026–1033.
54. Olsen, M. K.; Choi, M. H.; Kulseng, B.; Zhao, C.-M.; Chen, D., Time-restricted feeding on weekdays restricts weight gain: A study using rat models of high-fat diet-induced obesity. *Physiology & behavior* 2017, 173, 298–304.
55. Louie, J. C. Y.; Tan, S.-Y., Free Sugars Consumption and Type 2 Diabetes: What Are the Concerns and How Strong is the Evidence? *Current nutrition reports* 2026, 15, (1), 11.
56. Güney, C.; Akar, F., The possible mechanisms of high-fructose diet-induced pancreatic disturbances. *Journal of Research in Pharmacy* 2023, 27, (2), 753–761.
57. Yang, W.; Jiang, W.; Guo, S., Regulation of macronutrients in insulin resistance and glucose homeostasis during type 2 diabetes mellitus. *Nutrients* 2023, 15, (21), 4671.
58. Jiao, Z.-T.; Luo, Q., Molecular mechanisms and health benefits of ghrelin: a narrative review. *Nutrients* 2022, 14, (19), 4191.
59. Siboto, A.; Akinnuga, A. M.; Khumalo, B. N.; Ismail, M. B.; Booysen, I. N.; Sibiya, N. H.;

- Ngubane, P. S.; Khathi, A., The effects of a [3+ 1] oxo-free rhenium (V) compound with uracil-derived ligands on selected parameters of glucose homeostasis in diet-induced pre-diabetic rats. *Obesity Medicine* 2020, 19, 100258.
60. Khathi, A.; Luvuno, M.; Mabandla, M., Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. *PONTE International Journal of Science and Research* 2018, 74, (5).
61. Bertola, M. L.; Rimm, E. B.; Mukamal, K. J.; Hu, F. B.; Willett, W. C.; Cassidy, A., Dietary flavonoid intake and weight maintenance: three prospective cohorts of 124 086 US men and women followed for up to 24 years. *bmj* 2016, 352.
62. Rufino, A. T.; Costa, V. M.; Carvalho, F.; Fernandes, E., Flavonoids as antiobesity agents: A review. *Medicinal Research Reviews* 2021, 41, (1), 556–585.
63. Proença, C.; Ribeiro, D.; Freitas, M.; Fernandes, E., Flavonoids as potential agents in the management of type 2 diabetes through the modulation of α -amylase and α -glucosidase activity: A review. *Critical Reviews in Food Science and Nutrition* 2022, 62, (12), 3137–3207.
64. Shibib, L.; Al-Qaisi, M.; Guess, N.; Miras, A. D.; Greenwald, S. E.; Pelling, M.; Ahmed, A., Manipulation of post-prandial hyperglycaemia in type 2 diabetes: an update for practitioners. *Diabetes, Metabolic Syndrome and Obesity* 2024, 3111–3130.
65. Szekeres, Z.; Nagy, A.; Jahner, K.; Szabados, E., Impact of selected glucagon-like peptide-1 receptor agonists on serum lipids, adipose tissue, and muscle metabolism—A narrative review. *International Journal of Molecular Sciences* 2024, 25, (15), 8214.
66. Pillai, A. A.; Melo, L.; Frishman, W. H.; Aronow, W. S., The effects of metformin on weight loss, cardiovascular health, and longevity. *Cardiology in Review* 2024, 10.1097.
67. Dutta, S.; Shah, R. B.; Singhal, S.; Dutta, S. B.; Bansal, S.; Sinha, S.; Haque, M., Metformin: a review of potential mechanism and therapeutic utility beyond diabetes. *Drug design, development and therapy* 2023, 1907–1932.
68. Al-kuraishy, H. M.; Al-Gareeb, A. I.; Alexiou, A.; Papadakis, M.; Nadwa, E. H.; Albogami, S. M.; Alorabi, M.; Saad, H. M.; Batiha, G. E. S., Metformin and growth differentiation factor 15 (GDF15) in type 2 diabetes mellitus: A hidden treasure. *Journal of diabetes* 2022, 14, (12), 806–814.
69. Jensen, J.; Rustad, P. I.; Kolnes, A. J.; Lai, Y.-C., The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise. *Frontiers in physiology* 2011, 2, 112.
70. Röder, P. V.; Wu, B.; Liu, Y.; Han, W., Pancreatic regulation of glucose homeostasis. *Experimental & molecular medicine* 2016, 48, (3), e219–e219.
71. Norton, L.; Shannon, C.; Gastaldelli, A.; DeFronzo, R. A., Insulin: The master regulator of glucose metabolism. *Metabolism* 2022, 129, 155142.
72. van Gerwen, J.; Shun-Shion, A. S.; Fazakerley, D. J., Insulin signalling and GLUT4 trafficking in insulin resistance. *Biochemical Society Transactions* 2023, 51, (3), 1057–1069.

73. Merz, K. E.; Thurmond, D. C., Role of skeletal muscle in insulin resistance and glucose uptake. *Comprehensive Physiology* 2020, 10, (3), 785–809.
74. Abdul-Ghani, M. A.; DeFronzo, R. A., Pathogenesis of insulin resistance in skeletal muscle. *BioMed Research International* 2010, 2010, (1), 476279.
75. Akinnuga, A. M.; Siboto, A.; Khumalo, B.; Sibiya, N. H.; Ngubane, P.; Khathi, A., Evaluation of the effects of bredemolic acid on selected markers of glucose homeostasis in diet-induced prediabetic rats. *Archives of Physiology and Biochemistry* 2022, 128, (2), 306–312.
76. Niziński, P.; Hawrył, A.; Polak, P.; Kondracka, A.; Oniszcuk, T.; Soja, J.; Hawrył, M.; Oniszcuk, A., Potential of quercetin as a promising therapeutic agent against type 2 diabetes. *Molecules* 2025, 30, (15), 3096.
77. Zhou, M.; Konigsberg, W. H.; Hao, C.; Pan, Y.; Sun, J.; Wang, X., Bioactivity and mechanisms of flavonoids in decreasing insulin resistance. *Journal of Enzyme Inhibition and Medicinal Chemistry* 2023, 38, (1), 2199168.
78. Herman, R.; Kravos, N. A.; Jensterle, M.; Janež, A.; Dolžan, V., Metformin and insulin resistance: a review of the underlying mechanisms behind changes in GLUT4-mediated glucose transport. *International journal of molecular sciences* 2022, 23, (3), 1264.
79. Naeem, A.; Ming, Y.; Pengyi, H.; Jie, K. Y.; Yali, L.; Haiyan, Z.; Shuai, X.; Wenjing, L.; Ling, W.; Xia, Z. M., The fate of flavonoids after oral administration: A comprehensive overview of its bioavailability. *Critical reviews in food science and nutrition* 2022, 62, (22), 6169–6186.
80. Association, A. D., 2. Classification and diagnosis of diabetes: Standards of Care in Diabetes—2023. *Diabetes care* 2023, 46, S19–S40.
81. Mbatha, B.; Khathi, A.; Sibiya, N.; Booysen, I.; Ngubane, P., A Dioxidovanadium Complex cis-[VO₂ (obz) py] Attenuates Hyperglycemia in Streptozotocin (STZ)-Induced Diabetic Male Sprague-Dawley Rats via Increased GLUT4 and Glycogen Synthase Expression in the Skeletal Muscle. *Evidence-Based Complementary and Alternative Medicine* 2022, 2022, (1), 5372103.
82. Khathi, A.; Serumula, M. R.; Myburg, R. B.; Van Heerden, F. R.; Musabayane, C. T., Effects of Syzygium aromaticum-derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge. *PloS one* 2013, 8, (11), e81632.
83. Ngubane, P. S.; Masola, B.; Musabayane, C. T., The effects of Syzygium aromaticum-derived oleanolic acid on glycogenic enzymes in streptozotocin-induced diabetic rats. *Renal Failure* 2011, 33, (4), 434–439.

Bridge

The first manuscript presented in Chapter 4 showed that diet-induced prediabetes was associated with disturbed glucose homeostasis. This was evidenced by elevated fasting blood glucose levels, postprandial glucose levels, glycated haemoglobin and moderate insulin resistance, in conjunction with increased caloric intake, body weight gain, elevated plasma ghrelin levels and altered glycogen distribution between the liver and skeletal muscle. The administration of gossypetin (GTIN), in both the presence and absence of dietary intervention, significantly improved these metabolic parameters. The therapeutic effects of GTIN were slightly better than metformin in many respects, especially under the HFHC diet. Prediabetes is a well-established risk factor for cardiovascular disease and is linked to dyslipidaemia, oxidative stress, endothelial dysfunction and chronic low-grade inflammation. The findings presented in manuscript 1 prompted further investigation into the potential effects of GTIN on cardiovascular function during the prediabetic state. Accordingly, Chapter 5 presents the findings of manuscript 2 which aims to investigate the effects of GTIN on cardiovascular function, including plasma lipid profile, mean arterial pressure, adiposity indices, oxidative stress markers and systemic inflammatory mediators in diet-induced prediabetes.

CHAPTER 5: MANUSCRIPT 2

PROLOGUE

Literature has shown that cardiovascular complications associated with type 2 diabetes mellitus begin during the prediabetic state. Moderate insulin resistance, oxidative stress and low-grade inflammation during prediabetes contribute to endothelial dysfunction, elevated blood pressure and lipid abnormalities, which increase the risk of developing cardiovascular diseases. Current management strategies for prediabetes-associated cardiovascular complications involve a combination of dietary and pharmacological interventions. However, low patient adherence to dietary intervention compromises the health benefits of the combined interventions. Consequently, identifying compounds capable of attenuating prediabetes-associated cardiovascular complications, regardless of dietary intervention is warranted. Flavonoids have been extensively studied for their potent antioxidant and anti-inflammatory effects within the cardiovascular system. In manuscript 1, gossypetin (GTIN) significantly improved markers associated with glucose homeostasis in a diet-induced prediabetic rat model. However, the effects of GTIN on cardiovascular function in the prediabetic state was not investigated. Hence, in manuscript 2, we investigated the effects of GTIN on cardiovascular function in both the presence and absence of dietary intervention in a diet-induced prediabetic rat model.

The manuscript in Chapter 5 is titled “**Investigating the effects of gossypetin on cardiovascular function in diet-induced prediabetic male Sprague Dawley rats**” and is authored by Naidoo K and Khathi A.

The manuscript has been published in the “**International Journal of Molecular Sciences**” and formatted according to the journal’s guidelines for authors. See Appendix 4.

Candidate Contribution: Karishma Naidoo was responsible for study conceptualisation, study design, all experimental work, statistical analysis, first draft writing and manuscript editing.

Investigating the effects of gossypetin on cardiovascular function in diet-induced pre-diabetic male Sprague Dawley rats

Karishma Naidoo ^{1,2}, Andile Khathi¹

1. University of KwaZulu-Natal, Westville, KwaZulu-Natal South Africa
2. Corresponding Author: Karishma Naidoo

School of Laboratory Medicine & Medical Sciences

University of KwaZulu-Natal

Private Bag X54001

Durban,

SOUTH AFRICA

Phone: 0711607469 /27 (0) 31 260 7585, Fax: 27 (0) 31 260 7132

Email: karishma280199@gmail.com /khathia@ukzn.ac.za

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Abstract

Gossypetin (GTIN), a naturally derived flavonoid, has demonstrated promising biological activities; however, its influence on cardiovascular complications associated with the pre-diabetic state remains unclear. This study examined the effects of GTIN on cardiovascular function in a diet-induced pre-diabetic rat model, which has not been previously studied. Pre-diabetes was induced using a high-fat high-carbohydrate (HFHC) diet supplemented with 15% fructose water for 20 weeks. Thereafter, the pre-diabetic animals were sub-divided into five groups ($n = 6$), where they were either orally treated with GTIN (15 mg/kg) or metformin (MET) (500 mg/kg), both in the presence and absence of dietary intervention for 12 weeks. The results demonstrated that the pre-diabetic (PD) control group exhibited significantly higher plasma triglyceride, total cholesterol, low-density lipoprotein and very low-density lipoprotein levels, along with decreased high-density lipoprotein (HDL) levels in comparison to the non-pre-diabetic (NPD) group. This was accompanied by significantly higher mean arterial pressure (MAP), body mass index (BMI), waist circumference (WC) and plasma endothelial nitric oxide (eNOS) levels in PD control. Additionally, there were increased heart malondialdehyde levels, reduced heart superoxide dismutase and glutathione peroxidase activity as well as increased plasma interleukin-6, tumour necrosis factor alpha and c-reactive protein levels present in the PD control group. Notably, both GTIN-treated groups showed significantly reduced plasma lipid levels and increased HDL, as well as decreases in MAP, BMI, WC and eNOS levels in comparison to PD control. Additionally, GTIN significantly decreased heart lipid peroxidation, enhanced antioxidant activity and decreased plasma inflammation markers. These findings may suggest that GTIN administration in both the presence and absence of dietary intervention may offer therapeutic potential in ameliorating cardiovascular disturbances associated with the PD state. However, future studies are needed to determine the physiological mechanisms by which GTIN improves cardiovascular function in the PD state.

1.0 Introduction

High-caloric diets have been linked to an increased risk of metabolic disorders, including pre-diabetes, which is a multifactorial condition influenced by genetic, environmental and lifestyle factors [1-3]. Pre-diabetes is an intermediate state between normal glucose tolerance (NGT) and overt type 2 diabetes mellitus (T2DM) [4]. The International Diabetes Federation estimated that about 298 million adults worldwide have been diagnosed with pre-diabetes, with projections showing that this number will increase to about 414 million by the year 2045 [5]. This condition is characterised by moderate hyperglycaemia, moderate insulin resistance and impaired glucose tolerance [4]. Studies have shown that many of the complications seen in T2DM begin in the pre-diabetic (PD) state [6, 7]. Excess saturated fatty acids, trans fatty acids and pro-inflammatory adipokines have been shown to promote the development of insulin resistance, which is a risk factor for pre-diabetes [7-10]. Pre-diabetes has been shown to be associated with an increased risk of cardiovascular diseases (CVDs) and endothelial dysfunction [11]. This condition has been shown to promote adverse effects through multiple pathways by generating reactive oxygen species (ROS) [12]. Increased oxidative stress reduces the bioavailability of nitric oxide (NO), promoting increased blood pressure [13]. Chronic low-grade inflammation has been observed in the PD state, and this has been characterised by increased levels of pro-inflammatory markers, including tumour necrosis factor (TNF)- α , interleukin (IL)-6 and c-reactive protein (CRP) [14, 15]. This has further been accompanied by increased plasma levels of triglycerides (TG), low-density lipoproteins (LDL) and decreased high-density lipoproteins (HDL), which have been shown to promote foam cell accumulation in artery walls and, subsequently, atherosclerosis [16].

The current strategy for the management of pre-diabetes involves both the combination of lifestyle modification and pharmacological intervention [17]. However, patients often neglect lifestyle modifications due to the financial burden of dietary changes and ease of access to pharmacological treatment [18]. Furthermore, studies have shown that individuals with lower educational backgrounds may lack knowledge of healthy dietary habits and preventive behaviours [19-21]. Limited nutrition education is a contributing factor to the development of pre-diabetes, as individuals with insufficient knowledge tend to make unhealthy lifestyle choices, including the consumption of high-calorie, nutrient-deficient foods that contribute to insulin resistance and metabolic dysfunction [22-25]. Additionally, a lack of understanding about the importance of dietary changes and higher prices of healthier foods may lead to an over-reliance on pharmacological treatments instead of lifestyle modifications, delaying the potential benefits of dietary intervention in reducing the risk of T2DM development [18, 26-28]. Studies have shown that pharmacological intervention in the absence of dietary modification shows limited effectiveness in the treatment of pre-diabetes [29, 30]. This highlights the need for pharmacological treatments that can effectively manage pre-diabetes and its associated complications in both the presence and absence of dietary intervention [31]. Flavonoids

have received increasing attention as potential cardioprotective agents due to their antioxidant, anti-inflammatory, and vascular regulatory properties [32, 33]. Quercetin is a well-known flavonol that has shown efficacy in alleviating hyperglycaemia, hyperlipidaemia, hypertension and oxidative stress [34, 35]. Studies have shown that quercetin administration was associated with reduced CVD risk [36, 37]. Interestingly, GTIN (Figure 1) is structurally similar to quercetin, with an extra hydroxyl group [38, 39]. It exhibits many of the same biological activities as quercetin and has demonstrated more potent antioxidant activity [38, 40, 41]. The capacity of GTIN to scavenge free radicals assists in cell protection and may reduce the risk of CVDs [39, 42]. Furthermore, a recent study revealed that GTIN administration significantly enhanced insulin sensitivity, improved glucose tolerance and lowered fasting blood glucose levels [43]. Despite these findings, no studies have investigated the effects of GTIN on cardiovascular complications in the PD state. Hence, this study investigated the effects of diet-induced pre-diabetes on cardiovascular complications in both the presence and absence of dietary modification.

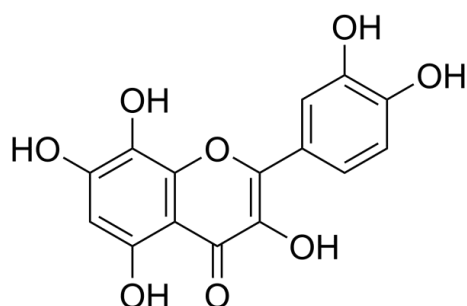


Figure 1. The chemical structure of gossypetin [44].

2. Results

2.1. Lipid Profile Markers

Lipid profile marker concentrations were measured in all experimental groups at week 32 of the treatment period ($n = 6$, per group). The results (Table 1) showed that the PD control group had significantly higher levels of plasma TG ($p = < 0.0001$), total cholesterol (TC) ($p = < 0.0001$), LDL ($p = < 0.0001$) and very low-density lipoproteins (VLDL) ($p = < 0.0001$), along with lower HDL levels ($p = 0.0004$) in comparison to the NPD group. However, PD animals receiving GTIN with a normal diet (GTIN + ND) ($p = < 0.0001$) and with a HFHC diet (GTIN + HFHC) ($p = < 0.0001$) showed a significant reduction in these parameters and an increase in plasma HDL ($p = < 0.0001$, $p = 0.0002$) levels in comparison to the PD control group. Additionally, PD animals receiving MET with a normal diet (MET + ND) ($p = < 0.0001$) and with a HFHC diet (MET + HFHC) ($p = < 0.0001$) exhibited significantly similar results as the GTIN-treated groups.

Table 1. The effects of GTIN on lipid profile markers in both the presence and absence of dietary modification at week 32 of the treatment period (n = 6, per group). Values are presented as mean $\pm$ SEM.

Parameters	Experimental Groups					
	NPD	PD	GTIN + ND	GTIN + HFHC	MET + ND	MET + HFHC
TG (mmol/L)	1.03 $\pm$ 0.077	2.27 $\pm$ 0.22 *	0.91 $\pm$ 0.04 #	1.18 $\pm$ 0.050 #	1.05 $\pm$ 0.071 #	1.19 $\pm$ 0.013 #
TC (mmol/L)	2.65 $\pm$ 0.12	4.36 $\pm$ 0.05 *	2.80 $\pm$ 0.058 #^	3.08 $\pm$ 0.091 #	2.84 $\pm$ 0.045 #	3.25 $\pm$ 0.076 #
HDL (mmol/L)	1.38 $\pm$ 0.084	0.70 $\pm$ 0.12 *	1.52 $\pm$ 0.093 #	1.41 $\pm$ 0.066 #	1.56 $\pm$ 0.11 #	1.22 $\pm$ 0.10 #
LDL (mmol/L)	0.74 $\pm$ 0.14	2.79 $\pm$ 0.14 *	0.72 $\pm$ 0.10 #^	1.06 $\pm$ 0.07 #	0.69 $\pm$ 0.094 #	1.30 $\pm$ 0.066 #
VLDL (mmol/L)	0.53 $\pm$ 0.024	0.87 $\pm$ 0.011 *	0.56 $\pm$ 0.012 #^	0.62 $\pm$ 0.018 #	0.56 $\pm$ 0.0065 #	0.64 $\pm$ 0.012 #

* = $p < 0.05$ denotes comparison with NPD, # = $p < 0.05$ denotes comparison with PD, ^ = $p < 0.05$ denotes comparison with MET + HFHC. NPD: non-pre-diabetic control; PD: pre-diabetic control; gossypetin and normal diet (GTIN + ND); gossypetin and high-fat high-carbohydrate diet (GTIN + HFHC); metformin and normal diet (MET + ND); metformin and high-fat high-carbohydrate group (MET + HFHC).

2.2. MAP Levels

The MAP levels of all the animals were determined from week 20 to week 32 of the experimental period (n = 6, per group). The results (Figure 2) showed that the MAP levels were significantly ($p = < 0.0001$) higher in the PD control group throughout the treatment period in comparison to the NPD. However, the GTIN+ND ($p = < 0.0001$, $p = 0.0017$) and GTIN+HFHC ($p = 0.0017$, $p = < 0.0001$) groups showed significant reductions to MAP levels at weeks 28 and 32 in comparison to the PD control. Additionally, the MET+ND ($p = 0.0148$, $p = < 0.0001$) and MET+HFHC ($p = < 0.0001$, $p = 0.0007$) groups exhibited significantly similar results as the GTIN-treated groups at weeks 28 and 32 of the treatment period.

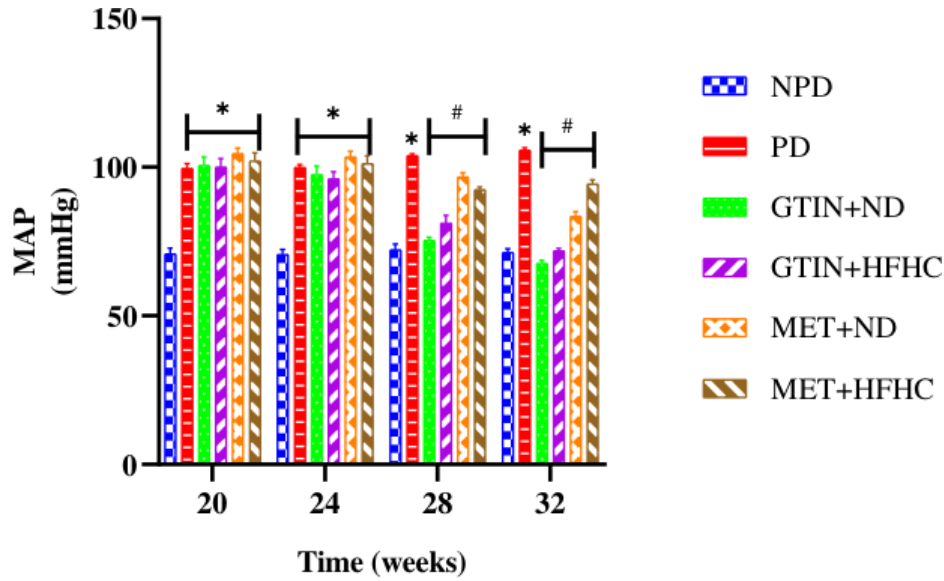


Figure 2. Effects of GTIN on mean arterial pressure (MAP) from week 20 to week 32. Data are presented as mean $\pm$ SEM ($n = 6$ per group). * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

2.3. BMI and WC

The BMI and WC of all the animals were determined from week 20 to week 32 of the experimental period ($n = 6$, per group). The results (Figure 3) showed that the BMI and WC in the PD control group were significantly ($p < 0.05$) higher throughout the treatment period in comparison to the NPD group. However, the GTIN+ND ($p = 0.0002$, $p = < 0.0001$) and GTIN+HFHC ($p = < 0.0001$, $p = < 0.0001$) groups exhibited a significant reduction in BMI and WC at week 28 and week 32 of the treatment period in comparison to the PD control. The MET + ND group showed significantly reduced BMI ($p = 0.0068$) and WC ($p = < 0.0001$) at week 28 in comparison to the PD control. Additionally, both MET-treated groups showed significantly ($p < 0.05$) similar results at week 32 in comparison to the PD control.

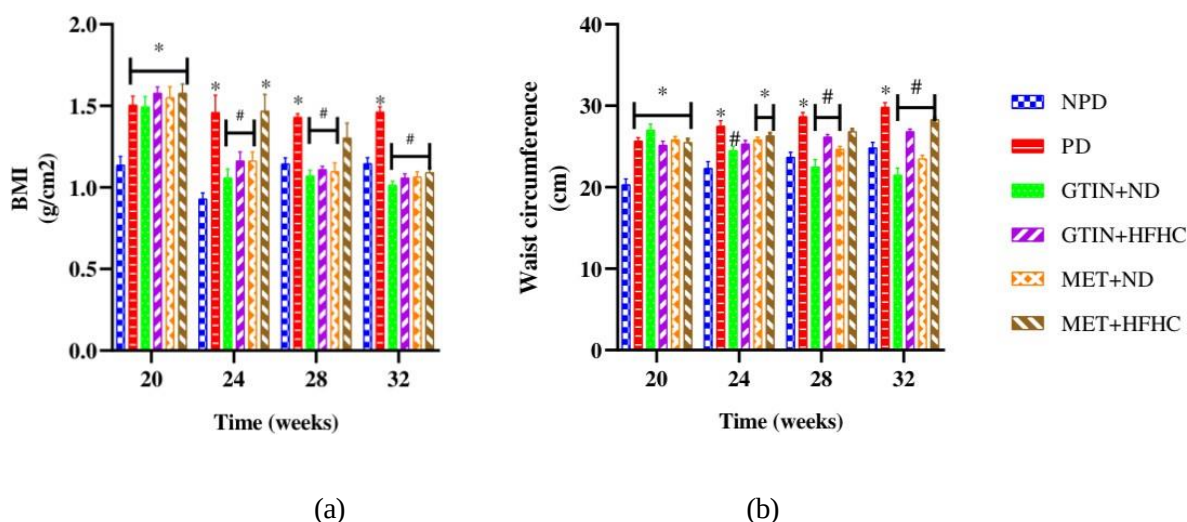


Figure 3. Effects of GTIN on (a) body mass index (BMI) and (b) waist circumference (WC) in rats with or without dietary modification from week 20 to week 32 (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

2.4. eNOS Levels

The plasma eNOS concentrations were measured in all experimental groups at week 32 of the treatment period (n = 6, per group). The results (Figure 4) showed significantly ($p = < 0.0001$) higher eNOS levels in the PD control group in comparison to the NPD group. However, the GTIN+ND ($p = < 0.0001$) and GTIN+HFHC ($p = 0.0180$) groups showed significantly lower eNOS levels in comparison to the PD control. The MET+ ND groups exhibited significantly ($p = < 0.0001$) similar results in comparison to the PD control. The GTIN + HFHC ($p = 0.0013$) and MET-HFHC ($p = 0.0001$) groups had significantly higher eNOS levels in comparison to the NPD.

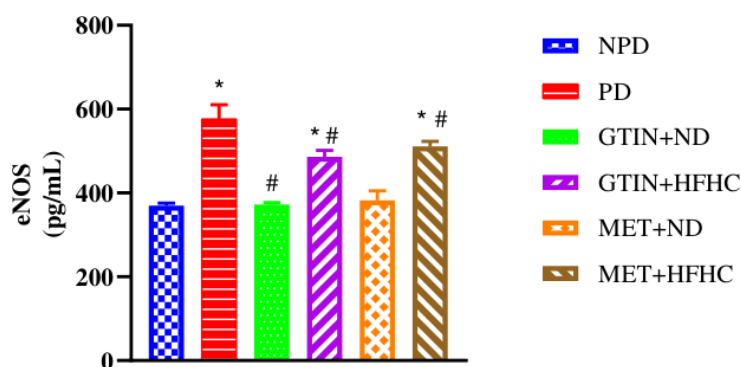


Figure 4. Effects of GTIN on plasma eNOS levels in rats with or without dietary modification at week 32 of the treatment period (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

2.5. Lipid Peroxidation, Antioxidant and Inflammatory Markers

The concentrations of heart lipid peroxidation, antioxidants and plasma inflammatory markers were assessed in the experimental groups at week 32 of the treatment period ($n = 6$ per group). The results (Table 2) indicated that the PD control group showed significantly ($p = < 0.0001$) higher malondialdehyde (MDA) levels along with reduced heart SOD ($p = < 0.0001$) and Gpx ($p = < 0.0001$) activity in comparison to the NPD group. Furthermore, the PD control group showed significantly higher plasma levels of IL-6 ($p = < 0.0001$), TNF- α ($p = < 0.0001$) and CRP ($p = < 0.0001$) in comparison to the NPD group. Although the GTIN+ND group exhibited lower heart SOD and GPx activity, as well as lower plasma IL-6 and TNF- α levels than the NPD group, these differences were not statistically significant. However, both GTIN-treated groups demonstrated a significant ($p < 0.05$) reversal in the level of these markers relative to the PD control. Additionally, the MET + ND group exhibited significantly similar effects on heart MDA ($p = < 0.0001$), SOD ($p = < 0.0001$), and Gpx ($p = < 0.0001$) activity in comparison to the GTIN-treated groups. Notably, both MET-treated groups significantly ($p < 0.05$) lowered plasma inflammatory markers in comparison to the PD control.

Table 2. The effects of GTIN on heart oxidative stress, antioxidant and plasma inflammatory markers in both the presence and absence of dietary modification at week 32 of the treatment period ($n = 6$, per group). Values are presented as mean $\pm$ SEM.

Parameters	Experimental Groups					
	NPD	PD	GTIN + ND	GTIN + HFHC	MET + ND	MET + HFHC
MDA						
(nmol/g protein)	3.95 $\pm$ 0.59	7.73 $\pm$ 0.18 *	4.42 $\pm$ 0.47 #^	5.38 $\pm$ 0.23 #	4.97 $\pm$ 0.28 #	6.68 $\pm$ 0.24 #
T-SOD Activity						
(U/mgprot)	19.88 $\pm$ 0.89	6.88 $\pm$ 0.83 *	17.65 $\pm$ 0.59 #^	13.74 $\pm$ 0.30 #	16.98 $\pm$ 0.73 #	9.80 $\pm$ 0.68
GPx Activity						
(U/mgprot)	941.00 $\pm$ 27.93	415.10 $\pm$ 9.69 *	932.60 $\pm$ 40.95 #^	837.3 $\pm$ 20.84 #	842.10 $\pm$ 16.60 #	463.00 $\pm$ 49.69
IL-6						
(pg/mL)	20.22 $\pm$ 3.55	54.98 $\pm$ 3.56 *	18.79 $\pm$ 1.10 #^	22.60 $\pm$ 0.65 #	22.80 $\pm$ 0.99 #	25.76 $\pm$ 0.89 #
TNF-α						
(pg/mL)	14.96 $\pm$ 0.31	31.77 $\pm$ 0.51 *	13.09 $\pm$ 0.31 #^	21.15 $\pm$ 3.03 #	13.77 $\pm$ 0.61 #	25.83 $\pm$ 0.81 #
hs-CRP						
(pg/mL)	4.98 $\pm$ 0.58	15.91 $\pm$ 0.18 *	5.12 $\pm$ 0.71 #^	6.58 $\pm$ 0.074 #^	6.03 $\pm$ 0.18 #	7.74 $\pm$ 0.11 #

* = $p < 0.05$ denotes comparison with NPD; # = $p < 0.05$ denotes comparison with PD, ^ = $p < 0.05$ denotes comparison with MET + HFHC. NPD: non-pre-diabetic control; PD: pre-diabetic control; gossypetin and normal diet (GTIN + ND); gossypetin and high-fat high-carbohydrate diet (GTIN +

HFHC); metformin and normal diet (MET + ND); metformin and high-fat high-carbohydrate group (MET + HFHC).

3. Discussion

High-calorie diets have been shown to lead to the development of pre-diabetes by promoting insulin resistance (IR) and hyperinsulinaemia [46]. This diet further contributes to the development of obesity, oxidative stress and low-grade inflammation [45]. This combination increases the risk of developing CVDs such as hypertension, myocardial infarction and stroke [46]. Various scientific studies recommend that a combination of lifestyle and pharmacological intervention is necessary to effectively manage pre-diabetes [17, 47]. However, the over-reliance on pharmacological interventions without making necessary dietary changes may hinder the benefits of dietary modifications [48, 49]. Flavonoids such as quercetin have been shown to mitigate the risk of CVDs during pre-diabetes by improving endothelial function, reducing inflammation and enhancing insulin sensitivity [50]. Gossypetin is present in various plant species, including *Hibiscus sabdariffa*, *Hibiscus vitifolius*, *Gossypium herbaceum* and *Gossypium arboreum*, from the Malvaceae family [51-54]. It has also been found in *Empetrum nigrum* from the Ericaceae family and *Acacia constricta* from the Fabaceae family [51, 52, 54]. Notably, the majority of studies have focused on the qualitative analysis of GTIN, with scarce studies quantifying the percentage content [55-58]. A previous study showed that of the seven compounds isolated from a *Hibiscus sabdariffa* phenolic extract, GTIN exhibited the highest yield of 23% [59]. Interestingly, the phenolic content in *Hibiscus sabdariffa* ranges from 24.36 to 44.43 mg of gallic acid equivalents/100 g of dried calyces [56, 60, 61]. Furthermore, studies have shown that GTIN exhibits potent antioxidant and anti-inflammatory properties [39, 62]. The multiple hydroxyl groups present within GTIN's structure facilitate its ability to mitigate oxidative stress [39, 62]. Despite these findings, the effects of this compound on cardiovascular function in the PD state have not been investigated.

Under normal physiological conditions, plasma lipid levels are regulated through the assistance of lipoproteins [63]. VLDLs transport hepatic TG while LDLs primarily transport cholesterol within the bloodstream [63]. HDLs facilitate reverse cholesterol transport to the liver [64]. In individuals with normal metabolic function, the lipid profile is characterised by TC levels below 5.2 mmol/L, LDL cholesterol concentrations less than 2.6 mmol/L, HDL cholesterol levels greater than 1.1 mmol/L, TG below 1.7 mmol/L and VLDL levels less than 0.8 mmol/L [65-67]. In contrast, individuals with pre-diabetes have been shown to exhibit disturbed lipid profiles, which may be characterised by elevated plasma TG, TC, LDL and VLDL, along with decreased HDL levels [68-72]. These abnormalities may include plasma TG levels ranging from 1.7 mmol/L to 2.2 mmol/L, TC levels exceeding 5.2 mmol/L, LDL levels greater than 2.6 mmol/L, VLDL concentrations between 0.8 and 1.2 mmol/L and HDL levels below 1.0 mmol/L in comparison to healthy individuals [68-72]. The chronic consumption of high-calorie diets, which include high sugar, saturated fats and processed foods, leads to

dyslipidaemia by inducing IR and oxidative stress [73-75]. These conditions stimulate increased lipolysis, which in turn promotes TG synthesis and raises plasma VLDL levels [76]. Additionally, increased ROS levels promote LDL oxidation which inhibits LDL clearance [77]. This has been associated with foam cell formation and an increased risk of developing arteriosclerosis [78]. Furthermore, oxidative stress may impair mitochondrial function, reducing fatty acid oxidation and exacerbating lipid accumulation, while activation of inflammatory pathways such as nuclear factor kappa B (NF- κ B) further contributes to dyslipidaemia and vascular injury [79, 80]. In this study, plasma TG, TC, LDL and VLDL levels were significantly higher along with lower plasma HDL levels in the PD control in comparison to the NPD group. This study's results corroborated with previous findings of dyslipidaemia present during pre-diabetes [30, 81]. However, the GTIN + ND and GTIN + HFHC groups significantly reduced these parameters while increasing plasma HDL levels in comparison to the PD control.

Flavonoids have been shown to reduce LDL oxidation by targeting oxidative stress and decrease TG levels by promoting fatty acid oxidation [82, 83]. Quercetin promotes HDL synthesis, which helps maintain cholesterol homeostasis [84]. A previous study has shown that GTIN reduced oxidised LDL-induced foam cell formation and intracellular lipid accumulation [85]. Similarly, in our study, GTIN may have improved the lipid profile during pre-diabetes by enhancing LDL clearance, promoting fatty acid oxidation and HDL synthesis [82]. These effects may be mediated by activation of AMP-activated protein kinase (AMPK) [86]. AMPK promotes fatty acid oxidation, inhibits hepatic lipid synthesis, and improves metabolic homeostasis [87]. In addition, GTIN may reduce oxidative stress [86]. This reduction may suppress NF- κ B signalling and thereby reduce inflammation-associated disturbances in lipid metabolism [88]. Additionally, the MET + ND and MET + HFHC groups demonstrated similar effects on lipid profiles compared to the GTIN-treated groups. MET has been shown to improve dyslipidaemia by enhancing insulin sensitivity and regulating hepatic lipid metabolism [89, 90]. MET exerts many of these effects through activation of AMPK [91]. In contrast, GTIN may provide additional cardioprotective benefits through its potent antioxidant and anti-inflammatory properties [39, 92]. Although their primary mechanisms may differ, both compounds appear to act on pathways that improve lipid homeostasis and reduce cardiovascular risk [42, 93]. This may explain the comparable improvements observed with GTIN and MET. While the MET + ND and MET + HFHC groups were effective in improving lipid profile markers in the PD state, GTIN exhibited more pronounced effects.

Under normal physiological conditions, the regulation of MAP levels involves the integration of neural, hormonal and local mechanisms [94]. NO is a vasodilator that is produced by eNOS and is important in the regulation of vascular tone [95]. However, intermediate hyperglycaemia promotes mitochondrial dysfunction and the formation of advanced glycated end products and triggers inflammatory pathways [96]. These mechanisms contribute to increased oxidative stress, which

damages endothelial cells [97, 98]. Endothelial dysfunction reduces NO availability, which subsequently increases vascular resistance and MAP levels [97, 98]. In this study, MAP levels were significantly higher throughout the treatment period in comparison to the NPD group. This was further accompanied by elevated plasma eNOS levels at week 32 in the PD control group. These results corroborated with previous studies, which have reported similar findings in the PD state [81, 99]. The findings may suggest that eNOS levels increased to compensate for elevated MAP levels. However, the GTIN + ND and GTIN + HFHC groups exhibited significantly lower MAP levels at week 28 and week 32 in comparison to the PD control. This was further accompanied by decreased plasma eNOS levels at week 32 of the treatment period.

Studies have shown that quercetin improves MAP levels by reducing oxidative stress and activating eNOS synthesis [100, 101]. Furthermore, previous literature has shown that 15 mg/kg and 20 mg/kg GTIN administration ameliorates intermediate hyperglycaemia, reduces oxidative stress and enhances AMP-activated protein kinase (AMPK) activity [39, 86]. AMPK activation enhances the NO pathway, which promotes vasodilation [86]. Similarly, in our study, 15 mg/kg GTIN may have decreased MAP levels by facilitating vasodilation through its effects on intermediate hyperglycaemia and oxidative stress. Reduced oxidative stress may also preserve endothelial nitric oxide signalling by improving NO bioavailability and limiting endothelial dysfunction [102]. Furthermore, attenuation of oxidative stress may suppress NF- κ B activation, thereby reducing vascular inflammation and contributing to improved endothelial function [103]. The plasma eNOS levels in the GTIN + ND and GTIN + HFHC groups may have been attributed to reduced oxidative stress. Additionally, both MET-treated groups exhibited effects on MAP levels that were similar to the GTIN-treated group. This was further accompanied by decreased plasma eNOS levels in the MET + ND group in comparison to the PD control. This may suggest that MET is effective in controlling MAP levels when used in conjunction with dietary intervention. While both GTIN and MET improved vascular function, MET is suggested to exert these effects predominantly through AMPK activation and improved insulin sensitivity, whereas GTIN may additionally protect the endothelium through its antioxidant and anti-inflammatory properties [39, 92, 93]. These findings suggest that GTIN may provide complementary cardiovascular benefits beyond its glucose-lowering effects.

Adiposity is influenced by caloric intake, energy expenditure and hormonal control [104]. This condition promotes chronic inflammation, which accelerates plaque buildup in the arteries and subsequently increases the risk of CVDs [105]. BMI and WC are useful in monitoring adiposity and visceral fat in the management of pre-diabetes [106, 107]. During chronic overnutrition, excess nutrient availability suppresses AMP-activated protein kinase (AMPK) activity [108]. This reduces fatty acid oxidation and mitochondrial energy expenditure, leading to increased lipid accumulation [108]. In contrast, increased nutrient availability enhances peroxisome proliferator-activated receptor gamma (PPAR γ) activation [109]. PPAR γ promotes the differentiation of pre-adipocytes into mature

adipocytes and increases lipid storage [109]. Similarly, persistent activation of mechanistic target of rapamycin complex 1 (mTORC1) in response to nutrient abundance stimulates anabolic processes, including lipogenesis, protein synthesis and adipocyte growth [110]. These metabolic disturbances may have contributed to the increased BMI and WC observed in the PD group.

However, the GTIN + ND and GTIN + HFHC groups showed significantly lower BMI and WC at week 28 and week 32 of the treatment period in comparison to the PD control. In a recent study, it was shown that GTIN administration significantly reduced caloric intake and weight gain by decreasing plasma ghrelin levels [43]. This may account for the reduced BMI and WC observed in the GTIN-treated groups. Additionally, flavonoids have been reported to reverse obesity-associated metabolic disturbances through activation of AMPK and inhibition of mTORC1 signalling [111]. This promotes fatty acid oxidation and mitochondrial energy expenditure while suppressing SREBP-1c-mediated lipogenesis [112]. Flavonoids may also attenuate excessive PPAR γ activation, thereby reducing adipocyte differentiation and lipid storage [113]. Through these mechanisms, flavonoids mimic aspects of caloric restriction by shifting metabolism from energy storage towards increased fat utilisation [111]. As GTIN is a flavonoid, it is plausible that the reductions in BMI and WC observed in the present study were mediated through these mechanisms [114]. Furthermore, improved mitochondrial function resulting from AMPK activation may enhance cellular energy utilisation, thereby contributing to the reductions in adiposity observed following GTIN treatment [86]. Interestingly, the MET + ND group showed significantly lower BMI at week 28 in comparison to the PD control. MET reduces body weight and adiposity through multiple metabolic mechanisms [115]. It activates AMPK, which improves cellular energy regulation and enhances fatty acid oxidation [116]. MET also inhibits hepatic gluconeogenesis, reducing excessive hepatic glucose production and improving insulin sensitivity [117]. Furthermore, MET suppresses appetite through hypothalamic pathways and increases secretion of gut-derived hormones, such as glucagon-like peptide-1 (GLP-1) [118]. This promotes satiety and reduces caloric intake [119]. MET may also decrease intestinal glucose absorption and enhance peripheral glucose uptake through increased GLUT4 translocation in skeletal muscle [120]. While both MET-treated groups were effective in reducing BMI and WC by week 32, the GTIN-treated groups had already shown effectiveness by week 28. However, dose-response and pharmacokinetic studies are required to determine GTIN therapeutic potential over MET. This may suggest that 15 mg/Kg GTIN administration both in the presence and absence of dietary modification exhibits a faster onset of effectiveness than MET.

