The Effect of the 14:10-Hour Time-Restricted Feeding Regimen on Selected Markers of Glucose Homeostasis in Diet-Induced Prediabetic Male Sprague Dawley Rats

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PREFACE

High-calorie diets are associated with disruptions in blood glucose homeostasis and have been implicated in the onset and progression of type 2 diabetes mellitus (T2DM). The onset of T2DM is often preceded by prediabetes, an intermediate stage between normal glucose regulation and the development of T2DM. Various interventions have been developed to manage T2DM, some of which have also shown potential in managing prediabetes. Among these, the 14:10 time-restricted feeding (TRF) regimen has demonstrated promise in mitigating glycaemic dysregulation associated with T2DM. However, it remains unclear whether the 14:10 TRF regimen can similarly alleviate dysregulated glycemia in the prediabetic state. To explore this, we used a prediabetic rat model that was developed in our laboratory using a high-fat, high-carbohydrate diet that mimics the human condition of prediabetes. This study utilized this diet-induced rat model of prediabetes to investigate the effects of the 14:10 TRF regimen on specific markers of glucose homeostasis. The research described in this dissertation was conducted at the University of KwaZulu-Natal, Westville Campus, Durban, South Africa, by Sthembiso Msane, under the supervision of Prof. A Khathi and Mr. A.M. Sosibo.

DECLARATION

I, Sthembiso Snegugu Msane hereby declare that the dissertation entitled:

"The effect of the 14:10-Hour Time-Restricted Feeding Regimen on Selected Markers of Glucose Homeostasis in Diet-Induced Prediabetic Male Sprague Dawley." 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.

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PLAGIARISM DECLARATION

School of Laboratory Medicine and Medical Sciences, College of Health Sciences

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1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's
2. I have used the Vancouver convention for citation and referencing. Each contribution to, and quotation in, this dissertation from the works of other people has been attributed and has been cited and referenced.
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4. I have not allowed and will not allow anyone to copy my work with the intention of passing it off as his or her own work.

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DEDICATION

This work is dedicated to God, my supportive parents, siblings, and colleagues.

ACKNOWLEDGEMENTS

First and foremost, I express my heartfelt gratitude to the Almighty God for granting me the strength and wisdom to complete this project.

To my family: thank you for your support and motivation.

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To the Biomedical Resource Unit (BRU): thank you to the BRU Technicians for their technical assistance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviations	Full name
ADA	American Diabetes Association
AREC	Animal Research Ethics Committee
Akt	Protein kinase B
AUC	Area Under the Curve
BRU	Biomedical Research Unit
ELISA	Enzyme-linked immunosorbent assay
BMI	Body Mass Index
HbA1C	Glycated hemoglobin
FG	Fasting glucose
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
OGTT	Oral glucose tolerance test
GLUT4	Glucose transporter 4
IRS1	Insulin Receptor Substrate 1
IRS2	Insulin Receptor Substrate 2
PI3K	Phosphoinositide 3-kinase
R	Reverse
F	Forward
mTORC 1	Mechanistic Target of Rapamycin Complex 1
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
IF	Intermittent fasting
HFHC	High fat high carbohydrate
TRF	time-restricted feeding
T2DM	Type 2 diabetes mellitus
Met	Metformin
SEM	Standard Error of Mean
NPD	non-prediabetic
PD	Prediabetes
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
RT-qPCR	Real-time quantitative polymerase chain reaction
cDNA	complementary Deoxyribonucleic acid
RNA	Ribonucleic acid
WHO	World Health Organization
NAFLD	Nonalcoholic Fatty Liver Disease
SOD	Superoxide Dismutase

H_2O2	Hydrogen Peroxide

LIST OF PUBLICATIONS FROM THE THESIS

1. Msane, S.; Khathi, A.; Sosibo, A. Therapeutic Potential of Various Intermittent Fasting Regimens in Alleviating Type 2 Diabetes Mellitus and Prediabetes: A Narrative Review. Nutrients 2024, 16, 2692. https://doi.org/10.3390/nu16162692.
2. Msane, S.; Khathi, A.; Sosibo, A.M. The Effect of the14:10-Hour Time-Restricted Feeding (TRF) Regimen on Selected Markers of Glucose Homeostasis in Diet-Induced Prediabetic Male Sprague Dawley Rats.Nutrients2025,17, 292. https://doi.org/10.3390/nu17020292

STUDY OUTLINE

This dissertation is presented in manuscript format and is organized into four chapters. **Chapter 1** consists of an introduction to the study, including its aims, research questions, objectives and hypothesis. **Chapter 2** offers a literature review in manuscript format, exploring the therapeutic potential of various intermittent fasting regimens in alleviating type 2 diabetes mellitus and prediabetes. This work, authored by S. Msane under the supervision of Prof. A. Khathi and Mr. A.M. Sosibo, has been published in *Nutrients* and formatted following the journal's author guidelines. **Chapter 3** details the second study, also presented in manuscript format, which investigates the effects of the 14:10-hour time-restricted feeding (TRF) regimen on selected markers of glucose homeostasis in diet-induced prediabetic male Sprague Dawley rats. This manuscript, authored by S. Msane and supervised by Prof. A. Khathi and Mr. A.M. Sosibo, has been published in *Nutrients* and follows the journal's formatting guidelines. Finally, **Chapter 4** synthesizes the findings of the study as well as provides a conclusion and possible future studies.

ABSTRACT

Background

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by persistent high blood glucose levels due to either impaired insulin secretion, reduced insulin sensitivity, or a combination of both. It is strongly linked to insulin resistance, leptin resistance, obesity, elevated fasting glucose levels, and glucose intolerance. The global incidence of T2DM is increasing rapidly, currently affecting approximately 500 million people worldwide. Projections indicate a 25% rise by 2030 and a 51% increase by 2045. Management of T2DM typically involves pharmacological interventions, such as metformin, combined with lifestyle changes, including enhanced physical activity and dietary modifications. However, poor patient adherence to these approaches often compromises their effectiveness, underscoring the need for alternative treatment options. Recently, intermittent fasting (IF) has garnered interest as a potential management strategy for T2DM. The IF protocols alternate between periods of eating and fasting, which may involve caloric restriction or complete fasting during the fasting phases. The duration and structure of fasting and eating windows differ across various IF regimens, including the 5:2 diet, alternate-day fasting, and time-restricted feeding (TRF). In TRF, individuals typically fast for 14 hours followed by a 10-hour eating window (14:10) or a 16-hour fasting period with an 8-hour eating window (16:8). Studies have demonstrated that the 14:10 TRF regimen effectively reduces body weight, leptin resistance, glucose intolerance, insulin resistance, and blood glucose levels in individuals with T2DM. While the positive effects of 14:10 TRF on T2DM are well-established, its impact on glycemic control during the prediabetic stage remains to be studied.

Materials and Methods

Twenty-four male (120g to 180g) Sprague Dawley rats were obtained and randomly divided into two dietary groups: Group 1 (n=6) received a standard diet and water. In contrast, group 2 (n=18) was provided a high-fat, high-carbohydrate (HFHC) diet supplemented with 15% fructose for 20 weeks to induce prediabetes. After confirming prediabetes, an additional 12 weeks of treatment took place during which an intermittent fasting (IF) regimen was assigned to the rats while also having untreated and metformin-treated prediabetic rats serving as controls. The IF regimen assigned in this study is TRF where Sprague Dawley rats were exposed to fasting for 14 hours, then had a 10-hour window to eat (14:10). Every 4 weeks during the treatment period, body weight, calorie intake, oral glucose response, and fasting glucose were measured for all groups. Following study termination after 12 weeks of treatment, organs such as the skeletal muscle and the liver were harvested from the animals along with blood. Glycogen concentrations were measured in the liver and skeletal muscle tissues. Additionally, the expression levels of insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), protein kinase A (Akt), Phosphoinositide 3-Kinase (PI3K), and Mechanistic Target of Rapamycin Complex 1 (mTORC1) were assessed in liver tissue, while glucose transporter 4 (GLUT4). Moreover, plasma was extracted to assess fasting insulin and leptin concentrations and computed as well as determined Homeostatic Model Assessment of Insulin Resistance (HOMA-IR).

Results

The untreated prediabetic group demonstrated impaired blood fasting glucose throughout the treatment period. However, introducing an IF regimen, even while maintaining an HFHC diet, effectively alleviated this dysregulation. The benefits of IF were evident through significant improvement in GLUT4 expression and skeletal muscle glycogen stores accompanied by a reduction in liver glycogen concentrations. Both the 14:10 hr TRF and HFHC-Met groups showed significantly lower levels of blood glucose, leptin, and BMI compared to the prediabetic group. Additionally, the IF group exhibited significantly lower insulin, HOMA-IR, and HbA1c levels than both control groups.

Conclusion

This study demonstrated that a 14:10 time-restricted feeding regimen positively impacts prediabetes management by improving glucose tolerance, enhancing insulin sensitivity, and upregulating markers associated with glucose homeostasis. While the diet-induced animal model of prediabetes has been shown to closely replicate the human condition, this study cannot conclude that the effects of IF would be identical in humans, as factors like genetic makeup and dietary habits may influence the outcomes. Additionally, it is recommended to conduct further research to explore its application in humans. This study also showed the potential of IF in alleviating prediabetes-related dysregulation of glucose homeostasis and therefore warrants further investigations into its use in the management of prediabetes.