Under normal conditions, there exists a balance between ROS levels and antioxidants [121]. However, intermediate hyperglycaemia has been shown to promote the accumulation of increased ROS through mitochondrial dysfunction [14, 122]. Furthermore, elevated advanced glycated end-products and free fatty acids observed in the PD state trigger inflammation-induced ROS production [123]. The accumulation of ROS contributes to oxidative stress, which triggers lipid peroxidation [124]. MDA is

the toxic by-product produced by lipid peroxidation and is responsible for damaging cellular functions [125]. The redox imbalances observed during pre-diabetes may contribute to the development of CVDs [126]. In this study, heart MDA levels were significantly higher in the PD control group compared to the NPD group. This was further accompanied by significantly lower heart SOD and Gpx activity in the PD control. These results may suggest that the reduced antioxidant capacity of heart tissue may have compromised its ability to maintain redox balance, leading to increased lipid peroxidation. However, the GTIN + ND and GTIN + HFHC groups showed significantly lower heart MDA levels in comparison to the PD control. This was further accompanied by increased heart SOD and Gpx activity in both GTIN-treated groups. Previous studies have shown that GTIN exhibits potent antioxidant effects, which have been attributed to its free radical scavenging capability [42, 127]. Interestingly, the specific arrangement of hydroxyl and methoxy groups on the flavonoid backbone structure of GTIN (Figure 1) may contribute to its antioxidant effects [42, 92]. This study's results may suggest that 15 mg/Kg GTIN administration in both the presence and absence of dietary modification reduces oxidative stress and lipid peroxidation by enhancing the antioxidant enzyme activity of heart tissue. By reducing oxidative stress, GTIN may also preserve mitochondrial function and limit activation of redox-sensitive inflammatory pathways such as NF- κ B, thereby providing additional cardioprotective effects [128]. Additionally, both MET-treated groups showed significantly similar effects on heart MDA levels as the GTIN-treated groups. This was further accompanied by increased heart SOD and GPx activity in only the MET + ND group. Previous studies have shown that MET decreases oxidative stress by promoting AMPK activation, improving insulin sensitivity, upregulating antioxidant enzyme expression and improving mitochondrial function [129, 130]. This may suggest that MET exhibits beneficial effects on antioxidant enzyme activity when used in conjunction with dietary modification. The antioxidant activity of GTIN may involve direct scavenging of ROS due to its polyhydroxylated flavonoid structure [131]. In contrast, MET mainly exerts antioxidant effects indirectly through improved metabolic regulation [132].

Under normal physiological conditions, inflammation is essential in the response to injury and tissue healing [133]. This has to be regulated to prevent chronic inflammation, which promotes tissue damage observed in CVDs [134]. However, during pre-diabetes, oxidative stress has been shown to activate pathways that release pro-inflammatory cytokines such as TNF- α , IL-6 and CRP [14, 135]. The inflammatory response recruits and activates immune cells at the site of injury [136]. Activated immune cells produce ROS and cytokines, which further worsen inflammation and tissue damage [137]. This chronic inflammatory state is partly mediated through activation of NF- κ B signalling, a key regulator of inflammatory gene expression [138]. Persistent NF- κ B activation promotes the production of cytokines such as TNF- α and IL-6, thereby contributing to endothelial dysfunction and cardiovascular complications associated with pre-diabetes [139]. In this study, levels for inflammatory markers, such as plasma IL-6, TNF- α and CRP, were significantly higher in the PD control group in comparison to the NPD group. This study's findings corroborate previous studies that reported the

presence of low-grade inflammation in the PD state [135, 140]. However, the GTIN + ND and GTIN + HFHC groups showed significantly lower inflammatory marker levels in comparison to the PD control. A previous study showed that GTIN reduces inflammation by targeting ROS accumulation [62]. Therefore, it may be speculated that GTIN reduces low-grade inflammation by targeting oxidative stress due to its potent antioxidant properties [86]. We speculate by reducing ROS accumulation, GTIN may indirectly attenuate NF- κ B activation, resulting in reduced inflammatory cytokine production and improved cardiovascular protection. Additionally, both MET-treated groups exhibited effects on these parameters similar to the GTIN-treated groups. Previous studies have shown that MET reduces inflammation by activating the AMPK, which inhibits NF- κ B signaling [141, 142]. By suppressing NF- κ B, MET reduces the production of TNF- α and IL-6, improving cardiovascular function [116]. Although both GTIN and MET demonstrated anti-inflammatory effects, their mechanisms may differ [90, 143]. MET primarily regulates inflammation through AMPK-dependent inhibition of NF- κ B signalling, whereas GTIN may provide additional anti-inflammatory effects through direct antioxidant activity and reduction of oxidative stress [90, 143]. The anti-inflammatory effects of the conventional anti-diabetic drug MET were comparable to GTIN.

This study highlights the potential of 15 mg/Kg GTIN as an effective compound for improving cardiovascular function in the PD state. The results demonstrated that GTIN significantly improved lipid profile markers, reducing plasma TC, TG, LDL and VLDL, while increasing HDL in comparison to the NPD group. Additionally, GTIN administration decreased MAP, BMI, WC and plasma eNOS levels. It also reduced heart oxidative stress by lowering MDA levels and enhanced antioxidant activity through increased SOD and Gpx activity. Moreover, GTIN decreased inflammation, as indicated by lower plasma IL-6 and TNF- α levels. Notably, these beneficial effects were observed both in the presence and absence of dietary intervention, highlighting GTIN's potential role in cardiovascular health management.

Although GTIN demonstrated promising cardiometabolic effects in this preclinical model, further investigations are required to determine its translational potential in humans. Important considerations include pharmacokinetic characteristics, oral bioavailability, metabolism, tissue distribution, toxicity and the establishment of an appropriate therapeutic dose. As flavonoids often exhibit limited bioavailability due to extensive metabolism, future studies should determine whether sufficient concentrations of GTIN can be achieved safely to produce biological effects in humans [144]. Furthermore, long-term toxicity studies and clinical trials are required to establish whether GTIN can complement or provide an alternative therapeutic strategy to established treatments such as MET in the prevention of cardiovascular complications associated with pre-diabetes.

4. Materials and Methods

4.1. Chemicals and Drugs

All chemicals and drugs were of analytical grade and purchased from standard commercial suppliers.

4.2. Animals and Housing

This study utilised 36 male Sprague Dawley rats (150–180 g), which were bred and housed in the Biomedical Research Unit (BRU) at the University of KwaZulu-Natal (UKZN), Westville campus. The animals were maintained under standard laboratory conditions, which included a constant temperature of $22 \pm 2^\circ\text{C}$, a carbon dioxide (CO_2) content of <5000 p.p.m., a relative humidity of $55 \pm 5\%$ and illumination (12 h light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels. All animals were allowed access to food and fluids ad libitum. The Animal Research Ethics Committee of the University of KwaZulu-Natal (ethics: AREC/00004965/2022) granted permission for all animal experimentation and protocols. Before exposure to the experimental diets, the rats were allowed to acclimatise to their new environment while consuming standard rat diet and tap water [145]. The care and handling procedures were followed in accordance with the Animal Research Ethics Committee (AREC) of UKZN.

4.3. Induction of Pre-Diabetes

The animals were randomly assigned into two dietary groups: group A ($n = 6$) and group B ($n = 30$). Experimental pre-diabetes was induced in the animals using a method previously outlined by Luvuno et al. [145]. Group A animals received a standard rat diet with tap water while group B received the HFHC diet supplemented with 15% fructose-enriched water for the induction of pre-diabetes (AVI Products (Pty) Ltd., Waterfall, South Africa). At week 20, the animals were assessed for pre-diabetes based on the American Diabetes Association (ADA) criteria [146]. Animals with a fasting blood glucose concentration of 5.6 to 6.9 mmol/L, 2 h oral glucose tolerance test glucose concentration of 7.8 to 11.0 mmol/L and a glycated haemoglobin concentration of 5.7 to 6.4% were classified as the pre-diabetic group. The animals that received the standard diet were also evaluated at week 20 to confirm normoglycaemia and were classified as the non-pre-diabetic group.

4.4. Experimental Design and Treatment

Following the induction of pre-diabetes, the pre-diabetic group ($n = 30$) was further divided into five sub-groups (Group B to Group F), consisting of six animals each. The pre-diabetic animals either continued the HFHC diet or changed to a normal standard diet (ND) while receiving either 15 mg/Kg GTIN or 500 mg/Kg MET orally once every third day for 12 weeks. We selected an oral dose of 15 mg/Kg GTIN based on previous studies showing its effectiveness and safety within the 10 mg/Kg–20 mg/Kg range [42, 43, 147, 148]. Similarly, 500 mg/Kg MET was chosen due to its proven therapeutic effects at this dose and minimal side effects [30, 149, 150]. The timing of dosage was chosen to minimise drug accumulation and toxicity [30, 117, 151]. The non-pre-diabetic control (Group A) animals continued the ND while the pre-diabetic control group (PD, Group B) remained on the HFHC

diet and received as the vehicle 3 mL/Kg of diluted dimethyl sulphoxide (2 mL DMSO: 19 mL normal saline, p.o.)(Merck, Darmstadt, Germany). Group C (GTIN + ND) animals received GTIN and were changed to the ND diet while Group D (GTIN + HFHC) received GTIN and continued the HFHC diet. The Group E (MET + ND) animals received MET and changed to the ND diet while Group F animals (MET + HFHC) received MET and continued the HFHC diet. Parameters such as body mass index (BMI), waist circumference (WC) and blood pressure were monitored at weeks 20, 24, 28 and 32 as described in the established protocol [30]. The systolic and diastolic pressure of all experimental animals was measured using a non-invasive tail-cuff method and the MAP was calculated using the formula $MAP = [(2 \times \text{diastolic}) + \text{systolic}]/3$ [30, 152].

4.5. Blood Collection and Tissue Harvesting

At the end of week 32, all animals were anaesthetised with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd., Roodeport, South Africa) for 3 min using a gas anaesthetic chamber (BRU, UKZN, Durban, South Africa). Once the animals were unconscious, blood samples were obtained from the animals through cardiac puncture and transferred into individual pre-cooled heparinised containers. The blood samples were centrifuged (Eppendorf AG, Eppendorf centrifuge 5403, Hamburg, Germany) at 4 °C, 503 g for 15 min to obtain plasma. Each plasma sample was aspirated into plain sample bottles and stored at –80 °C in a Bio Ultra freezer (Snijers Scientific, Tilburg, The Netherlands) until ready for biochemical analysis. Thereafter, the heart was removed and snap-frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, The Netherlands) at –80 °C until biochemical analysis.

4.6. Biochemical Analysis

At the end of week 32, the plasma lipid profile was analysed by measuring TG, TC and HDL according to manufacturer instructions using their respective assay kits (Elabsience Biotechnology Co., Ltd., Houston, TX, USA). The absorbance of the samples for TG and TC was measured at 510 nm, while HDL absorbance was measured at 600 nm using a Spectrostar Nanospectrophotometer (BMG Labtech, Ortenberg, Baden-Württemberg, LGBW, Germany). VLDL and LDL cholesterol levels were calculated using Friedewald's formula: $LDL = TC - HDL - (TG/2.2)$ and $VLDL = TG/2.2$ [153]. Heart malondialdehyde (MDA) levels were assessed following a previously published protocol [154]. Heart SOD and GPx activity as well as plasma eNOS, TNF- α , IL-6 and CRP were measured using their respective ELISA kits (Elabsience Biotechnology Co., Ltd., Houston, TX, USA).

4.7. Statistical Analysis

The statistical data were presented as mean $\pm$ SEM. Prior to statistical analysis, the assumptions of normality and homogeneity of variance were assessed to ensure the suitability of the data for parametric analyses. MAP, BMI and WC data were analysed using a two-way analysis of variance (ANOVA), whereas data collected at a single time point were analysed using one-way ANOVA. The

Tukey–Kramer post hoc test was used for multiple comparisons. Statistical significance was determined at $p < 0.05$.

5. Conclusions

Taken together, GTIN administration, both in the presence and absence of dietary modification, reduced elevated BMI, WC and MAP, attenuated oxidative stress and inflammation, improved endothelial function, and enhanced antioxidant enzyme activity in diet-induced prediabetes. The results were comparable to those observed with MET administration, indicating that GTIN may serve as a viable alternative for managing cardiovascular complications in the PD state. Nonetheless, further research is necessary to elucidate the mechanisms underlying the observed effects of GTIN administration.

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References

1. Ping, W. X.; Hu, S.; Su, J. Q.; Ouyang, S. Y., Metabolic disorders in prediabetes: From mechanisms to therapeutic management. *World J Diabetes* 2024, 15, (3), 361–377.
2. Lai, M.; Chandrasekera, P. C.; Barnard, N. D., You are what you eat, or are you? The challenges of

translating high-fat-fed rodents to human obesity and diabetes. *Nutrition & diabetes* 2014, 4, (9), e135–e135.

3. Meshram, I. I.; Nagalla, B.; Kodavanti, M. R.; Avula, L.; Veera, B. G. N., Overweight/obesity, pre-diabetes, diabetes and its association with hypertension and other factors among rural adults (≥ 18 years) in India. *Indian Heart Journal* 2024, 76, (3), 182–191.

4. Beulens, J.; Rutters, F.; Ryden, L.; Schnell, O.; Mellbin, L.; Hart, H.; Vos, R., Risk and management of pre-diabetes. *European journal of preventive cardiology* 2019, 26, (2_suppl), 47–54.

5. Rooney, M. R.; Fang, M.; Ogurtsova, K.; Ozkan, B.; Echouffo-Tcheugui, J. B.; Boyko, E. J.; Magliano, D. J.; Selvin, E., Global prevalence of prediabetes. *Diabetes care* 2023, 46, (7), 1388–1394.

6. Gumede, N.; Ngubane, P.; Khathi, A., Assessing the risk factors for myocardial infarction in diet-induced prediabetes: myocardial tissue changes. *BMC cardiovascular disorders* 2022, 22, (1), 350.

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

8. Abshirini, M.; Mahaki, B.; Bagheri, F.; Siassi, F.; Koohdani, F.; Qorbani, M.; Yavari, P.; Sotoudeh, G., Dietary fat quality and pre-diabetes: a case-control study. *International journal of preventive medicine* 2020, 11, 160.

9. Grundy, S. M., Pre-diabetes, metabolic syndrome, and cardiovascular risk. *Journal of the American College of Cardiology* 2012, 59, (7), 635–643.

10. Shen, X.-M.; Huang, Y.-Q.; Zhang, X.-Y.; Tong, X.-Q.; Zheng, P.-F.; Shu, L., Association between dietary patterns and prediabetes risk in a middle-aged Chinese population. *Nutrition journal* 2020, 19, (1), 77.

11. Brannick, B.; Dagogo-Jack, S., Prediabetes and cardiovascular disease: pathophysiology and interventions for prevention and risk reduction. *Endocrinology and metabolism clinics of North America* 2018, 47, (1), 33.

12. Bhatti, J. S.; Sehrawat, A.; Mishra, J.; Sidhu, I. S.; Navik, U.; Khullar, N.; Kumar, S.; Bhatti, G. K.; Reddy, P. H., Oxidative stress in the pathophysiology of type 2 diabetes and related complications: Current therapeutics strategies and future perspectives. *Free Radical Biology and Medicine* 2022, 184, 114–134.

13. Asmat, U.; Abad, K.; Ismail, K., Diabetes mellitus and oxidative stress—A concise review. *Saudi pharmaceutical journal* 2016, 24, (5), 547–553.

14. Luc, K.; Schramm-Luc, A.; Guzik, T.; Mikolajczyk, T., Oxidative stress and inflammatory markers in prediabetes and diabetes. *Journal of Physiology & Pharmacology* 2019, 70, (6), 1.

15. Weaver, J. R.; Odanga, J. J.; Breathwaite, E. K.; Treadwell, M. L.; Murchinson, A. C.; Walters, G.; Fuentes, D. P.; Lee, J. B., An increase in inflammation and islet dysfunction is a feature of

- prediabetes. *Diabetes/Metabolism Research and Reviews* 2021, 37, (6), e3405.
16. Neves, J. S.; Newman, C.; Bostrom, J. A.; Buyschaert, M.; Newman, J. D.; Medina, J. L.; Goldberg, I. J.; Bergman, M., Management of dyslipidemia and atherosclerotic cardiovascular risk in prediabetes. *Diabetes research and clinical practice* 2022, 190, 109980.
 17. Kanat, M.; DeFronzo, R. A.; Abdul-Ghani, M. A., Treatment of prediabetes. *World journal of diabetes* 2015, 6, (12), 1207.
 18. Rezaei, M.; Valiee, S.; Tahan, M.; Ebtekar, F.; Ghanei Gheshlagh, R., Barriers of medication adherence in patients with type-2 diabetes: a pilot qualitative study. *Diabetes, Metabolic Syndrome and Obesity* 2019, 589–599.
 19. Aaby, A.; Friis, K.; Christensen, B.; Rowlands, G.; Maindal, H. T., Health literacy is associated with health behaviour and self-reported health: A large population-based study in individuals with cardiovascular disease. *European journal of preventive cardiology* 2017, 24, (17), 1880–1888.
 20. Silva, P.; Araújo, R.; Lopes, F.; Ray, S., Nutrition and food literacy: framing the challenges to health communication. *Nutrients* 2023, 15, (22), 4708.
 21. Cusquisibán-Alcantara, Y.; Toledo-Garrido, C.; Calizaya-Milla, Y. E.; Carranza-Cubas, S. P.; Saintila, J., Impact of a nutrition education intervention on knowledge, healthy eating index, and biochemical profile in a rural community in Peru. *Journal of multidisciplinary healthcare* 2024, 1111–1125.
 22. Harrison, C.; Peyyety, V.; Rodriguez Gonzalez, A.; Chivate, R.; Qin, X.; Zupa, M. F.; Ragavan, M. I.; Vajravelu, M. E., Prediabetes prevalence by adverse social determinants of health in adolescents. *JAMA Network Open* 2024, 7, (6), e2416088.
 23. Ablah, E.; Dong, F.; Cupertino, A. P.; Konda, K.; Johnston, J. A.; Collins, T., Prevalence of diabetes and pre-diabetes in Kansas. *Ethnicity & Disease* 2013, 23, (4), 415–420.
 24. Aldossari, K. K.; Aldiab, A.; Al-Zahrani, J. M.; Al-Ghamdi, S. H.; Abdelrazik, M.; Batais, M. A.; Javad, S.; Nooruddin, S.; Razzak, H. A.; El-Metwally, A., Prevalence of prediabetes, diabetes, and its associated risk factors among males in Saudi Arabia: a population-based survey. *Journal of diabetes research* 2018, 2018, (1), 2194604.
 25. Ghaddar, R.; Hudson, E. A.; Jeans, M. R.; Vandyousefi, S.; Landry, M. J.; Davis, J. N., Ethnicity/race, parent educational attainment, and obesity associated with prediabetes in children. *Nutrition & Diabetes* 2023, 13, (1), 15.
 26. Hill-Briggs, F.; Adler, N. E.; Berkowitz, S. A.; Chin, M. H.; Gary-Webb, T. L.; Navas-Acien, A.; Thornton, P. L.; Haire-Joshu, D., Social determinants of health and diabetes: a scientific review. *Diabetes care* 2020, 44, (1), 258.
 27. Solini, A.; Tricò, D., Clinical efficacy and cost-effectiveness of metformin in different patient populations: A narrative review of real-world evidence. *Diabetes, Obesity and Metabolism* 2024, 26,

20–30.

28. Skoglund, G.; Nilsson, B. B.; Olsen, C. F.; Bergland, A.; Hilde, G., Facilitators and barriers for lifestyle change in people with prediabetes: a meta-synthesis of qualitative studies. *BMC public health* 2022, 22, (1), 553.

29. Perreault, L.; Færch, K., Approaching pre-diabetes. *Journal of Diabetes and its Complications* 2014, 28, (2), 226–233.

30. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Plant-derived oleanolic acid ameliorates markers associated with non-alcoholic fatty liver disease in a diet-induced pre-diabetes rat model. *Diabetes, Metabolic Syndrome and Obesity* 2019, 1953–1962.

31. Bansal, N., Prediabetes diagnosis and treatment: A review. *World journal of diabetes* 2015, 6, (2), 296.

32. Ciumărnean, L.; Milaciu, M. V.; Runcan, O.; Vesa, Ș. C.; Răchișan, A. L.; Negrean, V.; Perné, M.-G.; Donca, V. I.; Alexescu, T.-G.; Para, I., The effects of flavonoids in cardiovascular diseases. *Molecules* 2020, 25, (18), 4320.

33. Adegbola, P.; Aderibigbe, I.; Hammed, W.; Omotayo, T., Antioxidant and anti-inflammatory medicinal plants have potential role in the treatment of cardiovascular disease: a review. *American journal of cardiovascular disease* 2017, 7, (2), 19.

34. Bondonno, N. P.; Bondonno, C. P.; Hodgson, J. M.; Ward, N. C.; Croft, K. D., The efficacy of quercetin in cardiovascular health. *Current Nutrition Reports* 2015, 4, (4), 290–303.

35. Caro-Ordieres, T.; Marín-Royo, G.; Opazo-Ríos, L.; Jiménez-Castilla, L.; Moreno, J. A.; Gómez-Guerrero, C.; Egido, J., The coming age of flavonoids in the treatment of diabetic complications. *Journal of clinical medicine* 2020, 9, (2), 346.

36. Larson, A. J.; Symons, J. D.; Jalili, T., Therapeutic potential of quercetin to decrease blood pressure: review of efficacy and mechanisms. *Advances in nutrition* 2012, 3, (1), 39–46.

37. Papakyriakopoulou, P.; Velidakis, N.; Khattab, E.; Valsami, G.; Korakianitis, I.; Kadoglou, N. P., Potential pharmaceutical applications of quercetin in cardiovascular diseases. *Pharmaceuticals* 2022, 15, (8), 1019.

38. Jo, K. W.; Lee, D.; Cha, D. G.; Oh, E.; Choi, Y. H.; Kim, S.; Park, E. S.; Kim, J. K.; Kim, K.-T., Gossypetin ameliorates 5xFAD spatial learning and memory through enhanced phagocytosis against Aβ. *Alzheimer's research & therapy* 2022, 14, (1), 158.

39. Khan, A.; Manna, K.; Bose, C.; Sinha, M.; Das, D. K.; Kesh, S. B.; Chakrabarty, A.; Banerji, A.; Dey, S., Gossypetin, a naturally occurring hexahydroxy flavone, ameliorates gamma radiation-mediated DNA damage. *International journal of radiation biology* 2013, 89, (11), 965–975.

40. François-Haugrin, F.; Monan, M.; Nossin, E.; Smith-Ravin, J.; Marcelin, O., Antioxidant activity of an isomer of gossypitrin (gossypetin-3'-O-glucoside) isolated in the petals of *Talipariti elatum* Sw.,

and determination of total phenolic content of the total flower. *Journal of Pharmacognosy and Phytochemistry* 2016, 5, (5), 200–208.

41. Dutta, M. S.; Mahapatra, P.; Ghosh, A.; Basu, S., Estimation of the reducing power and electrochemical behavior of few flavonoids and polyhydroxybenzophenones substantiated by bond dissociation energy: A comparative analysis. *Molecular Diversity* 2022, 26, (2), 1101–1113.

42. Chen, J.-H.; Tsai, C.-W.; Wang, C.-P.; Lin, H.-H., Anti-atherosclerotic potential of gossypetin via inhibiting LDL oxidation and foam cell formation. *Toxicology and applied pharmacology* 2013, 272, (2), 313–324.

43. Naidoo, K.; Khathi, A., Effects of gossypetin on glucose homeostasis in diet-induced pre-diabetic rats. *Molecules* 2024, 29, (18), 4410.

44. Nix, A.; Paull, C.; Colgrave, M., Flavonoid profile of the cotton plant, *Gossypium hirsutum*: a review. *Plants* 2017, 6, (4), 43.

45. Small, L.; Brandon, A. E.; Turner, N.; Cooney, G. J., Modeling insulin resistance in rodents by alterations in diet: what have high-fat and high-calorie diets revealed? *American Journal of Physiology-Endocrinology and Metabolism* 2018, 314, (3), E251–E265.

46. Agarwal, S. K., Impact of Diet on Cardiovascular Diseases: Hypertension and Stroke. *Asian Journal of Cardiology Research* 2021, 4, (1), 285–297.

47. Sheng, Z.; Cao, J.-Y.; Pang, Y.-C.; Xu, H.-C.; Chen, J.-W.; Yuan, J.-H.; Wang, R.; Zhang, C.-S.; Wang, L.-X.; Dong, J., Effects of lifestyle modification and anti-diabetic medicine on prediabetes progress: a systematic review and meta-analysis. *Frontiers in endocrinology* 2019, 10, 455.

48. Yau, J. W.; Thor, S. M.; Ramadas, A., Nutritional strategies in prediabetes: A scoping review of recent evidence. *Nutrients* 2020, 12, (10), 2990.

49. Seidu, B. S.; Osman, H.; Seidu, S., Lifestyle or pharmacotherapy in cardio-metabolic disease prevention. *Therapeutic Advances in Cardiovascular Disease* 2023, 17, 17539447231177175.

50. Aghababaei, F.; Hadidi, M., Recent advances in potential health benefits of quercetin. *Pharmaceuticals* 2023, 16, (7), 1020.

51. Harborne, J., Gossypetin and herbacetin as taxonomic markers in higher plants. *Phytochemistry* 1969, 8, (1), 177–183.

52. Huang, K.; Liu, Z.; Kim, M.-O.; Kim, K.-R., Anticancer effects of gossypetin from *Hibiscus sabdariffa* in oral squamous cell carcinoma. *Journal of Applied Oral Science* 2023, 31, e20230243.

53. Salvamani, S.; Gunasekaran, B.; Shaharuddin, N. A.; Ahmad, S. A.; Shukor, M. Y., Antiatherosclerotic effects of plant flavonoids. *BioMed research international* 2014, 2014, (1), 480258.

54. Ismailov, A.; Karimdzhannov, A.; Islambekov, S. Y.; Rakhimkhanov, Z., Flavonoids of the cotton

plant and plants close to it. *Chemistry of Natural Compounds* 1994, 30, (1), 1–14.

55. Khalid, M. N.; Rasheed, Z.; Amjad, I.; Qasim, M.; Khan, M., An in-depth review of flavonoid profile in cotton (*Gossypium hirsutum* L.). *Indian J. Pure Appl. Biosci* 2021, 9, 202–216.

56. Hapsari, B. W.; Setyaningsih, W., Methodologies in the analysis of phenolic compounds in roselle (*Hibiscus sabdariffa* L.): Composition, biological activity, and beneficial effects on human health. *Horticulturae* 2021, 7, (2), 35.

57. Mohammed, M. A.; Amer, N. M.; Abdallah, H. M.; Saleh, M. S., A comprehensive tool in recycling plant-waste of *Gossypium barbadense* L agricultural and industrial waste extracts containing gossy pin and gossypol: Hepatoprotective, anti-inflammatory and antioxidant effects. *Plant Methods* 2024, 20, (1), 54.

58. Xing, A.; Wang, X.; Nazir, M. F.; Zhang, X.; Wang, X.; Yang, R.; Chen, B.; Fu, G.; Wang, J.; Ge, H., Transcriptomic and metabolomic profiling of flavonoid biosynthesis provides novel insights into petals coloration in Asian cotton (*Gossypium arboreum* L.). *BMC plant biology* 2022, 22, (1), 416.

59. Shoker, R. M. H., Analysis of the Pharmacological and Phytochemical constituents of the phenolic extracts of *Hibiscus sabdariffa* L. dried calyces plant by HPLC Techniques. *Journal of Wasit for Science and Medicine* 2017, 10, (1), 68–77.

60. Jafarian, S.; Mortazavi, A.; Kenari, R. S.; Rad, A. E., Total phenolic content & antioxidant activity of roselle (*Hibiscus sabdariffa* l.) calyces extracts. 2014.

61. Sirag, N.; Elhadi, M.; Algaili, A. M.; Hassan, H. M.; Ohaj, M., Determination of total phenolic content and antioxidant activity of roselle (*Hibiscus sabdariffa* L.) calyx ethanolic extract. *Standard Research Journal of Pharmacy and Pharmacology* 2014, 1, (2), 34–39.

62. Mounnissamy, V.; Gopal, V.; Gunasegaran, R.; Saraswathy, A., Antiinflammatory activity of gossypetin isolated from *Hibiscus sabdariffa*. *Indian Journal of Heterocyclic Chemistry* 2002, 12, (1), 85–86.

63. Feingold, K. R., Introduction to lipids and lipoproteins. *Endotext [internet]* 2024.

64. Trajkovska, K. T.; Topuzovska, S., High-density lipoprotein metabolism and reverse cholesterol transport: strategies for raising HDL cholesterol. *Anatolian journal of cardiology* 2017, 18, (2), 149.

65. Association, A. D., Standards of medical care for patients with diabetes mellitus. *Diabetes care* 2002, 25, (suppl_1), s33–s49.

66. Varias-Menor, A.; Michlin, A.; Stere, B.; Afifi, S.; Tasouli-Drakou, V.; Varras, J., Targeting lymphatic dysfunction in atherosclerosis: A state-of-the-art review on potential therapies and future directions. *Frontiers in Cardiovascular Medicine* 2026, 13, 1774468.

67. Kolovou, G. D.; Watts, G. F.; Mikhailidis, D. P.; Perez-Martinez, P.; Mora, S.; Bilianou, H.; Panotopoulos, G.; Katsiki, N.; Ooi, T. C.; Lopez-Miranda, J., Postprandial hypertriglyceridaemia revisited in the era of non-fasting lipid profile testing: a 2019 expert panel statement, main text.

68. Bhowmik, B.; Siddiquee, T.; Mujumder, A.; Afsana, F.; Ahmed, T.; Mdala, I. A.; do V. Moreira, N. C.; Khan, A. K. A.; Hussain, A.; Holmboe-Ottesen, G., Serum lipid profile and its association with diabetes and prediabetes in a rural Bangladeshi population. *International journal of environmental research and public health* 2018, 15, (9), 1944.
69. Jasim, O.; Mahmood, M.; Ad'hiah, A., Significance of lipid profile parameters in predicting pre-diabetes. *Archives of Razi Institute* 2022, 77, (1), 277.
70. Zhu, X.; Yang, Z.; He, Z.; Hu, J.; Yin, T.; Bai, H.; Li, R.; Cai, L.; Guo, H.; Li, M., Factors correlated with targeted prevention for prediabetes classified by impaired fasting glucose, impaired glucose tolerance, and elevated HbA1c: A population-based longitudinal study. *Frontiers in endocrinology* 2022, 13, 965890.
71. Nguyen, Q. M.; Srinivasan, S. R.; Xu, J.-H.; Chen, W.; Berenson, G. S., Changes in risk variables of metabolic syndrome since childhood in pre-diabetic and type 2 diabetic subjects: the Bogalusa Heart Study. *Diabetes care* 2008, 31, (10), 2044–2049.
72. Ryden, L.; Standl, E.; Bartnik, M.; Van den Berghe, G.; Betteridge, J.; De Boer, M.-J.; Cosentino, F.; Jönsson, B.; Laakso, M.; Malmberg, K., Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary: The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD). *European heart journal* 2007, 28, (1), 88–136.
73. Boden, G.; Homko, C.; Barrero, C. A.; Stein, T. P.; Chen, X.; Cheung, P.; Fecchio, C.; Koller, S.; Merali, S., Excessive caloric intake acutely causes oxidative stress, GLUT4 carbonylation, and insulin resistance in healthy men. *Science translational medicine* 2015, 7, (304), 304re7–304re7.
74. Fuhrman, J., The hidden dangers of fast and processed food. *American journal of lifestyle medicine* 2018, 12, (5), 375–381.
75. Azevedo-Martins, A. K.; Santos, M. P.; Abayomi, J.; Ferreira, N. J. R.; Evangelista, F. S., The impact of excessive fructose intake on adipose tissue and the development of childhood obesity. *Nutrients* 2024, 16, (7), 939.
76. Marí, M.; Morales, A.; Colell, A.; García-Ruiz, C.; Fernandez-Checa, J. C., Oxidative stress in nonalcoholic fatty liver disease. In *Studies on hepatic disorders*, Springer: 2015; pp 279–308.
77. Levitan, I.; Volkov, S.; Subbaiah, P. V., Oxidized LDL: diversity, patterns of recognition, and pathophysiology. *Antioxidants & redox signaling* 2010, 13, (1), 39–75.
78. Yang, H.; Mohamed, A. S. S.; Zhou, S.-h., Oxidized low density lipoprotein, stem cells, and atherosclerosis. *Lipids in health and disease* 2012, 11, (1), 85.
79. Li, L.; Guo, Z.; Zhao, Y.; Liang, C.; Zheng, W.; Tian, W.; Chen, Y.; Cheng, Y.; Zhu, F.; Xiang, X., The impact of oxidative stress on abnormal lipid metabolism-mediated disease development.

Archives of biochemistry and biophysics 2025, 766, 110348.

80. Olivares-Vicente, M.; Herranz-López, M., The interplay between oxidative stress and lipid composition in obesity-induced inflammation: antioxidants as therapeutic agents in metabolic diseases. *International Journal of Molecular Sciences* 2025, 26, (17), 8544.

81. Luvuno, M.; Khathi, A.; Mabandla, M. V., Diet-induced prediabetes: effects of exercise treatment on risk factors for cardiovascular complications. *Nutrition & metabolism* 2021, 18, (1), 45.

82. Mulvihill, E. E.; Huff, M. W., Protection from metabolic dysregulation, obesity, and atherosclerosis by citrus flavonoids: Activation of hepatic PGC1 α -Mediated fatty acid oxidation. *PPAR research* 2012, 2012, (1), 857142.

83. Rufino, A. T.; Costa, V. M.; Carvalho, F.; Fernandes, E., Flavonoids as antiobesity agents: A review. *Medicinal Research Reviews* 2021, 41, (1), 556–585.

84. Duan, R.; Guan, X.; Huang, K.; Zhang, Y.; Li, S.; Xia, J. a.; Shen, M., Flavonoids from whole-grain oat alleviated high-fat diet-induced hyperlipidemia via regulating bile acid metabolism and gut microbiota in mice. *Journal of Agricultural and Food Chemistry* 2021, 69, (27), 7629–7640.

85. Lin, H.-H., In vitro and in vivo atheroprotective effects of gossypetin against endothelial cell injury by induction of autophagy. *Chemical Research in Toxicology* 2015, 28, (2), 202–215.

86. Oh, E.; Lee, J.; Cho, S.; Kim, S. W.; Jo, K. W.; Shin, W. S.; Gwak, S. H.; Ha, J.; Jeon, S. Y.; Park, J.-H., Gossypetin prevents the progression of nonalcoholic steatohepatitis by regulating oxidative stress and AMP-activated protein kinase. *Molecular Pharmacology* 2023, 104, (5), 214–229.

87. Fang, C.; Pan, J.; Qu, N.; Lei, Y.; Han, J.; Zhang, J.; Han, D., The AMPK pathway in fatty liver disease. *Frontiers in physiology* 2022, 13, 970292.

88. Roşian, Ş. H.; Boarescu, I.; Boarescu, P.-M., Antioxidant and anti-inflammatory effects of bioactive compounds in atherosclerosis. *International Journal of Molecular Sciences* 2025, 26, (3), 1379.

89. Chen, D.; Jia, D.; Wu, X.; Shi, K.; Ren, C.; Dou, Y.; Guo, M.; Wang, J.; Ma, M.; Wu, Z., A novel metformin derivative showed improvement of lipid metabolism in obese rats with type 2 diabetes. *Clinical and Experimental Pharmacology and Physiology* 2020, 47, (8), 1382–1392.

90. Yasmin, T.; Rahman, M. M.; Khan, F.; Kabir, F.; Nahar, K.; Lasker, S.; Islam, M. D.; Hossain, M. M.; Hasan, R.; Rana, S., Metformin treatment reverses high fat diet-induced non-alcoholic fatty liver diseases and dyslipidemia by stimulating multiple antioxidant and anti-inflammatory pathways. *Biochemistry and Biophysics Reports* 2021, 28, 101168.

91. Rena, G.; Hardie, D. G.; Pearson, E. R., The mechanisms of action of metformin. *Diabetologia* 2017, 60, (9), 1577–1585.

92. Proença, C.; Rufino, A. T.; Santos, I.; Albuquerque, H. M.; Silva, A. M.; Fernandes, E.; Ferreira de Oliveira, J. M. P., Gossypetin is a novel modulator of inflammatory cytokine production and a

suppressor of osteosarcoma cell growth. *Antioxidants* 2023, 12, (9), 1744.

93. Bu, Y.; Peng, M.; Tang, X.; Xu, X.; Wu, Y.; Chen, A. F.; Yang, X., Protective effects of metformin in various cardiovascular diseases: clinical evidence and AMPK-dependent mechanisms. *Journal of Cellular and Molecular Medicine* 2022, 26, (19), 4886–4903.

94. Dampney, R. A., Central neural control of the cardiovascular system: current perspectives. *Advances in physiology education* 2016, 40, (3), 283–296.

95. Taggart, P.; Critchley, H.; van Duijvendoden, S.; Lambiase, P. D., Significance of neuro-cardiac control mechanisms governed by higher regions of the brain. *Autonomic Neuroscience* 2016, 199, 54–65.

96. Khalid, M.; Petroianu, G.; Adem, A., Advanced glycation end products and diabetes mellitus: mechanisms and perspectives. *Biomolecules* 2022, 12, (4), 542.

97. Jia, G.; DeMarco, V. G.; Sowers, J. R., Insulin resistance and hyperinsulinaemia in diabetic cardiomyopathy. *Nature Reviews Endocrinology* 2016, 12, (3), 144–153.

98. Vanessa Fiorentino, T.; Priolella, A.; Zuo, P.; Folli, F., Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Current pharmaceutical design* 2013, 19, (32), 5695–5703.

99. Mkhize, B. C.; Mosili, P.; Ngubane, P. S.; Sibiya, N. H.; Khathi, A., Diet-induced prediabetes: Effects on the activity of the renin–angiotensin–aldosterone system in selected organs. *Journal of Diabetes Investigation* 2022, 13, (5), 768–780.

100. David, A. V. A.; Arulmoli, R.; Parasuraman, S., Overviews of biological importance of quercetin: A bioactive flavonoid. *Pharmacognosy reviews* 2016, 10, (20), 84.

101. Ozgen, S.; Kilinc, O. K.; Selamoğlu, Z., Antioxidant activity of quercetin: a mechanistic review. *Turkish Journal of Agriculture-Food Science and Technology* 2016, 4, (12), 1134–1138.

102. Aleksandrowicz, M.; Konop, M.; Rybka, M.; Mazurek, Ł.; Stradczuk-Mazurek, M.; Kciuk, M.; Bądryńska, B.; Dobrowolski, L.; Kuczeriszka, M., Dysfunction of microcirculation in atherosclerosis: implications of nitric oxide, oxidative stress, and inflammation. *International Journal of Molecular Sciences* 2025, 26, (13), 6467.

103. Chen, X.; Xie, N.; Feng, L.; Huang, Y.; Wu, Y.; Zhu, H.; Tang, J.; Zhang, Y., Oxidative stress in diabetes mellitus and its complications: From pathophysiology to therapeutic strategies. *Chinese Medical Journal* 2025, 138, (01), 15–27.

104. Hopkins, M.; Blundell, J. E., Energy balance, body composition, sedentariness and appetite regulation: pathways to obesity. *Clinical Science* 2016, 130, (18), 1615–1628.

105. Kawai, T.; Autieri, M. V.; Scalia, R., Adipose tissue inflammation and metabolic dysfunction in obesity. *American journal of physiology-cell physiology* 2021, 320, (3), C375–C391.