CHAPTER 1: INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a condition marked by elevated blood glucose levels due to insufficient insulin action, impaired insulin secretion, or a combination of both (1, 2). T2DM is linked to leptin and insulin resistance, obesity, impaired fasting blood glucose concentration and glucose intolerance (2, 4-9). The prevalence of T2DM is increasing at a concerning rate, currently affecting nearly half a billion people worldwide (3). This figure is projected to increase by 25% in the year 2030 and by 51% by the year 2045 (3). Managing T2DM involves a combination of pharmacological interventions such as metformin with lifestyle modifications that include increased physical activity and dietary modification (10, 11). However, poor patient compliance with these strategies has been noted to diminish the effectiveness of these strategies therefore alternative interventions are required (12-14). Thus, intermittent fasting has recently emerged as a possible alternative management strategy.

Prediabetes is a metabolic state with an elevated risk of diabetes, characterized by glycemic levels that are above normal but below the threshold for T2DM diagnosis (24). A recent study indicated that the global prevalence of prediabetes will exceed 400 million individuals by the year 2045 (25). The World Health Organization and the American Diabetes Association (ADA) define prediabetes as having an impaired fasting blood glucose (IFBG) level of 5.6–6.9 mmol/L and having an impaired glucose tolerance (IGT) range of 7.8–11.0 mmol/L. The ADA has an additional criterion of glycated hemoglobin (HbA1c) levels between 5.7–6.4% (26). Prediabetes has been linked to the onset of leptin and insulin resistance, elevated HbA1c levels, obesity, intermediate hyperglycemia, moderate glucose intolerance and impaired fasting glucose (27, 28). While the effects of 14:10 TRF on T2DM are well-known, no study has yet examined the impact of this regimen on glycaemic regulation during the prediabetic state.

Intermittent fasting protocols consist of dietary approaches that alternate eating periods with fasting, which may involve reduced calorie intake or complete abstinence from calories during fasting (15, 16). The duration and schedule of fasting and eating periods differ across various intermittent fasting protocols, including the 5:2 diet, alternate-day fasting and time-restricted feeding (TRF) (17). In TRF, individuals can choose to fast for 14 hours each day and eat within a 10-hour window (14:10) or opt for a 16-hour fasting period followed by an 8-hour eating window (16:8) (18, 19). Studies have demonstrated that the 14:10 fasting approach effectively reduces body weight, leptin resistance, glucose intolerance, insulin resistance and blood glucose levels in individuals with T2DM (20-22). However, the onset of T2DM is often preceded by a condition known as prediabetes (23).

Studies show that animal models are commonly used to investigate the metabolic changes linked to both T2DM and prediabetes, as well as to evaluate the impact of different intervention strategies for

managing these conditions (29, 30). In the laboratory, a diet-induced animal model of prediabetes was used, which has been demonstrated to mimic the human condition both in terms of development and progression (31). Therefore, using this model, the current study sought to investigate the effects of a 14:10-hour TRF regimen on markers associated with glycaemic control in diet-induced prediabetic male Sprague Dawley rats.

1. Aim

The aim of the study was to evaluate the effects of a 14:10-hour TRF regimen on markers associated with glycaemic control in diet-induced prediabetic male Sprague Dawley rats

2. Research question

What are the effects of a 14:10-hour TRF regimen on glucose regulation in diet-induced prediabetic male Sprague Dawley rats?

3. Objectives

The specific objectives of this study were to:

1. determine the effect of 14:10 TRF on obesity by measuring body weight and BMI in diet-induced prediabetic rats
2. evaluate the effect of 14:10 TRF on glycaemic control by measuring the fasting glucose, oral glucose tolerance responses and HbA1c in diet-induced prediabetic rats
3. assess the effect of 14:10 TRF on the insulin signaling pathway by measuring the expression of Akt, IRS1, IRS2, mTORC1, PI3K, and GLUT 4 in diet-induced prediabetic rats
4. evaluate the effect of 14:10 TRF on insulin sensitivity by measuring plasma insulin concentrations and evaluating HOMA-IR in diet-induced prediabetic rats
5. determine the effect of 14:10 hour TRF regimen on leptin concentration in diet-induced prediabetic rats

4. Hypothesis

The 14:10 TRF will alleviate diet-induced prediabetic related glucose dysregulation in male Sprague Dawley rats

5. Null hypothesis

The 14:10 TRF does not alleviate diet-induced prediabetic-related glucose dysregulation in male Sprague Dawley rats.

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CHAPTER 2: LITERATURE REVIEW

PROLOGUE

Type 2 diabetes mellitus has been linked to the chronic consumption of high-calorie diets and is characterized by glycemic dysregulation. The onset of this condition is often preceded by prediabetes, a state of intermediate hyperglycemia also associated with excessive calorie intake. Several studies have shown that prediabetes is reversible and therefore it is being targeted for therapeutic intervention to prevent the onset of T2DM. Various studies have shown intermittent fasting to be a promising strategy for managing both T2DM and prediabetes. These intermittent fasting regimens include alternate-day fasting (24 h of fasting followed by 24 h of eating), time-restricted fasting (fasting for 14 h and eating within a 10 h window), and the 5:2 diet (fasting for two days and eating normally for the other five days). This literature review explores the current state of the art on the effects of various intermittent fasting regimens and highlights the gap in the literature as no study has looked at the effects of the time restricted fasting regimen during the prediabetic state.

The literature review in Chapter 2 was published as a manuscript titled "**Therapeutic Potential of Various Intermittent Fasting Regimens in Alleviating Type 2 Diabetes Mellitus and Prediabetes: A Narrative Review"** and is authored by S. Msane, A. Khathi, and A.M Sosibo.

The manuscript is published **https://doi.org/10.3390/nu16162692** in the journal Nutrients **(ISSN: 2072- 6643)** and has been formatted according to the journal's guidelines for authors. **See Appendix 4.** This journal is accredited by the Department of Higher Education and Training South Africa and appears in the Scopus accredited list (2024).

Candidate Contribution: S. Msane was responsible for study conceptualization, study design, first draft writing, and manuscript editing.

Therapeutic Potential of Various Intermittent Fasting Regimens in Alleviating Type 2 Diabetes Mellitus and Prediabetes: A Narrative Review

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Abstract

Intermittent fasting has drawn significant interest in the clinical research community due to its potential to address metabolic complications such as obesity and type 2 diabetes mellitus. Various intermittent fasting regimens include alternate-day fasting (24 h of fasting followed by 24 h of eating), time-restricted fasting (fasting for 14 h and eating within a 10 h window), and the 5:2 diet (fasting for two days and eating normally for the other five days). Intermittent fasting is associated with a reduced risk of type 2 diabetes mellitus-related complications and can slow their progression. The increasing global prevalence of type 2 diabetes mellitus highlights the importance of early management. Since prediabetes is a precursor to type 2 diabetes mellitus, understanding its progression is essential. However, the long-term effects of intermittent fasting on prediabetes are not yet well understood. Therefore, this review aimed to comprehensively compile existing knowledge on the therapeutic effects of intermittent fasting in managing type 2 diabetes mellitus and prediabetes.

Keywords: intermittent fasting; type 2 diabetes mellitus; prediabetes; HbA1c; glucose tolerance

1. Introduction

Intermittent fasting (IF) is a broad term describing several eating regimens in which individuals alternate long periods of normal calorie intake with periods of minimal or no energy intake [1]. In 1935, McCay elucidated the correlation between calorie restriction and lifespan or longevity [2]. Since then, studies have extensively investigated calorie restriction, evolving into the practice of intermittent fasting [1,3–5]. Research findings have highlighted the use of intermittent fasting and its efficacy in metabolic-related disorders [1,3–6]. Several intermittent fasting protocols have been recognized for their capacity to mitigate metabolic disorders [7–15].

The IF protocols include dietary approaches that involve alternating eating periods with either fasting by restricting calorie intake or zero calorie intake during the fasting period [6]. The timing of fasting and feeding periods varies among different IF protocols, such as the 5:2 diet, alternate-day fasting, and time-restricted feeding [16]. The 5:2 fasting diet is a dietary regimen where individuals eat without restrictions for five days, followed by two days per week during which they consume a very-low-calorie diet;fewer than 800 calories per day [17]. Alternate day fasting (ADF) involves alternating between a 24 h fasting period, during which individuals consume less than 25% of their usual energy needs, and a 24 h eating period, where they can eat normally [15]. Time-restricted feeding (TRF) is an IF protocol with a specified time of prolonged fasting practiced by adhering to 16 h of abstinence from food and 8 h of food intake within 24 h [6]. IF has gained popularity in body weight management and alleviating metabolic-related disorders [18,19]. Therefore, this review aims to synthesize existing knowledge on the therapeutic effects of intermittent fasting on the management of T2DM and prediabetes, providing a critical overview of its current state of understanding. This review will begin by examining the impact of various intermittent fasting protocols on general metabolic issues, particularly those associated with type 2 diabetes (T2DM). Given that T2DM is often preceded by prediabetes, both metabolic states will be discussed. The effects and limitations of conventional management strategies for T2DM and prediabetes will be briefly described. Subsequently, this review will explore the impact of all intermittent fasting regimens on T2DM and prediabetes. Then, future recommendations and conclusions about the possible use of IF regimens in diabetes management will be made.