106. Jabłonowska-Lietz, B.; Wrzosek, M.; Włodarczyk, M.; Nowicka, G., New indexes of body fat distribution, visceral adiposity index, body adiposity index, waist-to-height ratio, and metabolic disturbances in the obese. *Polish Heart Journal (Kardiologia Polska)* 2017, 75, (11), 1185–1191.
107. Amato, M. C.; Giordano, C., Visceral adiposity index: an indicator of adipose tissue dysfunction. *International journal of endocrinology* 2014, 2014, (1), 730827.
108. Janssen, J. A., Hypothesis: Nutrient Off-Loading and Ectopic Fat Reduction Reverse Insulin Resistance and Improve Cardiovascular Outcomes in Type 2 Diabetes—A Narrative Review. *International Journal of Molecular Sciences* 2026, 27, (5), 2150.
109. Dutta, B.; Tripathy, A.; Archana, P.; Kamath, S. U., Unraveling the complexities of diet induced obesity and glucolipid dysfunction in metabolic syndrome. *Diabetology & Metabolic Syndrome* 2025, 17, (1), 292.
110. Moazed, K. T., mTOR Signaling Pathways. In *mTOR Signaling in Ophthalmology: Understanding the Essentials*, Springer: 2025; pp 105–166.
111. Saikia, L.; Talukdar, N. C.; Dutta, P. P., Exploring the therapeutic role of flavonoids through AMPK activation in metabolic syndrome: a narrative review. *Phytotherapy Research* 2025, 39, (3), 1403–1421.
112. Guzmán-Quevedo, O.; Araújo, A. N. V. d.; Romero-Juárez, P. A.; Costa Silva, J. C.; Soria-Melgarejo, G.; Torner, L.; de Souza Aquino, J., Impact of the flavonoid-induced intestinal microbiota modulation on global energy metabolism: implication for obesity. *Frontiers in Nutrition* 2025, 12, 1696152.
113. Chen, J.; Zhang, K.; Yu, X.; Ren, J.; Kan, C.; Sheng, S.; Han, F.; Zhang, Y.; Chen, J.; Sun, X., Flavonoids in metabolic disease: a narrative review of mechanisms and therapeutic potential. *Phytotherapy Research* 2025, 39, (11), 5365–5377.
114. Ijaz, M. U.; Alvi, K.; Khan, H. A.; Imran, M.; Afsar, T.; Almajwal, A.; Amor, H.; Razak, S., Gossypetin mitigates doxorubicin-induced nephrotoxicity: A histopathological and biochemical evaluation. *Journal of King Saud University-Science* 2023, 35, (7), 102830.
115. González-Casanova, J. E.; Navarro-Marquez, M.; Saez-Tamayo, T.; Angarita, L.; Durán-Agüero, S.; Fuentes-Barría, H.; Bermúdez, V.; Rojas-Gómez, D. M., New perspectives on the molecular action of metformin in the context of cellular transduction and adipogenesis. *International Journal of Molecular Sciences* 2025, 26, (8), 3690.
116. Kang, L.; Yi, J.; Lau, C.-W.; He, L.; Chen, Q.; Xu, S.; Li, J.; Xia, Y.; Zhang, Y.; Huang, Y., AMPK-dependent YAP inhibition mediates the protective effect of metformin against obesity-associated endothelial dysfunction and inflammation. *Antioxidants* 2023, 12, (9), 1681.
117. Bailey, C. J., Metformin: Therapeutic profile in the treatment of type 2 diabetes. *Diabetes, Obesity and Metabolism* 2024, 26, 3–19.

118. Dutta, S.; Shah, R. B.; Singhal, S.; Dutta, S. B.; Bansal, S.; Sinha, S.; Haque, M., Metformin: a review of potential mechanism and therapeutic utility beyond diabetes. *Drug design, development and therapy* 2023, 1907–1932.
119. Herman, R.; Kravos, N. A.; Jensterle, M.; Janež, A.; Dolžan, V., Metformin and insulin resistance: a review of the underlying mechanisms behind changes in GLUT4-mediated glucose transport. *International journal of molecular sciences* 2022, 23, (3), 1264.
120. Eldamarawi, M.; Abdelazeem, M., Effect of quercetin and metformin on glucose transporter-4 expression, oxidative stress, inflammation markers and insulin resistance in type 2 diabetes mellitus. *Bulletin of Egyptian Society for Physiological Sciences* 2020, 40, (2), 70–85.
121. Pisoschi, A. M.; Pop, A., The role of antioxidants in the chemistry of oxidative stress: A review. *European journal of medicinal chemistry* 2015, 97, 55–74.
122. Yousef, H.; Khandoker, A. H.; Feng, S. F.; Helf, C.; Jelinek, H. F., Inflammation, oxidative stress and mitochondrial dysfunction in the progression of type II diabetes mellitus with coexisting hypertension. *Frontiers in endocrinology* 2023, 14, 1173402.
123. Rani, A. J.; Mythili, S., Study on total antioxidant status in relation to oxidative stress in type 2 diabetes mellitus. *Journal of clinical and diagnostic research: JCDR* 2014, 8, (3), 108.
124. Tangvarasittichai, S., Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. *World journal of diabetes* 2015, 6, (3), 456.
125. Jadoon, S.; Malik, A., A review article on the formation, mechanism and biochemistry of MDA and MDA as a biomarker of oxidative stress. *Int. J. Adv. Res* 2017, 5, (12), 811–818.
126. Korac, B.; Kalezic, A.; Pekovic-Vaughan, V.; Korac, A.; Jankovic, A., Redox changes in obesity, metabolic syndrome, and diabetes. *Redox Biology* 2021, 42, 101887.
127. Puthanveedu, V.; Muraleedharan, K., Study on structural detailing of gossypetin and its medicinal application in UV filtering, radical scavenging, and metal chelation open up through NCI, TD-DFT, QTAIM, ELF, and LOL analysis. *Computational and Theoretical Chemistry* 2023, 1225, 114126.
128. Shang, W.; Li, X.-H.; Zeng, L.-H.; Li, Z.; Hu, Y.; Wen, H.-M.; Cao, F.-J.; Wan, G.-X., Mechanistic insights into flavonoid subclasses as cardioprotective agents against doxorubicin-induced cardiotoxicity: a comprehensive review. *Drug design, development and therapy* 2025, 5553–5596.
129. Diniz Vilela, D.; Gomes Peixoto, L.; Teixeira, R. R.; Belele Baptista, N.; Carvalho Caixeta, D.; Vieira de Souza, A.; Machado, H. L.; Pereira, M. N.; Sabino-Silva, R.; Espindola, F. S., The role of metformin in controlling oxidative stress in muscle of diabetic rats. *Oxidative medicine and cellular longevity* 2016, 2016, (1), 6978625.
130. Wu, S.-B.; Wu, Y.-T.; Wu, T.-P.; Wei, Y.-H., Role of AMPK-mediated adaptive responses in human cells with mitochondrial dysfunction to oxidative stress. *Biochimica et Biophysica Acta (BBA)-*

General Subjects 2014, 1840, (4), 1331–1344.

131. MUHAMMAD, I.; UNUOHA, S.; AJINGI, Y.; MADUNGURUM, M.; BELLO, A.; ABUBAKAR, S.; USMAN, B. In *MINERAL ELEMENTS AND LIPID PROFILE OF Hibiscus Sabdariffa SEEDS AND IN SILICO STUDY OF INHIBITORY ACTIVITY OF CALYX-CONTAINING GOSSYPETIN AGAINST HUMAN ANGIOTENSIN CONVERTING ENZYME (ACE)*, Faculty of Agriculture International Conference Book of Proceedings, 2025; 2025; pp 294–302.
132. Hu, Y.; Zhang, X.; Yang, C.; Wu, X.; Ye, X.; Yu, J.; Yang, Y., Metformin: Historical Origins, Mechanisms of Action, and Emerging Clinical Applications. *Diabetes/Metabolism Research and Reviews* 2026, 42, (4), e70171.
133. Nguyen, D. V.; Shaw, L. C.; Grant, M. B., Inflammation in the pathogenesis of microvascular complications in diabetes. *Frontiers in endocrinology* 2012, 3, 170.
134. Henein, M. Y.; Vancheri, S.; Longo, G.; Vancheri, F., The role of inflammation in cardiovascular disease. *International journal of molecular sciences* 2022, 23, (21), 12906.
135. Grossmann, V.; Schmitt, V. H.; Zeller, T.; Panova-Noeva, M.; Schulz, A.; Laubert-Reh, D.; Juenger, C.; Schnabel, R. B.; Abt, T. G.; Laskowski, R., Profile of the immune and inflammatory response in individuals with prediabetes and type 2 diabetes. *Diabetes care* 2015, 38, (7), 1356–1364.
136. Chawla, A.; Chawla, R.; Jaggi, S., Microvascular and macrovascular complications in diabetes mellitus: Distinct or continuum? *Indian journal of endocrinology and metabolism* 2016, 20, (4), 546.
137. Barone, E.; Di Domenico, F.; Perluigi, M.; Butterfield, D. A., The interplay among oxidative stress, brain insulin resistance and AMPK dysfunction contribute to neurodegeneration in type 2 diabetes and Alzheimer disease. *Free Radical Biology and Medicine* 2021, 176, 16–33.
138. Yesupatham, A.; Saraswathy, R., Role of oxidative stress in prediabetes development. *Biochemistry and Biophysics Reports* 2025, 43, 102069.
139. Anagnostopoulou, L.; Ktenopoulos, N.; Apostolos, A.; Fragoulis, C.; Vlachakis, P.; Karakasis, P.; Sagris, M.; Milaras, N.; Drakopoulou, M.; Synetos, A., Intersecting molecular pathways in cardiovascular disease and diabetes mellitus: Emerging roles of inflammation and therapeutics. *Diabetes/Metabolism Research and Reviews* 2026, 42, (4), e70167.
140. Marinković, D. M.; Dragović, T.; Stanojević, I.; Đurić, P.; Dejanović, B.; Rakočević, J.; Kiković, S.; Malović, D.; Stevanović, I.; Ristić, P., Low-grade inflammation and inflammatory mediators in individuals with prediabetes. *Vojnosanitetski pregled* 2024, 81, (9), 547–554.
141. Bjornstad, P.; Schäfer, M.; Truong, U.; Cree-Green, M.; Pyle, L.; Baumgartner, A.; Garcia Reyes, Y.; Maniatis, A.; Nayak, S.; Wadwa, R. P., Metformin improves insulin sensitivity and vascular health in youth with type 1 diabetes mellitus: randomized controlled trial. *Circulation* 2018, 138, (25), 2895–2907.
142. Bosi, E., Metformin—the gold standard in type 2 diabetes: what does the evidence tell us?

Diabetes, Obesity and Metabolism 2009, 11, 3–8.

143. Khan, A.; Manna, K.; Das, D. K.; Kesh, S. B.; Sinha, M.; Das, U.; Biswas, S.; Sengupta, A.; Sikder, K.; Datta, S., Gossypetin ameliorates ionizing radiation-induced oxidative stress in mice liver—a molecular approach. *Free Radical Research* 2015, 49, (10), 1173–1186.

144. Bas, T. G., Dietary polyphenols (flavonoids) derived from plants for use in therapeutic health: Antioxidant performance, ROS, molecular mechanisms, and bioavailability limitations. *International journal of molecular sciences* 2026, 27, (3), 1404.

145. Khathi, A.; Luvuno, M.; Mabandla, M., Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. *PONTE International Journal of Science and Research* 2018, 74, (5).

146. Care, D., 2. Classification and diagnosis of diabetes: standards of care in. *Diabetes care* 2023, 46, S19.

147. Samant, N. P.; Gupta, G. L., Gossypetin-based therapeutics for cognitive dysfunction in chronic unpredictable stress-exposed mice. *Metabolic Brain Disease* 2022, 37, (5), 1527–1539.

148. Mustafa, S.; Anwar, H.; Ain, Q. U.; Ahmed, H.; Iqbal, S.; Ijaz, M. U., Therapeutic effect of gossypetin against paraquat-induced testicular damage in male rats: a histological and biochemical study. *Environmental Science and Pollution Research* 2023, 30, (22), 62237–62248.

149. Siboto, A.; Akinnuga, A. M.; Khumalo, B. N.; Ismail, M. B.; Booysen, I. N.; Sibiya, N. H.; Ngubane, P. S.; Khathi, A., The effects of a [3+ 1] oxo-free rhenium (V) compound with uracil-derived ligands on selected parameters of glucose homeostasis in diet-induced pre-diabetic rats. *Obesity Medicine* 2020, 19, 100258.

150. Meng, X.-M.; Ma, X.-X.; Tian, Y.-L.; Jiang, Q.; Wang, L.-L.; Shi, R.; Ding, L.; Pang, S.-G., Metformin improves the glucose and lipid metabolism via influencing the level of serum total bile acids in rats with streptozotocin-induced type 2 diabetes mellitus. *European Review for Medical & Pharmacological Sciences* 2017, 21, (9), 2232.

151. Fan, N.; Du, L.; Guo, T.; Liu, M.; Chen, X., Pharmacokinetic Interaction Between Imatinib and Metformin in Rats: N. Fan et al. *European Journal of Drug Metabolism and Pharmacokinetics* 2024, 49, (2), 171–179.

152. Khanna, A. K.; Kinoshita, T.; Natarajan, A.; Schwager, E.; Linn, D. D.; Dong, J.; Ghosh, E.; Vicario, F.; Maheshwari, K., Association of systolic, diastolic, mean, and pulse pressure with morbidity and mortality in septic ICU patients: a nationwide observational study. *Annals of intensive care* 2023, 13, (1), 9.

153. Lee, J.; Jang, S.; Jeong, H.; Ryu, O.-H., Validation of the Friedewald formula for estimating low density lipoprotein cholesterol: the Korea National Health and Nutrition Examination Survey, 2009 to 2011. *The Korean Journal of Internal Medicine* 2018, 35, (1), 150.

154. Luvuno, M.; Khathi, A.; Mabandla, M. V., The effects of exercise treatment on learning and

memory ability, and cognitive performance in diet-induced prediabetes animals. *Scientific Reports* 2020, 10, (1), 15048.

Bridge

The second manuscript presented in Chapter 5 showed that diet-induced prediabetes was associated with impaired cardiovascular function. This was evidenced by elevated plasma triglycerides, total cholesterol, low-density lipoprotein, very low-density lipoprotein, increased mean arterial pressure, body mass index, waist circumference and heightened markers of oxidative stress and systemic inflammation. The administration of gossypetin (GTIN), in both the presence and absence of dietary intervention, significantly improved cardiovascular function in diet-induced prediabetes. The findings presented in manuscript 2 prompted further investigation into the effects of GTIN on liver function, due to the shared mechanisms underlying cardiovascular disease and non-alcoholic fatty liver disease in prediabetes. Accordingly, Chapter 6 presents the findings of manuscript 3, which aims to assess the effects of GTIN on liver function, including liver triglycerides, liver weight, oxidative stress markers, inflammatory mediators, liver injury enzymes and histopathological changes in diet-induced prediabetes.

CHAPTER 6: MANUSCRIPT 3

PROLOGUE

Literature has shown that liver complications associated with type 2 diabetes mellitus (T2DM) begin during the prediabetic state. Moderate hyperglycaemia and insulin resistance, as seen in prediabetes, induce hepatic oxidative stress, lipid accumulation and liver injury. This has been shown to contribute to the development and progression of non-alcoholic fatty liver disease. Liver dysfunction in prediabetes further exacerbates systemic insulin resistance, creating a vicious cycle that promotes the development of T2DM. Despite the importance of managing liver dysfunction in prediabetes, effective treatment remains a challenge. Dietary modification is essential for preventing liver complications in prediabetic patients. However, poor adherence to dietary modifications remains a significant barrier to attaining optimal therapeutic outcomes. Pharmacological treatments such as metformin have demonstrated benefits in improving liver health, but limitations in efficacy and inconsistent clinical outcomes highlight the need for alternative therapies. Flavonoids derived from natural sources have emerged as promising candidates due to their antioxidant and anti-inflammatory properties. In the previous manuscripts, gossypetin (GTIN) has been shown to significantly improve glucose homeostasis and cardiovascular function in a diet-induced prediabetic rat model. However, the effects of GTIN on prediabetes-associated liver complications were not explored. Hence, in manuscript 3, we investigated the effects of GTIN on liver health in both the presence and absence of dietary intervention in a diet-induced prediabetic rat model.

The manuscript in Chapter 6 is titled “**Investigating the effects of gossypetin on liver health in diet-induced prediabetic male Sprague Dawley rats**” and is authored by Naidoo K and Khathi A.

The manuscript has been published in the journal “**Molecules**” and formatted according to the journal’s guidelines for authors. See Appendix 5.

Candidate Contribution: Karishma Naidoo was responsible for study conceptualisation, study design, all experimental work, statistical analysis, first draft writing and manuscript editing.

Investigating the effects of gossypetin on liver function in diet-induced pre-diabetic male

Sprague Dawley rats

Karishma Naidoo^{1,2}, Andile Khathi¹

1. University of KwaZulu-Natal, Westville, KwaZulu-Natal South Africa

2. Corresponding Author: Karishma Naidoo

School of Laboratory Medicine & Medical Sciences

University of KwaZulu-Natal

Private Bag X54001

Durban,

SOUTH AFRICA

Phone: 0711607469 /27 (0) 31 260 7585, Fax: 27 (0) 31 260 7132

Email: karishma280199@gmail.com /khathia@ukzn.ac.za

Keywords: Gossypetin; NAFLD; liver injury; flavonoids; steatosis; high-caloric diets

Abstract

The rising prevalence of non-alcoholic fatty liver disease among patients with type 2 diabetes mellitus has emerged as a global health challenge. Gossypetin (GTIN) is a natural flavonoid which has recently demonstrated antihyperglycaemic, antioxidant, and anti-inflammatory effects. Despite these findings, no studies have investigated its effects on liver health in the pre-diabetic state. Hence, this study aimed to investigate the effects of GTIN on liver health in diet-induced pre-diabetic male rats in the presence and absence of dietary intervention and to compare these effects with those of metformin (MET). Following 20 weeks of pre-diabetes induction, the animals were divided into six groups ($n = 6$) as follows: non-pre-diabetic (NPD) control, pre-diabetic (PD) control, and PD groups treated with GTIN (15 mg/kg body weight (bw)) or metformin (500 mg/kg bw) on either a normal diet or a high-fat, high-carbohydrate diet for 12 weeks. significantly higher liver triglycerides (TAG), liver weights, sterol regulatory binding element regulatory protein-1c (SREBP-1c), malondialdehyde (MDA) levels, and liver injury enzyme levels, along with decreased liver superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, and plasma bilirubin levels in comparison to NPD. Histologically, there was an increased lipid droplet accumulation and structural disarray in the PD group. GTIN treatment significantly reduced liver TAGs, liver weights, and plasma SREBP-1c levels, as well as improved liver SOD and GPx activity while decreasing liver MDA levels and liver injury enzymes in comparison to the PD control. Notably, GTIN treatment increased plasma bilirubin levels. Liver histology in the GTIN-treated groups revealed decreased lipid droplet accumulation and improved tissue integrity. Similar results were observed for the liver parameters in the MET-treated groups. The findings of this study may suggest that GTIN and MET exhibit therapeutic effects on liver health in diet-induced pre-diabetes in both the presence and absence of diet intervention. Dietary intervention may confer beneficial effects on liver health, with the most favorable therapeutic outcomes observed through a combination of treatment with dietary intervention. Additionally, GTIN may exhibit greater hepatoprotective effects than MET in rats without dietary intervention.

1.0 Introduction

The increasing prevalence of calorie-dense dietary patterns has contributed to the growing burden of diabetes and associated liver complications such as non-alcoholic fatty liver disease (NAFLD) [1]. Pre-diabetes precedes the onset of overt type 2 diabetes mellitus (T2DM), with previous studies showing that around 70% of untreated pre-diabetic individuals progress to T2DM [2, 3]. Insulin resistance and hyperinsulinemia associated with pre-diabetes contribute to the development of liver complications [4]. Insulin resistance promotes increased liver triglyceride accumulation by disrupting the ability of insulin to suppress adipose tissue lipolysis [5]. Hyperinsulinemia, elevated glucose, and free fatty acid flux into the liver upregulates hepatic de novo lipogenesis through steroid regulatory elementary binding protein-1c (SREBP-1c) [6]. The accumulation of lipids in the liver contributes to hepatic insulin resistance and the development of NAFLD [7]. This triggers a cascade of oxidative and inflammatory events which leads to liver damage [7]. Moreover, it has been shown that liver injury marker levels, which include alanine transaminase and aspartate transaminase, were significantly higher in the pre-diabetic state [8]. Histopathological changes observed in NAFLD include lipid droplet accumulation, inflammation, and lobular disarray [9]. This condition may not only disrupt the normal functioning of the liver but can also create a feedback loop in which liver impairment further exacerbates the pre-diabetic state [10].

Lifestyle modification remains the cornerstone of NAFLD management, although pharmacological therapy is frequently required [11, 12]. There has been low patient compliance with lifestyle modifications due to the challenges of maintaining long-term dietary change and exercise [13, 14]. Metformin is primarily used as a first-line treatment for T2DM and has been shown to improve liver health in the diabetic state [15]. However, there have been some challenges with the current drugs under investigation, which include inconsistent findings and a lack of evidence supporting their effectiveness in humans [16]. Thus, there is a need to identify compounds which are effective in treating diabetic liver complications in the presence and absence of dietary modifications. Flavonoids found in plant sources have been shown to improve T2DM and associated liver complications through their multifactorial effects, low toxicity, and vast availability [17, 18]. Gossypetin (GTIN) is a naturally occurring flavonoid that serves as a structural analog of quercetin with an additional hydroxyl group [19]. This compound has been isolated from the calyx and flowers of *Hibiscus sabdariffa* [20]. Previous studies have highlighted GTIN for its potent antioxidant and anti-inflammatory properties [21, 22]. Recent studies have demonstrated that GTIN improves glucose homeostasis and cardiovascular function in the pre-diabetic state [23, 24]. Despite these current findings, no studies have explored the effects of GTIN on liver health in the pre-diabetic state. Hence, this study aimed to investigate the effects of GTIN on liver health in both the presence and absence of dietary intervention in a diet-induced pre-diabetic male rat model and to compare these effects with those of metformin. The oral dose of 15 mg/kg GTIN was selected to be used in our study based on

previous studies, demonstrating its efficacy and safety within the 10–20 mg/kg range [25, 26]. The 500 mg/kg dose of MET was selected due to its established therapeutic benefits and minimal adverse effects [4, 27].

2. Results

2.1. Liver Triglycerides and Liver Weights

Liver triglyceride levels and liver weights were measured to assess the extent of lipid accumulation and liver enlargement. A one-way ANOVA showed significant differences in liver TAG levels ($F(5, 30) = 68.11, p < 0.0001$) and liver weights ($F(5, 30) = 78.87, p < 0.0001$) between the groups. Post hoc comparisons showed (Figure 1) that the pre-diabetic (PD) control group exhibited significantly ($p < 0.0001$) higher liver TAG levels and liver weights ($p = 0.0007$) in comparison to the non-pre-diabetic (NPD) group. However, the PD animals receiving gossypetin with a normal diet (GTIN+ND) and a high-fat, high-carbohydrate diet (GTIN+HFHC) exhibited significantly lower liver TAG levels ($p < 0.0001, p < 0.0001$) and liver weights ($p = 0.0006, p = 0.0360$) in comparison to the PD control. Similar results were observed in the PD animals receiving metformin with a normal diet (MET+ND) and high-fat, high-carbohydrate diet (MET+HFHC) for liver TAG levels ($p < 0.0001, p < 0.0001$) and liver weights ($p = 0.0026, p < 0.0001$). A two-way ANOVA revealed that both treatment ($F(1, 20) = 45.16, p < 0.0001$) and diet ($F(1, 20) = 67.03, p < 0.0001$) exhibited significant main effects on liver TAGs. Similarly, significant main effects of treatment ($F(1, 20) = 18.08, p < 0.0004$) and diet ($F(1, 20) = 79.37, p < 0.0001$) were observed for liver weight. There was a significant interaction between treatment and diet on liver TAG ($F(1, 20) = 10.73, p = 0.0038$). However, there was no significant interaction between treatment and diet on liver weight ($F(1, 20) = 3.76, p = 0.0667$). Post hoc comparisons showed that GTIN treatment (GTIN+HFHC) resulted in significantly lower liver TAG ($p < 0.0001$) levels and liver weights ($p < 0.0001$) in comparison to MET treatment (MET+HFHC). Animals on an ND (GTIN+ND and MET+ND) exhibited significantly ($p < 0.05$) lower levels of both parameters in comparison to those on a HFHC diet (GTIN+HFHC and MET+HFHC).

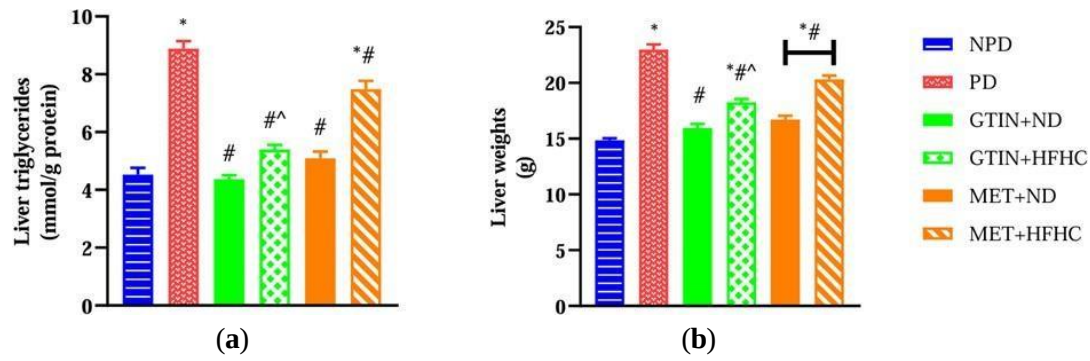


Figure 1. Effects of GTIN on (a) liver TAG levels and (b) liver weight in rats with or without dietary intervention at week 32 of the treatment period (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD; ^ $p < 0.05$ vs. MET+HFHC

2.2. Plasma SREBP-1c Levels

Plasma SREBP-1c levels were measured to assess the regulation of lipogenesis in the liver. A one-way ANOVA showed significant differences ($F(5, 30) = 74.47$, $p < 0.0001$) in plasma SREBP-1c levels between the groups. Post hoc comparisons showed (Figure 2) that the PD control group exhibited significantly ($p < 0.0001$) higher plasma SREBP-1c levels in comparison to the NPD group. However, the GTIN+ND ($p < 0.0001$) and GTIN+HFHC ($p < 0.0001$) groups exhibited significantly lower plasma SREBP-1c levels in comparison to the PD control. Similar results were observed for plasma SREBP-1c levels in the MET+ND ($p < 0.0001$) and MET+HFHC ($p < 0.0001$) groups. A two-way ANOVA revealed that both treatment ($F(1, 20) = 24.86$, $p < 0.0001$) and diet ($F(1, 20) = 223.30$, $p < 0.0001$) exhibited significant main effects on plasma SREBP-1c levels. There was a significant ($F(1, 20) = 10.74$, $p = 0.0038$) interaction between treatment and diet on plasma SREBP-1c levels. Post hoc comparisons showed that GTIN treatment (GTIN+ND) resulted in significantly ($p < 0.05$) lower plasma SREBP-1c levels in comparison to MET treatment (MET+ND). Animals on an ND (GTIN+ND and MET+ND) exhibited significantly ($p < 0.05$) lower levels of this parameter in comparison to those on a HFHC diet (GTIN+HFHC and MET+HFHC).

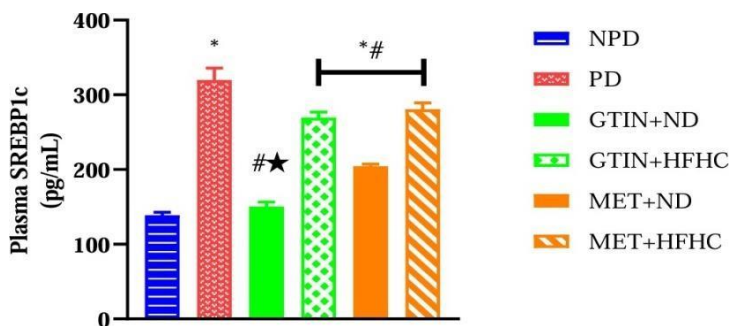


Figure 2. Effects of GTIN on plasma SREBP-1c levels in rats with or without dietary intervention at week 32 of the treatment period (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD; ★ $p < 0.05$ vs. MET+ND.

2.3. Liver Oxidative Status

Liver oxidative status was assessed by measuring MDA levels for lipid peroxidation and SOD and GPx activities for enzymatic antioxidant defense. A one-way ANOVA showed significant differences in liver MDA ($F(5, 30) = 14.44, p < 0.0001$), SOD activity ($F(5, 30) = 211.50, p < 0.0001$), and GPx activity ($F(5, 30) = 29.17, p < 0.0001$) between the groups. Post hoc comparisons showed (Figure 3) that the PD control group exhibited significantly ($p < 0.0001$) higher liver MDA levels with reduced SOD ($p < 0.0001$) and GPx activity ($p < 0.0001$) in comparison to the NPD group. However, the GTIN+ND and GTIN+HFHC groups exhibited significantly lower liver MDA levels ($p < 0.0001, p = 0.0018$) with improved SOD ($p < 0.0001, p < 0.0001$) and GPx activity ($p < 0.0001, p < 0.0001$) in comparison to the PD control. Similar results were observed for liver MDA levels and GPx activity in the MET+ND ($p < 0.0001$) and MET+HFHC ($p = 0.0057$) groups. The MET+ND group showed significantly ($p < 0.0001$) higher liver GPx activity in comparison to the PD control.

A two-way ANOVA revealed that both treatment ($F(1, 20) = 10.51, p < 0.0041$) and diet ($F(1, 20) = 34.04, p < 0.0001$) exhibited significant main effects on liver MDA levels. Similarly, both treatment ($F(1, 20) = 252.90, p < 0.0001$) and diet ($F(1, 20) = 138.50, p < 0.0001$) exhibited significant main effects on liver SOD activity. Furthermore, both treatment ($F(1, 20) = 10.59, p = 0.0040$) and diet ($F(1, 20) = 21.54, p = 0.0002$) exhibited significant main effects on liver GPx activity. There was no significant ($F(1, 20) = 0.42, p = 0.5242$) interaction between treatment and diet on liver MDA levels. However, there was a significant interaction between treatment and diet on liver SOD activity ($F(1, 20) = 1.05, p = 0.3174$) and GPx activity ($F(1, 20) = 4.54, p = 0.0457$). Post hoc comparisons showed that GTIN treatment (GTIN+ND and GTIN+HFHC) resulted in significantly ($p = 0.0002, p < 0.0001$) higher liver SOD activity in comparison to MET treatment (MET+ND and MET+HFHC). Additionally, GTIN treatment (GTIN+HFHC) resulted in significantly ($p < 0.05$) higher liver GPx activity in comparison to MET treatment (MET+HFHC). Animals on an ND (GTIN+ND and MET+ND) exhibited significantly ($p < 0.05$) lower liver MDA levels and higher SOD activity in comparison to those on a HFHC diet (GTIN+HFHC and MET+HFHC).

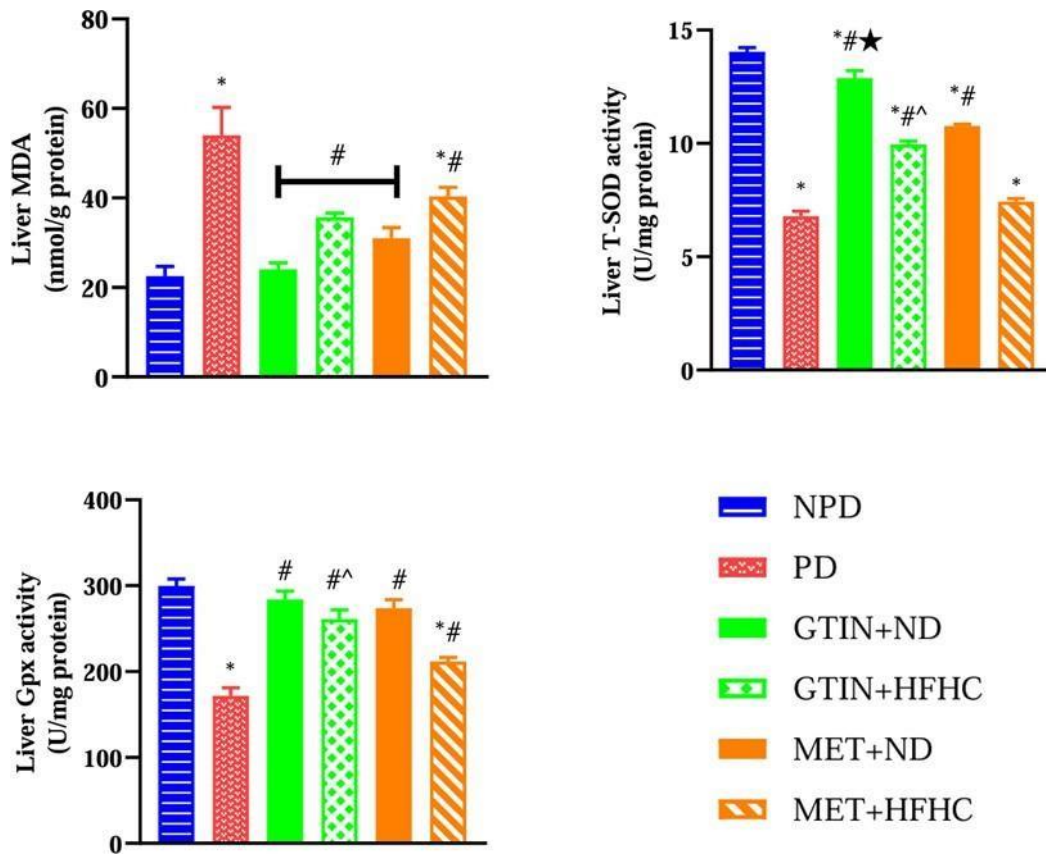


Figure 3. Effects of GTIN on liver oxidative stress markers in rats with or without dietary intervention at week 32 of the treatment period (n = 6 per group). Data are presented as mean ± SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD; ★ $p < 0.05$ vs. MET+ND; ^ $p < 0.05$ vs. MET+HFHC.

2.4. Plasma Liver Injury Enzymes

Plasma aspartate transaminase (AST) and alanine transaminase (ALT) were measured to assess liver function and injury. A one-way ANOVA showed significant differences in plasma AST ($F(5, 30) = 59.97$, $p < 0.0001$) and ALT ($F(5, 30) = 122.50$, $p < 0.0001$) levels between the groups. Post hoc comparisons showed (Figure 4) that the PD control group exhibited significantly ($p < 0.0001$) higher plasma AST and ALT levels in comparison to the NPD group. However, the GTIN+ND and GTIN+HFHC groups exhibited significantly ($p < 0.0001$) lower plasma AST and ALT levels in comparison to the PD control. Similar results ($p < 0.0001$) were observed for plasma AST and ALT levels in the MET+ND and MET+HFHC groups. A two-way ANOVA revealed that both treatment ($F(1, 20) = 33.80$, $p < 0.0001$) and diet ($F(1, 20) = 105.60$, $p < 0.0001$) exhibited significant main effects on plasma AST levels. Similarly, both treatment ($F(1, 20) = 27.82$, $p < 0.0001$) and diet ($F(1,$

20) = 79.67, $p < 0.0001$) exhibited significant main effects on plasma ALT levels. There was a significant ($F(1, 20) = 14.33$, $p = 0.0012$) interaction between treatment and diet on plasma AST levels. However, there was no significant ($F(1, 20) = 3.33$, $p = 0.0829$) interaction between treatment and diet on plasma ALT levels. Post hoc comparisons showed that GTIN treatment (GTIN+HFHC) resulted in significantly ($p < 0.05$) lower plasma AST and ALT levels in comparison to MET treatment (MET+HFHC). Animals on an ND (GTIN+ND and MET+ND) exhibited significantly ($p < 0.05$) lower levels of both these parameters in comparison to those on a HFHC diet (GTIN+HFHC and MET+HFHC).

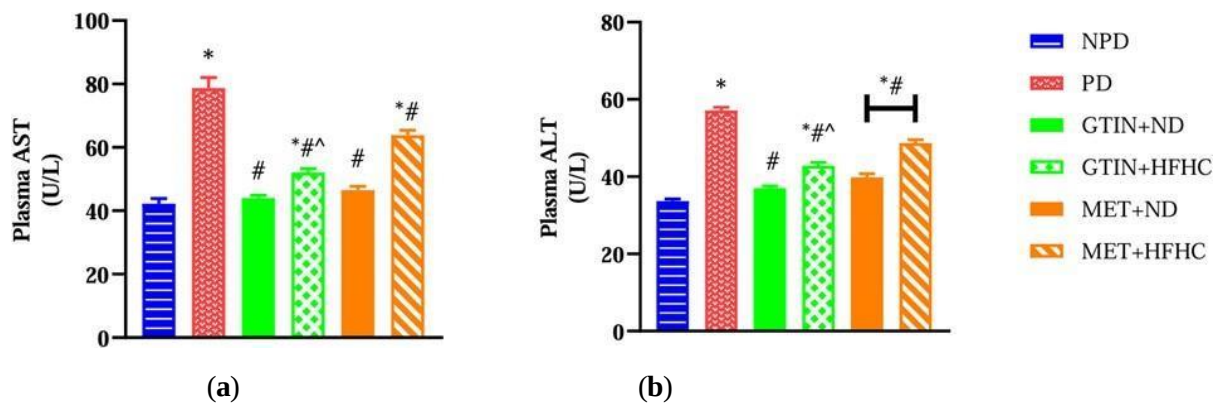


Figure 4. Effects of GTIN on plasma (a) AST and (b) ALT levels in rats with or without dietary intervention at week 32 of the treatment period ($n = 6$ per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD; ^ $p < 0.05$ vs. MET+HFHC.

2.5. Plasma Bilirubin

Plasma bilirubin levels were measured to assess liver function and antioxidant status in the pre-diabetic state. A one-way ANOVA showed significant ($F(5, 30) = 62.93$, $p < 0.0001$) differences in plasma bilirubin levels between the groups. Post hoc comparisons showed (Figure 5) that the PD control group exhibited significantly ($p < 0.05$) lower plasma bilirubin levels in comparison to the NPD group. However, the GTIN+ND ($p = < 0.0001$) and GTIN+HFHC ($p = < 0.0001$) groups exhibited significantly higher plasma bilirubin in comparison to the PD control. Similar results observed for plasma bilirubin levels in the MET+ND ($p = < 0.0001$) and MET+HFHC ($p = < 0.0001$) groups. A two-way ANOVA revealed that both treatment ($F(1, 20) = 14.85$, $p < 0.0010$) and diet ($F(1, 20) = 51.21$, $p < 0.0001$) exhibited significant main effects on plasma bilirubin levels. There was a significant ($F(1, 20) = 4.85$, $p = 0.0396$) interaction between treatment and diet on plasma bilirubin levels. Post hoc comparisons showed that GTIN treatment (GTIN+HFHC) resulted in significantly ($p < 0.05$) higher plasma bilirubin levels in comparison to MET treatment (MET+HFHC). Animals on an ND (GTIN+ND and MET+ND) exhibited significantly ($p < 0.05$) higher levels of this parameter in comparison to those on a HFHC diet (GTIN+HFHC and MET+HFHC).

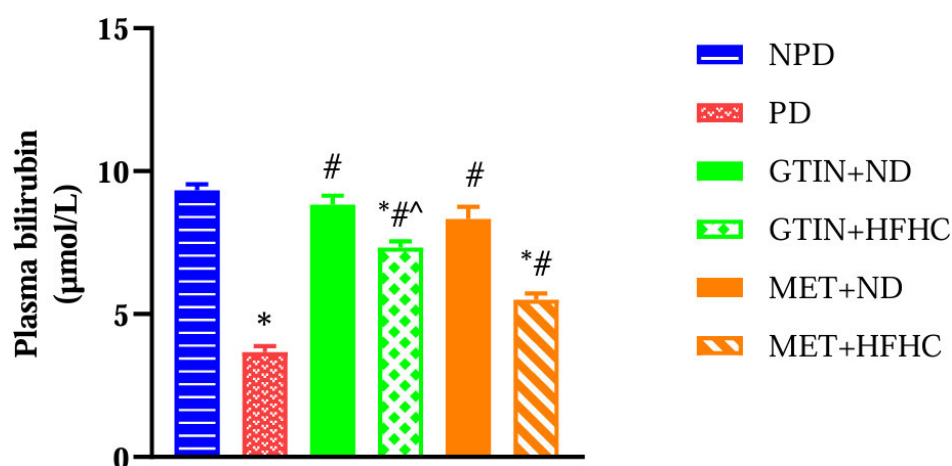


Figure 5. Effects of GTIN on plasma bilirubin levels in rats with or without dietary intervention at week 32 of the treatment period (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD; ^ $p < 0.05$ vs. MET+HFHC.

2.6. Liver Histology

Liver histology was measured to assess liver architecture and cellular damage. The results (Figure 6B) showed increased lipid droplet accumulation, hepatocyte nucleus displacement, disturbed sinusoidal space, and hepatocyte plate arrangement in the PD group in comparison to the NPD group. There was no remarkable difference in the number of binucleated hepatocytes in the PD group in comparison to the NPD group. The GTIN+ND (Figure 6C) and GTIN+HFHC (Figure 6D) groups showed a decrease in lipid droplet accumulation and hepatocyte nucleus displacement, as well as improved sinusoidal space and hepatocyte arrangement in comparison to the PD group. Similar results were observed in the MET+ND (Figure 6E) and MET+HFHC (Figure 6F). There was no remarkable difference in the number of binucleated hepatocytes in the treatment groups (GTIN+ND, GTIN+HFHC, MET+ND, MET+HFHC) in comparison to the PD group. The histopathological scores for each parameter are shown in Table 1. For each histological parameter, the scores were the same for all animals within each group.