2. Effects of IF on Metabolic Complications

Obesity has emerged as a notable issue. For example, a study reported an obesity-related death rate of 62,000 out of every 100,000 individuals in the population [20]. Epi-epidemiological studies utilize Body Mass Index (BMI) as a tool to identify individuals who are either obese or overweight [21,22]. Obesity has been correlated with the development of several physiological disorders, including type 2 diabetes, inflammation, cardiovascular disease, hypertension, dyslipidemia, non-alcohol fatty liver disease, and insulin resistance (IR)[21,22]. Energy imbalance, leading to excess body fat (≥20%), defines obesity [23–25]. Therapeutic interventions such as fasting and the use of antidiabetic medications have been linked to substantial weight loss, suggesting improvements in clinical

factors associated with metabolic complications [26–29].

The fasting regimens mentioned have demonstrated efficacy in eliciting favorable metabolic alterations. The changes include improved glucose control, reduced glycogen storage, release of fatty acids and ketones, decreased levels of leptin, and increased levels of adiponectin [1,30–32]. In overweight or obese adults, IF has reported a decrease in BMI, body weight, waist circumference, and fat mass [3,17,33–35]. Interestingly, a study conducted in obese middle-aged female Wistar rats found that ADF and TRF did not lead to reductions in blood lipid profiles, adiposity, or insulin resistance. Instead, these dietary interventions increased inflammatory biomarkers, potentially elevating the risk of obesity-associated comorbidities [36]. Other studies reported different outcomes of insulin resistance, blood lipids, adiposity, inflammatory markers, and glycemic control upon IF adherence [37,38].

IR occurs when the main target tissues for insulin action in glucose metabolism do not respond to insulin as they should due to chronic energy surpluses [39]. Therefore, weight loss is essential for regulating disordered glucose and lipid metabolism, notably IR and hyperinsulinemia caused by central obesity [30,31,40]. Furthermore, IR has been implicated in T2DM [41,42]. T2DM is a chronic hyperglycaemic condition triggered by a preceding loss of β-cell insulin secretion and IR[43]. The onset of a metabolic switch brought on by fasting is due to the negative energy balance caused by the depletion of glycogen stores and metabolized fatty acids.

The metabolic switch from using glucose to fatty acid-derived ketones represents a gradual change in the metabolism from lipid/cholesterol synthesis and fat storage to fat mobilization through fatty acid β-oxidation and fatty acid-derived ketones. As a result, the metabolic switch aids in maintaining muscle mass and its function, which promotes weight loss [16]. During fasting, ketones continuously increase while glucose decreases, and this is inverse to the postprandial state, where glucose levels increase while ketones diminish [44]. The outcomes of the metabolic transition observed during fasting may ameliorate insulin sensitivity and glycaemic regulation detected in persons with non-insulin-dependent diabetes.

3. Type 2 Diabetes Mellitus

Diabetes mellitus continues to be a significant contributor to mortality and morbidity rates globally [45]. It is expected that 578 million people will have diabetes by 2030, increasing by 51% (700 million) by 2045 from 463 million in 2019 [45,46]. Approximately 90% to 95% of all diabetes diagnoses are classified as type 2 diabetes mellitus [47]. According to both the American Diabetes Association (ADA) and World Health Organisation (WHO), diabetes can be diagnosed if a person has a fasting plasma glucose level of ≥126 mg/dL (7.0 mmol/L) after fasting for at least 8 h and a plasma glucose level of ≥200 mg/dL (11.1 mmol/L) during a 75 g oral glucose tolerance test (OGTT) [48]. The ADA also uses a glycated hemoglobin (HbA1c) level of at least 6.5% (48 mmol/mol Hb)

to diagnose diabetes [49,50]. Both the ADA and the WHO approve diagnosing glucose in plasma, although it can also be measured in serum and whole blood [51,52].

Multiple organs are involved in the pathophysiology of T2DM. Disruption in the pathways connecting the endocrine pancreas, liver, skeletal muscles, and adipose tissues can disrupt glucose regulation, leading to the onset of T2DM, as illustrated in Figure 1. The primary causes of the disruption include chronic inflammatory markers (e.g., interleukin-6 and C-reactive protein) and overnutrition, especially through diets high in carbohydrates and saturated fats [53]. They disrupt the insulin signaling pathway in muscle, liver, and adipose tissues, resulting in hyperglycemia. Progressive hyperglycemia further damages the β-cells of pancreatic islets by inducing oxidative stress within the β-cells of pancreatic islets [54].

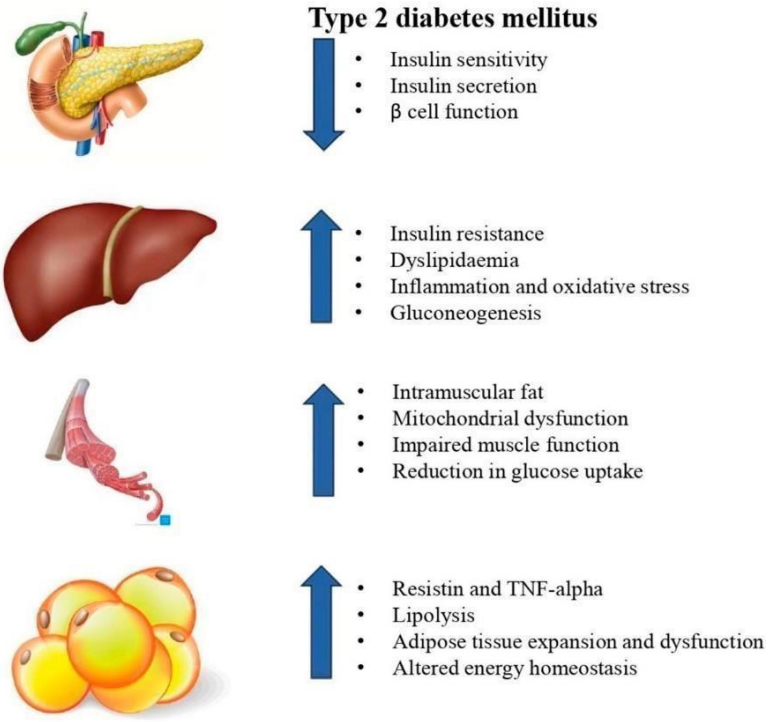

Figure 1. Illustrates the primary organs and molecules whose modifications contribute to the progression of type 2 diabetes mellitus.

4. Conventional Management of T2DM

4.1. Insulin Therapy

Insulin therapy serves as an injectable medication for diabetes mellitus [55]. The use of exogenous insulin is primarily employed to regulate blood glucose levels and alleviate symptoms of T2DM by replenishing or complementing the body's natural insulin production from the pancreas [55,56]. Insulin therapy directly activates the insulin receptor, leading to increased glucose uptake, decreased production of glucose by the liver, and decreased breakdown of fats [57]. Hence, insulin therapy is positively associated with a decrease in fasting glucose levels, glucose tolerance, and HbA1c [58–60]. Nevertheless, diminishing fat breakdown may lead to the buildup of triglycerides in both the bloodstream and fatty tissues, resulting in an elevated likelihood of gaining weight [61]. The weight gain mechanism in insulin-treated T2DM involves factors such as hypoglycemia-associated snacking, inhibited glucose excretion (glycosuria), and decreased metabolic rates [62].

4.2. Glucagon-like Peptide-1 Receptor Agonists

Glucagon-like peptide-1 (GLP-1) is a hormone primarily located in the gastrointestinal tract, secreted in response to the ingestion of nutrients (carbohydrates and fats) [63]. Glucagon-like Peptide-1 Receptor Agonists (GLP-1Ra) functions by controlling elevated blood glucose levels after meals by increasing the release of insulin from the βcells [64]. These drugs have been associated with decreased FG, HbA1c, and GT [65–67]. Additional benefits encompass suppressing glucagon release, delaying stomach emptying, promoting insulin release, and reducing appetite [63,64,68]. The disadvantages of using GLP-1Ra have been associated not only with gastrointestinal effects but also with gallbladder diseases, attributed to reduced gallbladder refilling [69,70].

4.3. Dipeptidyl Peptidase-4 Inhibitors

Dipeptidyl peptidase-4 inhibitors (DPP4i) function by inhibiting the activity of DPP-4 [71]. DPP4i enhances insulin secretion from pancreatic β-cell in a glucose-dependent manner through the action of GLP-1 while concurrently decreasing glucagon release from alpha cells [68,72]. DPP-4 inhibitors have been demonstrated to effectively lower FG, post- prandial glucose, and HbA1c levels while maintaining a low risk of hypoglycemia [72,73]. Nonetheless, there has been a noted rise in the occurrence of acute pancreatitis linked to their utilization [71,74].