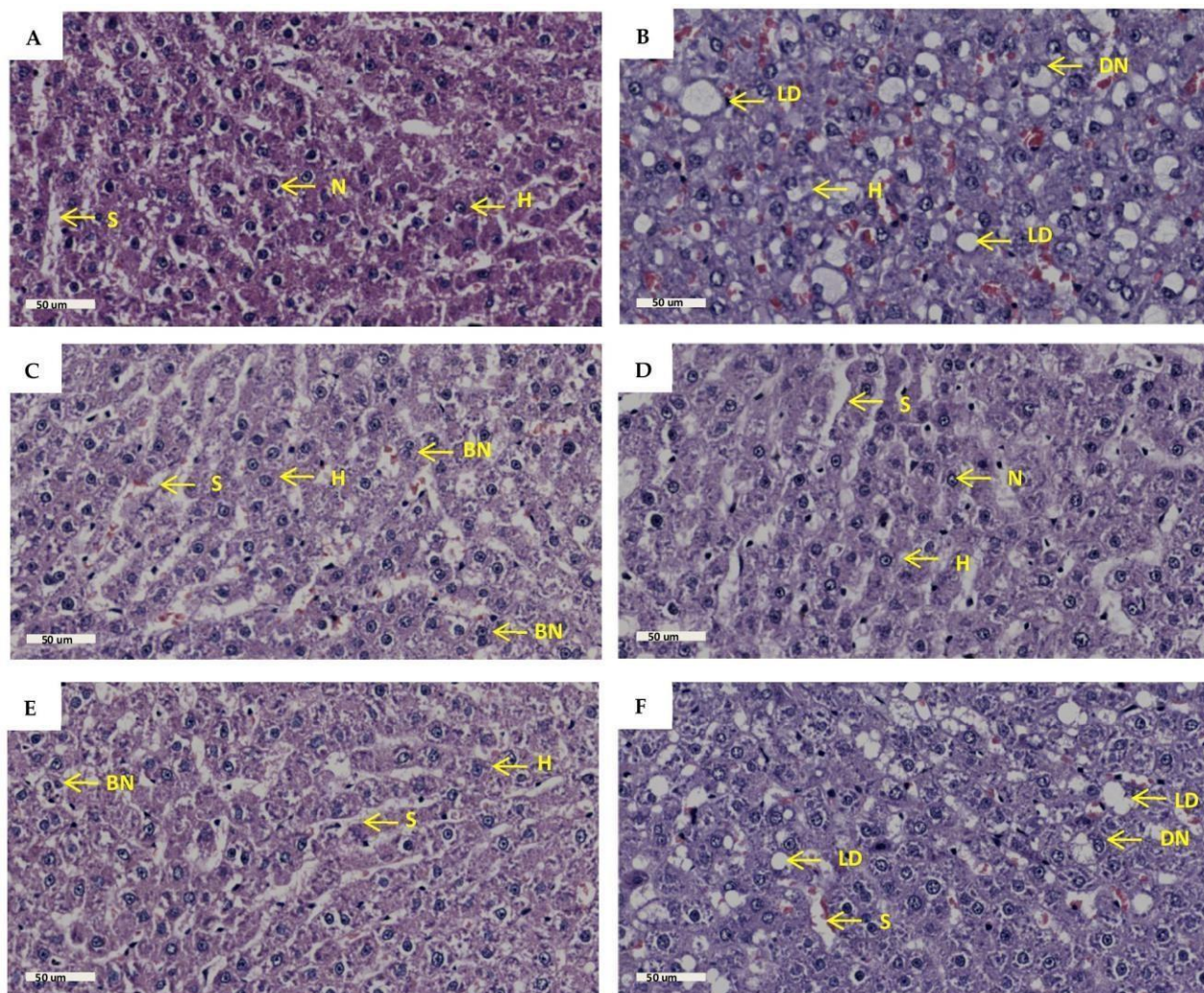


Figure 6. Effects of GTIN on liver histopathology in rats with or without dietary intervention at week 32 of the treatment period (n = 2 per group). Representative H&E-stained liver sections (40×; scale bar = 50 µm). (A) NPD; (B) PD; (C) GTIN+ND; (D) GTIN+HFHC; (E) MET+ND; (F) MET+HFHC. BN = binuclear; DN = displaced nucleus; H = hepatocyte; LD = lipid droplet; N = nucleus; S = sinusoid

Table 1. Histopathological scoring of liver tissue at week 32 of the experimental period ($n = 2$, per group).

Groups	Lipid Droplets	Nuclei	Sinusoid	Hepatocyte Plate	Binuclear
		Displacement	Arrangement	Arrangement	Hepatocytes
NPD	0	0	0	0	1
PD	3	2	3	3	1
GTIN+ND	1	1	1	1	1
GTIN+HFHC	1	1	1	1	1
MET+ND	1	1	1	1	1
MET+HFHC	2	1	2	2	1

3. Discussion

Excess glucose influx into the liver triggers the activation of lipogenic transcription factor SREBP-1c, which drives de novo lipogenesis and leads to increased TAG accumulation [27]. In this study, the liver TAG levels, liver weights, and plasma SREBP-1c levels were significantly higher in the PD group in comparison to the NPD group. The development of NAFLD has been associated with unhealthy dietary patterns characterized by high saturated fat and carbohydrate content along with a low intake of fruits and vegetables [28]. Additionally, fructose has been identified as a key factor in the development of NAFLD in humans and rodents [29]. The high-fat, high-carbohydrate (HFHC) diet and fructose-supplemented water used to induce pre-diabetes in our study may have promoted insulin resistance, contributing to hyperinsulinemia and elevated glucose levels, as previously reported [30]. Furthermore, as demonstrated in Chapter 4, the PD animals exhibited increased caloric intake. Under insulin-resistant conditions, skeletal muscle glucose uptake and glycogen storage are impaired, allowing excess glucose to remain in the circulation and be delivered to the liver [31]. This impaired peripheral glucose disposal may be associated with reduced insulin signalling and decreased GLUT4 translocation in skeletal muscle, resulting in increased hepatic glucose availability [32]. The increased hepatic glucose availability may have stimulated SREBP-1c activation, promoting the conversion of carbohydrates into fatty acids and increasing hepatic TAG accumulation [33]. Furthermore, excessive nutrient availability during pre-diabetes suppresses AMPK activity, which normally inhibits lipogenesis and promotes fatty acid oxidation [34]. Reduced AMPK activity may therefore contribute to enhanced SREBP-1c activation and hepatic lipid accumulation [34]. These metabolic alterations may have contributed to the elevated liver TAG levels and liver weights observed in the PD group.

However, both GTIN-treated groups exhibited significantly lower liver TAG levels, liver weights, and plasma SREBP-1c levels in comparison to the PD control. A recent study showed that the

administration of 15 mg/kg body weight (bw) of GTIN promoted weight loss and improved insulin sensitivity by reducing ghrelin levels and caloric intake [23]. Additionally, another study found that the administration of 20 mg/kg bw of GTIN to mice on a methionine–choline-deficient diet for 4 weeks reduced hepatic lipid accumulation by inhibiting lipogenic pathways and promoting β -oxidation [25]. They also found that GTIN attenuates hepatic lipid accumulation by promoting AMP-activated protein kinase (AMPK) activation [25]. Therefore, in our study, we speculate that GTIN may have reduced liver TAG accumulation by improving insulin sensitivity, promoting β -oxidation, and inhibiting lipogenic pathways, as evidenced by the low plasma SREBP1c levels in the GTIN-treated groups. Activation of AMPK by GTIN may have contributed to these effects by inhibiting SREBP-1c-mediated lipogenesis while enhancing mitochondrial fatty acid oxidation [25]. Improved mitochondrial function may reduce excessive lipid accumulation by promoting efficient energy utilisation and preventing lipid-induced metabolic stress [35].

Furthermore, as demonstrated in Chapter 4, GTIN improved peripheral glucose handling by promoting skeletal muscle glucose uptake and glycogen storage. Increased glucose disposal by skeletal muscle may reduce the amount of circulating glucose delivered to the liver [36]. Consequently, less glucose may be available to activate SREBP-1c and drive the conversion of carbohydrates into fatty acids [36]. This may explain the reductions in plasma SREBP-1c levels, liver TAG accumulation, and liver weight observed in the GTIN-treated groups. These findings suggest coordinated regulation of glucose metabolism between skeletal muscle and the liver during GTIN treatment.

Similar results were observed for liver TAG levels, liver weights, and plasma SREBP-1c levels in MET-treated groups. Studies have shown that MET was effective in reducing intrahepatic lipid accumulation [15, 37]. In skeletal muscle, MET has been reported to activate AMPK, which increases glucose transporter-4 translocation and improves glucose uptake [15, 37]. Similar to GTIN, improved peripheral glucose disposal may reduce hepatic glucose availability, limiting SREBP-1c-mediated de novo lipogenesis and hepatic TAG accumulation. These mechanisms may explain the lower liver TAG levels, liver weights, and plasma SREBP-1c levels observed in the MET-treated groups [15, 37].

Although GTIN and MET may share overlapping mechanisms through AMPK activation and improved insulin sensitivity, their modes of action may differ [15, 25, 37]. Metformin primarily improves metabolic homeostasis by reducing hepatic gluconeogenesis and enhancing insulin sensitivity, whereas GTIN may additionally exert hepatoprotective effects through antioxidant and anti-inflammatory activity [21, 38, 39]. Therefore, the comparable improvements observed following GTIN and MET treatment may reflect convergence on AMPK-related metabolic pathways, while GTIN may provide additional protection against oxidative and inflammatory damage associated with NAFLD progression [21, 38, 39].

Animals maintained on a normal diet exhibited significantly lower levels of liver TAGs, liver weights, and plasma SREBP-1c levels in comparison to those on the HFHC diet. Our findings corroborated a previous study which showed that the consumption of a HFHC diet consisting of 30% fat and 55% carbohydrate for 10 weeks promoted increased de novo lipogenesis [40]. Another study showed that mice fed a high-fat diet (HFD) composed of 42% fat and 42.7% carbohydrates for 3 months significantly increased liver weight [41]. Additionally, a study showed that the consumption of a HFD composed of 60% fats for 2 weeks significantly increased liver TAG levels [42]. A significant interaction between treatment and diet was observed for liver TAG levels and plasma SREBP-1c concentrations, whereas no significant interaction was observed for liver weight. These findings may suggest that the therapeutic effects of treatment on liver TAG and SREBP-1c levels may be enhanced when combined with dietary intervention. Furthermore, GTIN treatment exhibited significantly lower liver TAG levels and liver weights in comparison to MET in HFHC-fed animals. GTIN treatment also exhibited significantly lower plasma SREBP-1c levels than MET in animals on a normal diet. These findings may suggest that GTIN exerts greater therapeutic effects on liver TAGs, liver weights, and SREBP-1c levels than MET treatment, particularly in animals without dietary intervention.

Reactive oxygen species (ROS) are generated from various endogenous and exogenous sources, including mitochondrial respiration, enzymatic reactions, and xenobiotics [43]. The body utilizes enzymatic and non-enzymatic antioxidant systems to counteract the harmful effects of ROS production [43]. Enzymatic antioxidants include SOD, GPx, and catalase, while non-enzymatic antioxidants include glutathione, vitamin C, vitamin E, and bilirubin, among others [43]. In the pre-diabetic state, liver MDA levels, which are a by-product of lipid peroxidation, were significantly higher in comparison to the control group [44, 45]. Insulin resistance associated with NAFLD increases the flow of free fatty acids (FFAs) into the liver which triggers β -oxidation to manage the excess lipids [46]. Mitochondria adapt to lipid accumulation in hepatocytes by increasing β -oxidation [43]. However, increased substrate delivery to the electron transport chain (ETC) leads to increased ROS production [43]. In NAFLD, elevated ROS levels not only promote lipid peroxidation but also deplete antioxidant molecules and impair antioxidant enzyme activity [47]. Patients with NAFLD exhibit a diminished antioxidant capacity in hepatocytes, as evidenced by a significant decrease in enzymatic antioxidants such as SOD, GPx, and catalase [47]. Moreover, oxidative stress plays a pivotal role in the progression from simple steatosis to NASH [48].

In this study, the liver MDA levels were significantly higher in the PD group in comparison to the NPD group. This was further accompanied by significantly lower liver SOD and Gpx activity. This may suggest increased hepatic lipid peroxidation and decreased enzymatic antioxidant activity in the liver during the pre-diabetic state. The increased oxidative burden observed in the PD group may indicate impaired mitochondrial homeostasis, as excessive nutrient availability can overload

mitochondrial oxidative pathways and increase ROS generation [49]. Mitochondrial dysfunction may further worsen hepatic insulin resistance by impairing energy metabolism and promoting inflammatory signalling [49].

However, both GTIN-treated groups showed significantly lower liver MDA levels in comparison to the PD control. This was further accompanied by significantly increased liver SOD and Gpx activity. Flavonoids are natural antioxidants present in the human diet and have gained attention as promising treatment options for NAFLD due to their potent antioxidant effects and safety profile [50, 51]. Their therapeutic effects on oxidative stress have been attributed to the activation of AMPK signaling and the nuclear factor erythroid 2-related factor 2 (Nrf2) pathways [52]. AMPK signaling reduces ROS production, while Nrf2 activation promotes the upregulation of GPx and catalase [53]. A recent study showed that the administration of 20 mg/kg bw of GTIN for 4 weeks directly reduces oxidative stress in the liver rather than upregulating Nrf2-dependent antioxidant responses [25]. However, another study showed that the oral administration of 30 mg/kg bw of GTIN for 3 days upregulated SOD expression by increasing the nuclear translocation of Nrf2 [54].

Therefore, the antioxidant effects of GTIN may involve multiple complementary mechanisms, including direct ROS scavenging due to its flavonoid structure, AMPK-mediated improvement of mitochondrial function, and modulation of endogenous antioxidant defence pathways [21, 25, 54]. By reducing mitochondrial oxidative stress, GTIN may prevent further lipid peroxidation and preserve hepatocyte function during pre-diabetes [49].

The observed improvement on liver SOD and GPx activity in the GTIN-treated groups of our study may have been due to the longer treatment duration of 12 weeks as compared to the 4-week treatment period reported in the previous study [25]. Similar results were observed for liver MDA levels and SOD activity in the MET-treated groups, while the MET+ND showed comparable Gpx activity. Our study's results corroborated previous studies showing that MET alleviates oxidative stress in the liver by enhancing the activity of SOD and GPx in a high-fat diet-fed rat model [55, 56]. Although both treatments reduced oxidative stress, MET primarily achieves this through AMPK activation and improved metabolic regulation, whereas GTIN may additionally provide direct antioxidant protection through its phenolic structure [25, 57]. This difference may explain the enhanced antioxidant enzyme activity observed in the GTIN-treated groups compared with MET.

Animals maintained on a normal diet exhibited significantly lower levels of liver MDA as well as higher activities of SOD and GPx in comparison to those on the HFHC diet. The results of this study corroborated a previous study which showed that consumption of a diet containing 44.3% fat and 20% fructose for 4 weeks resulted in increased liver MDA levels and decreased SOD and Gpx activity [58]. Interestingly, a significant interaction between diet and treatment was observed for liver Gpx activities despite small differences in mean values. There was no significant interaction detected for liver MDA

and SOD activity. These findings suggest that the most pronounced reductions in liver Gpx activities may be achieved through a combination of pharmacological and dietary intervention. The lack of interaction between treatment and diet on liver MDA levels and SOD activity may suggest that the effects of treatment are not influenced by diet. Furthermore, GTIN treatment demonstrated significantly higher SOD activity in comparison to MET in rats fed a normal diet and HFHC diet. Additionally, GTIN exhibited significantly higher GPx activity in comparison to MET in HFHC-fed animals. This may suggest that GTIN treatment exerts greater beneficial effects than MET on liver SOD and GPx activity in rats without dietary intervention. However, these findings should be interpreted cautiously, as antioxidant effects observed in animal models may not directly translate to humans [59]. Future studies investigating GTIN pharmacokinetics, tissue availability, metabolism and optimal therapeutic dosing are required to determine whether sufficient concentrations can be achieved in humans to produce similar hepatoprotective effects.

Under normal physiological conditions, ALT and AST are enzymes found in hepatocytes [60]. The increased levels of AST and ALT in circulation serve as a marker for liver injury, since these enzymes are released when hepatocytes are damaged [60]. Studies have shown increased plasma ALT and AST levels in the progression of NAFLD [61, 62]. Liver injury is driven by insulin resistance, oxidative stress, and inflammation, all of which have been associated with high-caloric diets [63]. Hepatic fatty acid accumulation during insulin resistance enhances β -oxidation, which increases ROS production and promotes oxidative stress [64]. This damages cell membranes of hepatocytes, which leads to liver tissue injury [64]. Furthermore, oxidative stress-induced activation of inflammatory pathways, including NF- κ B signalling, may promote the release of pro-inflammatory cytokines such as TNF- α and IL-6, further contributing to hepatocyte injury and NAFLD progression [65].

In this study, the plasma AST and ALT levels were significantly higher in the PD control group in comparison to the NPD group. This may suggest that diet-induced pre-diabetes damages hepatocytes, and this may have been due to hepatic FFA accumulation and lipid peroxidation, as seen in the PD group. However, both GTIN-treated groups showed significantly lower plasma AST and ALT levels in comparison to the PD control. Our results corroborated two recent studies which showed that GTIN administration reduced liver injury [25, 66]. In our study, GTIN may have reduced liver damage due to its ability to regulate lipid metabolism and mitigate oxidative stress [25]. The hepatoprotective effects of GTIN may therefore be attributed to the combined reduction of hepatic lipid accumulation, attenuation of oxidative stress, and suppression of inflammation-associated damage [38, 66]. By reducing ROS-mediated activation of NF- κ B signalling, GTIN may limit inflammatory cytokine production and preserve hepatocyte integrity. Similar results were observed for plasma AST and ALT levels in the MET-treated groups. Previous studies have shown that MET reduces liver injury by enhancing insulin sensitivity and decreasing hepatic lipid accumulation through activation of the

AMPK pathway Animals maintained on a normal diet exhibited significantly lower levels of plasma AST and ALT levels in comparison to those on the HFHC diet. Our findings corroborated a previous study which has shown that consumption of a HFD composed of 49% fat for 8 weeks promoted significantly elevated plasma AST and ALT levels [67]. Another study showed that the consumption of a high-carbohydrate diet composed of 80% carbohydrates also promoted an increase in plasma AST and ALT levels [68]. A significant interaction between diet and treatment was observed for plasma AST levels, whereas no significant interaction was detected for plasma ALT levels. These findings may suggest that dietary intervention enhances the beneficial effects of treatment on plasma AST levels. The lack of interaction between treatment and diet on plasma ALT may suggest that the effects of treatment are not influenced by diet. Furthermore, GTIN treatment exhibited significantly lower plasma AST and ALT levels in comparison to MET in HFHC-fed animals. This may suggest that GTIN treatment exerts greater beneficial effects than MET on plasma AST and ALT levels in rats without dietary intervention.

Bilirubin is formed in the reticuloendothelial cells of the liver, spleen, and bone marrow through the breakdown of heme [68]. Studies have shown that bilirubin exerts potent antioxidant, anti-inflammatory, and cytoprotective effects in T2DM, hepatic steatosis, and cardiovascular diseases [69, 70]. In this study, the plasma bilirubin levels were significantly lower in the PD control in comparison to the NPD group. This study's results corroborated previous studies which have shown similar results in the pre-diabetic state [4, 71]. Plasma bilirubin levels have been shown to be inversely associated with T2DM and NAFLD [72, 73]. Bilirubin has been explored for its therapeutic effects in managing NAFLD in T2DM due to a lack of approved pharmacological treatments [73]. Bilirubin has been shown to enhance insulin sensitivity in diet-induced obese mice by suppressing endoplasmic reticulum stress and chronic inflammation in adipose tissue and the liver [74]. Furthermore, a previous study showed that bilirubin was more effective in protecting against lipid peroxidation than glutathione [75]. Notably, plasma bilirubin contributes to the total antioxidant capacity of the blood [76]. In this study, both GTIN-treated groups exhibited significantly higher plasma bilirubin levels in comparison to the PD control. The increased plasma bilirubin may have contributed to the reduction in hepatic oxidative stress. These findings suggest that GTIN may contribute to improved liver health by enhancing antioxidant plasma bilirubin levels. The increase in bilirubin following GTIN treatment may represent an additional endogenous antioxidant response that complements GTIN's direct free-radical scavenging activity, thereby reducing oxidative damage associated with NAFLD progression.

Similar results were observed for plasma bilirubin levels in the MET-treated groups. This study's results corroborated a previous study showing elevated plasma bilirubin levels after MET administration [71]. Notably, animals maintained on a normal diet exhibited significantly higher plasma bilirubin levels in comparison to those on the HFHC diet. Our findings corroborated a

previous study which has shown that plasma bilirubin decreased after consumption of a HFD [77]. A significant interaction between diet and treatment was observed for plasma bilirubin levels. This may suggest that the effects of pharmacological treatment on plasma bilirubin levels may be enhanced when combined with dietary intervention. Furthermore, GTIN treatment exhibited significantly higher plasma bilirubin levels in comparison to MET in HFHC-fed animals. This may suggest that GTIN treatment exerts greater beneficial effects than MET on plasma bilirubin in rats without dietary intervention.

Under normal physiological conditions, the liver is composed of hexagonal lobules with hepatocytes arranged in radial plates around the central vein [78]. Sinusoids are located between hepatocytes and facilitate the exchange of nutrients and waste [78]. Macrovesicular steatosis is a key histological feature of NAFLD and has been observed in the pre-diabetic state [72, 79]. The accumulation of fat in hepatocytes displaces the nucleus to the periphery and lipid-laden hepatocytes reduce sinusoidal space in NAFLD [80]. High-caloric diets and insulin resistance promotes increased hepatic FFA influx, which contributes to hepatic steatosis [46]. In this study, the PD group exhibited notable features such as increased lipid droplet accumulation, the displacement of hepatocyte nuclei, disturbed sinusoidal space, and hepatocyte plate arrangement in comparison to the NPD group. These histological findings are consistent with the elevated liver TAG and plasma SREBP-1c levels observed in the PD group. Increased SREBP-1c-mediated de novo lipogenesis may have promoted hepatic lipid accumulation, leading to structural liver damage [81]. Furthermore, the hepatocyte plate arrangement may have been disrupted due to fat deposition, leading to disarray within the liver lobules.

However, both GTIN-treated groups showed reductions in lipid droplet accumulation, displaced hepatocyte nuclei, and improved sinusoidal space and hepatocyte arrangement in comparison to the PD group. These improvements are consistent with the reductions in plasma SREBP-1c levels and liver TAG accumulation observed following GTIN treatment. This suggests that reduced hepatic lipogenesis may have contributed to the restoration of normal liver architecture. These improvements may have been attributed to the antilipidaemic, antioxidant, and anti-inflammatory properties of GTIN [21, 22]. The ability of GTIN to improve multiple pathological features of NAFLD may be associated with its flavonoid structure, which allows modulation of oxidative stress, inflammatory pathways and metabolic signalling [66, 82]. However, direct molecular confirmation of pathways such as AMPK activation, NF- κ B inhibition and mitochondrial protection was not performed in this study and should be investigated in future studies.

Additionally, the MET+ND and MET+HFHC groups showed similar histological improvements as the GTIN-treated groups. The improved hepatic architecture observed in the MET-treated groups may be attributed to the reductions in plasma SREBP-1c levels and hepatic TAG accumulation. However, the MET+HFHC group showed increased lipid droplet accumulation, reduced sinusoidal space, and

moderate disruption to hepatocyte plate arrangement in comparison to the MET+ND group. The HFHC diet may have limited the ability of MET to effectively reduce hepatic lipid accumulation [83]. There was no remarkable difference in the number of binucleated hepatocytes among all six groups, which may suggest that hepatocyte regeneration may be occurring at similar rates.

Although GTIN demonstrated comparable or greater effects than MET in several liver-related outcomes, translation of these findings into clinical applications requires consideration of important pharmacological factors. Flavonoids often exhibit limited oral bioavailability due to poor absorption, rapid metabolism and extensive conjugation reactions [84, 85]. Therefore, future studies should investigate GTIN pharmacokinetics, including absorption, plasma half-life, metabolic conversion and tissue distribution, to determine whether effective concentrations observed in animal models can be achieved in humans. Furthermore, long-term toxicity studies are required to establish the safety profile and therapeutic window of GTIN before clinical application.

This study highlights the potential of GTIN as an effective compound for ameliorating liver complications associated with the pre-diabetic state. The results demonstrated that GTIN administration in the presence and absence of dietary intervention significantly improved liver TAG levels, liver weights, plasma SREBP-1c, and bilirubin levels in comparison to the PD group. Additionally, GTIN administration reduced liver MDA levels and increased liver SOD and Gpx activity. It also decreased liver injury, demonstrated by reduced plasma AST and ALT levels. Moreover, GTIN improved liver histology by reducing steatosis and restoring liver architecture in comparison to the PD group. The effects of GTIN on liver parameters were comparable to MET treatment. Moreover, dietary intervention may positively influence liver health in pre-diabetes. However, the combination of pharmacological and dietary intervention may exhibit greater therapeutic outcomes on liver health than when used independently. Notably, GTIN may exert greater protective effects than MET in rats without dietary intervention.

4. Materials and Methods

4.1. Chemicals and Drugs

The chemicals employed were of analytical grade and sourced from standard commercial suppliers (Merck, Darmstadt, Germany). Gossypetin was purchased from INDOFINE Chemical Company (Hillsborough, NJ, USA). Metformin was purchased from Sigma-Aldrich (St. Louis, MI, USA).

4.2. Animals and Housing

The study involved 36 male Sprague Dawley rats (150–180 g), which were bred and kept in the Biomedical Research Unit (BRU) at the University of KwaZulu-Natal (UKZN), Westville campus. Under standard laboratory conditions, the animals were housed at a constant temperature of 22 ± 2 °C,

a carbon dioxide (CO₂) content of <5000 p.p.m, relative humidity of 55 ± 5%, and a 12 h light/dark cycle (lights on at 07:00). Noise levels were kept under 65 decibels. Food and water were provided ad libitum to all animals. All animal experiments and handling were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (ethics number: AREC/00004965/2022). Pain and discomfort were closely monitored in accordance with the humane endpoint criteria outlined by the UKZN Institutional Animal Ethics Committee. The rats were housed in well-ventilated polycarbonate plastic cages (Techniplast, Labotec, Midrand, South Africa). Before the experimental diet (high fat high carbohydrate) was introduced, the rats were allowed to acclimatize to their new environment while consuming a standard rat diet (Meadow Feeds, Lanseria, South Africa) and tap water for 1 week [30].

4.3. Induction of Pre-Diabetes

The animals were randomly distributed into two groups based on diet (Figure 7) as follows: group 1 ($n = 6$) and group 2 ($n = 30$). The induction of experimental pre-diabetes in the animals was carried out using the protocol outlined by Luvuno et al. [30]. Group 1 animals were given a standard rat diet and tap water, while group 2 animals received a high-fat, high-carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, Johannesburg, South Africa) supplemented with 15% fructose-enriched water to induce pre-diabetes for 20 weeks. The HFHC diet was composed of 55% carbohydrates, 30% fats, and 15% proteins of the total energy content [30]. Following 20 weeks, the animals were assessed for pre-diabetes using the American Diabetes Association criteria [86]. Animals with a fasting blood glucose concentration of 5.6 to 6.9 mmol/L and a 2 h oral glucose tolerance test glucose concentration of 7.8 to 11.0 mmol/L were classified as the pre-diabetic group. The animals on the standard diet were also assessed at week 20 to confirm normoglycemia and were categorized as the non-pre-diabetic group. To measure glucose concentration, blood was collected using the tail-prick method [87]. Glucose levels were assessed using a OneTouch Select glucometer (LifeScan, Malta, Kent, UK). Blood samples were taken at week 20 and every 4 weeks during the treatment period to monitor changes in glucose levels. The sample size was selected based on similar studies and power analysis to ensure adequate statistical power while ensuring animal welfare [71, 88].

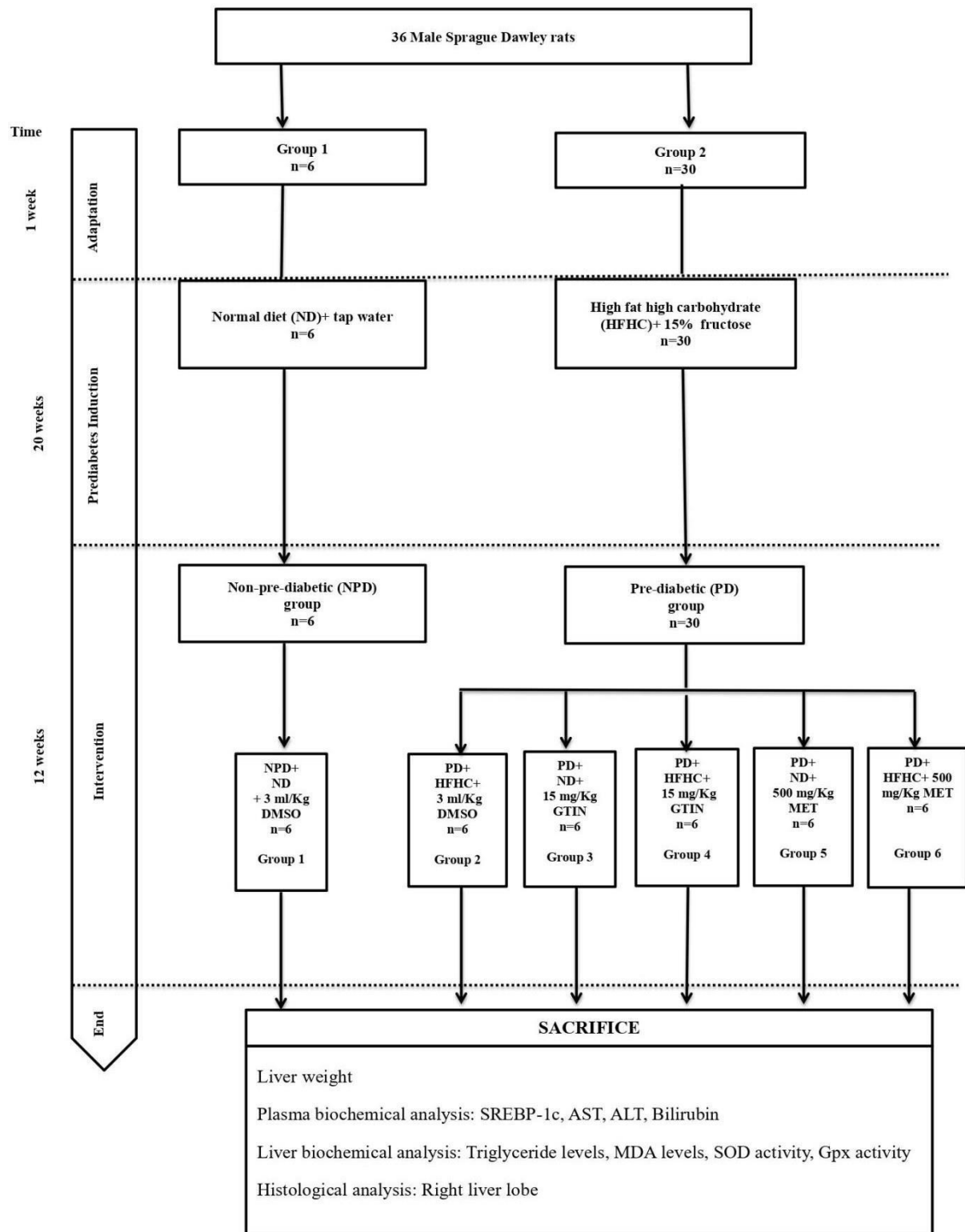


Figure 7. Layout of experimental design and grouping.

4.4. Experimental Design and Treatment

Following the induction of pre-diabetes, the pre-diabetic group ($n = 30$) was randomly subdivided into five groups (Groups 2 to 6), each group comprising six animals each. The pre-diabetic animals either

continued the HFHC diet or changed to a normal standard diet (ND) while receiving either 15 mg/kg body weight (b.w) gossypetin (GTIN) or 500 mg/kg bw metformin (MET) orally once every third day at 9:00 a.m. for 12 weeks. The doses of 15 mg/kg GTIN and 500 mg/kg MET were selected based on previous studies, demonstrating their effectiveness in similar pre-diabetic rat models [4, 24]. The non-pre-diabetic control (NPD, Group 1) animals were continuously fed on a normal diet (ND). The pre-diabetic control (PD, Group 2) group consisted of untreated pre-diabetic rats that were continuously fed a HFHC diet. Group 3 (GTIN+ND) animals were changed to the ND and received GTIN, whereas Group 4 (GTIN+HFHC) animals remained on the HFHC diet and received GTIN. Group 5 (MET+ND) animals were changed to the ND and were given MET, while Group 6 (MET+HFHC) animals continued on the HFHC diet and received MET. In all treatment groups, the drugs were dissolved in 3 mL/kg of diluted dimethyl sulfoxide (DMSO) (prepared as 2 mL DMSO in 19 mL normal saline, p.o). To ensure consistency and control for any potential effects of the vehicle, the control groups were also administered the same dose of DMSO without any dissolved drug. We have used 10% DMSO as a vehicle, as it has proven to dissolve organic compounds based on previous studies in our laboratory [89, 90]. The amount of DMSO administered was 3 mL/kg every third day. We have selected this volume based on the recommended safe volume to be administered to rodents [91].

4.5. Blood Collection and Tissue Harvesting

At the end of week 32 of the experimental period, all animals were anesthetized for 3 min with Isofor (100 mg/kg bw) (Safeline Pharmaceuticals (Pty) Ltd., Roodeport, South Africa) using a gas anesthetic chamber (BRU, UKZN, Durban, South Africa). After the animals were anesthetized, blood was obtained via cardiac puncture and placed into pre-cooled heparinized containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) at 4 °C, at 503 g for 15 min to obtain plasma. Each plasma sample was transferred into Eppendorf tubes and stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, The Netherlands) until ready for biochemical analysis. The liver was removed, weighed, and rinsed with a cold normal saline solution. The left lobe was immediately snap-frozen in liquid nitrogen and stored at -80 °C in a BioUltra freezer (Snijers Scientific, Tilburg, The Netherlands) for biochemical analysis, while the right lobe was preserved in 10% formalin buffer for histopathological examination.

4.6. Biochemical Analysis

At the end of week 32 of the experimental period, various biochemical parameters were assessed.

4.6.1. Plasma Biochemical Analysis

Plasma aspartate transaminase (AST), alanine transaminase (ALT), and bilirubin levels were analyzed with IDEXX Catalyst One Chemistry Analyzer (IDEXX Laboratories Inc. Westbrook, ME, USA).

Plasma sterol regulatory element binding protein 1c (SREBP-1c) levels were measured using the relevant ELISA kit (catalog no.: EKC39725) according to the manufacturer's instructions (Biomatik USA, LLC, Wilmington, DE, USA) (Supplementary Materials S1). The absorbance was measured at 450 nm using the Spectrostar Nanospectrophotometer (BMG Labtech, Ortenberg, Baden-Württemberg, LGBW, Germany).

4.6.2. Liver Biochemical Analysis

Liver tissue samples and the homogenate medium used for determining liver triglycerides levels, superoxide dismutase, and glutathione peroxidase activity were prepared according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). Briefly, 20 mg of liver tissue was harvested for each assay. The tissue was washed with ice-cold phosphate-buffered saline (PBS, 0.01 M, pH 7.4) and then homogenized on ice in 180 μ L of PBS. The homogenate was centrifuged at 10,000 $\times$ *g* for 10 min at 4 °C. The supernatant was collected and kept on ice for immediate analysis or stored at -80 °C for later use.

Liver triglyceride (TAG) levels were quantified using the triglyceride colorimetric assay kit (GPO-PAP method, catalog no.: E-BC-K238) according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) (Supplementary Materials S2). The activity of superoxide dismutase (SOD) was determined using the total superoxide dismutase (T-SOD) activity assay kit (WST-1 method, catalog no.: E-BC-K020-M) according to the manufacturer's instructions (Supplementary Materials S3). The activity of glutathione peroxidase (Gpx) was measured using the glutathione peroxidase (GSH-Px) activity assay kit (catalog no.: E-BC-K096-S) (Supplementary Materials S4). The Spectrostar Nanospectrophotometer (BMG Labtech, Ortenberg, Baden-Württemberg, LGBW, Germany) was used to measure the absorbance for TAG at 510 nm, SOD at 450 nm, and GPx at 412 nm.

4.7. MDA Assay

The liver malondialdehyde (MDA) concentration was quantified following a previously established laboratory protocol [92]. Briefly, 50 mg of liver tissue was homogenized in 500 μ L of 0.2% phosphoric acid. The homogenate was centrifuged at 400 $\times$ *g* for 10 min. Thereafter, 400 μ L of the homogenate was combined with 400 μ L of 2% phosphoric acid and then divided into two glass tubes, each receiving equal volumes of the solution. Then, 200 μ L of 7% phosphoric acid was added to both tubes, followed by the addition of 400 μ L of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) solution to the sample tube and 400 μ L of 3 mM hydrochloric acid (HCl) to the blank tube. To adjust the pH to 1.5, 200 μ L of 1 M HCl was added to both tubes. The tubes were heated at 100 °C for 15 min and allowed to cool to room temperature. After cooling, 1.5 mL of butanol was added, the solution was vortexed for 1 min for thorough mixing, and then allowed to settle until two phases could be distinguished. The upper butanol phase was transferred to Eppendorf tubes and centrifuged

at $13,200\times g$ for 6 min. The samples were aliquoted into a 96-well microtiter plate in triplicate, and the absorbance was measured at 532 nm (reference 600 nm) using a Spectrostar Nanospectrophotometer (BMG Labtech, Ortenberg, Baden-Württemberg, LGBW, Germany). The absorbance from these wavelengths was used to calculate the concentration of MDA using Beer's Law as follows: MDA concentration = Average Absorbance/Absorption coefficient (156 nm) [93].

4.8. Histological Analysis

A histological examination of the liver was performed on two randomly selected animals per group, following a previously documented protocol [92]. Due to budget constraints, histological assessment was limited to two animals per group. Histological findings were interpreted in conjunction with the biochemical and molecular markers to provide an integrated assessment of liver injury. Following removal from the animals, liver tissues were immediately fixed in 10% formalin buffer. The samples were dehydrated with alcohol and embedded in paraffin wax. A microtome was used to slice 5 μm sections of liver tissue, which were then stained with hematoxylin and eosin (H&E). The processed tissue sections were visualized and captured with a Leica SCN400 scanner and analyzed using Aperio *ImageScope* software (version 12.4.6.5003; Leica Biosystems, Buffalo Grove, IL, USA). Prior to histopathological assessment, all tissue sections were assigned random identification codes, and histopathological scoring was performed by the investigator using predefined scoring criteria while blinded to the treatment groups, thereby minimising observer bias. As all tissue sections were evaluated by a single observer, interobserver variability was not applicable.

Lipid droplet scores were assigned using the Brunt method [94] as follows: Grade 0: none; Grade 1: <33% hepatocytes affected; Grade 2: 33–66% hepatocytes affected; Grade 3: >66% hepatocytes affected.

Nuclei displacement scores were assigned as follows: Grade 0: none; Grade 1: 1–24% displaced nuclei; Grade 2: 25–50% displaced nuclei; Grade 3: >50% displaced nuclei.

Sinusoid disruption scores were assigned as follows: Grade 0: normal well-defined sinusoids; Grade 1: mild changes with some sinusoidal dilation but no significant loss; Grade 2: moderate disruption with sinusoidal dilation and focal areas of loss; Grade 3: severe disruption with large loss of sinusoidal space.

Hepatocyte plate arrangement scores were assigned as follows: Grade 0: radial plate arrangement; Grade 1: mild disruption where some plates are irregular but general organization is maintained; Grade 2: moderate disruption where plates are disorganized with gaps; Grade 3: severe disruption with no clear plate formation.

Binuclear hepatocyte scores were assigned as follows: Grade 0: none; Grade 1: <5% binucleated hepatocytes; Grade 2: 5–20% binucleated hepatocytes; Grade 3: >20% binucleated hepatocytes.

4.9. Statistical Analysis

Data were presented as the mean $\pm$ standard error of the mean (SEM). The Shapiro–Wilk test was used to evaluate the normality of the data and Bartlett’s test was used to test for homogeneity of variance. A one-way analysis of variance (ANOVA) was used to compare differences among all groups for the dependent variable. An ordinary two-way ANOVA was conducted on the GTIN+ND, GTIN+HFHC, MET+ND, and MET+HFHC groups. This analysis assessed the effects of two factors, namely diet (ND vs. HFHC) and treatment (GTIN vs. MET) on the dependent variable. Post hoc comparisons were made using the Tukey test via GraphPad Prism 5 software GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was determined at a value of $p < 0.05$.

5. Limitations and Future Recommendations

A limitation of this study was the restricted sample size for histological analysis, as only two animals per group were examined due to logistical constraints and shortages of laboratory supplies. Histopathological scoring was performed by a single blinded observer using predefined scoring criteria to minimise observer bias. However, interobserver variability could not be assessed because the tissue sections were not independently evaluated by a second observer. Despite this limitation, the histological findings were interpreted alongside the biochemical and molecular data, which showed consistent trends and strengthened the overall assessment of hepatic injury. Additionally, while lipid peroxidation and antioxidant activity were assessed, only selected markers were measured. Future studies should investigate a broader range of oxidative stress markers and antioxidant enzymes. Quantitative histological analysis should also be conducted on a larger sample size, and control animals without DMSO should be included in future studies.

6. Conclusions

Taken together, GTIN may serve as an effective candidate for managing liver complications in the pre-diabetic state in the presence and absence of dietary modification. This was evidenced by reduced liver TAGs, liver weights, and plasma SREBP-1c levels in the GTIN-treated animals. It also reduced liver MDA levels and injury while improving plasma bilirubin levels, SOD activity, Gpx activity, and liver histology. Similar results were observed for the liver parameters in the MET-treated groups. Although combined pharmacological treatment and dietary modification produced the most favourable hepatic outcomes, GTIN demonstrated notable hepatoprotective effects even in the absence of dietary intervention. Further studies investigating the molecular mechanisms underlying GTIN-mediated hepatic protection are warranted.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/doi/s1>, Supplementary Materials S1: Protocol for SREBP-1c assay. Supplementary Materials S2: Protocol for triglyceride assay. Supplementary Materials S3: Protocol for superoxide dismutase activity assay. Supplementary Materials S4: Protocol for glutathione

peroxidase activity assay.