4.4. Sodium-Glucose Co-Transport 2 Inhibitors

Sodium-glucose co-transport (SGLT)-2 is a kidney transporter responsible for the reabsorption of glucose from the renal filtrate, thus hindering the excretion of glucose through urine [75]. SGLT2 inhibitors represent a newer class of antihyperglycemic medications that function independently of insulin, providing effects beyond simply lowering glucose levels. These drugs promote urinary glucose excretion and natriuresis by inhibiting the reabsorption of glucose and sodium in the proximal tubule of the kidney [75]. This class of antidiabetic drugs has been revealed to reduce HbA1c levels (~0.51–1.01%), FG, and postprandial glucose [76–78]. The disadvantages related to the administration of SGLT2i include increased risk of urinary tract infection, genital infection, and lower limb amputation [79,80].

4.5. Biguanide (Metformin)

It a commonly prescribed antidiabetic drug, is widely acknowledged as a biguanide with properties that enhance insulin sensitivity [81]. It improves glucose utilization and sensitivity to insulin in tissues outside the liver [82]. At therapeutic doses, metformin utilizes multiple mechanisms to reduce blood glucose levels [83]. This antidiabetic drug has been reported to improve GT, HbA1c, and FG [83]. While the liver is the main target organ for metformin action, there is also evidence suggesting involvement of the intestines [84].

Metformin's effects in the gastrointestinal tract encompass increased intestinal absorption and lactate generation, elevated concentrations of GLP-1, and modification of bile acid pools, consequently impacting the microbiome's composition [84]. However, the use of metformin has been associated with the occurrence of vitamin B_{12} deficiency, which may contribute to the manifestation of diabetic neuropathy symptoms [81,85].

Furthermore, changes in gut flora, alterations in gut motility, competitive inhibition of absorption, and impairment of calcium-dependent membrane actions in the terminal ileum have been proposed as mechanisms contributing to the development of vitamin B_{12} deficiency associated with metformin use [85,86]. Regular monitoring of vitamin B_{12} levels and appropriate supplementation may be necessary for individuals on long- term metformin therapy to address this potential concern. Different types of antidiabetic drugs and their mode of action are shown in Table 1.

Table 1. Shows the different types of antidiabetic drugs and their mode of action, effects on glucose parameters, and shortfalls.

Types of antidiabetic drug(s)	Mode of Action	Effects on glucose parameters	Shortfall(s)
Insulin therapy	Direct glucose-lowering effect Facilitation of glucose uptake by cells Inhibition of hepatic glucose production Promotion of glycogen synthesis	Reduced FG Reduced GT Reduced HbA1c	Weight gain
GLP-1RA	Slowing of gastric emptying Suppression of glucagon secretion Enhancement of glucose-dependent insulin secretion Improvement in Beta Cell Function Reduction of Appetite and Food Intake	Reduced FG Reduced GT Reduced HbA1c	Gastrointestinal effects Gallbladder disease
DPP4i	Inhibition of DPP-4 enzyme Reduction in Blood Glucose Levels Prolongation of incretin hormone activity Weight management	Reduced FG Reduced GT Reduced HbA1c	Acute pancreatitis
SGL2i	Inhibition of SGLT2 in the kidneys Increased urinary glucose excretion Reduction in blood glucose levels Caloric loss and weight reduction Osmotic Diuresis	Reduced FG Reduced GT Reduced HbA1c	Urinary tract infection, Genital infection Lower limb amputation
Metformin	Enhanced peripheral glucose uptake Inhibition of intestinal glucose transport Improvement of lipid metabolism.	Reduced FG Reduced GT Reduced HbA1c	Vitamin B12 deficiency Lactic acidosis

5. Lifestyle Intervention

Lifestyle intervention is widely recognized for its effectiveness in reducing the risks associated with T2DM [87]. Lifestyle intervention has been linked to reduced occurrences of T2DM and lower incidences of cardiovascular events, microvascular complications, cardiovascular mortality, and all-cause mortality, leading to increased life expectancy in patients with IGT [87]. The application of lifestyle intervention has also been reported as cost-

effective for patients who adhere to it [88]. A DPP 10-year follow-up diabetes study showed that at 2.8 years, there was a 58% reduction in the incidence of diabetes among high-risk adults with lifestyle intervention, which was superior to the 31% reduction observed with metformin [89]. In the initial year visit, a mean weight loss of 7 kg was observed [89]. This confirms the superiority of lifestyle intervention over the established first-line drug, metformin. Intervention strategies encompass physical activity, exercise, and dietary plans [90].