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References

1. Mourouti, N.; Kantaras, P.; Mouratidou, T.; Karaglani, M.; Giménez-Legarre, N.; de Miguel-Etayo, P.; Rurik, I.; Torzsa, P.; Iotova, V.; Tankova, T., Determinants of BMI underreporting in adults from families at high risk for type 2 diabetes in Europe: The Feel4Diabetes study. *Journal of Public Health* 2025, 1–9.
2. Khan, R. M. M.; Chua, Z. J. Y.; Tan, J. C.; Yang, Y.; Liao, Z.; Zhao, Y., From pre-diabetes to diabetes: diagnosis, treatments and translational research. *Medicina* 2019, 55, (9), 546.
3. Hostalek, U., Global epidemiology of prediabetes-present and future perspectives. *Clinical diabetes and endocrinology* 2019, 5, (1), 5.
4. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Plant-derived oleanolic acid ameliorates markers associated with non-alcoholic fatty liver disease in a diet-induced pre-diabetes rat model. *Diabetes, Metabolic Syndrome and Obesity* 2019, 1953–1962.
5. Chen, Z.; Yu, R.; Xiong, Y.; Du, F.; Zhu, S., A vicious circle between insulin resistance and

- inflammation in nonalcoholic fatty liver disease. *Lipids in health and disease* 2017, 16, (1), 203.
6. Smith, G. I.; Shankaran, M.; Yoshino, M.; Schweitzer, G. G.; Chondronikola, M.; Beals, J. W.; Okunade, A. L.; Patterson, B. W.; Nyangau, E.; Field, T., Insulin resistance drives hepatic de novo lipogenesis in nonalcoholic fatty liver disease. *The Journal of clinical investigation* 2020, 130, (3), 1453–1460.
 7. Marušić, M.; Paić, M.; Knobloch, M.; Liberati Pršo, A.-M., NAFLD, insulin resistance, and diabetes mellitus type 2. *Canadian Journal of Gastroenterology and Hepatology* 2021, 2021, (1), 6613827.
 8. Kumar, M.; Tripathi, N., Metamorphic Changes of Liver Enzymes in Prediabetic Young Adult.
 9. Tamaki, N.; Ajmera, V.; Loomba, R., Non-invasive methods for imaging hepatic steatosis and their clinical importance in NAFLD. *Nature Reviews Endocrinology* 2022, 18, (1), 55–66.
 10. Jung, I.; Koo, D.-J.; Lee, W.-Y., Insulin resistance, non-alcoholic fatty liver disease and type 2 diabetes mellitus: clinical and experimental perspective. *Diabetes & metabolism journal* 2024, 48, (3), 327–339.
 11. Kim, K.-S.; Lee, B.-W., Beneficial effect of anti-diabetic drugs for nonalcoholic fatty liver disease. *Clinical and molecular hepatology* 2020, 26, (4), 430.
 12. Lin, C.-Y.; Adhikary, P.; Cheng, K., Cellular protein markers, therapeutics, and drug delivery strategies in the treatment of diabetes-associated liver fibrosis. *Advanced drug delivery reviews* 2021, 174, 127–139.
 13. Shibayama, K.; Furushima, C.; Saka, M.; Sakamoto, T.; Takahashi, H., Barriers to lifestyle modification in patients with non-alcoholic fatty liver disease: a scoping review. *Journal of Rural Medicine* 2024, 19, (1), 1–9.
 14. Parry, S. A.; Hodson, L., Managing NAFLD in type 2 diabetes: the effect of lifestyle interventions, a narrative review. *Advances in Therapy* 2020, 37, (4), 1381–1406.
 15. Perazza, F.; Leoni, L.; Colosimo, S.; Musio, A.; Bocedi, G.; D’Avino, M.; Agnelli, G.; Nicastri, A.; Rossetti, C.; Sacilotto, F., Metformin and the liver: unlocking the full therapeutic potential. *Metabolites* 2024, 14, (4), 186.
 16. Genua, I.; Cusi, K., Pharmacological approaches to nonalcoholic fatty liver disease: current and future therapies. *Diabetes Spectrum* 2024, 37, (1), 48–58.
 17. Bell, W.; Jennings, A.; Thompson, A. S.; Bondonno, N. P.; Tresserra-Rimbau, A.; Kühn, T.; Cassidy, A., A flavonoid-rich diet is associated with lower risk and improved imaging biomarkers of nonalcoholic fatty liver disease: a prospective cohort study. *The American journal of clinical nutrition* 2024, 120, (6), 1325–1334.
 18. Yi, X.; Dong, M.; Guo, N.; Tian, J.; Lei, P.; Wang, S.; Yang, Y.; Shi, Y., Flavonoids improve type 2 diabetes mellitus and its complications: A review. *Frontiers in Nutrition* 2023, 10, 1192131.

19. Lee, M. S.; Tsai, C. W.; Wang, C. P.; Chen, J. H.; Lin, H. H., Anti-prostate cancer potential of gossypetin via inducing apoptotic and autophagic cell death. *Molecular carcinogenesis* 2017, 56, (12), 2578–2592.
20. Huang, K.; Liu, Z.; Kim, M.-O.; Kim, K.-R., Anticancer effects of gossypetin from *Hibiscus sabdariffa* in oral squamous cell carcinoma. *Journal of Applied Oral Science* 2023, 31, e20230243.
21. Proença, C.; Rufino, A. T.; Santos, I.; Albuquerque, H. M.; Silva, A. M.; Fernandes, E.; Ferreira de Oliveira, J. M. P., Gossypetin is a novel modulator of inflammatory cytokine production and a suppressor of osteosarcoma cell growth. *Antioxidants* 2023, 12, (9), 1744.
22. Lin, H.-H., In vitro and in vivo atheroprotective effects of gossypetin against endothelial cell injury by induction of autophagy. *Chemical Research in Toxicology* 2015, 28, (2), 202–215.
23. Naidoo, K.; Khathi, A., Effects of gossypetin on glucose homeostasis in diet-induced pre-diabetic rats. *Molecules* 2024, 29, (18), 4410.
24. Naidoo, K.; Khathi, A., Investigating the effects of gossypetin on cardiovascular function in diet-induced pre-diabetic male Sprague Dawley rats. *International Journal of Molecular Sciences* 2024, 25, (22), 12105.
25. Oh, E.; Lee, J.; Cho, S.; Kim, S. W.; Jo, K. W.; Shin, W. S.; Gwak, S. H.; Ha, J.; Jeon, S. Y.; Park, J.-H., Gossypetin prevents the progression of nonalcoholic steatohepatitis by regulating oxidative stress and AMP-activated protein kinase. *Molecular Pharmacology* 2023, 104, (5), 214–229.
26. Gulsheen; Kumar, A.; Sharma, A., Antianxiety and antidepressant activity guided isolation and characterization of gossypetin from *Hibiscus sabdariffa* Linn. calyces. *Journal of Biologically Active Products from Nature* 2019, 9, (3), 205–214.
27. Meng, X.-M.; Ma, X.-X.; Tian, Y.-L.; Jiang, Q.; Wang, L.-L.; Shi, R.; Ding, L.; Pang, S.-G., Metformin improves the glucose and lipid metabolism via influencing the level of serum total bile acids in rats with streptozotocin-induced type 2 diabetes mellitus. *European Review for Medical & Pharmacological Sciences* 2017, 21, (9), 2232.
28. Vancells Lujan, P.; Vinas Esmel, E.; Sacanella Meseguer, E., Overview of non-alcoholic fatty liver disease (NAFLD) and the role of sugary food consumption and other dietary components in its development. *Nutrients* 2021, 13, (5), 1442.
29. Yu, S.; Li, C.; Ji, G.; Zhang, L., The contribution of dietary fructose to non-alcoholic fatty liver disease. *Frontiers in Pharmacology* 2021, 12, 783393.
30. Khathi, A.; Luvuno, M.; Mabandla, M., Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. *PONTE International Journal of Science and Research* 2018, 74, (5).
31. Dlamini, M.; Khathi, A., Prediabetes-associated changes in skeletal muscle function and their possible links with diabetes: a literature review. *International Journal of Molecular Sciences* 2023, 25, (1), 469.

32. Hulett, N. A.; Scalzo, R. L.; Reusch, J. E., Glucose uptake by skeletal muscle within the contexts of type 2 diabetes and exercise: an integrated approach. *Nutrients* 2022, 14, (3), 647.
33. Bo, T.; Gao, L.; Yao, Z.; Shao, S.; Wang, X.; Proud, C. G.; Zhao, J., Hepatic selective insulin resistance at the intersection of insulin signaling and metabolic dysfunction-associated steatotic liver disease. *Cell Metabolism* 2024, 36, (5), 947–968.
34. Ping, W.-X.; Hu, S.; Su, J.-Q.; Ouyang, S.-Y., Metabolic disorders in prediabetes: from mechanisms to therapeutic management. *World journal of diabetes* 2024, 15, (3), 361.
35. Zong, Y.; Li, H.; Liao, P.; Chen, L.; Pan, Y.; Zheng, Y.; Zhang, C.; Liu, D.; Zheng, M.; Gao, J., Mitochondrial dysfunction: mechanisms and advances in therapy. *Signal transduction and targeted therapy* 2024, 9, (1), 124.
36. Scoditti, E.; Sabatini, S.; Carli, F.; Gastaldelli, A., Hepatic glucose metabolism in the steatotic liver. *Nature reviews gastroenterology & hepatology* 2024, 21, (5), 319–334.
37. Green, C. J.; Marjot, T.; Walsby-Tickle, J.; Charlton, C.; Cornfield, T.; Westcott, F.; Pinnick, K. E.; Moolla, A.; Hazlehurst, J. M.; McCullagh, J., Metformin maintains intrahepatic triglyceride content through increased hepatic de novo lipogenesis. *European journal of endocrinology* 2022, 186, (3), 367–377.
38. Chen, J.-H.; Tsai, C.-W.; Wang, C.-P.; Lin, H.-H., Anti-atherosclerotic potential of gossypetin via inhibiting LDL oxidation and foam cell formation. *Toxicology and applied pharmacology* 2013, 272, (2), 313–324.
39. Bailey, C. J., Metformin: Therapeutic profile in the treatment of type 2 diabetes. *Diabetes, Obesity and Metabolism* 2024, 26, 3–19.
40. Swarbrick, M. M.; Stanhope, K. L.; Elliott, S. S.; Graham, J. L.; Krauss, R. M.; Christiansen, M. P.; Griffen, S. C.; Keim, N. L.; Havel, P. J., Consumption of fructose-sweetened beverages for 10 weeks increases postprandial triacylglycerol and apolipoprotein-B concentrations in overweight and obese women. *British Journal of Nutrition* 2008, 100, (5), 947–952.
41. VanSaun, M. N.; Lee, I. K.; Washington, M. K.; Matrisian, L.; Gorden, D. L., High fat diet induced hepatic steatosis establishes a permissive microenvironment for colorectal metastases and promotes primary dysplasia in a murine model. *The American journal of pathology* 2009, 175, (1), 355–364.
42. St-Amand, R.; Ngo Sock, É. T.; Quinn, S.; Lavoie, J.-M.; St-Pierre, D. H., Two weeks of western diet disrupts liver molecular markers of cholesterol metabolism in rats. *Lipids in health and Disease* 2020, 19, (1), 192.
43. Afzal, S.; Abdul Manap, A. S.; Attiq, A.; Albokhadaim, I.; Kandeel, M.; Alhojaily, S. M., From imbalance to impairment: the central role of reactive oxygen species in oxidative stress-induced disorders and therapeutic exploration. *Frontiers in pharmacology* 2023, 14, 1269581.

44. Mulla, I. G.; Anjankar, A.; Pratinidhi, S. A.; Lambe, S. D.; Agrawal, S. V.; Pratinidhi, S., Oxidative Stress in Prediabetic Young Adults. *Cureus* 2024, 16, (6).
45. Saghir, F. S.; Ibrahim, F. S.; Amom, Z.; Othman, L., The role of oxidative stress and inflammation in prediabetes: a review. *Malaysian Journal of Medicine and Health Sciences* 2023, 19, (4), 326–331.
46. Tanase, D. M.; Gosav, E. M.; Costea, C. F.; Ciocoiu, M.; Lacatusu, C. M.; Maranduca, M. A.; Ouatu, A.; Floria, M., The intricate relationship between type 2 diabetes mellitus (T2DM), insulin resistance (IR), and nonalcoholic fatty liver disease (NAFLD). *Journal of diabetes research* 2020, 2020, (1), 3920196.
47. Gonzalez, A.; Huerta-Salgado, C.; Orozco-Aguilar, J.; Aguirre, F.; Tacchi, F.; Simon, F.; Cabello-Verrugio, C., Role of oxidative stress in hepatic and extrahepatic dysfunctions during nonalcoholic fatty liver disease (NAFLD). *Oxidative Medicine and Cellular Longevity* 2020, 2020, (1), 1617805.
48. Chen, Z.; Tian, R.; She, Z.; Cai, J.; Li, H., Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease. *Free Radical Biology and Medicine* 2020, 152, 116–141.
49. Yesupatham, A.; Saraswathy, R., Role of oxidative stress in prediabetes development. *Biochemistry and Biophysics Reports* 2025, 43, 102069.
50. Tan, P.; Jin, L.; Qin, X.; He, B., Natural flavonoids: Potential therapeutic strategies for non-alcoholic fatty liver disease. *Frontiers in Pharmacology* 2022, 13, 1005312.
51. Liao, Y.; Lv, F.; Quan, T.; Wang, C.; Li, J., Flavonoids in natural products for the therapy of liver diseases: progress and future opportunities. *Frontiers in pharmacology* 2024, 15, 1485065.
52. Zhou, G.; Myers, R.; Li, Y.; Chen, Y.; Shen, X.; Fenyk-Melody, J.; Wu, M.; Ventre, J.; Doebber, T.; Fujii, N., Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of clinical investigation* 2001, 108, (8), 1167–1174.
53. Petsouki, E.; Cabrera, S. N. S.; Heiss, E. H., AMPK and NRF2: Interactive players in the same team for cellular homeostasis? *Free Radical Biology and Medicine* 2022, 190, 75–93.
54. Khan, A.; Manna, K.; Bose, C.; Sinha, M.; Das, D. K.; Kesh, S. B.; Chakrabarty, A.; Banerji, A.; Dey, S., Gossypetin, a naturally occurring hexahydroxy flavone, ameliorates gamma radiation-mediated DNA damage. *International journal of radiation biology* 2013, 89, (11), 965–975.
55. de la Rosa, L. C.; Vrenken, T. E.; Buist-Homan, M.; Faber, K. N.; Moshage, H., Metformin protects primary rat hepatocytes against oxidative stress-induced apoptosis. *Pharmacology research & perspectives* 2015, 3, (2), e00125.
56. Cahova, M.; Palenickova, E.; Dankova, H.; Sticova, E.; Burian, M.; Drahota, Z.; Cervinkova, Z.; Kucera, O.; Gladkova, C.; Stopka, P., Metformin prevents ischemia reperfusion-induced oxidative stress in the fatty liver by attenuation of reactive oxygen species formation. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2015, 309, (2), G100–G111.
57. Fujita, Y.; Hosokawa, M.; Fujimoto, S.; Mukai, E.; Abudukadier, A.; Obara, A.; Ogura, M.;

- Nakamura, Y.; Toyoda, K.; Nagashima, K., Metformin suppresses hepatic gluconeogenesis and lowers fasting blood glucose levels through reactive nitrogen species in mice. *Diabetologia* 2010, 53, (7), 1472–1481.
58. Jarukamjorn, K.; Jearapong, N.; Pimson, C.; Chatuphonprasert, W., A high-fat, high-fructose diet induces antioxidant imbalance and increases the risk and progression of nonalcoholic fatty liver disease in mice. *Scientifica* 2016, 2016, (1), 5029414.
59. Halliwell, B., Understanding mechanisms of antioxidant action in health and disease. *Nature Reviews Molecular Cell Biology* 2024, 25, (1), 13–33.
60. Lala, V.; Zubair, M.; Minter, D. A., Liver function tests. In *StatPearls [internet]*, StatPearls Publishing: 2023.
61. Sogabe, M.; Okahisa, T.; Kurihara, T.; Takehara, M.; Kagemoto, K.; Okazaki, J.; Kida, Y.; Hirao, A.; Tanaka, H.; Tomonari, T., Differences among patients with and without nonalcoholic fatty liver disease having elevated alanine aminotransferase levels at various stages of metabolic syndrome. *PLoS One* 2020, 15, (8), e0238388.
62. Lahmi, A.; Oryan, S.; Eidi, A.; Rohani, A. H., Comparative effects of thymol and vitamin E on nonalcoholic fatty liver disease in male Wistar rats. *Brazilian journal of biology* 2023, 84, e268781.
63. Yang, K.; Chen, J.; Zhang, T.; Yuan, X.; Ge, A.; Wang, S.; Xu, H.; Zeng, L.; Ge, J., Efficacy and safety of dietary polyphenol supplementation in the treatment of non-alcoholic fatty liver disease: A systematic review and meta-analysis. *Frontiers in immunology* 2022, 13, 949746.
64. Ma, Y.; Lee, G.; Heo, S.-Y.; Roh, Y.-S., Oxidative stress is a key modulator in the development of nonalcoholic fatty liver disease. *Antioxidants* 2021, 11, (1), 91.
65. Yang, Y. M.; Cho, Y. E.; Hwang, S., Crosstalk between oxidative stress and inflammatory liver injury in the pathogenesis of alcoholic liver disease. *International journal of molecular sciences* 2022, 23, (2), 774.
66. Khan, A.; Manna, K.; Das, D. K.; Kesh, S. B.; Sinha, M.; Das, U.; Biswas, S.; Sengupta, A.; Sikder, K.; Datta, S., Gossypetin ameliorates ionizing radiation-induced oxidative stress in mice liver—a molecular approach. *Free Radical Research* 2015, 49, (10), 1173–1186.
67. Gao, Y.; Hua, R.; Peng, K.; Yin, Y.; Zeng, C.; Guo, Y.; Wang, Y.; Li, L.; Li, X.; Qiu, Y., High-starchy carbohydrate diet aggravates NAFLD by increasing fatty acids influx mediated by NOX2. *Food Science and Human Wellness* 2023, 12, (4), 1081–1101.
68. Guerra Ruiz, A. R.; Crespo, J.; López Martínez, R. M.; Iruzubieta, P.; Casals Mercadal, G.; Lalana Garcés, M.; Lavin, B.; Morales Ruiz, M., Measurement and clinical usefulness of bilirubin in liver disease. *Advances in Laboratory Medicine/Avances en Medicina de Laboratorio* 2021, 2, (3), 352–361.
69. Ramírez-Mejía, M. M.; Castillo-Castañeda, S. M.; Pal, S. C.; Qi, X.; Méndez-Sánchez, N., The

multifaceted role of bilirubin in liver disease: a literature review. *Journal of clinical and translational hepatology* 2024, 12, (11), 939.

70. Jayanti, S.; Dalla Verde, C.; Tiribelli, C.; Gazzin, S., Inflammation, dopaminergic brain and bilirubin. *International Journal of Molecular Sciences* 2023, 24, (14), 11478.

71. Mabuza, L. P.; Gamede, M. W.; Maikoo, S.; Booysen, I. N.; Nguban, P. S.; Khathi, A., Hepatoprotective effects of a ruthenium (II) Schiff base complex in rats with diet-induced prediabetes. *Current Therapeutic Research* 2019, 91, 66–72.

72. Yang, M.; Ni, C.; Chang, B.; Jiang, Z.; Zhu, Y.; Tang, Y.; Li, Z.; Li, C.; Li, B., Association between serum total bilirubin levels and the risk of type 2 diabetes mellitus. *Diabetes research and clinical practice* 2019, 152, 23–28.

73. Wei, Y.; Liu, C.; Lai, F.; Dong, S.; Chen, H.; Chen, L.; Shi, L.; Zhu, F.; Zhang, C.; Lv, X., Associations between serum total bilirubin, obesity and type 2 diabetes. *Diabetology & Metabolic Syndrome* 2021, 13, (1), 143.

74. Dong, H.; Huang, H.; Yun, X.; Kim, D.-s.; Yue, Y.; Wu, H.; Sutter, A.; Chavin, K. D.; Otterbein, L. E.; Adams, D. B., Bilirubin increases insulin sensitivity in leptin-receptor deficient and diet-induced obese mice through suppression of ER stress and chronic inflammation. *Endocrinology* 2014, 155, (3), 818–828.

75. Vitek, L., The role of bilirubin in diabetes, metabolic syndrome, and cardiovascular diseases. *Frontiers in pharmacology* 2012, 3, 55.

76. Žibera, L.; Jenko-Pražnikar, Z.; Petelin, A., Serum bilirubin levels in overweight and obese individuals: the importance of anti-inflammatory and antioxidant responses. *Antioxidants* 2021, 10, (9), 1352.

77. Pallister, T.; Jackson, M.; Martin, T.; Glastonbury, C.; Jennings, A.; Beaumont, M.; Mohny, R.; Small, K.; MacGregor, A.; Steves, C., Untangling the relationship between diet and visceral fat mass through blood metabolomics and gut microbiome profiling. *International journal of obesity* 2017, 41, (7), 1106–1113.

78. Trefts, E.; Gannon, M., Wasserman DJCbC. *The liver* 2017, 27, R1147–R1151.

79. Brown, G. T.; Kleiner, D. E., Histopathology of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Metabolism* 2016, 65, (8), 1080–1086.

80. Leow, W.-Q.; Chan, A. W.-H.; Mendoza, P. G. L.; Lo, R.; Yap, K.; Kim, H., Non-alcoholic fatty liver disease: the pathologist's perspective. *Clinical and Molecular Hepatology* 2022, 29, (Suppl), S302.

81. Li, B.; Piao, S.; Fu, Y.; Fu, Q.; Qin, P.; Kong, W.; Ma, Y.; Zhang, Z.; Fang, X.; Hu, X., Lipid metabolism-MAFLD crosstalk: mechanisms and therapy. *Frontiers in Endocrinology* 2026, 17, 1785178.

82. Mounnissamy, V.; Gopal, V.; Gunasegaran, R.; Saraswathy, A., Antiinflammatory activity of gossypetin isolated from *Hibiscus sabdariffa*. *Indian Journal of Heterocyclic Chemistry* 2002, 12, (1), 85–86.
83. Chen, L.; Deng, H.; Cui, H.; Fang, J.; Zuo, Z.; Deng, J.; Li, Y.; Wang, X.; Zhao, L., Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget* 2017, 9, (6), 7204.
84. Naeem, A.; Ming, Y.; Pengyi, H.; Jie, K. Y.; Yali, L.; Haiyan, Z.; Shuai, X.; Wenjing, L.; Ling, W.; Xia, Z. M., The fate of flavonoids after oral administration: A comprehensive overview of its bioavailability. *Critical reviews in food science and nutrition* 2022, 62, (22), 6169–6186.
85. Chen, L.; Cao, H.; Huang, Q.; Xiao, J.; Teng, H., Absorption, metabolism and bioavailability of flavonoids: A review. *Critical reviews in food science and nutrition* 2022, 62, (28), 7730–7742.
86. Care, D., 2. Classification and diagnosis of diabetes: standards of care in. *Diabetes care* 2023, 46, S19.
87. Lee, G.; Goosens, K. A., Sampling blood from the lateral tail vein of the rat. *JoVE (Journal of Visualized Experiments)* 2015, (99), e52766.
88. Siboto, A.; Akinnuga, A. M.; Khumalo, B. N.; Ismail, M. B.; Booysen, I. N.; Sibiya, N. H.; Ngubane, P. S.; Khathi, A., The effects of a [3+ 1] oxo-free rhenium (V) compound with uracil-derived ligands on selected parameters of glucose homeostasis in diet-induced pre-diabetic rats. *Obesity Medicine* 2020, 19, 100258.
89. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Plant-derived oleanolic acid ameliorates markers of subclinical inflammation and innate immunity activation in diet-induced pre-diabetic rats. *Therapeutic Advances in Endocrinology and Metabolism* 2020, 11, 2042018820935771.
90. Khumalo, B.; Siboto, A.; Akinnuga, A. M.; Sibiya, N.; Khathi, A.; Ngubane, P. S., *Momordica balsamina* improves glucose handling in a diet-induced prediabetic rat model. *PLoS One* 2023, 18, (12), e0295498.
91. Jones-Bolin, S., Guidelines for the care and use of laboratory animals in biomedical research. *Current Protocols in Pharmacology* 2012, 59, (1), A. 4B. 1–A. 4B. 9.
92. Mzimela, N. C.; Ngubane, P. S.; Khathi, A., The changes in immune cell concentration during the progression of pre-diabetes to type 2 diabetes in a high-fat high-carbohydrate diet-induced pre-diabetic rat model. *Autoimmunity* 2019, 52, (1), 27–36.
93. Mkhwanazi, B. N.; Serumula, M. R.; Myburg, R. B.; Van Heerden, F. R.; Musabayane, C. T., Antioxidant effects of maslinic acid in livers, hearts and kidneys of streptozotocin-induced diabetic rats: effects on kidney function. *Renal Failure* 2014, 36, (3), 419–431.
94. Santiago-Rolón, A.; Purcell, D.; Rosado, K.; Toro, D. H., A comparison of brunt criteria, the non alcoholic fatty liver disease activity score (NAS) & a proposed NAS-including fibrosis as valid diagnostic scores for NASH. *Puerto Rico health sciences journal* 2015, 34, (4), 189.

Bridge

The third manuscript presented in Chapter 6 showed that diet-induced prediabetes was associated with altered liver function. This was evidenced by elevated liver triglycerides, increased liver weight, heightened levels of oxidative stress markers, inflammatory mediators and liver injury enzymes, along with histopathological disturbances. The administration of gossypetin (GTIN), in both the presence and absence of dietary intervention, significantly ameliorated these liver disturbances. The findings presented in manuscript 3 prompted further investigation into the effects of GTIN on kidney function, given the interconnected pathophysiology of hepatic and renal complications in prediabetes. Accordingly, Chapter 7 presents the findings of manuscript 4, which aims to investigate the effects of GTIN on kidney function, including kidney weight, glomerular filtration rate, renal oxidative stress markers, inflammatory mediators, electrolyte handling and markers of kidney injury in diet-induced prediabetes.

CHAPTER 7: MANUSCRIPT 4

PROLOGUE

Emerging evidence indicates that renal complications associated with type 2 diabetes mellitus begin during the prediabetic state. In prediabetes, renal complications arise from moderate hyperglycaemia-induced oxidative stress and increased pro-inflammatory cytokine production, along with glomerular hyperfiltration driven by elevated intraglomerular pressure. As seen in prediabetes, over-activation of the renin-angiotensin-aldosterone system, further contributes to podocyte injury, electrolyte imbalances and impaired glomerular filtration efficiency. The management of renal impairment during prediabetes typically involves a combination of pharmacological and dietary interventions. However, patient compliance with long-term dietary intervention is often poor, reducing the effectiveness of pharmacological treatments. Consequently, there is a critical demand for therapeutic agents capable of improving renal function independently of dietary intervention. Flavonoids have been shown to improve diabetic nephropathy by reducing oxidative stress and inflammation. In the previous manuscripts, gossypetin (GTIN) has been shown to improve markers associated with glucose homeostasis, cardiovascular function and liver health in a diet-induced prediabetic rat model. However, the effects of GTIN on prediabetes-associated kidney complications were not explored. Hence, in manuscript 4, we investigated the effects of GTIN on kidney function in both the presence and absence of dietary intervention in a diet-induced prediabetic rat model.

The manuscript in Chapter 7 is titled “**Investigating the effects of gossypetin on kidney function in diet-induced prediabetic male Sprague Dawley rats**” and is authored by Naidoo K and Khathi A.

The manuscript is currently under review in the journal “**Pharmaceutics**” and has been formatted according to the journal’s guidelines for authors.

Candidate Contribution: Karishma Naidoo was responsible for study conceptualisation, study design, all experimental work, statistical analysis, first draft writing and manuscript editing.

**Investigating the effects of gossypetin on selected markers of kidney function in diet-induced
prediabetic male Sprague Dawley rats**

Karishma Naidoo^{1,2}, Andile Khathi¹

1. University of KwaZulu-Natal, Westville, KwaZulu-Natal South Africa

2. Corresponding Author: Karishma Naidoo

School of Laboratory Medicine & Medical Sciences

University of KwaZulu-Natal

Private Bag X54001

Durban,

SOUTH AFRICA

Phone: 0711607469 /27 (0) 31 260 7585, Fax: 27 (0) 31 260 7132

Email: karishma280199@gmail.com /khathia@ukzn.ac.za

Keywords: Gossypetin, high fat high carbohydrate, pre-diabetes, kidney function

Abstract

Background/Objectives: Renal dysfunction is increasingly recognised as an early complication of metabolic disturbances associated with pre-diabetes. Gossypetin (GTIN) is a natural derivative of the well-known flavonoid, quercetin; however, studies regarding its biological effects remain limited. This study sought to investigate the effects of GTIN on selected markers of kidney function in both the presence and absence of dietary intervention in diet-induced prediabetes. **Methods:** Experimental prediabetes was induced by administering a high-fat, high-carbohydrate (HFHC) diet alongside drinking water supplemented with 15% fructose for a duration of 20 weeks. Following induction of the pre-diabetic state, animals were assigned into five groups (n = 6), receiving either gossypetin (15 mg/kg) or metformin (500 mg/kg) with a normal or HFHC diet for 12 weeks. **Results:** GTIN administration in both the presence and absence of dietary intervention significantly decreased kidney weight, fluid intake and urine output while also promoting renal function, as evidenced by an elevated glomerular filtration rate and improved biochemical markers, including creatinine, albumin, urea and uric acid, compared to the prediabetic (PD) group. The GTIN-treated group also showed improved sodium and potassium handling, along with reduced plasma aldosterone levels. Decreased malondialdehyde, reduced inflammatory markers as well as elevated total antioxidant status levels in the kidneys were also observed. Plasma kidney injury molecule-1 and urinary podocin mRNA expression were significantly decreased. **Conclusions:** GTIN administration, even without dietary intervention, may exhibit renoprotective effects in diet-induced prediabetes.

1. Introduction

The chronic consumption of calorie-dense diets consisting of refined carbohydrates and saturated fats, combined with sedentary lifestyles contribute to the development of prediabetes [1]. Prediabetes is an intermediate hyperglycaemic state that often precedes the development of type 2 diabetes mellitus (T2DM) [2]. It has been reported that around one-third of type 2 diabetic individuals exhibit renal damage [3]. Studies have shown that early markers of renal dysfunction are present during the prediabetic state [4, 5]. The prevalence of renal damage in prediabetes was reported to be 70% higher compared to normal glucose-tolerant individuals [4]. In prediabetes, renal dysfunction arises from altered blood flow and nephron damage leading to reduced filtration, proteinuria and kidney injury [4, 6]. Elevated plasma glucose levels damage the glomerular filtration membrane, leading to albuminuria [7]. It was reported that glomerular filtration rate (GFR) increases as a compensatory response to moderate hyperglycaemia and insulin resistance [8]. However, sustained hyperfiltration raises intraglomerular pressure, promoting glomerular injury [9]. Plasma creatinine, urea and uric acid levels may be altered in prediabetes due to impaired renal filtration efficiency [5, 10]. Sodium and potassium imbalances have been reported due to increased tubular reabsorption, impaired filtration and renin-angiotensin-aldosterone system (RAAS) activation in prediabetes [11, 12].

Moderate hyperglycaemia, as seen during prediabetes, has been shown to induce oxidative stress and activate the RAAS, leading to increased expression of inflammatory cytokines [11]. RAAS activation raises plasma aldosterone levels, leading to sodium and water retention [13]. Individuals with prediabetes have shown increased urine output and water intake as a compensatory response to excessive fluid loss [14]. Kidney total antioxidant status (TAS) is reduced in prediabetes due to oxidative stress and diminished antioxidant defenses [15]. In prediabetes, podocyte injury and initial glomerular damage are reflected by higher levels of kidney injury molecule-1 (KIM-1) and urinary podocin [10]. Kidney weight has been shown to be increased during prediabetes due to glomerular hypertrophy and inflammation [16].

Current management of diabetic nephropathy relies on pharmacological therapy supported by lifestyle modifications [17]. However, patients often rely solely on pharmacological intervention without implementing necessary lifestyle changes [18]. This has been shown to limit the effectiveness of pharmacological interventions [18]. Consequently, there is a growing need to identify affordable pharmacological agents that can ameliorate renal dysfunction in prediabetes in the absence of dietary intervention. Natural compounds with antioxidant and antidiabetic properties have gained attention as potential therapeutic options to treat renal dysfunction [19]. Quercetin has demonstrated insulin-sensitizing, antioxidant and renoprotective effects in models of T2DM [20]. Gossypetin (GTIN) is a natural hydroxylated flavone found in *Hibiscus sabdariffa* and is structurally similar to quercetin [21, 22]. Emerging research has shown that GTIN exhibited glucose lowering, cardioprotective and

hepatoprotective effects [23-25]. However, the effects of GTIN on renal function during the pre-diabetic state remain unexplored. Accordingly, this study evaluates the effects of GTIN on kidney function in both the presence and absence of dietary intervention in a diet-induced rat model.

2. Materials and Methods

2.1 Chemicals and drugs

All chemicals and drugs were of analytical grade and purchased from standard commercial suppliers (Merck, Darmstadt, Germany).

2.2 Animals and Housing

Thirty-six male Sprague Dawley rats (weighing 150–180 g) were bred and maintained at the Biomedical Research Unit (BRU) located at the University of KwaZulu-Natal, Westville campus. The animals were kept under controlled laboratory conditions with a stable temperature of 22 ± 2 °C, carbon dioxide levels below 5000 ppm, relative humidity maintained at $55 \pm 5\%$, and a 12-hour light/dark cycle with lights on at 07:00 a.m. Noise was limited to less than 65 decibels. All animals had free access to food and water. All procedures involving animals and their handling received approval from the Animal Research Ethics Committee at the University of KwaZulu-Natal (ethics approval number: AREC/00004965/2022). Pain and distress were carefully observed following the humane endpoint guidelines set by the UKZN Institutional Animal Ethics Committee. The rats were kept in well-ventilated polycarbonate cages (Techniplast, Labotec, Midrand, South Africa). Before introducing the high fat high carbohydrate diet, the rats were acclimated to their new surroundings for one week while being fed a standard rat diet (Meadow Feeds, Lanseria, South Africa) and provided with tap water.

2.3 Induction of prediabetes

Animals were randomly divided into two groups according to diet: group 1 (n = 6) and group 2 (n = 30). Prediabetes was induced in the animals using the protocol from Luvuno *et al.* [26]. Group 1 consumed a standard rat diet and tap water, while group 2 was provided a high-fat high-carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, Johannesburg, South Africa) supplemented with 15% fructose water over a period of 20 weeks to induce prediabetes. Following 20 weeks, the animals were assessed for prediabetes using the American Diabetes Association criteria [27]. Animals with a fasting blood glucose concentration of 5.6 to 6.9 mmol/L and a 2-hour oral glucose tolerance test glucose concentration of 7.8 to 11.0 mmol/L were classified as the prediabetic group. The animals on the standard diet were also assessed at week 20 to confirm normoglycaemia and were categorised as

the non-prediabetic group. To ensure appropriate statistical power and uphold animal welfare standards, the sample size was chosen in accordance with similar studies and power analysis [10, 28].

2.4 Experimental design and treatment

Following prediabetes induction, the prediabetic group (n = 30) was further divided into five subgroups (Group 2-6), with each group comprising six rats. These animals either remained on the high-fat high-carbohydrate (HFHC) diet or were switched to a standard normal diet (ND), while being administered either 15 mg/kg of gossypetin (GTIN) or 500 mg/kg of metformin (MET) orally every third day over a 12-week period. The selected GTIN dose of 15 mg/kg was based on prior studies demonstrating its efficacy and safety within the 10–20 mg/kg range [29-31]. Likewise, the 500 mg/kg MET dosage was chosen for its established therapeutic benefits and low risk of adverse effects [4, 32]. Group 1 consisted of non-prediabetic control (NPD) animals maintained on a normal diet (ND). Group 2 consisted of prediabetic control (PD) animals that continued on the HFHC diet. Animals in Group 3 (GTIN + ND) received GTIN treatment and were switched to the ND, while Group 4 (GTIN + HFHC) were administered GTIN but remained on the HFHC diet. Group 5 (MET + ND) animals were treated with metformin (MET) and transitioned to the ND, whereas Group 6 (MET + HFHC) received MET and continued on the HFHC diet.

2.5 Determination of fluid intake and urine output

The animals were placed in individual metabolic cages for 24 hours to measure fluid intake and urine output at weeks 20, 24, 28 and 32. During this period, the rats were not fasted but had free access to water. Fluid intake was determined by measuring the difference between the initial and final volumes of water in graduated drinking bottles over the 24-hour period. Urine was collected in the metabolic cage collection tubes, centrifuged at 13,000 rpm for 5 minutes at 4 °C, and the resulting supernatants were stored at –80 °C in a Bio Ultra freezer (Snijders Scientific, Tilburg, The Netherlands) until analysis of kidney function parameters.

2.6 Blood collection and tissue harvesting

At the end of week 32, the animals were put under anesthesia with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd., Roodepoort, South Africa) for 3 minutes using a gas anesthesia chamber (BRU, UKZN, Durban, South Africa). Upon the animals becoming unconscious, blood samples were collected by cardiac puncture and placed into individually cooled heparinised tubes. Blood samples were spun at 503×g for 15 minutes at 4 °C using an Eppendorf 5403 centrifuge (Eppendorf AG, Hamburg, Germany) to isolate plasma. The separated plasma was aliquoted into Eppendorf tubes and preserved at –80 °C in a Bio Ultra freezer (Snijders Scientific, Tilburg, The Netherlands) for later biochemical evaluation. After removal, the kidneys were weighed and rinsed with normal saline, then

rapidly frozen in liquid nitrogen and stored at -80°C for subsequent biochemical analysis.

2.7 Biochemical analysis

At the end of week 32, creatinine, urea and uric acid levels were determined in both plasma and urine samples (Elabsience Biotechnology Co., Ltd., Houston, TX, USA). Plasma albumin, sodium and potassium concentrations were also measured using the same manufacturer's assay kits (Elabsience Biotechnology Co., Ltd., Houston, TX, USA). Urine albumin and electrolytes (Na^+ and K^+) levels were measured by Global Clinical and Viral Laboratories (Amanzimtoti, Durban, South Africa).

The glomerular filtration rate (GFR) of all the animals was determined from the estimation of creatinine in the plasma and urine (creatinine clearance) as follows [33]:

$$\text{GFR [mL/min]} = \frac{\text{Urine creatinine } \left(\frac{\text{mg}}{\text{dl}} \right) \times 24 \text{ hrs urine volume (mL)}}{\text{Plasma creatinine (mg/dl)} \times 60 \text{ min} \times 24 \text{ hours}}$$

Kidney total antioxidant status (TAS) levels were measured using the assay kit as instructed by the manufacturer (Elabsience Biotechnology Co., Ltd., Houston, TX, USA). Kidney inflammatory markers (IL-6, TNF- α) as well as plasma kidney injury molecule (KIM-1) and aldosterone concentrations were determined from their specific ELISA kits as instructed by the manufacturer (Elabsience Biotechnology Co., Ltd., Houston, TX, USA).

2.8 Quantitative real-time PCR

Ribonucleic acid (RNA) was isolated from urine by using the ZR Urine RNA Isolation Kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocol. The purity of the RNA was confirmed by the relative absorbance ratio of 260/280 nm via a Nanodrop 1000 spectrophotometer (Thermo Scientific, Santa Clara, CA, USA).

The cDNA synthesis kit was used to produce reverse transcription reactions using 2 μg of urine RNA as described by the manufacturer (New England Biolabs, Ipswich, MA, USA). The Luna Universal qPCR Master Mix was utilized for amplification (New England Biolabs, Ipswich, MA, USA). All samples were run in duplicate and β -actin mRNA was used as a housekeeping gene. Table 1 below shows the primer sequences (Inqaba Biotec, Pretoria, South Africa) that were utilised in this study. The cycling conditions were as follows: initial denaturation at 95°C for 60 seconds, followed by 45 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 30 seconds. A melt curve analysis was performed from 60°C to 95°C according to instrument-specific recommendations. The $2^{-\Delta\Delta\text{Ct}}$ relative quantification method was used to represent gene expression values.

Table 1. List of primers used in this study

Gene of interest	Sequence
Podocin	Forward: 5'- TGGAAAGCTGAGG CACAAAGA-3'
	Reverse: 5'- AGAATCTCAGCCGCCATCCT-3'
β -actin	Forward: 5'-AGTGCCAGCCTCGTCTCATA-3'
	Reverse: 5'-GATGGTGATGGGTTTCCCGT-3'

2.9 Statistical analysis

The results were expressed as mean $\pm$ SEM. Normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was assessed using the Bartlett's test. Fluid intake and urine output, were analysed using a two-way analysis of variance (ANOVA), whereas data collected at a single time point were analysed using one-way ANOVA. The Tukey–Kramer post hoc test was used for multiple comparisons. Statistical significance was determined at $p < 0.05$.

3. Results

3.1 Kidney weights

Kidney weights were assessed in all experimental animals at the end of the study period ($n = 6$ per group). As shown in Figure 1, the PD control group exhibited a significant increase ($p = < 0.0001$) in kidney weight compared to the NPD group. In contrast, both the GTIN + ND ($p = < 0.0001$) and GTIN + HFHC ($p = < 0.0001$) groups demonstrated significantly reduced kidney weights relative to the PD group. A similar reduction ($p = < 0.0001$) was observed in the MET + ND group when compared to the PD group.

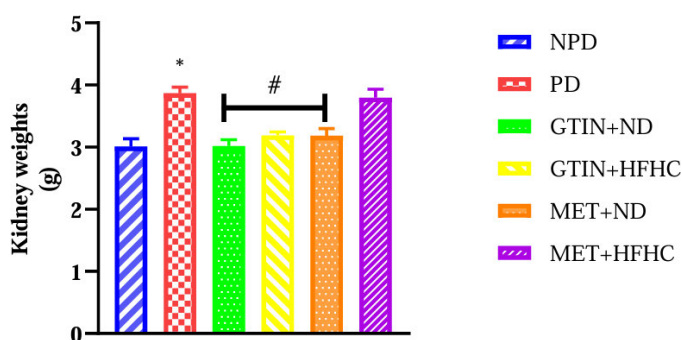


Figure 1. Effects of GTIN and MET with or without dietary intervention on kidney weight in diet-induced prediabetes after 12 weeks of treatment ($n = 6$ per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.2 Fluid intake and urine output

Fluid intake and urine output were monitored in all groups from week 20 to week 32 of the study period ($n = 6$ per group). As illustrated in Figure 2, the PD group displayed a significant increase both fluid intake ($p = < 0.0001$) and urine output ($p = < 0.0001$) throughout the treatment period compared to the NPD group. In contrast, the GTIN + ND and GTIN + HFHC groups exhibited significantly reduced ($p < 0.05$) fluid intake and urine output from week 24 to week 32 relative to the PD group. Similarly, both the MET + ND and MET + HFHC groups showed significant reductions ($p < 0.05$) in these parameters over the same time frame compared to the PD group.

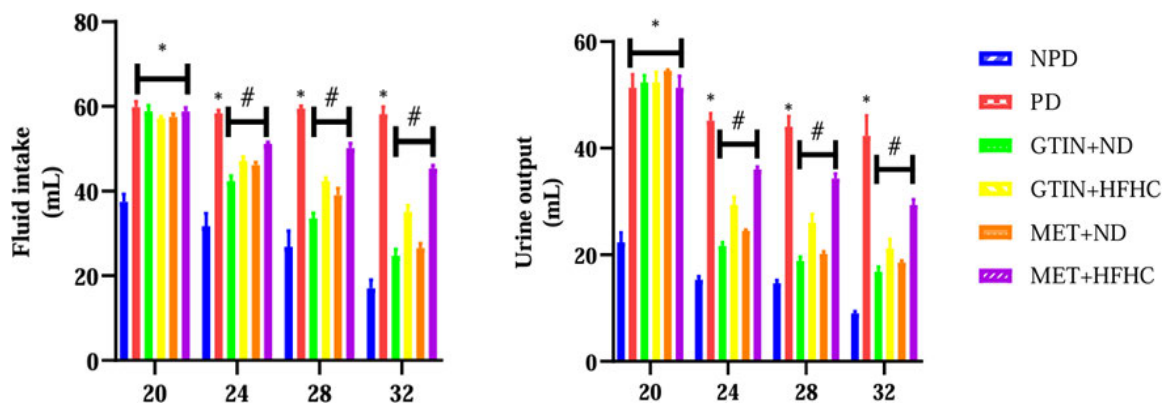


Figure 2. Effects of GTIN and MET with or without dietary intervention on fluid intake and urine output in diet-induced prediabetes after 12 weeks of treatment ($n = 6$ per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.3 Glomerular filtration rate (GFR)

Glomerular filtration rate (GFR) was assessed in all animals at the end of the study period ($n = 6$ per group). As shown in Figure 3, the PD group exhibited a significant reduction ($p = < 0.0001$) in GFR compared to the NPD group. In contrast, both the GTIN + ND ($p = < 0.0001$) and GTIN + HFHC ($p = < 0.0001$) groups demonstrated significantly increased GFR values relative to the PD group. Similarly, the MET + ND ($p = < 0.0001$) and MET + HFHC ($p = < 0.0001$) groups showed significantly elevated GFR compared to the PD group.

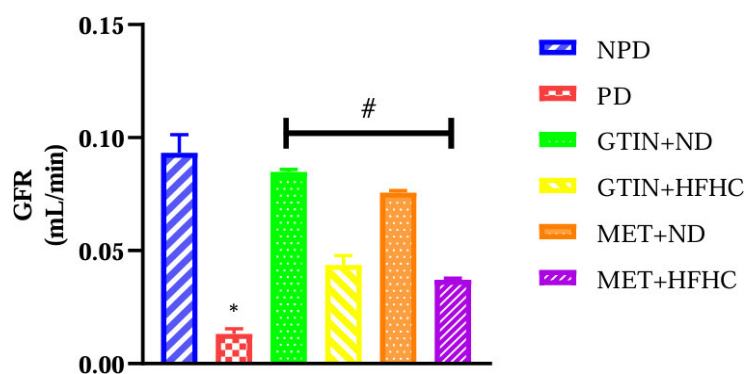


Figure 3. Effects of GTIN and MET with or without dietary intervention on glomerular filtration rate (GFR) in diet-induced prediabetes after 12 weeks of treatment (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.4 Plasma and urine creatinine

Plasma and urine creatinine levels were evaluated in all animals at the end of the study period (n = 6 per group). As presented in Figure 4, the PD group showed a significant increase in plasma creatinine ($p < 0.0001$) and a decrease in urine creatinine levels ($p < 0.0001$) compared to the NPD group. In contrast, the GTIN + ND and GTIN + HFHC groups exhibited significantly lower plasma creatinine ($p < 0.0001$, $p < 0.0001$) and higher urine creatinine levels ($p < 0.0001$, $p < 0.0001$) relative to the PD group. Similarly, both the MET + ND and MET + HFHC groups demonstrated significant improvements ($p < 0.05$) in these parameters when compared to the PD group.

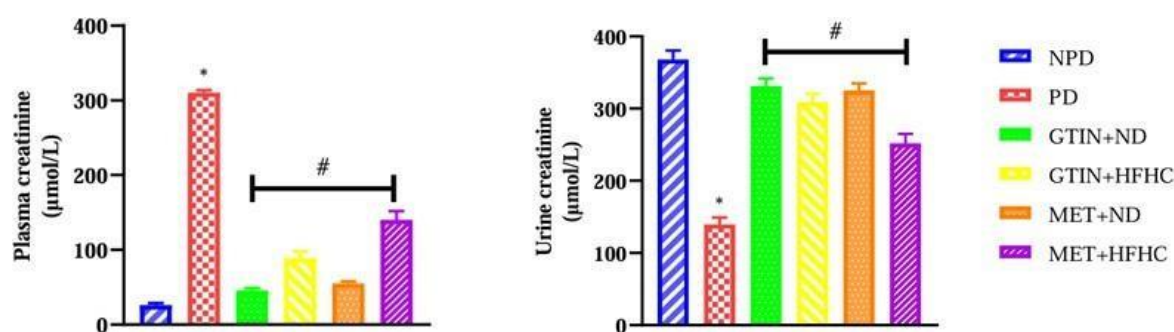


Figure 4. Effects of GTIN and MET with or without dietary intervention on plasma and urine creatinine levels in diet-induced prediabetes after 12 weeks of treatment (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.5 Plasma and urine albumin

Plasma and urine albumin levels were assessed in all animals at the end of the study period (n = 6 per group). As illustrated in Figure 5, the PD group exhibited significantly lower plasma albumin ($p < 0.0001$) and higher urine albumin ($p < 0.0001$) levels compared to the NPD group. In contrast, both

the GTIN + ND and GTIN + HFHC groups showed significantly increased plasma albumin ($p = < 0.0001$, $p = < 0.0001$) and decreased urine albumin levels ($p = < 0.0001$, $p = < 0.0001$) relative to the PD group. Similarly, the MET + ND group demonstrated a significant improvement in both parameters ($p < 0.05$) when compared to the PD group.

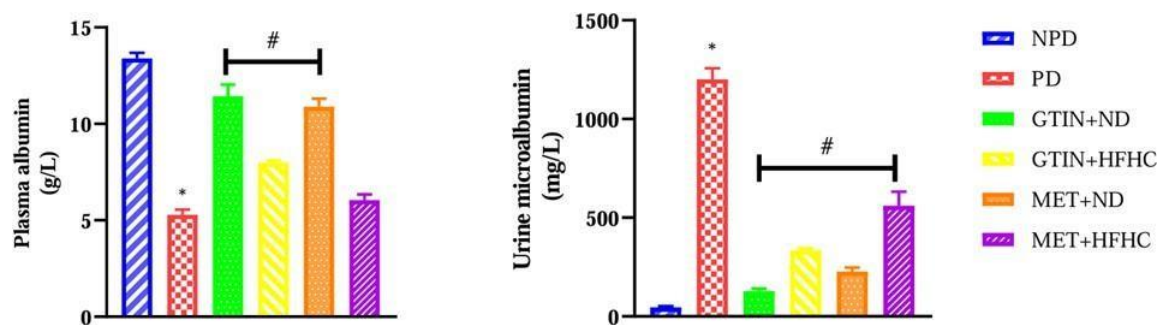


Figure 5. Effects of GTIN and MET with or without dietary intervention on plasma and urine albumin levels in diet-induced prediabetes after 12 weeks of treatment ($n = 6$ per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.6 Plasma and urine urea

Plasma and urine urea levels were evaluated in all animals at the end of the study period ($n = 6$ per group). As depicted in Figure 6, the PD group exhibited a significant increase in plasma urea ($p = < 0.0001$) and a decrease in urine urea levels ($p = < 0.0001$) compared to the NPD group. In contrast, both the GTIN + ND and GTIN + HFHC groups demonstrated significantly lower plasma urea ($p = < 0.0001$, $p = < 0.0001$) and higher urine urea levels ($p = < 0.0001$, $p = < 0.0001$) relative to the PD group. Similarly, the MET + ND and MET + HFHC groups also showed significant improvements in these parameters ($p < 0.05$) when compared to the PD group.

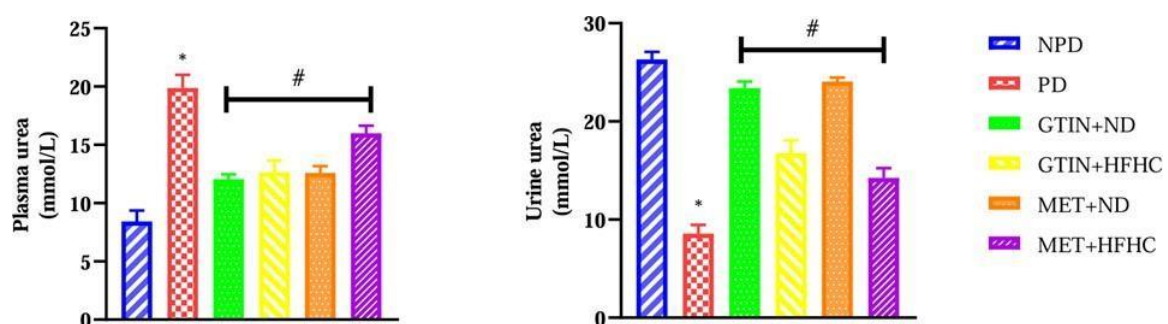


Figure 6. Effects of GTIN and MET with or without dietary intervention on plasma and urine urea levels in diet-induced prediabetes after 12 weeks of treatment ($n = 6$ per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.7 Plasma and urine uric acid

Plasma and urine uric acid levels were measured in all animals at the end of the study period ($n = 6$ per group). As illustrated in Figure 7, the PD group showed significantly elevated plasma uric acid ($p = < 0.0001$) and reduced urine uric acid ($p = < 0.0001$) levels compared to the NPD group. In contrast, both the GTIN+ ND and GTIN + HFHC groups exhibited significantly lower plasma uric acid ($p = < 0.0001$, $p = < 0.0001$) and higher urine uric acid levels ($p = < 0.0001$, $p = < 0.0001$) relative to the PD group. Similarly, the MET + ND and MET + HFHC groups demonstrated significant improvements in these parameters ($p < 0.05$) when compared to the PD group.

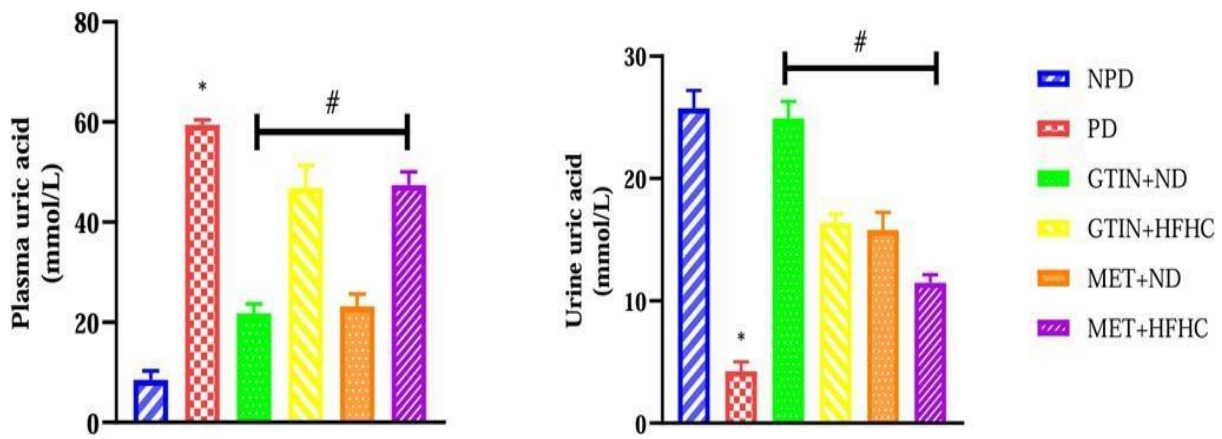


Figure 7. Effects of GTIN and MET with or without dietary intervention on plasma and urine uric acid levels in diet-induced prediabetes after 12 weeks of treatment ($n = 6$ per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.8 Plasma and urine sodium

Plasma and urine sodium levels were assessed in all animals at the end of the study period ($n = 6$ per group). As shown in Figure 8, the PD group exhibited significantly elevated plasma sodium ($p = < 0.0001$) and reduced urine sodium levels ($p = < 0.0001$) compared to the NPD group. The GTIN + ND group demonstrated significantly lower plasma sodium ($p = < 0.0001$) and higher urine sodium levels ($p = < 0.0001$) relative to the PD group. Similarly, the MET + ND group showed a significant reduction in plasma sodium levels ($p < 0.05$) compared to the PD group. Although both the MET + ND and MET + HFHC groups displayed higher urine sodium levels than the PD group, these differences were not statistically significant ($p > 0.05$).

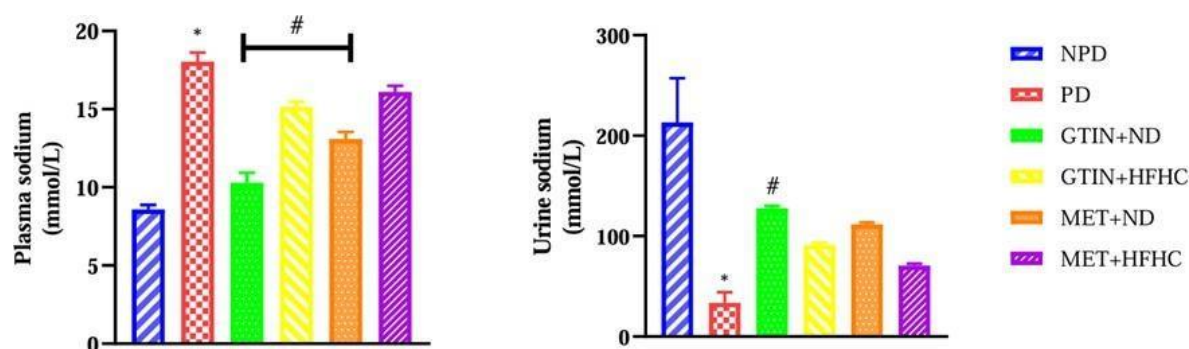


Figure 8. Effects of GTIN and MET with or without dietary intervention on plasma and urine sodium levels in diet-induced prediabetes after 12 weeks of treatment (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.9 Plasma and urine potassium

Plasma and urine potassium levels were measured in all animals at the end of the study period (n = 6 per group). As depicted in Figure 9, the PD group exhibited significantly lower plasma potassium ($p = < 0.0001$) and higher urine potassium levels ($p = < 0.0001$) compared to the NPD group. In contrast, the GTIN + ND and GTIN + HFHC groups showed significantly increased plasma potassium ($p = < 0.0001$, $p = < 0.0001$) and decreased urine potassium levels ($p = < 0.0001$, $p = < 0.0001$) relative to the PD group. Similarly, the MET + ND and MET + HFHC groups demonstrated significant improvements in potassium regulation, with higher plasma and lower urine potassium levels ($p < 0.05$) compared to the PD group.

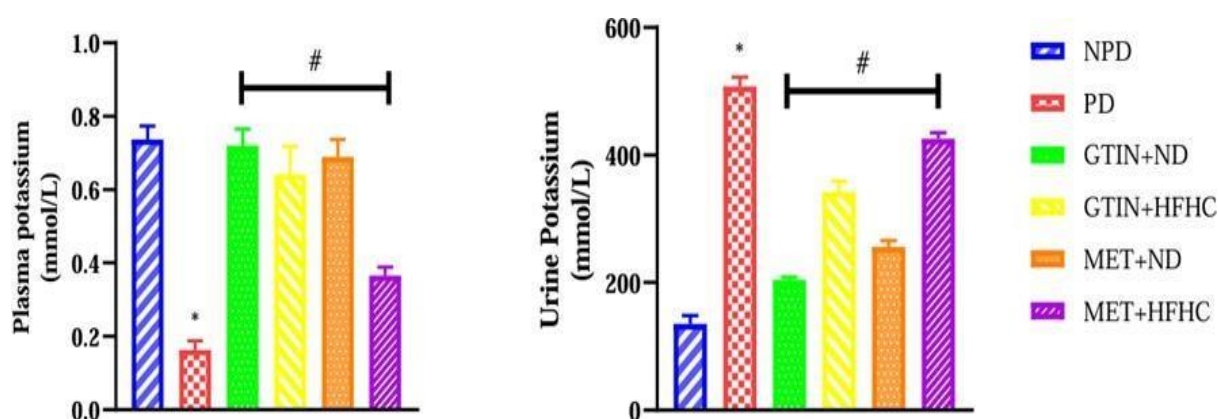


Figure 9. Effects of GTIN and MET with or without dietary intervention on plasma and urine sodium levels in diet-induced prediabetes after 12 weeks of treatment (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.10 Plasma aldosterone

Plasma aldosterone levels were measured in all animals at the end of the study period ($n = 6$ per group). As shown in Figure 10, the PD group exhibited significantly elevated plasma aldosterone levels ($p = < 0.0001$) compared to the NPD group. In contrast, both the GTIN + ND ($p = < 0.0001$) and GTIN + HFHC ($p = < 0.0001$) groups demonstrated significantly reduced plasma aldosterone levels relative to the PD group. Similarly, the MET + ND group showed a significant decrease ($p = < 0.0001$) in plasma aldosterone compared to the PD group.

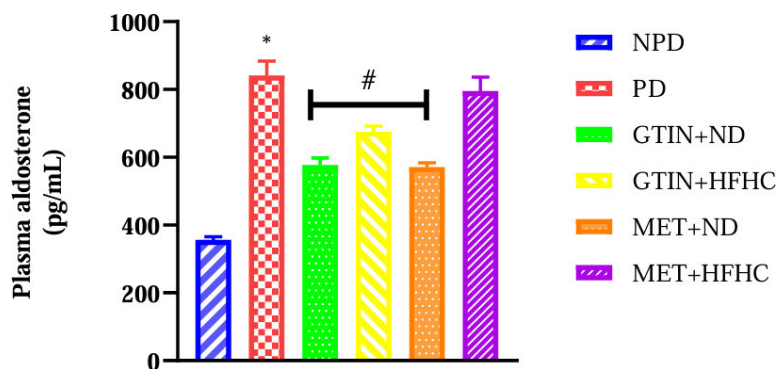


Figure 10. Effects of GTIN and MET with or without dietary intervention on plasma aldosterone levels in diet-induced prediabetes after 12 weeks of treatment ($n = 6$ per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.11 Kidney oxidative status and inflammatory markers

Kidney oxidative status and inflammatory marker levels in all animals were measured at the end of the study period ($n = 6$ per group). The results (Table 2) showed that the PD group had significantly higher kidney MDA levels ($p = < 0.0001$) and lower total antioxidant status (TAS) ($p = < 0.0001$) in comparison to the NPD group. This was accompanied by significantly higher kidney IL-6 ($p = < 0.0001$) and TNF- α levels ($p = < 0.0001$) in comparison to the NPD group. However, the GTIN + ND and GTIN + HFHC groups showed significantly lower kidney MDA ($p = < 0.0001$, $p = < 0.0001$) levels and higher TAS ($p = < 0.0001$, $p = < 0.0001$) in comparison to the PD group. This was accompanied by significantly lower kidney IL-6 and TNF- α levels in comparison to the PD control. Similarly, the MET + ND and MET+HFHC groups showed significantly ($p < 0.05$) lower kidney MDA levels and higher TAS levels. This was accompanied by significantly ($p < 0.05$) lower kidney IL-6 and TNF- α levels in comparison to PD group.

Table 2. Effects of GTIN and MET with or without dietary intervention on kidney oxidative status and inflammatory markers in diet-induced prediabetes after 12 weeks of treatment (n=6, in each group). Values are expressed as mean $\pm$ SEM.

Parameters	Experimental groups					
	NPD	PD	GTIN+ND	GTIN+HFHC	MET+ND	MET+HFHC
MDA (mmol/L)	121.80 $\pm$ 6.92	408.00 $\pm$ 25.48*	136.40 $\pm$ 5.29#	158.30 $\pm$ 11.81#	151.30 $\pm$ 13.58#	173.70 $\pm$ 13.84#
TAS (mmol Trolox/ kg wet wgt)	1.28 $\pm$ 0.070	0.28 $\pm$ 0.018*	0.91 $\pm$ 0.017#	0.52 $\pm$ 0.03#	0.80 $\pm$ 0.016#	0.44 $\pm$ 0.021#
IL-6 (pg/mL)	21.53 $\pm$ 1.51	345.60 $\pm$ 7.34*	39.00 $\pm$ 0.94#	60.01 $\pm$ 0.76#	52.50 $\pm$ 1.30#	243.90 $\pm$ 22.82#
TNF-α (pg/mL)	25.12 $\pm$ 1.22	447.80 $\pm$ 113.40*	62.64 $\pm$ 1.75#	66.24 $\pm$ 2.30#	40.99 $\pm$ 2.20#	246.30 $\pm$ 33.90#

* = $p < 0.05$ compared to NPD; # = $p < 0.05$ compared to PD.

3.12 Plasma kidney injury molecule-1 (KIM-1)

Plasma KIM-1 levels were measured in all animals at the end of the study period (n = 6 per group). As illustrated in Figure 11, the PD group exhibited significantly elevated plasma KIM-1 levels ($p = < 0.0001$) compared to the NPD group. In contrast, both the GTIN + ND ($p = < 0.0001$) and GTIN + HFHC groups ($p = < 0.0001$) showed significantly reduced plasma KIM-1 levels relative to the PD group. Similarly, the MET + ND ($p = < 0.0001$) and MET + HFHC ($p = < 0.0001$) groups demonstrated significant decreases in plasma KIM-1 compared to the PD group.

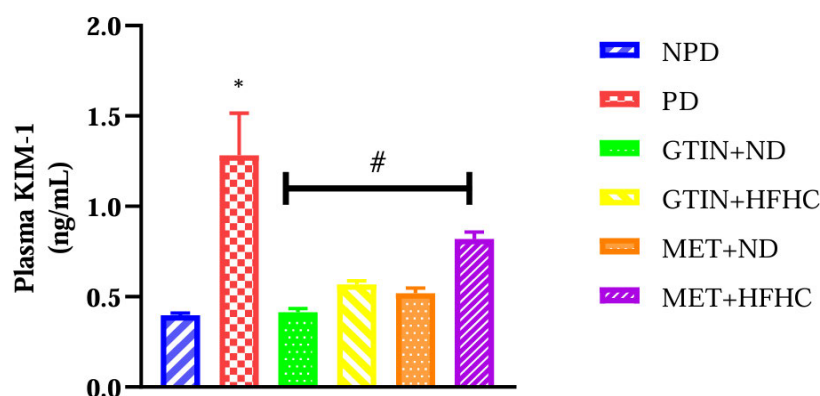


Figure 11. Effects of GTIN and MET with or without dietary intervention on plasma KIM-1 levels in diet-induced prediabetes after 12 weeks of treatment (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

3.13 Urine podocin mRNA relative expression

Urine podocin mRNA relative expression levels were measured in all animals at the end of the study period (n = 6 per group). As shown in Figure 12, the PD group exhibited significantly higher urine podocin mRNA expression ($p = < 0.0001$) compared to the NPD group. In contrast, both the GTIN + ND ($p = < 0.0001$) and GTIN + HFHC ($p = < 0.0001$) groups demonstrated significantly reduced urine podocin mRNA levels relative to the PD group. Similarly, the MET + ND ($p = < 0.0001$) and MET + HFHC groups ($p = < 0.0001$) showed significant decreases in podocin mRNA expression compared to the PD group ($p < 0.05$).

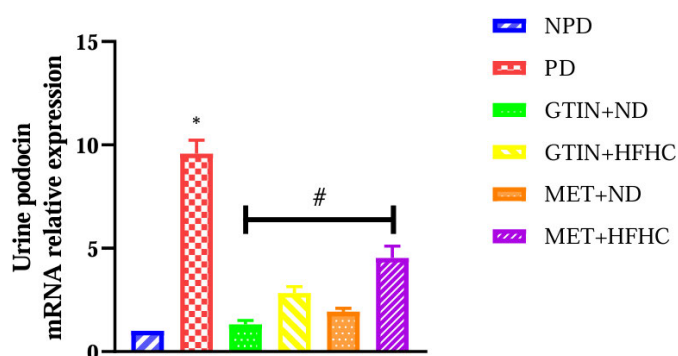


Figure 12. Effects of GTIN and MET with or without dietary intervention on urine podocin mRNA relative expression in diet-induced prediabetes after 12 weeks of treatment (n = 6 per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. NPD; # $p < 0.05$ vs. PD.

4. Discussion

High-calorie diets commonly seen in westernized lifestyles have been shown to promote the development of PD [34]. Several studies have shown that renal complications observed in T2DM begin during the prediabetic state [5, 10]. It was reported that approximately 4.5%–26% of

individuals with prediabetes exhibit early signs of kidney dysfunction [35]. The combination of dietary and pharmacological interventions has been widely accepted for managing diabetes-associated renal complications [36, 37]. However, poor compliance to dietary intervention by patients has limited the effectiveness of pharmacological therapies [38]. Thus, there is a need to identify compounds that are effective in both the presence and absence of dietary intervention. Flavonoids have previously been shown to exhibit reno-protective effects by reducing oxidative stress and inflammation in the kidneys [39, 40]. GTIN is a natural flavonoid found in *Hibiscus* plant species, which exhibits therapeutic potential [41]. Recent studies have demonstrated that GTIN possesses potent antioxidant, anti-inflammatory and antihyperglycaemic effects in the prediabetic state [22, 31, 42]. However, no studies have investigated the effects of GTIN on kidney function in diet-induced prediabetes. Hence, this study investigated the effects of GTIN on kidney function in the presence and absence of dietary intervention in a diet-induced prediabetic rat model.

Fluid intake and urine output are essential markers of fluid balance and kidney function [43]. In the PD state, fluid intake and urine output increase due to disruptions in glucose metabolism [10, 14]. Moderate hyperglycaemia promotes mild glucosuria which triggers osmotic diuresis and increases thirst [44]. Moreover, prediabetes-induced oxidative stress and inflammation impairs the ability of the kidneys to concentrate urine, contributing to polyuria and polydipsia [45]. These functional disturbances have been shown to be accompanied by structural changes in the kidneys [6]. Notably, kidney weight increases during prediabetes due to renal hypertrophy, inflammation and extracellular matrix accumulation [5, 6]. In this study, the PD group showed significantly higher fluid intake and urine output throughout the treatment period. This was accompanied by significantly higher kidney weights in comparison to the NPD group. Our findings corroborated with previous studies which have reported increased fluid intake, urine output and kidney weights during prediabetes [10, 46]. However, the GTIN-treated groups showed significantly lower fluid intake and urine output throughout the treatment period. This was accompanied by significantly lower kidney weights in comparison to the PD group. We speculate that GTIN may have reduced fluid intake and urine output by improving the concentrating ability of the kidneys and limiting glucosuria. The observed reduction in kidney weights may be attributed to the antioxidant and anti-inflammatory effects of GTIN which may have mitigated renal hypertrophy [22, 31]. Additionally, improved peripheral glucose handling following GTIN treatment may have contributed to reduced renal glucose exposure. Flavonoids such as quercetin have been shown to enhance insulin signalling and GLUT4 translocation in insulin-responsive tissues, thereby increasing glucose uptake and reducing circulating glucose levels [47, 48]. Although GLUT4 expression and translocation were not measured in this study, improved glucose disposal may have indirectly reduced renal stress associated with hyperglycaemia.

Similarly, the MET-treated groups showed significantly lower fluid intake and urine output throughout the treatment period. Additionally, the MET+ND group showed significantly lower kidney

weights in comparison to the PD group. Studies have shown that MET lowers blood glucose, thereby reducing renal glucose filtration and urine output [49, 50]. Metformin also reduces oxidative stress and inflammation-induced renal hypertrophy in T2DM [51]. While the MET-treated groups were effective in reducing these parameters in the PD state, GTIN exhibited more pronounced effects.

GFR reflects the volume of plasma filtered through the glomeruli into Bowman's capsule per unit time [52]. In prediabetes, GFR is elevated as a compensatory response to moderate insulin resistance and elevated blood glucose levels [53]. However, sustained glomerular hyperfiltration induces oxidative stress on glomerular structures, contributing to renal injury [54]. Notably, studies have shown that albuminuria may be present before the onset of T2DM [8, 28]. Low-grade inflammation and oxidative stress, as seen in prediabetes, compromises the glomerular filtration barrier, promoting albumin leakage into urine [55]. In this study, the PD group showed significantly lower GFR in comparison to the NPD group. The PD group also showed significantly higher plasma creatinine and lower urine creatinine levels. This was accompanied by significantly lower plasma albumin and higher urine albumin in comparison to the NPD group. The low GFR may suggest that the PD group has progressed beyond compensatory hyperfiltration and exhibits early renal impairment. The disturbed plasma and urine creatinine levels may suggest impaired renal clearance and filtration capacity in the PD state. The reduced plasma albumin and increased urine albumin may suggest glomerular barrier dysfunction.

However, the GTIN-treated groups showed significantly higher GFR in comparison to the PD group. The GTIN-treated groups also showed significantly lower plasma creatinine and higher urine creatinine levels. This was accompanied by significantly higher plasma albumin and lower urine albumin in comparison to the PD group. These findings suggest that GTIN may enhance GFR, improve renal clearance and exert renoprotective effects on the glomerular filtration membrane. This improvement may be associated with reduced oxidative damage to glomerular endothelial cells and preservation of filtration barrier integrity. Oxidative stress-induced mitochondrial dysfunction contributes to renal injury by impairing ATP production and promoting inflammatory signalling [56]. Therefore, the antioxidant activity of GTIN may protect renal cells against mitochondrial dysfunction associated with prediabetic metabolic stress [57].

Our findings corroborated with a recent study which showed that GTIN improves GFR in a nephrotoxicity model [29]. This may be due to the ability of GTIN to mitigate insulin resistance, oxidative stress and inflammation [31, 42]. The MET-treated groups showed similar effects on GFR and creatinine levels to the GTIN-treated groups. This was accompanied by significantly higher plasma albumin and lower urine albumin levels in comparison to the PD group. Studies have shown that MET improves GFR and renal clearance by activating adenosine monophosphate-activated protein kinase (AMPK) [51, 58]. AMPK activation promotes vasodilation which enhances renal blood

flow and filtration [58]. MET also reduces oxidative stress and preserves glomerular barrier integrity, thereby decreasing albuminuria [59]. Further studies comparing GTIN directly with MET using molecular markers of AMPK activation, mitochondrial function and inflammatory pathways are required to confirm these mechanisms.

Urea and uric acid are waste products of amino acid and purine metabolism, primarily excreted by the kidneys [60]. In prediabetes, low-grade inflammation and oxidative stress have been shown to impair kidney function [61]. Hyperinsulinaemia associated with prediabetes promotes uric acid reabsorption via upregulation of sodium–hydrogen exchangers in the proximal tubules [62]. Moreover, moderate hyperglycaemia and insulin resistance seen in prediabetes has been shown to increase uric acid synthesis [63]. In this study, the PD group showed significantly higher plasma urea and lower urine urea levels in comparison to the NPD group. This was accompanied by significantly higher plasma uric acid and lower urine uric acid levels. This study's results corroborated with previous studies which have shown similar results in the PD state [5, 63]. The GTIN-treated groups exhibited notably reduced plasma urea concentrations alongside increased urea levels in the urine in comparison to the PD group. Similarly, plasma uric acid levels were significantly decreased, while urinary uric acid levels were elevated. The observed alterations in plasma and urinary levels of urea and uric acid suggest that GTIN may improve renal clearance in the context of prediabetes. It is proposed that the improvement in renal clearance observed with GTIN treatment may be attributed to its modulatory effects on hyperinsulinaemia and moderate insulin resistance. Additionally, renal clearance may have been improved due to the antioxidant and anti-inflammatory effects of GTIN [31, 64]. The improvement in insulin sensitivity following GTIN administration may reduce excessive renal exposure to glucose and metabolic stress. This may subsequently limit mitochondrial dysfunction and ROS generation within renal tubular cells, thereby preserving renal transport processes involved in waste excretion. However, further studies evaluating renal mitochondrial activity and glucose transporter regulation are required to confirm these mechanisms.

The MET-treated groups showed similar effects on plasma and urine urea and uric acid levels as the GTIN-treated groups. Studies have shown that MET enhances renal handling of urea and uric acid by improving insulin sensitivity and glycaemic control [65, 66]. Another study showed that MET reduces uric acid reabsorption by down-regulating sodium–hydrogen exchangers, consequently decreasing purine breakdown [67]. The comparable effects of GTIN and MET suggest that both compounds may improve renal clearance through improved metabolic control. However, GTIN may provide additional benefits due to its antioxidant and anti-inflammatory properties, whereas MET primarily improves renal outcomes through metabolic regulation and AMPK-dependent pathways [68].

Plasma and urine levels of sodium and potassium reflect renal electrolyte regulation [69]. This regulation is controlled by hormonal systems such as the RAAS [69]. Hyperinsulinaemia has been shown to alter sodium and potassium balance [70]. Hyperinsulinaemia increases the activity of

sodium transporters in the proximal tubules, promoting sodium reabsorption and contributing to fluid retention [71]. Moreover, RAAS activation as seen in prediabetes raises aldosterone levels, which increases sodium retention and renal potassium excretion [11]. In our study, the PD group showed significantly higher plasma sodium and lower urine sodium levels in comparison to the NPD group. The PD group also showed significantly lower plasma potassium levels and higher urine potassium levels in comparison to the NPD group. This was accompanied by significantly higher plasma aldosterone levels in the PD group. The altered electrolyte levels in prediabetes may suggest impaired renal filtration. Hyperinsulinaemia may have upregulated renal sodium transporters leading to increased sodium retention. The increased urinary potassium levels in the PD group may be due to RAAS activation and elevated aldosterone.

However, the GTIN+ND group showed significantly lower plasma sodium and higher urine sodium levels in comparison to the PD group. The GTIN+ND and GTIN+HFHC groups also showed significantly lower plasma potassium and higher urine potassium levels in comparison to the PD group. This was accompanied by significantly lower plasma aldosterone levels in comparison to the PD group. This may suggest that GTIN improves renal handling of sodium and potassium during prediabetes. The reduced plasma aldosterone levels in the GTIN-treated groups may have contributed to decreased sodium retention and potassium excretion. The reduction in aldosterone levels following GTIN treatment may be associated with decreased oxidative stress and inflammatory activation, as oxidative stress has been implicated in excessive RAAS activation [72]. Additionally, improved insulin sensitivity may reduce hyperinsulinaemia-mediated sodium retention [73]. The MET+ND group showed significantly lower plasma sodium with non-significantly higher urine sodium in comparison to the PD group. The

MET-treated groups showed similar effects on plasma and urine potassium levels as the GTIN-treated groups. This was accompanied by significantly lower plasma aldosterone in comparison to the PD group. Studies have shown that MET enhances insulin sensitivity and exerts stimulatory effects on renal sodium transporters [66, 73]. This leads to decreased sodium reabsorption in the proximal tubules [73]. Additionally, MET inhibits oxidative stress and inflammation-induced RAAS activation [74]. This reduces renal sodium retention and potassium excretion [73]. It was observed that the most favourable health outcomes in renal electrolyte handling may be achieved when treatment is combined with dietary intervention.

Malondialdehyde (MDA) levels in the kidney is a key marker of lipid peroxidation and oxidative stress [75]. Kidney total antioxidant status (TAS) reflects the overall enzymatic and non-enzymatic antioxidant capacity of renal tissue [76]. In prediabetes, moderate hyperglycaemia and insulin resistance stimulate reactive oxygen species (ROS) production through mitochondrial dysfunction, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and advanced glycation end-products (AGEs) formation [77]. Excess ROS in prediabetes damage renal cells and deplete

antioxidant defenses evidenced by reduced kidney TAS levels [78]. The decline in TAS contributes to cellular injury, fibrosis and promotes inflammatory responses [78]. Studies have shown the presence of low-grade inflammation in kidney tissue during the PD state, evidenced by elevated interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) [6, 79]. In this study, the PD group showed significantly higher kidney MDA and lower TAS levels in comparison to the NPD group. This was accompanied by significantly higher kidney IL-6 and TNF- α levels in comparison to the NPD group. These findings may suggest the presence of renal oxidative stress and low-grade inflammation in the PD state.

Renal tissue from GTIN-treated groups demonstrated lower MDA and higher TAS levels in comparison to PD group. This was accompanied by significantly lower kidney inflammatory markers in comparison to the PD group. These findings may suggest that GTIN reduces lipid peroxidation and improves antioxidant status in the kidney during prediabetes. Our results corroborated with a recent study which showed that GTIN significantly escalated the antioxidant enzymes activities accompanied by alleviation in MDA levels in a nephrotoxicity model [29]. The significant decrease in inflammatory markers may suggest that GTIN exerts anti-inflammatory effects within renal tissue. The antioxidant effects of GTIN may be mediated through activation of endogenous antioxidant pathways and suppression of oxidative stress-induced inflammatory signalling. In contrast to MET, which reduces inflammation mainly through AMPK activation and inhibition of NF- κ B signalling, GTIN may suppress inflammatory pathways through its flavonoid-mediated free radical scavenging and antioxidant actions [80, 81]. The MET-treated groups showed similar effects on kidney MDA, TAS and inflammatory markers in comparison to the PD group. Studies have shown that MET activates AMPK, which enhances antioxidant enzyme expression and reduces ROS production [82, 83]. It also suppresses nuclear factor kappa B (NF- κ B) signalling and limits immune cell infiltration, reducing IL-6 and TNF- α levels [84].

KIM-1 is an early biomarker of tubular damage which is expressed following injury [85]. Podocin is a crucial podocyte protein that maintains the selective permeability of the glomerular filtration barrier [86]. In prediabetes, moderate insulin resistance, oxidative stress and low-grade inflammation contribute to subclinical tubular injury [87, 88]. This down-regulates the expression of key slit diaphragm proteins such as podocin which compromises podocyte function [88]. Consequently, this leads to podocyte detachment and increased urinary podocin excretion [88]. In this study, the PD group showed significantly higher plasma KIM-1 levels and urine podocin mRNA expression in comparison to the NPD group. These findings may suggest that the PD state is associated with early tubular and podocyte injury. However, the GTIN-treated groups showed significantly lower plasma KIM-1 levels and urine podocin mRNA expression in comparison to the PD group. This may suggest that GTIN attenuates early tubular injury and podocyte injury in the PD state. The renoprotective effects of GTIN may be attributed to its ability to reduce oxidative stress-induced cellular injury [22, 64]. Mitochondrial dysfunction caused by excessive ROS production is an important contributor to

tubular epithelial injury and podocyte damage [89]. By improving antioxidant capacity and reducing inflammatory activation, GTIN may preserve renal cellular integrity. Furthermore, inhibition of inflammatory pathways such as NF- κ B may contribute to reduced cytokine production and protection against progressive renal injury [29, 81]. Similarly, the MET-treated groups showed significantly lower plasma KIM-1 levels and urine podocin mRNA levels in comparison to the PD group. Studies have shown that MET preserves renal tubular and podocyte integrity by lowering hyperglycaemia-induced oxidative stress and inflammation through AMPK activation [58, 59]. The comparable effects of GTIN and MET on KIM-1 and podocin suggest that both compounds may prevent early renal damage associated with prediabetes. However, MET has a well-established clinical profile, whereas GTIN requires further investigation to determine whether similar renal protective effects can be achieved in humans [90, 91].

Improvements in renal function parameters following gossypetin treatment were apparent both in the presence and absence of dietary intervention. The results demonstrated that GTIN significantly reduced fluid intake, urine output and kidney weights in both the presence and absence of dietary intervention in comparison to the PD group. Additionally, GTIN administration improved GFR, creatinine, albumin, urea and uric acid levels. It also improved renal sodium and potassium handling along with reduced RAAS activation. Gossypetin also decreased lipid peroxidation and inflammation in the kidney, evidenced by lower kidney MDA, IL-6, TNF- α and higher TAS levels. Additionally, GTIN reduced kidney injury markers including plasma KIM-1 and urinary podocin mRNA expression. This may suggest that GTIN may exhibit reno-protective effects in both the presence and absence of diet intervention during prediabetes.

However, translation of these findings from animal models to human application requires further investigation. Flavonoids frequently demonstrate limited systemic availability due to poor absorption and rapid conjugation; therefore, studies are required to determine whether effective renal concentrations of GTIN can be achieved following oral administration in humans [92, 93]. Furthermore, long-term toxicity studies are required to establish the safety profile of GTIN, particularly with chronic administration. Although the dose used in this study (15 mg/kg body weight) demonstrated beneficial effects in rats, dose-response studies are required to determine the optimal therapeutic range and whether equivalent doses can be safely translated to humans [23, 24].

5. Conclusions

In both the presence and absence of dietary modification, GTIN administration, reduces fluid intake, urine output and kidney weight, while improving GFR, creatinine, albumin, urea and uric acid levels in diet-induced prediabetes. Furthermore, GTIN contributed to improved fluid balance and renal electrolyte regulation, while attenuating activation of the RAAS. The renoprotective effects of GTIN were further supported by reductions in renal oxidative stress, inflammatory responses, and markers

of kidney injury. Importantly, these beneficial effects were observed irrespective of dietary intervention, suggesting that GTIN may represent a potential adjunctive strategy for limiting early renal dysfunction associated with the pre-diabetic state.

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Abbreviations

The following abbreviations are used in this manuscript:

GFR	Glomerular filtration rate
GTIN	Gossypetin
HFHC	High fat high carbohydrate
IL-6	Interleukin-6
KIM-1	Kidney injury molecule-1
MDA	Malondialdehyde
MET	Metformin
RAAS	Renin-angiotensin-aldosterone system
TAS	Total antioxidant status
TNF- α	Tumour Necrosis Factor-alpha

References

1. Clemente-Suárez, V. J.; Beltrán-Velasco, A. I.; Redondo-Flórez, L.; Martín-Rodríguez, A.; Tornero-Aguilera, J. F., Global impacts of western diet and its effects on metabolism and health: a narrative review. *Nutrients* 2023, 15, (12), 2749.
2. Beulens, J.; Rutters, F.; Ryden, L.; Schnell, O.; Mellbin, L.; Hart, H.; Vos, R., Risk and management of pre-diabetes. *European journal of preventive cardiology* 2019, 26, (2_suppl), 47–54.
3. He, P.; Deng, Y.; Dong, S.; Li, H.; Liu, C.; Ma, Y.; Tang, C.; Zhang, M., Association of different domains of physical activity with diabetic kidney disease: a population-based study. *Frontiers in Endocrinology* 2024, 15, 1364028.
4. Siboto, A.; Akinnuga, A. M.; Khumalo, B.; Ismail, M. B.; Booysen, I. N.; Sibiya, N. H.; Ngubane, P.; Khathi, A., Ameliorative Effects of a Rhenium (V) Compound with Uracil-Derived Ligand Markers Associated with Hyperglycaemia-Induced Renal Dysfunction in Diet-Induced Prediabetic Rats. *International Journal of Molecular Sciences* 2022, 23, (23), 15400.
5. Shilpasree, A. S.; Patil, V. S.; Revanasiddappa, M.; Patil, V. P.; Ireshnavar, D., Renal dysfunction in prediabetes: confirmed by glomerular hyperfiltration and albuminuria. *Journal of Laboratory Physicians* 2021, 13, (03), 257–262.
6. Fontalvo, J. R.; Soler, M. J.; Arnedo, R. D.; Navarro-Blackaller, G.; Medina-González, R.; Yáñez, T. R.; Cardona-Blanco, M.; Cabrales-Juan, J.; Uparrela-Gulfo, I.; Chávez-Iñiguez, J. S., Prediabetes and CKD: Does a causal relationship exist. *Nefrología (English Edition)* 2024, 44, (5), 628–638.
7. Vallon, V.; Nakagawa, T., Renal tubular handling of glucose and fructose in health and disease. *Comprehensive Physiology* 2022, 12, (1), 2995–3044.
8. Melsom, T.; Schei, J.; Stefansson, V. T.; Solbu, M. D.; Jenssen, T. G.; Mathisen, U. D.; Wilsgaard, T.; Eriksen, B. O., Prediabetes and Risk of Glomerular Hyperfiltration and Albuminuria in the General Nondiabetic Population: A Prospective Cohort Study. *Am J Kidney Dis* 2016, 67, (6), 841–50.
9. Kanbay, M.; Copur, S.; Bakir, C. N.; Covic, A.; Ortiz, A.; Tuttle, K. R., Glomerular

hyperfiltration as a therapeutic target for CKD. *Nephrology Dialysis Transplantation* 2024, 39, (8), 1228–1238.

10. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Preventing the onset of diabetes-induced chronic kidney disease during prediabetes: The effects of oleanolic acid on selected markers of chronic kidney disease in a diet-induced prediabetic rat model. *Biomed Pharmacother* 2021, 139, 111570.

11. Mkhize, B. C.; Mosili, P.; Ngubane, P. S.; Sibiyi, N. H.; Khathi, A., Diet-induced prediabetes: Effects on the activity of the renin–angiotensin–aldosterone system in selected organs. *Journal of Diabetes Investigation* 2022, 13, (5), 768–780.

12. Kim, G. S.; Oh, H. H.; Kim, S. H.; Kim, B. O.; Byun, Y. S., Association between prediabetes (defined by HbA1(C), fasting plasma glucose, and impaired glucose tolerance) and the development of chronic kidney disease: a 9-year prospective cohort study. *BMC Nephrol* 2019, 20, (1), 130.

13. Colafella, K. M. M.; Bovée, D. M.; Danser, A. J., The renin-angiotensin-aldosterone system and its therapeutic targets. *Experimental eye research* 2019, 186, 107680.

14. Kayumov, U. K.; Kalandarova, U. A., Clinical symptoms in patients with prediabetes. *J Multidiscip Eng Sci Technol (JMEST)* 2019, 6, (7).

15. Ferreira, G.; Vieira, P.; Alves, A.; Nunes, S.; Preguiça, I.; Martins-Marques, T.; Ribeiro, T.; Girão, H.; Figueirinha, A.; Salgueiro, L.; Pintado, M.; Gomes, P.; Viana, S.; Reis, F., Effect of Blueberry Supplementation on a Diet-Induced Rat Model of Prediabetes—Focus on Hepatic Lipid Deposition, Endoplasmic Stress Response and Autophagy. In *Nutrients*, 2024; Vol. 16, p 513.

16. Shevalye, H.; Lupachyk, S.; Watcho, P.; Stavniichuk, R.; Khazim, K.; Abboud, H. E.; Obrosova, I. G., Prediabetic nephropathy as an early consequence of the high-calorie/high-fat diet: relation to oxidative stress. *Endocrinology* 2012, 153, (3), 1152–1161.

17. Giannese, D.; D'Alessandro, C.; Panichi, V.; Pellegrino, N.; Cupisti, A., Nutritional treatment as a synergic intervention to pharmacological therapy in CKD patients. *Nutrients* 2023, 15, (12), 2715.

18. Onyenwenyi, C.; Ricardo, A. C., Impact of lifestyle modification on diabetic kidney disease. *Current diabetes reports* 2015, 15, (9), 60.

19. Natesan, V.; Kim, S.-J., Natural compounds in kidney disease: therapeutic potential and drug development. *Biomolecules & Therapeutics* 2024, 33, (1), 39.

20. Gomes, I. B.; Porto, M. L.; Santos, M. C. L.; Campagnaro, B. P.; Pereira, T. M.; Meyrelles, S. S.; Vasquez, E. C., Renoprotective, anti-oxidative and anti-apoptotic effects of oral low-dose quercetin in the C57BL/6J model of diabetic nephropathy. *Lipids in Health and Disease* 2014, 13, (1), 184.

21. Gulshreen; Kumar, A.; Sharma, A., Antianxiety and antidepressant activity guided isolation and characterization of gossypetin from *Hibiscus sabdariffa* Linn. calyces. *Journal of Biologically Active Products from Nature* 2019, 9, (3), 205–214.

22. Khan, A.; Manna, K.; Das, D. K.; Kesh, S. B.; Sinha, M.; Das, U.; Biswas, S.; Sengupta, A.;

- Sikder, K.; Datta, S., Gossypetin ameliorates ionizing radiation-induced oxidative stress in mice liver—a molecular approach. *Free Radical Research* 2015, 49, (10), 1173–1186.
23. Naidoo, K.; Khathi, A., Effects of gossypetin on glucose homeostasis in diet-induced pre-diabetic rats. *Molecules* 2024, 29, (18), 4410.
24. Naidoo, K.; Khathi, A., Investigating the effects of gossypetin on cardiovascular function in diet-induced pre-diabetic male Sprague Dawley rats. *International Journal of Molecular Sciences* 2024, 25, (22), 12105.
25. Naidoo, K.; Khathi, A., Investigating the effects of gossypetin on liver health in diet-induced pre-diabetic male Sprague Dawley rats. *Molecules* 2025, 30, (8), 1834.
26. Khathi, A.; Luvuno, M.; Mabandla, M., Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. *PONTE International Journal of Science and Research* 2018, 74, (5).
27. Hostalek, U., Global epidemiology of prediabetes-present and future perspectives. *Clinical diabetes and endocrinology* 2019, 5, (1), 5.
28. Khumalo, B.; Siboto, A.; Akinnuga, A. M.; Sibiya, N.; Khathi, A.; Ngubane, P. S., Momordica balsamina improves glucose handling in a diet-induced prediabetic rat model. *PLoS One* 2023, 18, (12), e0295498.
29. Ijaz, M. U.; Alvi, K.; Khan, H. A.; Imran, M.; Afsar, T.; Almajwal, A.; Amor, H.; Razak, S., Gossypetin mitigates doxorubicin-induced nephrotoxicity: A histopathological and biochemical evaluation. *Journal of King Saud University-Science* 2023, 35, (7), 102830.
30. Lin, H.-H., In vitro and in vivo atheroprotective effects of gossypetin against endothelial cell injury by induction of autophagy. *Chemical Research in Toxicology* 2015, 28, (2), 202–215.
31. Proença, C.; Rufino, A. T.; Santos, I.; Albuquerque, H. M.; Silva, A. M.; Fernandes, E.; Ferreira de Oliveira, J. M. P., Gossypetin is a novel modulator of inflammatory cytokine production and a suppressor of osteosarcoma cell growth. *Antioxidants* 2023, 12, (9), 1744.
32. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Plant-derived oleanolic acid ameliorates markers associated with non-alcoholic fatty liver disease in a diet-induced pre-diabetes rat model. *Diabetes, Metabolic Syndrome and Obesity* 2019, 1953–1962.
33. Siboto, A.; Sibiya, N.; Khathi, A.; Ngubane, P., The Effects of Momordica balsamina Methanolic Extract on Kidney Function in STZ-Induced Diabetic Rats: Effects on Selected Metabolic Markers. *Journal of diabetes research* 2018, 2018, (1), 7341242.
34. Mourouti, N.; Kantaras, P.; Mouratidou, T.; Karaglani, M.; Giménez-Legarre, N.; de Miguel-Etayo, P.; Rurik, I.; Torzsa, P.; Iotova, V.; Tankova, T., Determinants of BMI underreporting in adults from families at high risk for type 2 diabetes in Europe: The Feel4Diabetes study. *Journal of Public Health* 2025, 1–9.
35. Branda, J. I. F.; de Almeida-Pititto, B.; Ferreira, S. R. G., Prevalence of diabetic kidney disease in

prediabetes. *Obesity Medicine* 2019, 15, 100105.

36. Jha, R.; Lopez-Trevino, S.; Kankanamalage, H. R.; Jha, J. C., Diabetes and renal complications: an overview on pathophysiology, biomarkers and therapeutic interventions. *Biomedicines* 2024, 12, (5), 1098.

37. Pradhan, D.; Sahu, P. K.; Purohit, S.; Ranajit, S. K.; Acharya, B.; Sangam, S.; Shrivastava, A. K., Therapeutic interventions for diabetes mellitus-associated complications. *Current Diabetes Reviews* 2025, 21, (8), e030524229631.

38. Menini, S.; Iacobini, C.; Pugliese, G.; Pesce, C., Dietary interventions to contrast the onset and progression of diabetic nephropathy: a critical survey of new data. *Critical reviews in food science and nutrition* 2018, 58, (10), 1671–1680.

39. Vargas, F.; Romecín, P.; García-Guillén, A. I.; Wangesteen, R.; Vargas-Tendero, P.; Paredes, M. D.; Atucha, N. M.; García-Estañ, J., Flavonoids in kidney health and disease. *Frontiers in physiology* 2018, 9, 394.

40. Wati, D. P.; Ilyas, S., Renoprotective potential of flavonoids-rich against doxorubicin-induced in animal models: A review. *International Journal of Applied Pharmaceutics* 2024, 16, (6), 28–37.

41. Diyah, N. W.; Isnaeni; Hidayati, S. W.; Purwanto, B. T.; Siswandono, Design of gossypetin derivatives based on naturally occurring flavonoid in Hibiscus sabdariffa and the molecular docking as antibacterial agents. *Journal of Basic and Clinical Physiology and Pharmacology* 2021, 32, (4), 707–714.

42. Chen, J.-H.; Tsai, C.-W.; Wang, C.-P.; Lin, H.-H., Anti-atherosclerotic potential of gossypetin via inhibiting LDL oxidation and foam cell formation. *Toxicology and applied pharmacology* 2013, 272, (2), 313–324.

43. Gounden, V.; Bhatt, H.; Jialal, I., Renal function tests. In *StatPearls [Internet]*, StatPearls Publishing: 2024.

44. Baynes, K., Diabetes mellitus, obesity, lipoprotein disorders and other metabolic diseases. In *Medicine for Finals and Beyond*, CRC Press: 2022; pp 429–470.

45. Mas-Fontao, S.; Civantos, E.; Boukichou-Abdelkader, N.; Soto-Catalan, M.; Romeo-Colas, M.; Marco, A.; Gomez-Guerrero, C.; Moreno, J. A.; Tuomilehto, J.; Gabriel, R., Oxidative stress and inflammation on metabolic abnormalities and renal involvement in prediabetic subjects across Europe. *Nefrología (English Edition)* 2025, 45, (3), 238–248.

46. Mabuza, L. P.; Gamede, M. W.; Maikoo, S.; Booysen, I. N.; Ngubane, P. S.; Khathi, A., Amelioration of risk factors associated with diabetic nephropathy in diet-induced pre-diabetic rats by an uracil-derived diimine ruthenium (II) compound. *Biomedicine & Pharmacotherapy* 2020, 129, 110483.

47. Martín, M. Á.; Ramos, S., Dietary flavonoids and insulin signaling in diabetes and obesity. *Cells*

2021, 10, (6), 1474.

48. M. Eid, H.; S. Haddad, P., The antidiabetic potential of quercetin: underlying mechanisms. *Current medicinal chemistry* 2017, 24, (4), 355–364.
49. Hsu, W.-H.; Hsiao, P.-J.; Lin, P.-C.; Chen, S.-C.; Lee, M.-Y.; Shin, S.-J., Effect of metformin on kidney function in patients with type 2 diabetes mellitus and moderate chronic kidney disease. *Oncotarget* 2017, 9, (4), 5416.
50. Song, A.; Zhang, C.; Meng, X., Mechanism and application of metformin in kidney diseases: An update. *Biomedicine & Pharmacotherapy* 2021, 138, 111454.
51. Pan, Q.; Lu, X.; Zhao, C.; Liao, S.; Chen, X.; Guo, F.; Yang, C.; Liu, H.-f., Metformin: the updated protective property in kidney disease. *Aging (Albany NY)* 2020, 12, (9), 8742.
52. Leatherby, R. J.; Theodorou, C.; Dhanda, R., Renal physiology: blood flow, glomerular filtration and plasma clearance. *Anaesthesia & Intensive Care Medicine* 2021, 22, (7), 439–442.
53. de Boer, I. H.; Zelnick, L.; Afkarian, M.; Ayers, E.; Curtin, L.; Himmelfarb, J.; Ikizler, T. A.; Kahn, S. E.; Kestenbaum, B.; Utzschneider, K., Impaired glucose and insulin homeostasis in moderate-severe CKD. *Journal of the American Society of Nephrology: JASN* 2016, 27, (9), 2861.
54. Cortinovis, M.; Perico, N.; Ruggenenti, P.; Remuzzi, A.; Remuzzi, G., Glomerular hyperfiltration. *Nature reviews nephrology* 2022, 18, (7), 435–451.
55. Bahar, A.; Makhloogh, A.; Yousefi, A.; Kashi, Z.; Abediankenari, S., Correlation between prediabetes conditions and microalbuminuria. *Nephro-urology monthly* 2013, 5, (2), 741.
56. Jha, J. C.; Banal, C.; Chow, B. S.; Cooper, M. E.; Jandeleit-Dahm, K., Diabetes and kidney disease: role of oxidative stress. *Antioxidants & redox signaling* 2016, 25, (12), 657–684.
57. Szeto, H. H.; Liu, S.; Soong, Y.; Alam, N.; Prusky, G. T.; Seshan, S. V., Protection of mitochondria prevents high-fat diet–induced glomerulopathy and proximal tubular injury. *Kidney international* 2016, 90, (5), 997–1011.
58. Rogacka, D.; Piwkowska, A., Beneficial effects of metformin on glomerular podocytes in diabetes. *Biochemical Pharmacology* 2021, 192, 114687.
59. Lehtonen, S., Metformin protects against podocyte injury in diabetic kidney disease. *Pharmaceuticals* 2020, 13, (12), 452.
60. Zhang, B.; Li, Q., Urine. In *Clinical Molecular Diagnostics*, Springer: 2021; pp 241–252.
61. Markus, M.; Ittermann, T.; Baumeister, S. E.; Huth, C.; Thorand, B.; Herder, C.; Roden, M.; Siewert-Markus, U.; Rathmann, W.; Koenig, W., Prediabetes is associated with microalbuminuria, reduced kidney function and chronic kidney disease in the general population: The KORA (Cooperative Health Research in the Augsburg Region) F4-Study. *Nutrition, Metabolism and Cardiovascular Diseases* 2018, 28, (3), 234–242.

62. Castañeda, A. M.; Dutra-Rufato, A.; Juarez, M. J.; Grosembacher, L.; Gonzalez-Torres, H.; Musso, C. G., Sodium-glucose cotransporter 2 inhibitors (SGLT2i): renal implications. *International Urology and Nephrology* 2021, 53, (2), 291–299.
63. Shimodaira, M.; Minemura, Y.; Nakayama, T., Elevated serum uric acid is a risk factor for progression to prediabetes in Japanese women: A 5-year retrospective cohort study. *Journal of Diabetes Investigation* 2023, 14, (11), 1237–1245.
64. Khan, A.; Manna, K.; Bose, C.; Sinha, M.; Das, D. K.; Kesh, S. B.; Chakrabarty, A.; Banerji, A.; Dey, S., Gossypetin, a naturally occurring hexahydroxy flavone, ameliorates gamma radiation-mediated DNA damage. *International journal of radiation biology* 2013, 89, (11), 965–975.
65. Za'abi, M. A.; Ali, B. H.; Al Suleimani, Y.; Adham, S. A.; Ali, H.; Manoj, P.; Ashique, M.; Nemmar, A., The effect of metformin in diabetic and non-diabetic rats with experimentally-induced chronic kidney disease. *Biomolecules* 2021, 11, (6), 814.
66. Kawanami, D.; Takashi, Y.; Tanabe, M., Significance of metformin use in diabetic kidney disease. *International Journal of Molecular Sciences* 2020, 21, (12), 4239.
67. Liu, Z.; Kong, H.; Zhang, B., Narrative literature review of antidiabetic drugs' effect on hyperuricemia: elaborating actual data and mechanisms. *Endocrine Connections* 2024, 13, (6).
68. Bu, Y.; Peng, M.; Tang, X.; Xu, X.; Wu, Y.; Chen, A. F.; Yang, X., Protective effects of metformin in various cardiovascular diseases: clinical evidence and AMPK-dependent mechanisms. *Journal of Cellular and Molecular Medicine* 2022, 26, (19), 4886–4903.
69. Mohamed, E.; Mahmood, F.; Merie, G.; Saadi, A., The medical importance of sodium and potassium. *Int. J. Pharma Growth Res. Rev* 2025, 2, (2), 01–10.
70. DiNicolantonio, J. J.; O'Keefe, J. H., Sodium restriction and insulin resistance: a review of 23 clinical trials. *Journal of Insulin Resistance* 2023, 6, (1), 78.
71. Horita, S.; Seki, G.; Yamada, H.; Suzuki, M.; Koike, K.; Fujita, T., Insulin resistance, obesity, hypertension, and renal sodium transport. *International journal of hypertension* 2011, 2011, (1), 391762.
72. Valentini, A.; Heilmann, R. M.; Kühne, A.; Biagini, L.; De Bellis, D.; Rossi, G., The renin–angiotensin–aldosterone system (RAAS): Beyond cardiovascular regulation. *Veterinary sciences* 2025, 12, (8), 777.
73. Hashimoto, H.; Nomura, N.; Shoda, W.; Isobe, K.; Kikuchi, H.; Yamamoto, K.; Fujimaru, T.; Ando, F.; Mori, T.; Okado, T., Metformin increases urinary sodium excretion by reducing phosphorylation of the sodium-chloride cotransporter. *Metabolism* 2018, 85, 23–31.
74. Deshmukh, A.; Manjalkar, P., Synergistic effect of micronutrients and metformin in alleviating diabetic nephropathy and cardiovascular dysfunctioning in diabetic rat. *Journal of Diabetes & Metabolic Disorders* 2021, 20, (1), 533–541.

75. Vodošek Hojs, N.; Bevc, S.; Ekart, R.; Hojs, R., Oxidative stress markers in chronic kidney disease with emphasis on diabetic nephropathy. *Antioxidants* 2020, 9, (10), 925.
76. Rada, F. H., Oxidative stress assessment in patients with diabetic nephropathy. *Age (years)* 2018, 48, (5), 50–8.
77. Khalid, M.; Petroianu, G.; Adem, A., Advanced glycation end products and diabetes mellitus: mechanisms and perspectives. *Biomolecules* 2022, 12, (4), 542.
78. Balci, B.; Tekce, B. K.; Aktas, G., Evaluation of serum oxidative stress levels and antioxidant capacity in prediabetes. *Advances in Redox Research* 2024, 12, 100106.
79. Luc, K.; Schramm-Luc, A.; Guzik, T.; Mikolajczyk, T., Oxidative stress and inflammatory markers in prediabetes and diabetes. *Journal of Physiology & Pharmacology* 2019, 70, (6), 1.
80. Lin, H.; Ao, H.; Guo, G.; Liu, M., The Role and Mechanism of Metformin in Inflammatory Diseases. *J Inflamm Res* 2023, 16, 5545–5564.
81. Mounnissamy, V.; Gopal, V.; Gunasegaran, R.; Saraswathy, A., Antiinflammatory activity of gossypetin isolated from *Hibiscus sabdariffa*. *Indian Journal of Heterocyclic Chemistry* 2002, 12, (1), 85–86.
82. Buczyńska, A.; Sidorkiewicz, I.; Krętowski, A. J.; Adamska, A., Examining the clinical relevance of metformin as an antioxidant intervention. *Frontiers in pharmacology* 2024, 15, 1330797.
83. Ren, H.; Shao, Y.; Wu, C.; Ma, X.; Lv, C.; Wang, Q., Metformin alleviates oxidative stress and enhances autophagy in diabetic kidney disease via AMPK/SIRT1-FoxO1 pathway. *Molecular and cellular endocrinology* 2020, 500, 110628.
84. Ravindran, S.; Kuruvilla, V.; Wilbur, K.; Munusamy, S., Nephroprotective effects of metformin in diabetic nephropathy. *Journal of cellular physiology* 2017, 232, (4), 731–742.
85. Anandkumar, D. G.; Dheerendra, P. C.; Vellakampadi, D.; Ramanathan, G., Kidney injury molecule-1; is it a predictive marker for renal diseases? *Journal of Nephropharmacology* 2023, 12, (2), e10572–e10572.
86. Daehn, I. S.; Duffield, J. S., The glomerular filtration barrier: a structural target for novel kidney therapies. *Nature Reviews Drug Discovery* 2021, 20, (10), 770–788.
87. Thakur, V.; Chattopadhyay, M., Early urinary markers for diabetic and other kidney diseases. *Current drug targets* 2018, 19, (7), 825–831.
88. Adeva-Andany, M. M.; Fernández-Fernández, C.; Adeva-Contreras, L.; Carneiro-Freire, N.; Domínguez-Montero, A.; Mouriño-Bayolo, D., Insulin resistance is associated with subclinical vascular injury in patients with a kidney disease. *Current Cardiology Reviews* 2021, 17, (5), 58–73.
89. Menon, S. V.; Kumud; Pandya, S.; Dwivedi, S. K.; Sharma, P.; Tomar, B. S.; Sharma, A.; Wal, A., Potentiating Role of Flavonoids in Diabetes and its Complications: Preclinical and Clinical Studies.

Current Chemical Biology 2026.

90. Tombulturk, F. K.; Demirci, H. D., Reducing oxidative stress and enhancing antioxidant defenses for faster healing in diabetic wounds: The role of topical metformin. *International Journal of Medical Biochemistry* 2025, 8, (2), 89.
91. Caturano, A.; Nilo, D.; Nilo, R.; Sircana, M. C.; Erul, E.; Zielińska, K.; Russo, V.; Santonastaso, E.; Sasso, F. C., Old drug, new science: Metformin and the future of pharmaceuticals. *Pharmaceutics* 2026, 18, (1), 77.
92. Hu, L.; Luo, Y.; Yang, J.; Cheng, C., Botanical Flavonoids: Efficacy, Absorption, Metabolism and Advanced Pharmaceutical Technology for Improving Bioavailability. *Molecules* 2025, 30, (5), 1184.
93. Bas, T. G., Dietary polyphenols (flavonoids) derived from plants for use in therapeutic health: Antioxidant performance, ROS, molecular mechanisms, and bioavailability limitations. *International journal of molecular sciences* 2026, 27, (3), 1404.

Bridge

The fourth manuscript presented in Chapter 7 showed that diet-induced prediabetes was associated with early kidney dysfunction. This was evidenced by increased kidney weight, altered fluid balance, reduced glomerular filtration rate, electrolyte imbalances, elevated oxidative stress, inflammation and increased markers of kidney injury. The administration of gossypetin (GTIN), in both the presence and absence of dietary intervention, significantly improved the markers associated with renal dysfunction. The findings of manuscript 4 and the previous manuscripts highlight the beneficial effects of GTIN on glucose homeostasis, cardiovascular function, liver health and renal function in the prediabetic state. Chapter 8 presents an integrated synthesis and conclusion of the research.

CHAPTER 8: SYNTHESIS AND CONCLUSION

The chronic consumption of high-calorie diets combined with sedentary lifestyles has been shown to promote the development of prediabetes [1]. Prediabetes is an intermediate hyperglycaemic state associated with impaired fasting glucose levels and moderate glucose intolerance [2]. Studies have shown that prediabetes induces oxidative stress and low-grade inflammation which contribute to cardiovascular, hepatic and renal dysfunction [3 - 5] . The global prevalence of prediabetes in 2021 was 9.1% and is projected to increase to 10.0% by 2045 [6]. A combination of pharmacological and dietary interventions is recommended to manage prediabetes [7]. However, patients have been shown to rely solely on pharmacological intervention without implementing the necessary lifestyle changes [8]. This has been shown to limit the effectiveness of the pharmacological interventions [6]. Consequently, there is a growing need to identify affordable pharmacological agents that can manage prediabetes and its associated complications even in the absence of dietary intervention. Flavonoids are natural polyphenolic compounds produced by secondary metabolism in plants [8]. Gossypetin (GTIN) is a flavonoid found in various medicinal plants including *Hibiscus* species [9, 10]. GTIN possesses high structural similarity with quercetin which is well-known to ameliorate diabetes mellitus and its associated complications due to its antioxidant nature and anti-glycation ability [11, 12]. Recent studies have shown that GTIN exhibits antioxidant, anti-inflammatory, anti-atherosclerotic and antimutagenic effects [13, 14]. However, the effects of GTIN on glucose homeostasis, cardiovascular, liver and renal function during prediabetes have not yet been investigated. Hence, this study sought to investigate the effects of GTIN on glucose homeostasis in a diet-induced prediabetic rat model in both the presence and absence of dietary intervention. We further investigated the effects of GTIN on markers associated with cardiovascular, hepatic and renal function during prediabetes.

During prediabetes, glucose homeostasis is disturbed as evidenced by elevated fasting blood glucose (FBG), elevated postprandial blood glucose, elevated plasma insulin and homeostatic model assessment of insulin resistance (HOMA-IR) values as well as elevated levels of glycated haemoglobin (HbA1c) in the blood [15]. Studies have shown increased hepatic glycogen and decreased skeletal muscle glycogen levels during prediabetes due to reduced peripheral glucose uptake [16, 17]. Elevated plasma ghrelin levels during prediabetes promote increased caloric intake and weight gain [18]. Furthermore, moderate hyperglycaemia and insulin resistance promote the development of cardiovascular, hepatic and renal complications [3-5]. Hence, targeting moderate hyperglycaemia and insulin resistance may restore glucose homeostasis and prevent the onset of prediabetes-associated complications. In our laboratory, a high fat high carbohydrate (HFHC) diet-induced prediabetic rat model was used to mimic the human condition of prediabetes [19]. In our study, a high fat high carbohydrate (HFHC) diet was used to induce prediabetes. The onset of this

condition resulted in the elevation of FBG, postprandial glucose levels, increased HOMA2-IR index, increased blood HbA1c levels and plasma insulin in comparison to the non-prediabetic (NPD) group. The prediabetic (PD) group also showed significantly increased caloric intake, body weight gain and plasma ghrelin levels. This was accompanied by significantly lower skeletal muscle glycogen levels and higher liver glycogen levels in comparison to the NPD group. These findings corroborated with previous studies showing disturbed glucose homeostasis during prediabetes [19, 20]. The elevated FBG, postprandial glucose, HOMA2-IR index, HbA1c and plasma insulin levels suggest reduced insulin sensitivity and compromised glycaemic control. Elevated ghrelin levels may have driven the increased caloric intake and body weight gain in the PD group. The disturbed glycogen levels may suggest impaired skeletal muscle glucose uptake and a compensatory increase in hepatic glycogen storage. In this study, the GTIN-treated groups significantly improved markers of glucose homeostasis during the prediabetic state. Gossypetin administration in both the presence and absence of dietary intervention significantly reduced FBG, postprandial glucose, HOMA2-IR and HbA1c levels in comparison to the PD group. It also significantly lowered caloric intake, body weight gain and plasma ghrelin levels. Additionally, GTIN restored glycogen distribution by increasing skeletal muscle glycogen levels and reducing liver glycogen levels. Similarly, our findings corroborated with previous reports on flavonoids demonstrating improved insulin sensitivity, lowered blood glucose levels, enhanced muscle glycogen storage and reduced ghrelin levels [21, 22]. The findings of this study may suggest that GTIN improves glucose homeostasis during prediabetes by increasing insulin sensitivity and peripheral glucose uptake as well as restoring glycogen distribution and regulating energy balance. Metformin (MET) administration significantly improved glucose homeostasis by lowering FBG, postprandial glucose, HOMA2-IR and HbA1c levels in comparison to the PD group. MET administration also significantly increased skeletal muscle glycogen and reduced liver glycogen levels. The group where metformin was combined with dietary intervention was more effective than the group where metformin was administered without dietary intervention in reducing HOMA2-IR, caloric intake and body weight gain. These results corroborated with previous studies showing the effectiveness of MET in improving insulin sensitivity, enhancing skeletal muscle glucose utilisation and attenuating weight gain during prediabetes [17, 23]. This has been shown to be primarily mediated through adenosine monophosphate-activated protein kinase (AMPK) activation, which enhances insulin signalling and promotes glucose uptake in peripheral tissues [24]. MET has also been shown to suppress hepatic gluconeogenesis as well as regulate appetite and energy expenditure [25, 26]. Taken together, this study is the first to demonstrate that GTIN administration, in both the presence and absence of dietary intervention, may offer therapeutic potential in ameliorating disturbed glucose homeostasis during prediabetes, as seen in study 1.

Prediabetes has been characterised by disturbances in glucose homeostasis, including moderate hyperglycaemia, insulin resistance and hyperinsulinaemia [2]. These metabolic abnormalities promote

oxidative stress and chronic low-grade inflammation, which in turn contribute to cardiovascular, hepatic and renal dysfunction [3, 4, 27]. In study 1, GTIN significantly improved glucose homeostasis in a diet-induced prediabetic model, prompting further investigation into its potential effects on cardiovascular function. Hence, study 2 investigated the effects of GTIN on cardiovascular function in a diet-induced prediabetic rat model.

Studies have shown that pre-diabetes induces oxidative stress and low-grade inflammation which contributes to cardiovascular dysfunction, including increased blood pressure, endothelial injury and atherosclerosis [28, 29]. In this study, the PD group showed higher triglycerides (TAG), total cholesterol (TC), low-density lipoprotein (LDL), very low density lipoprotein (VLDL), mean arterial pressure (MAP), body mass index (BMI), waist circumference (WC), endothelial nitric oxide (eNOS), heart malondialdehyde (MDA) and inflammatory markers, with lower high density lipoprotein (HDL) levels. This was accompanied by reduced heart superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in comparison to the NPD group. These findings may suggest that diet-induced prediabetes may promote dyslipidaemia, endothelial dysfunction, oxidative stress and inflammation, contributing to cardiovascular dysfunction. In this study, the GTIN-treated groups significantly improved markers of cardiovascular function during the PD state. Gossypetin significantly reduced plasma TAGs, TC, LDL, VLDL, MAP, BMI, WC, eNOS and heart MDA levels. This was accompanied by significantly higher plasma HDL levels, SOD activity and GPx activity. Additionally, GTIN lowered pro-inflammatory cytokines, including interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α) and C-reactive protein (CRP), suggesting reduced oxidative stress and inflammation. Similarly, the improvements observed in the GTIN-treated groups aligned with existing literature supporting the use of flavonoids to manage cardiovascular complications during prediabetes [30, 31]. We speculated that the cardioprotective effects of GTIN may have been attributed to its antioxidant and anti-inflammatory properties as shown in recent studies [11, 15]. Notably, in our study, these beneficial effects were observed regardless of dietary intervention, highlighting GTIN's potential as a cardioprotective agent during pre-diabetes. The MET-treated groups demonstrated significant improvements in lipid profiles, blood pressure, adiposity, oxidative stress and inflammation, comparable to the effects seen in the GTIN-treated groups. These results corroborated with previous studies showing the effectiveness of MET in enhancing insulin sensitivity, regulating lipid metabolism, reducing oxidative stress and lowering inflammation in cardiovascular diseases associated with diabetes [32, 33]. Taken together, this study is the first to demonstrate that GTIN administration, in both the presence and absence of dietary intervention, may offer therapeutic potential in mitigating cardiovascular disturbances associated with the PD state, as seen in study 2.

Studies have shown that prediabetes leads to liver complications such as non-alcoholic fatty liver disease (NAFLD) due to insulin resistance and triglyceride accumulation [34, 35]. Insulin resistance promotes hepatic triglyceride accumulation by enhancing adipose tissue lipolysis and increasing

hepatic de novo lipogenesis via sterol regulatory element-binding protein 1c (SREBP-1c) activation [36, 37]. Excessive hepatic lipid accumulation promotes oxidative stress, inflammation and liver injury, contributing to the development of NAFLD [38]. Hepatic dysfunction has been shown to further exacerbate systemic insulin resistance in the PD state [39]. In this study, the PD group had significantly higher liver triglyceride levels, liver weights and plasma SREBP-1c levels in comparison to the NPD group. Liver MDA levels were significantly increased, while SOD and GPx activities were significantly decreased. Plasma aspartate transaminase (AST) and alanine transaminase (ALT) levels were significantly elevated and plasma bilirubin was significantly reduced. Liver histology showed increased lipid droplet accumulation, displaced hepatocyte nuclei, disrupted sinusoids and altered hepatocyte arrangement in the PD group. The findings of our study aligned with previous studies reporting early liver dysfunction associated with NAFLD during prediabetes [16, 35]. These findings may suggest that prediabetes induces early liver dysfunction marked by increased hepatic lipid accumulation, oxidative stress, impaired antioxidant defense and hepatocellular injury. In this study, the GTIN-treated groups showed reduced liver TAG levels, liver weights and plasma SREBP-1c. This was accompanied by significantly lower hepatic MDA levels and higher antioxidant enzyme activity. The GTIN-treated groups also showed significantly decreased plasma AST and ALT levels as well as increased bilirubin and improved liver histology. These findings corroborated a recent study demonstrating hepatoprotective effects of GTIN in a non-alcoholic steatohepatitis disease model [40]. The recent study showed that GTIN significantly reduced hepatic lipid accumulation and attenuated oxidative stress [40]. Additionally, in our study, GTIN enhanced the activity of SOD and GPx as well as improved liver histology. Our findings suggest that GTIN may exert hepatoprotective effects during prediabetes by reducing hepatic lipid accumulation and oxidative stress, even without dietary intervention. The hepatoprotective effects of GTIN may have been mediated due to its reported antioxidant, anti-inflammatory and antilipidaemic properties [10, 14]. In the MET-treated groups, liver TAG levels, liver weights and plasma SREBP-1c were significantly reduced in comparison to the PD group. Metformin administration also reduced liver MDA and increased Gpx activity in the presence and absence of dietary intervention. Plasma AST and ALT levels were significantly reduced along with increased plasma bilirubin in the MET-treated groups. Liver histology was improved, particularly characterised by reduced lipid droplets, improved sinusoid and hepatocyte plate arrangement in the MET-treated groups. These results corroborated previous studies, showing the effectiveness of MET in ameliorating hepatic steatosis, oxidative stress and liver injury through its insulin-sensitising, antioxidant and lipid-lowering effects in a NAFLD model [41, 42]. Taken together, this study is the first to demonstrate that GTIN administration, in both the presence and absence of dietary intervention, may mitigate hepatic disturbances associated with the PD state, as seen in study 3.

Studies have shown that prediabetes impairs kidney function causing glomerular damage, albuminuria and nephropathy [43, 44]. In prediabetes, renal dysfunction develops due to altered blood flow,

glomerular injury and impaired filtration [45, 46]. Impaired renal filtration contributes to disturbances in creatinine, urea, uric acid and electrolyte handling [45]. Moderate hyperglycaemia in prediabetes induces oxidative stress and activates the renin–angiotensin–aldosterone system (RAAS), promoting inflammation and renal injury [45, 46]. Early renal dysfunction in prediabetes has been characterised by compensatory increases in glomerular filtration rate (GFR), fluid intake and kidney hypertrophy [47]. In this study, the PD group showed significantly increased kidney weights, fluid intake and urine output in comparison to the NPD group. This was accompanied by significantly reduced GFR and altered levels of plasma creatinine, albumin, urea and uric acid. The PD group also exhibited disrupted sodium and potassium balance alongside elevated plasma aldosterone. Elevated MDA, inflammatory markers and reduced total antioxidant status (TAS) levels in the kidneys were observed in the PD group. Plasma kidney injury molecule-1 (KIM-1) and urinary podocin mRNA expression was significantly increased. The findings of our study corroborated previous studies reporting similar renal disturbances during prediabetes [44, 46]. This study's results may suggest the presence of early renal dysfunction during prediabetes evidenced by low GFR, increased oxidative damage, inflammatory markers and early kidney injury. In this study, GTIN administration significantly reduced fluid intake, urine output and kidney weights in the presence and absence of dietary intervention in comparison to the PD group. Additionally, GTIN administration improved GFR, creatinine, albumin, urea and uric acid levels. It also improved renal sodium and potassium handling as well as reduced plasma aldosterone levels. Gossypetin administration significantly decreased lipid peroxidation and inflammation in the kidney, evidenced by lower kidney MDA, IL-6, TNF- α and higher TAS levels. Additionally, GTIN reduced kidney injury markers including plasma KIM-1 and urinary podocin mRNA expression. These findings corroborated previous studies demonstrating the reno-protective effects of flavonoids in metabolic disease models [48, 49]. Flavonoids have been shown to enhance glomerular filtration, reduce oxidative stress and inflammation as well as regulate electrolyte balance and protect against early kidney injury [48]. Our findings suggest that GTIN may exert reno-protective effects by improving glomerular filtration, decreasing RAAS activity and restoring renal electrolyte handling. Moreover, the reduction in oxidative stress, inflammation and early kidney injury markers in the GTIN-treated groups may suggest improved renal function. We propose that GTIN may serve as a potential alternative to conventional treatments for diabetic nephropathy. The MET-treated groups improved renal function by enhancing glomerular filtration and restoring creatinine, urea, uric acid and albumin levels. Notably, significant reductions in kidney weight, plasma aldosterone levels and improved sodium handling were observed in the MET+ND group. These findings suggest that the HFHC diet may have limited the efficacy of MET in mitigating renal disturbances associated with prediabetes. Metformin also significantly reduced oxidative stress, inflammation and renal injury markers in this study. The findings of our study aligned with previous studies demonstrating reno-protective effects of MET administration [50, 51]. Metformin's effects are primarily mediated through AMPK activation, which improves insulin sensitivity, reduces RAAS

activity, oxidative stress and inflammation [52]. Taken together, this study is the first to demonstrate that GTIN administration in the presence and absence of dietary intervention, may mitigate renal disturbances associated with the PD state, as seen in study 4.

In summary, prediabetes induces significant disturbances in glucose homeostasis, cardiovascular, hepatic and renal function, driven by moderate insulin resistance, oxidative stress and inflammation. The administration of gossypetin, in both the presence and absence of dietary intervention, significantly improved markers of glucose homeostasis as well as cardiovascular, liver and renal dysfunction (Figure 1). It is plausible that these beneficial effects were mediated by the antioxidant and anti-inflammatory properties of GTIN. Similarly, metformin administration exhibited therapeutic effects comparable to those of GTIN. This further supports MET's well-established role in the management of prediabetes. Notably, the efficacy of GTIN even in the absence of dietary intervention highlights its potential as an alternative anti-diabetic agent, particularly in populations with limited adherence to lifestyle modifications. The positive health outcomes of GTIN across multiple organ systems further highlights its multi-targeted protective role in prediabetes. Therefore, based on the findings of this study, the hypothesis that GTIN improves the biomarkers associated with glucose homeostasis as well as cardiovascular, liver and renal function in diet-induced pre-diabetic male Sprague Dawley rats is accepted.

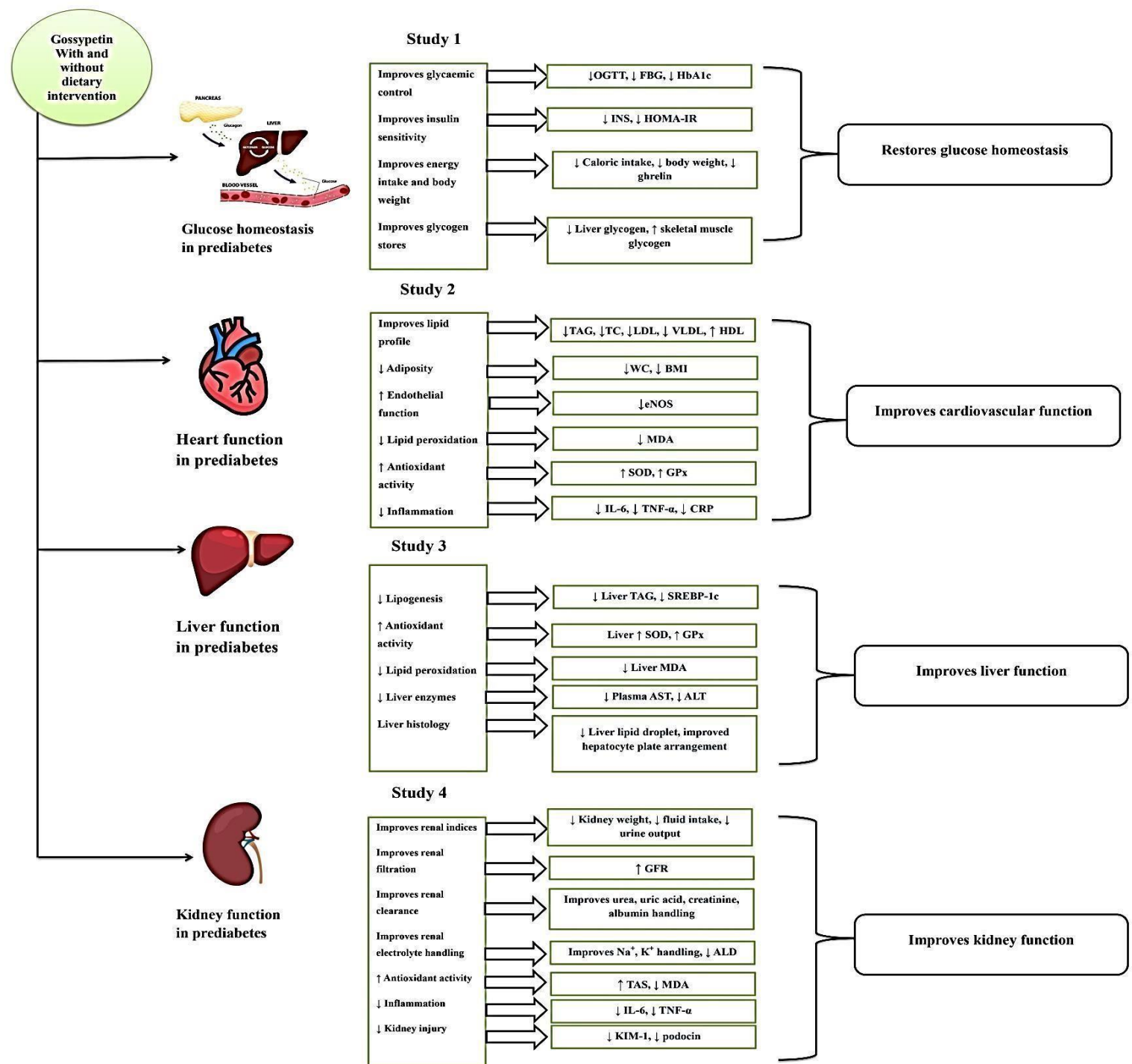


Figure 1: Diagram illustrating the effects of gossypetin on glucose homeostasis as well as cardiovascular, hepatic and renal function in a diet-induced pre-diabetic rat model.

Conclusion

Taken together, GTIN administration in presence and absence of dietary intervention showed significant improvements to glucose homeostasis, cardiovascular function, liver health and renal function in diet-induced prediabetes. Gossypetin improved glucose homeostasis by enhancing insulin sensitivity and regulating blood glucose levels, as seen in study 1. It also exerted protective effects on cardiovascular function by reducing oxidative stress and inflammation, as seen in study 2. Gossypetin improved prediabetes-induced liver dysfunction by reducing lipid accumulation and oxidative

damage, as seen in study 3. Furthermore, GTIN enhanced renal function by improving glomerular filtration and attenuating renal injury, as seen in study 4. These findings highlight the therapeutic potential of GTIN as a potential alternative to manage prediabetes and associated cardiovascular, hepatic and renal dysfunction.

Limitations and future studies

Lifestyle modification encompasses both dietary intervention and increased physical activity such as exercise [53]. However, the current study focused on dietary intervention as a preventative strategy for prediabetes. Therefore, future research should investigate the effects of physical activity and dietary intervention in the management of prediabetes.

Another limitation of the present study is the relatively small sample size ($n = 6$ per experimental group). Although this sample size was determined a priori using statistical power analysis and was sufficient to detect significant differences between treatment groups, larger sample sizes would improve the precision of the findings. Future studies should therefore include larger numbers of animals to strengthen the reliability of the observed therapeutic effects of GTIN.

Additionally, this study identified some limitations that provide opportunities for further investigation. Although the present study investigated several biochemical, oxidative stress and inflammatory markers, the molecular mechanisms underlying the therapeutic effects of GTIN were not completely explored. Future investigations employing more molecular approaches, including transcriptomic, proteomic and metabolomic analyses, are warranted to elucidate the mechanisms influenced by GTIN. Understanding these mechanisms will aid in the development of targeted therapies for prediabetes and its associated complications.

A limitation of the present study is that only male Sprague Dawley rats were used. This limits the generalisability of the findings to females. Sex-specific differences in the pathophysiology of prediabetes and type 2 diabetes are well documented [54]. Men are more likely to develop impaired fasting glucose, whereas women are more likely to develop impaired glucose tolerance [54]. These differences indicate that males and females develop dysglycaemia through different mechanisms [54]. Premenopausal females are relatively protected against metabolic dysfunction because of endogenous oestrogen [55]. Oestrogen improves insulin sensitivity, preserves pancreatic β -cell function, promotes skeletal muscle glucose uptake, improves mitochondrial function, suppresses oxidative stress and inflammation, and regulates hepatic lipid metabolism through pathways such as oestrogen receptor α (ER α) and AMP-activated protein kinase (AMPK) [55]. As a result, females are generally more resistant to diet-induced obesity, insulin resistance and hyperglycaemia than males [56]. However, this protection declines after menopause as oestrogen levels decrease [56]. Several of these pathways are also proposed mechanisms through which GTIN exerts its beneficial effects, including its antioxidant,

anti-inflammatory and AMPK-activating actions [10, 57]. Consequently, the beneficial effects of GTIN observed in male rats may not be directly applicable to females because hormonal differences can influence treatment responses. Future studies should include female animals at different reproductive stages, including pre- and post-menopausal models. This would help determine whether sex hormones affect the effectiveness of GTIN. It would also improve the applicability of these findings to both males and females.

Another limitation is that only a single GTIN dose (15 mg/kg body weight) was evaluated. Although this dose was selected based on previous evidence demonstrating its biological efficacy, the dose-response relationship of GTIN could not be established [58, 59]. Different doses may produce different therapeutic effects and safety profiles [60]. Therefore, it is unclear whether the selected dose represents the minimum effective dose, the optimal therapeutic dose or the maximum safe dose. Consequently, although GTIN significantly improved glucose homeostasis, cardiovascular, hepatic, and renal function in this study, these findings are specific to the 15 mg/kg dose and cannot be assumed to occur at lower or higher doses. Future studies should evaluate multiple GTIN doses across a wider dose range. This would help establish the dose-response profile, identify the optimal therapeutic dose and support future clinical dose recommendations.

Another limitation of the present study is that GTIN treatment was evaluated over a relatively short intervention period of 12 weeks. Therefore, the long-term efficacy and safety of GTIN could not be determined. Prediabetes is a progressive metabolic disorder, and the beneficial effects observed following short-term treatment may not be sustained with prolonged administration [61]. Furthermore, chronic administration may result in cumulative toxicity or organ-specific adverse effects that cannot be detected during a relatively short treatment period [62, 63]. Consequently, although GTIN significantly improved glucose homeostasis, cardiovascular, hepatic and renal function during the 12-week treatment period, it remains unclear whether these therapeutic benefits would be maintained with long-term administration. Future studies should evaluate GTIN over longer intervention periods and include chronic toxicity assessments to establish its long-term safety profile.

Another limitation of the present study is that the pharmacokinetic profile of GTIN was not investigated. Flavonoids generally have low oral bioavailability due to poor aqueous solubility, limited intestinal absorption, and extensive metabolism in the intestine and liver [64]. In addition, factors such as chemical structure, diet and gut microbiota can influence their absorption and metabolism [65]. The absorption, distribution, metabolism and excretion (ADME) profile of GTIN remains unknown. These pharmacokinetic data are essential to determine the appropriate human-equivalent dose, optimal dosing regimen and suitable formulation for clinical use [66]. Therefore, although GTIN produced significant improvements in glucose homeostasis, cardiovascular, hepatic, and renal function in this preclinical model, it is unclear whether similar therapeutic effects could be achieved in humans

because the bioavailability of GTIN has not been established. Future pharmacokinetic studies are therefore required before its clinical potential can be fully evaluated.

References

1. Papakonstantinou, E.; Oikonomou, C.; Nychas, G.; Dimitriadis, G. D., Effects of Diet, Lifestyle, Chrononutrition and Alternative Dietary Interventions on Postprandial Glycemia and Insulin Resistance. *Nutrients* 2022, 14, (4).
2. Khan, R. M. M.; Chua, Z. J. Y.; Tan, J. C.; Yang, Y.; Liao, Z.; Zhao, Y., From pre-diabetes to diabetes: diagnosis, treatments and translational research. *Medicina* 2019, 55, (9), 546.
3. Gamede, M.; Mabuza, L.; Ngubane, P.; Khathi, A., Plant-derived oleanolic acid ameliorates markers associated with non-alcoholic fatty liver disease in a diet-induced pre-diabetes rat model. *Diabetes, Metabolic Syndrome and Obesity* 2019, 1953–1962.
4. Gumede, N.; Ngubane, P.; Khathi, A., Assessing the risk factors for myocardial infarction in diet-induced prediabetes: myocardial tissue changes. *BMC cardiovascular disorders* 2022, 22, (1), 350.
5. Mabuza, L. P.; Gamede, M. W.; Maikoo, S.; Booysen, I. N.; Ngubane, P. S.; Khathi, A., Amelioration of risk factors associated with diabetic nephropathy in diet-induced pre-diabetic rats by an uracil-derived diimine ruthenium (II) compound. *Biomedicine & Pharmacotherapy* 2020, 129, 110483.
6. Rooney, M. R.; Fang, M.; Ogurtsova, K.; Ozkan, B.; Echouffo-Tcheugui, J. B.; Boyko, E. J.; Magliano, D. J.; Selvin, E., Global Prevalence of Prediabetes. *Diabetes Care* 2023, 46, (7), 1388–1394.
7. Beulens, J.; Rutters, F.; Ryden, L.; Schnell, O.; Mellbin, L.; Hart, H.; Vos, R., Risk and management of pre-diabetes. *European journal of preventive cardiology* 2019, 26, (2_suppl), 47–54.
8. Mutha, R. E.; Tatiya, A. U.; Surana, S. J., Flavonoids as natural phenolic compounds and their role in therapeutics: an overview. *Futur J Pharm Sci* 2021, 7, (1), 25.
9. Diyah, N. W.; Isnaeni; Hidayati, S. W.; Purwanto, B. T.; Siswandono, Design of gossypetin derivatives based on naturally occurring flavonoid in Hibiscus sabdariffa and the molecular docking as antibacterial agents. *J Basic Clin Physiol Pharmacol* 2021, 32, (4), 707–714.
10. Khan, A.; Manna, K.; Das, D. K.; Kesh, S. B.; Sinha, M.; Das, U.; Biswas, S.; Sengupta, A.; Sikder, K.; Datta, S., Gossypetin ameliorates ionizing radiation-induced oxidative stress in mice liver—a molecular approach. *Free Radical Research* 2015, 49, (10), 1173–1186.
11. Abdelmoaty, M. A.; Ibrahim, M.; Ahmed, N.; Abdelaziz, M., Confirmatory studies on the antioxidant and antidiabetic effect of quercetin in rats. *Indian Journal of Clinical Biochemistry* 2010, 25, (2), 188–192.
12. Bule, M.; Abdurahman, A.; Nikfar, S.; Abdollahi, M.; Amini, M., Antidiabetic effect of quercetin:

A systematic review and meta-analysis of animal studies. *Food and chemical toxicology* 2019, 125, 494–502.

13. Chen, J.-H.; Tsai, C.-W.; Wang, C.-P.; Lin, H.-H., Anti-atherosclerotic potential of gossypetin via inhibiting LDL oxidation and foam cell formation. *Toxicology and applied pharmacology* 2013, 272, (2), 313–324.

14. Proença, C.; Rufino, A. T.; Santos, I.; Albuquerque, H. M.; Silva, A. M.; Fernandes, E.; Ferreira de Oliveira, J. M. P., Gossypetin is a novel modulator of inflammatory cytokine production and a suppressor of osteosarcoma cell growth. *Antioxidants* 2023, 12, (9), 1744.

15. Basu, R.; Barosa, C.; Jones, J.; Dube, S.; Carter, R.; Basu, A.; Rizza, R. A., Pathogenesis of prediabetes: role of the liver in isolated fasting hyperglycemia and combined fasting and postprandial hyperglycemia. *The Journal of Clinical Endocrinology & Metabolism* 2013, 98, (3), E409–E417.

16. Arias-Fernández, M.; Fresneda, S.; Abbate, M.; Torres-Carballo, M.; Huguet-Torres, A.; Sánchez-Rodríguez, C.; Bennasar-Veny, M.; Yañez, A. M.; Busquets-Cortés, C., Fatty Liver Disease in Patients with Prediabetes and Overweight or Obesity. *Metabolites* 2023, 13, (4).

17. Khumalo, B.; Siboto, A.; Akinnuga, A. M.; Sibiya, N.; Khathi, A.; Ngubane, P. S., Momordica balsamina improves glucose handling in a diet-induced prediabetic rat model. *PLoS One* 2023, 18, (12), e0295498.

18. Allameh, A.; Niayesh-Mehr, R.; Aliarab, A.; Sebastiani, G.; Pantopoulos, K., Oxidative Stress in Liver Pathophysiology and Disease. *Antioxidants* 2023, 12, (9), 1653.

19. Khathi, A.; Luvuno, M.; Mabandla, M., Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. *PONTE International Journal of Science and Research* 2018, 74, (5).

20. Chakarova, N.; Dimova, R.; Grozeva, G.; Tankova, T., Assessment of glucose variability in subjects with prediabetes. *Diabetes research and clinical practice* 2019, 151, 56–64.

21. Martín, M. Á.; Ramos, S., Dietary flavonoids and insulin signaling in diabetes and obesity. *Cells* 2021, 10, (6), 1474.

22. Alkhalidy, H.; Wang, Y.; Liu, D., Dietary flavonoids in the prevention of T2D: An overview. *Nutrients* 2018, 10, (4), 438.

23. Huang, H.; Lorenz, B. R.; Zelmanovitz, P. H.; Chan, C. B., Metformin preserves β -cell compensation in insulin secretion and mass expansion in prediabetic Nile rats. *International Journal of Molecular Sciences* 2021, 22, (1), 421.

24. Herman, R.; Kravos, N. A.; Jensterle, M.; Janež, A.; Dolžan, V., Metformin and insulin resistance: a review of the underlying mechanisms behind changes in GLUT4-mediated glucose transport. *International journal of molecular sciences* 2022, 23, (3), 1264.

25. Agius, L.; Ford, B. E.; Chachra, S. S., The metformin mechanism on gluconeogenesis and AMPK activation: the metabolite perspective. *International journal of molecular sciences* 2020, 21, (9), 3240.

26. Szymczak-Pajor, I.; Wenclewska, S.; Śliwińska, A., Metabolic action of metformin. *Pharmaceuticals* 2022, 15, (7), 810.
27. Mkhize, B. C.; Mosili, P.; Ngubane, P. S.; Sibiyi, N. H.; Khathi, A., Diet-induced prediabetes: Effects on the activity of the renin–angiotensin–aldosterone system in selected organs. *Journal of Diabetes Investigation* 2022, 13, (5), 768–780.
28. Luc, K.; Schramm-Luc, A.; Guzik, T.; Mikolajczyk, T., Oxidative stress and inflammatory markers in prediabetes and diabetes. *Journal of Physiology & Pharmacology* 2019, 70, (6), 1.
29. Brannick, B.; Dagogo-Jack, S., Prediabetes and cardiovascular disease: pathophysiology and interventions for prevention and risk reduction. *Endocrinology and metabolism clinics of North America* 2018, 47, (1), 33.
30. Chen, Z.; Zhang, S.-L., The role of flavonoids in the prevention and management of cardiovascular complications: a narrative review. *Annals of palliative medicine* 2021, 10, (7), 8254263–8258263.
31. Yi, X.; Dong, M.; Guo, N.; Tian, J.; Lei, P.; Wang, S.; Yang, Y.; Shi, Y., Flavonoids improve type 2 diabetes mellitus and its complications: A review. *Frontiers in Nutrition* 2023, 10, 1192131.
32. Luo, F.; Das, A.; Chen, J.; Wu, P.; Li, X.; Fang, Z., Metformin in patients with and without diabetes: a paradigm shift in cardiovascular disease management. *Cardiovascular diabetology* 2019, 18, (1), 54.
33. Zhou, J.; Massey, S.; Story, D.; Li, L., Metformin: an old drug with new applications. *International journal of molecular sciences* 2018, 19, (10), 2863.
34. Sakurai, Y.; Kubota, N.; Yamauchi, T.; Kadowaki, T., Role of insulin resistance in MAFLD. *International journal of molecular sciences* 2021, 22, (8), 4156.
35. Vesa, C. M.; Behl, T.; Nemeth, S.; Bratu, O. G.; Diaconu, C. C.; Moleriu, R. D.; Negrut, N.; Zaha, D. C.; Bustea, C.; Radu, F. I., Prediction of NAFLD occurrence in prediabetes patients. *Experimental and Therapeutic Medicine* 2020, 20, (6), 190.
36. Ferré, P.; Phan, F.; Foufelle, F., SREBP-1c and lipogenesis in the liver: an update. *Biochemical journal* 2021, 478, (20), 3723–3739.
37. Smith, G. I.; Shankaran, M.; Yoshino, M.; Schweitzer, G. G.; Chondronikola, M.; Beals, J. W.; Okunade, A. L.; Patterson, B. W.; Nyangau, E.; Field, T., Insulin resistance drives hepatic de novo lipogenesis in nonalcoholic fatty liver disease. *The Journal of clinical investigation* 2020, 130, (3), 1453–1460.
38. Masarone, M.; Rosato, V.; Dallio, M.; Gravina, A. G.; Aglitti, A.; Loguercio, C.; Federico, A.; Persico, M., Role of oxidative stress in pathophysiology of nonalcoholic fatty liver disease. *Oxidative medicine and cellular longevity* 2018, 2018, (1), 9547613.
39. Niranjana, S.; Phillips, B. E.; Giannoukakis, N., Uncoupling hepatic insulin resistance–hepatic inflammation to improve insulin sensitivity and to prevent impaired metabolism-associated fatty liver

disease in type 2 diabetes. *Frontiers in endocrinology* 2023, 14, 1193373.

40. Oh, E.; Lee, J.; Cho, S.; Kim, S. W.; Jo, K. W.; Shin, W. S.; Gwak, S. H.; Ha, J.; Jeon, S. Y.; Park, J.-H., Gossypetin prevents the progression of nonalcoholic steatohepatitis by regulating oxidative stress and AMP-activated protein kinase. *Molecular Pharmacology* 2023, 104, (5), 214–229.

41. Huang, Y.; Wang, X.; Yan, C.; Li, C.; Zhang, L.; Zhang, L.; Liang, E.; Liu, T.; Mao, J., Effect of metformin on nonalcoholic fatty liver based on meta-analysis and network pharmacology. *Medicine* 2022, 101, (43), e31437.

42. Pinyopornpanish, K.; Leerapun, A.; Pinyopornpanish, K.; Chattipakorn, N., Effects of metformin on hepatic steatosis in adults with nonalcoholic fatty liver disease and diabetes: insights from the cellular to patient levels. *Gut and liver* 2021, 15, (6), 827.

43. Melsom, T.; Schei, J.; Stefansson, V. T. N.; Solbu, M. D.; Jenssen, T. G.; Mathisen, U. D.; Wilsgaard, T.; Eriksen, B. O., Prediabetes and risk of glomerular hyperfiltration and albuminuria in the general nondiabetic population: a prospective cohort study. *American Journal of Kidney Diseases* 2016, 67, (6), 841–850.

44. Fontalvo, J. R.; Soler, M. J.; Arnedo, R. D.; Navarro-Blackaller, G.; Medina-González, R.; Yáñez, T. R.; Cardona-Blanco, M.; Cabrales-Juan, J.; Uparrela-Gulfo, I.; Chávez-Iñiguez, J. S., Prediabetes and CKD: Does a causal relationship exist. *Nefrología (English Edition)* 2024, 44, (5), 628–638.

45. Shilpasree, A. S.; Patil, V. S.; Revanasiddappa, M.; Patil, V. P.; Ireshnavar, D., Renal dysfunction in prediabetes: confirmed by glomerular hyperfiltration and albuminuria. *Journal of Laboratory Physicians* 2021, 13, (03), 257–262.

46. Markus, M.; Ittermann, T.; Baumeister, S. E.; Huth, C.; Thorand, B.; Herder, C.; Roden, M.; Siewert-Markus, U.; Rathmann, W.; Koenig, W., Prediabetes is associated with microalbuminuria, reduced kidney function and chronic kidney disease in the general population: The KORA (Cooperative Health Research in the Augsburg Region) F4-Study. *Nutrition, Metabolism and Cardiovascular Diseases* 2018, 28, (3), 234–242.

47. Chen, C.; Liu, G.; Yu, X.; Yu, Y.; Liu, G., Association between prediabetes and renal dysfunction from a community-based prospective study. *International Journal of Medical Sciences* 2020, 17, (11), 1515.

48. Vargas, F.; Romecín, P.; García-Guillén, A. I.; Wangesteen, R.; Vargas-Tendero, P.; Paredes, M. D.; Atucha, N. M.; García-Estañ, J., Flavonoids in kidney health and disease. *Frontiers in physiology* 2018, 9, 394.

49. Hu, Q.; Qu, C.; Xiao, X.; Zhang, W.; Jiang, Y.; Wu, Z.; Song, D.; Peng, X.; Ma, X.; Zhao, Y., Flavonoids on diabetic nephropathy: advances and therapeutic opportunities. *Chinese medicine* 2021, 16, (1), 74.

50. Kawanami, D.; Takashi, Y.; Tanabe, M., Significance of metformin use in diabetic kidney disease.

International Journal of Molecular Sciences 2020, 21, (12), 4239.

51. Lehtonen, S., Metformin protects against podocyte injury in diabetic kidney disease. *Pharmaceuticals* 2020, 13, (12), 452.
52. Scheen, A.; Esser, N.; Paquot, N., Antidiabetic agents: potential anti-inflammatory activity beyond glucose control. *Diabetes & metabolism* 2015, 41, (3), 183–194.
53. Minnetti, M.; Barazzoni, R.; Batsis, J. A.; Busetto, L.; Yumuk, V.; Poggiogalle, E.; Weijs, P. J.; Donini, L. M., The integration of lifestyle modification advice and diet and physical exercise interventions: cornerstones in the management of obesity with incretin mimetics. *Obesity Facts* 2026, 19, (3), 310–326.
54. Onishi, Y.; Ichihashi, R.; Kasuga, M., Sex Differences in Fasting Glucose Levels among Non-diabetic Japanese: Men Exhibit Higher Levels Than Women. *Internal Medicine* 2026, 65, (3), 379–385.
55. Kautzky-Willer, A.; Leutner, M.; Harreiter, J., Sex differences in type 2 diabetes. *Diabetologia* 2023, 66, (6), 986–1002.
56. Ciarambino, T.; Crispino, P.; Guarisco, G.; Giordano, M., Gender Differences in Insulin Resistance: New Knowledge and Perspectives. *Curr Issues Mol Biol* 2023, 45, (10), 7845–7861.
57. Lin, H.-H.; Hsieh, M.-C.; Wang, C.-P.; Yu, P.-R.; Lee, M.-S.; Chen, J.-H., Anti-atherosclerotic effect of gossypetin on abnormal vascular smooth muscle cell proliferation and migration. *Antioxidants* 2021, 10, (9), 1357.
58. Gulsheen; Kumar, A.; Sharma, A., Antianxiety and antidepressant activity guided isolation and characterization of gossypetin from *Hibiscus sabdariffa* Linn. calyces. *Journal of Biologically Active Products from Nature* 2019, 9, (3), 205–214.
59. Ijaz, M. U.; Alvi, K.; Khan, H. A.; Imran, M.; Afsar, T.; Almajwal, A.; Amor, H.; Razak, S., Gossypetin mitigates doxorubicin-induced nephrotoxicity: A histopathological and biochemical evaluation. *Journal of King Saud University-Science* 2023, 35, (7), 102830.
60. Smith, D. A.; Obach, R. S., Metabolites in safety testing (MIST): considerations of mechanisms of toxicity with dose, abundance, and duration of treatment. *Chemical research in toxicology* 2009, 22, (2), 267–279.
61. Ping, W.-X.; Hu, S.; Su, J.-Q.; Ouyang, S.-Y., Metabolic disorders in prediabetes: from mechanisms to therapeutic management. *World journal of diabetes* 2024, 15, (3), 361.
62. Jennings, P.; Chandrasekaran, V.; Hardy, B.; Langemeijer, E.; Doktorova, T.; Madia, F.; Prieto, P., Mechanistic Analysis of Repeated Dose Toxicity Studies—Key Characteristics of Chemical-Induced Toxicity in the Liver, Lung, Cardiovascular System and Kidney. *Publications Office of the European Union* 2025, 239.
63. Cakir, L., Polypharmacy in type 2 diabetes mellitus and related conditions: a double-edged sword:

a narrative review. *Advances in Clinical Pharmacology and Therapeutics* 2025, 2, (2), 39–46.

64. Bas, T. G., Dietary polyphenols (flavonoids) derived from plants for use in therapeutic health: Antioxidant performance, ROS, molecular mechanisms, and bioavailability limitations. *International journal of molecular sciences* 2026, 27, (3), 1404.

65. Xiong, H. H.; Lin, S. Y.; Chen, L. L.; Ouyang, K. H.; Wang, W. J., The Interaction between Flavonoids and Intestinal Microbes: A Review. *Foods* 2023, 12, (2).

66. Afridi, S., *Use of Pharmacokinetics in the Drug Development Process*. 2024.

APPENDICES

APPENDIX 1: ETHICAL CLEARANCE



15 February 2023

Miss Karishma Naidoo (217000603)
School Of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Miss Naidoo,

Protocol reference number: AREC/00004965/2022

Project title: Investigating the effects of gossypetin on glucose homeostasis, cardiovascular, renal and hepatic dysfunctions in high-fat high-carbohydrate diet-induced pre-diabetic male Sprague Dawley rats.

Full Approval granted

This letter serves to notify you that your application, in connection with the above, was reviewed by the: Animal Research Ethics Committee (AREC) on 13 February 2023.

This approval is valid for one year from 13 February 2023.

To ensure uninterrupted approval of this study beyond the approval expiry date, a progress report must be submitted to the Research Office on the appropriate form 2 - 3 months before the expiry date.

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.

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

Yours faithfully

Dr. Doreen Vosloo, PhD, F.R. Nat. Sci.
Chair: Animal Research Ethics Committee

/kr

cc **Supervisor:** Dr Andile Khathi, Dr Phikelelani Ngubane
cc **BRU Manager:** Dr N Jaka

APPENDIX 2: COMPOSITION OF HIGH FAT HIGH CARBOHYDRATE (HFHC) DIET

Ingredient	Incl(%)	Mix(kg)
Maize	38.98	390.000
Palm Oil	20.99	210.000
Soya Full Fat	14.99	150.000
Wheat Gluten	6.50	65.000
Flour	6.00	60.000
Monodex	5.00	50.000
Sugar - White	5.00	50.000
Limestone	1.00	10.000
Dicalcium Phosphate	0.50	5.000
Vitamin Premix	0.35	3.500
Salt - Fine	0.30	3.000
Amino Acid - DL Methionine	0.30	3.000
Mineral Premix	0.10	1.000
	100.01	1000.50

Article

Effects of Gossypetin on Glucose Homeostasis in Diet-Induced Pre-Diabetic Rats

Karishma Naidoo  and Andile Khathi 

Department of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa; khathia@ukzn.ac.za

* Correspondence: karishma280199@gmail.com; Tel.: +27-31-260-7132

Abstract: Natural flavonoids exert many potential health benefits, including anti-hyperglycaemic effects. However, the effects of gossypetin (GTIN) on glucose homeostasis in pre-diabetes have not yet been investigated. This study examined the effects of GTIN on key markers of glucose homeostasis in a diet-induced pre-diabetic rat model. Pre-diabetes was induced by allowing the animals to feed on a high-fat high-carbohydrate (HFHC) diet supplemented with 15% fructose water for 20 weeks. Following pre-diabetes induction, the pre-diabetic animals were sub-divided into five groups ($n = 6$), where they were either orally treated with GTIN (15 mg/kg) or metformin (MET) (500 mg/kg), both with and without dietary intervention, over a 12-week period. The results demonstrated that animals in the untreated pre-diabetic (PD) control group exhibited significantly higher fasting and postprandial blood glucose levels, as well as elevated plasma insulin concentrations and increased homeostatic model assessment for insulin resistance (HOMA2-IR) index, relative to the non-pre-diabetic (NPD) group. Similarly, increased caloric intake, body weight and plasma ghrelin levels were observed in the PD control group. Notably, these parameters were significantly reduced in the PD animals receiving GTIN treatment. Additionally, glycogen levels in the liver and skeletal muscle, which were disturbed in the PD control group, showed significant improvement in both GTIN-treated groups. These findings may suggest that GTIN administration, with or without dietary modifications, may offer therapeutic benefits in ameliorating glucose homeostasis disturbances associated with the PD state.

Keywords: gossypetin; flavonoids; diet-induced pre-diabetes; glucose homeostasis; natural compound



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1. Introduction

Pre-diabetes represents a state of intermediate hyperglycaemia characterised by blood glucose levels that are elevated beyond normoglycaemia but do not meet the diagnostic criteria for type 2 diabetes mellitus (T2DM) [1]. This condition is associated with early insulin resistance and hyperinsulinaemia [2,3]. The prevalence of pre-diabetes has been linked to dietary patterns, particularly those high in fats and carbohydrates, commonly observed in “Westernized” diets [4]. As of 2017, the global prevalence of impaired glucose tolerance (IGT) was estimated at 7.3% among adults, affecting approximately 352.1 million individuals, with projections indicating an increase to 8.3% by 2045 [1,4]. Untreated pre-diabetes is recognised as a precursor to T2DM development and its associated complications [4,5]. The pathophysiology of pre-diabetes involves enhanced hepatic glucose storage and diminished glucose uptake by skeletal muscles [5]. Additionally, decreased cellular glucose uptake stimulates increased food intake, mediated by elevated ghrelin levels [6]. The progression from pre-diabetes to T2DM is significantly influenced by lifestyle factors, particularly dietary habits and adherence to prescribed interventions. Despite the proven efficacy of dietary modifications and pharmacological treatments in mitigating the risk of T2DM, adherence to these lifestyle changes remains a challenge [7]. Thus, there is a need for alternative treatments that remain effective even in the absence of dietary modifications [6].

Article

Investigating the Effects of Gossypetin on Cardiovascular Function in Diet-Induced Pre-Diabetic Male Sprague Dawley Rats

Karishma Naidoo  and Andile Khathi * 

Department of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa; karishma280199@gmail.com

* Correspondence: khathi@ukzn.ac.za; Tel.: +27-31-260-7132

Abstract: Gossypetin (GTIN) is a naturally occurring flavonoid recognised for its pharmacological properties. This study examined the effects of GTIN on cardiovascular function in a diet-induced pre-diabetic rat model, which has not been previously studied. Pre-diabetes was induced using a high-fat high-carbohydrate (HFHC) diet supplemented with 15% fructose water for 20 weeks. Thereafter, the pre-diabetic animals were sub-divided into five groups ($n = 6$), where they were either orally treated with GTIN (15 mg/kg) or metformin (MET) (500 mg/kg), both in the presence and absence of dietary intervention for 12 weeks. The results demonstrated that the pre-diabetic (PD) control group exhibited significantly higher plasma triglyceride, total cholesterol, low-density lipoprotein and very low-density lipoprotein levels, along with decreased high-density lipoprotein (HDL) levels in comparison to the non-pre-diabetic (NPD) group. This was accompanied by significantly higher mean arterial pressure (MAP), body mass index (BMI), waist circumference (WC) and plasma endothelial nitric oxide (eNOS) levels in PD control. Additionally, there were increased heart malondialdehyde levels, reduced heart superoxide dismutase and glutathione peroxidase activity as well as increased plasma interleukin-6, tumour necrosis factor alpha and c-reactive protein levels present in the PD control group. Notably, both GTIN-treated groups showed significantly reduced plasma lipid levels and increased HDL, as well as decreases in MAP, BMI, WC and eNOS levels in comparison to PD control. Additionally, GTIN significantly decreased heart lipid peroxidation, enhanced antioxidant activity and decreased plasma inflammation markers. These findings may suggest that GTIN administration in both the presence and absence of dietary intervention may offer therapeutic potential in ameliorating cardiovascular disturbances associated with the PD state. However, future studies are needed to determine the physiological mechanisms by which GTIN improves cardiovascular function in the PD state.

Keywords: Gossypetin; natural flavonoid; cardiovascular; diet-induced pre-diabetes



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1. Introduction

High-caloric diets have been linked to an increased risk of metabolic disorders, including pre-diabetes, which is a multifactorial condition influenced by genetic, environmental and lifestyle factors [1–3]. Pre-diabetes is an intermediate state between normal glucose tolerance [4] and overt type 2 diabetes mellitus (T2DM) [5]. The International Diabetes Federation estimated that about 298 million adults worldwide have been diagnosed with pre-diabetes, with projections showing that this number will increase to about 414 million by the year 2045 [6]. This condition is characterised by moderate hyperglycaemia, moderate insulin resistance and impaired glucose tolerance [5]. Studies have shown that many of the complications seen in T2DM begin in the pre-diabetic (PD) state [7,8]. Excess saturated fatty acids, trans fatty acids and pro-inflammatory adipokines have been shown to promote the development of insulin resistance, which

Article

Investigating the Effects of Gossypetin on Liver Health in Diet-Induced Pre-Diabetic Male Sprague Dawley Rats

Karishma Naidoo *  and Andile Khathi 

Department of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa; khathia@ukzn.ac.za

* Correspondence: karishma280199@gmail.com; Tel.: +27-31-260-7132

Abstract: The rising prevalence of non-alcoholic fatty liver disease among patients with type 2 diabetes mellitus has emerged as a global health challenge. Gossypetin (GTIN) is a natural flavonoid which has recently demonstrated antihyperglycaemic, antioxidant, and anti-inflammatory effects. Despite these findings, no studies have investigated its effects on liver health in the pre-diabetic state. Hence, this study aimed to investigate the effects of GTIN on liver health in diet-induced pre-diabetic male rats in the presence and absence of dietary intervention and to compare these effects with those of metformin (MET). Following 20 weeks of pre-diabetes induction, the animals were divided into six groups ($n = 6$) as follows: non-pre-diabetic (NPD) control, pre-diabetic (PD) control, and PD groups treated with GTIN (15 mg/kg body weight (bw)) or metformin (500 mg/kg bw) on either a normal diet or a high-fat, high-carbohydrate diet for 12 weeks. The results showed that the PD group had significantly higher liver triglycerides (TAG), liver weights, sterol regulatory binding element regulatory protein-1c (SREBP-1c), malondialdehyde (MDA) levels, and liver injury enzyme levels, along with decreased liver superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, and plasma bilirubin levels in comparison to NPD. Histologically, there was an increased lipid droplet accumulation and structural disarray in the PD group. GTIN treatment significantly reduced liver TAGs, liver weights, and plasma SREBP-1c levels, as well as improved liver SOD and GPx activity while decreasing liver MDA levels and liver injury enzymes in comparison to the PD control. Notably, GTIN treatment increased plasma bilirubin levels. Liver histology in the GTIN-treated groups revealed decreased lipid droplet accumulation and improved tissue integrity. Similar results were observed for the liver parameters in the MET-treated groups. The findings of this study may suggest that GTIN and MET exhibit therapeutic effects on liver health in diet-induced pre-diabetes in both the presence and absence of diet intervention. Dietary intervention may confer beneficial effects on liver health, with the most favorable therapeutic outcomes observed through a combination of treatment with dietary intervention. Additionally, GTIN may exhibit greater hepatoprotective effects than MET in rats without dietary intervention.

Keywords: gossypetin; NAFLD; liver injury; flavonoids; steatosis; high-caloric diets



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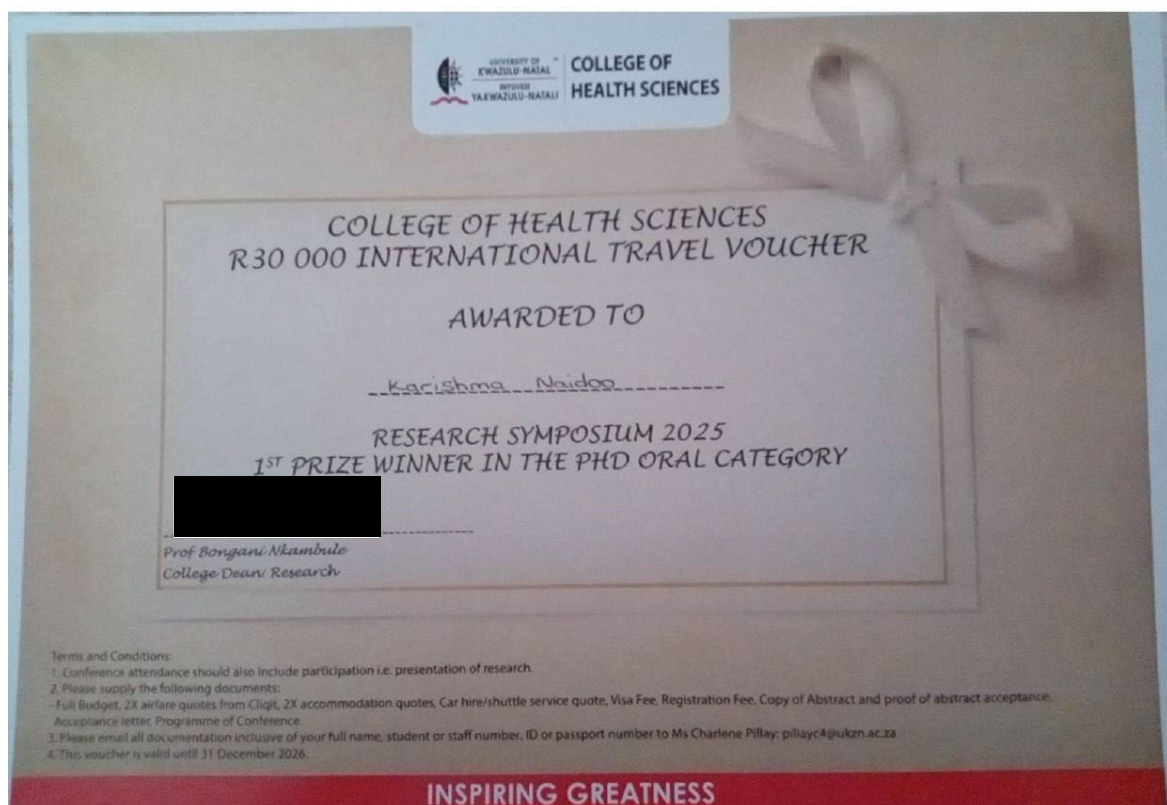
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1. Introduction

The chronic intake of unhealthy diets coupled with sedentary lifestyles contribute to the development of diabetes and associated liver complications such as non-alcoholic fatty liver disease (NAFLD) [1]. Pre-diabetes precedes the onset of overt type 2 diabetes mellitus (T2DM), with previous studies showing that around 70% of untreated pre-diabetic individuals progress to T2DM [2,3]. Insulin resistance and hyperinsulinemia associated with

APPENDIX 6: COLLEGE OF HEALTH SCIENCE SYMPOSIUM



APPENDIX 7: NEXT GENERATION AND EMERGING RESEARCHERS SYMPOSIUM



Aerobics		06:15-06:45
Arrival and Registration		08:00-08:30
Invited Speaker	"Interdisciplinary Research: Solving RealWorld Problems" Dr Nomcebo Mthombeni: Interim Director, Research and Postgraduate Support (DUT)	08:30-09:10
Invited Speaker	"Shaping the Future Professoriate" Prof Kirti Menon: Director, Future Professors Programme Phase 2 (UJ) Dr Andrew Kaniki: Academic Advisor, Future Professors Programme Phase 2 (UJ)	09:10-09:50
Mental Rest Break	"A Break for the Mind in Motion" Tebogo Digwamatje	09:50-10:00
Tea Break		10:00-10:20
Invited Speaker	"Making Your Research Talk to the Public" Dr Nozipho Gumbi: UNISA	10:20-11:00
S1: Agriculture and Environment	Oral Presentations	11:00-12:20 (15 min)
Lunch Break		12:30-13:30
Breakaway Sessions (Oral presentations)		
S2: Health Innovation	13:30-15:30 (20 min)	S3: Humanities and Society 13:30-15:30 (20 min)
Tea Break		15:30-15:45
S3: Energy Innovation	15:45-16:45 (15 min)	S4: ICT 15:45-16:45 (20 min)
Gala Dinner		18:30-23:00



APPENDIX 8: 3MT COMPETITION



3 Minute Thesis Competition - 3 Oct 2025

From Kholeka Busisa Dlamini <DlaminiK5@ukzn.ac.za>
Date Mon 10/6/2025 12:20 PM
To Karishma Naidoo (217000603) <217000603@stuukznac.onmicrosoft.com>
Cc Michelle Premjit <Premjit@ukzn.ac.za>

Dear Karishma

Happy Monday!

Congratulations on being the 1st Runner-up in the 2025 3MT Competition.

To facilitate the transfer of your R5,000, please forward a copy of your ID and proof of your banking details.

As you will be representing UKZN at the nationals, we are ordering golf shirts and jackets for you. Please provide your size (S, M, L, XL, or XXL).

To register for the nationals, please follow the link below. Registration needs to be done by Friday, 10th October 2025:

<https://events.ufs.ac.za/event/38993/>

Thank you,

Miss Kholeka Dlamini
Personal Assistant to the Deputy Vice-Chancellor of Research
Research
Research

University of KwaZulu-Natal, Durban, 4041
Campus: Westville
Email: DlaminiK5@ukzn.ac.za
Website: www.ukzn.ac.za
Tel: (+27) 31 260 2381
Cell: 0635723298

INSPIRING GREATNESS

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