5.1. Dietary Intervention

Dietary intervention encompasses the banting diet, the ketogenic diet, and the Mediterranean diet [91–93]. The Banting diet is characterized by high protein intake, whereas the ketogenic diet focuses on low carbohydrates, high fat, and adequate protein, and the Mediterranean diet emphasizes a higher consumption of vegetables [91,93,94]. To achieve long-term weight loss, factors such as meal timing and macronutrient composition must counteract compensatory changes in hunger, cravings, and ghrelin suppression mechanisms. These factors can serve as a boost for weight gain after a previous loss [95]. However, dietary intervention has been linked with positive effects on FG, GT, and HbA1c [96–98]. Abstinence from food or fasting entails the breakdown of lipids, carbohydrates, and proteins to regulate plasma glucose within the normal range. Progressive accumulation of fats in the pancreas and liver may lead to dysfunction of β cells, resulting in hyperglycemia. This condition can be reversed by reducing fats in the liver and pancreas [5].

5.2. Increased Physical Activity

Physical activity has demonstrated antidiabetic effects in individuals with T2DM [99]. Physical activity consists of body movements driven by the contraction of skeletal muscles, resulting in increased energy expenditure [99]. Increased physical activity, such as exercise interventions, has been implemented to alleviate hyperglycemia [100]. Research has shown that low- and moderate-intensity exercise can lower FG, GT, and HbA1c levels [101,102]. Qualitative research revealed that obstacles to physical activity can include health issues (such as breathing problems), difficulties with time and lifestyle management (such as lack of time and motivation), and various environmental, social, and cultural factors [103].

6. Effect of Intermittent Fasting on T2DM

6.1 Alternate Day Fasting

Many investigations have been carried out to assess the safety and tolerability of alternate-day fasting regimens, showing promising clinical outcomes related to T2DM [104–106]. Research findings suggest that alternate-day fasting can serve as an alternative approach to continuous CR, with superior effects observed in the retention of lean mass [105,107]. The utilization of alternate-day fasting resulted in a significant decrease in total cholesterol and serum triglycerides [108]. Another study, supported by evidence, demonstrated that adherence to alternate-day fasting can positively impact glucose tolerance within 3 weeks via heightened expression of the SIRT1 gene [109]. Despite a notable reduction in total intra-abdominal fat mass, the alternate-day fasting group reported a failure to alleviate diet-induced muscle insulin resistance caused by a high-fat diet [110].

The potential cause may be attributed to a decrease in the expression of GLUT-4 protein in both high-fat *ad libitum* (HF-AL) and high-fat alternate-day fasting (HF-ADF) rats compared to the Chow group [110]. Conversely, another study showed a positive impact on glycemic control in genetically obese mice undergoing alternate-day fasting despite the absence of significant weight loss [111]. In mice, fructose-induced insulin resistance was alleviated by a 100% restriction on chow food but allowing *ad libitum* access to a fructose drink during the fasting days of alternate day fasting. This group exhibited significant improvements in insulin sensitivity compared to the control group [112]. A study revealed a direct correlation between alterations in body weight and improvements in glycemic control, insulin sensitivity, and insulin secretion in obese males with and without T2DM [113]. Alternate-day fasting produces superior outcomes, specifically a decrease in fasting insulin levels and insulin resistance, compared to continuous CR in individuals with insulin resistance [34].

Alternate fasting has also been associated with adverse effects, including hunger, impaired cognitive function, and irritability, which may diminish within a month of adherence [114,115]. On the contrary, a six-month study found that combining ADF with a low-carbohydrate diet did not result in changes in appetite [116]. This suggests that various alternate-day fasting protocols may lead to diverse outcomes upon adherence. Therefore, further research is necessary to assess the impact of different alternate-day fasting approaches on the body's physiological functions.

6.2. The 5:2 Fasting Diet

The 5:2 diet regimen may serve as an alternative approach to continuous CR, exhibiting reported comparable efficacy in weight management and glycemic control [117]. A 600 kcal/day diet has been demonstrated to yield significant improvements in endocrine pancreatic β cell function and hepatic insulin sensitivity, potentially leading to a reversal of T2DM [118]. A 12-week study comparing consecutive versus non-consecutive fasting days utilizing the 5:2 diet regimen demonstrated significant reductions in weight and glycemic levels among individuals with T2DM [119]. Dietary restriction of energy intake was associated with substantial improvements in various markers, including reductions in HbA1c levels, improved results in OGTT, decreased pancreatic and liver triacylglycerol stores, and lowered FG levels [118]. However, cautious measures may be necessary for continuous Very Low-Calorie Diet (VLCD) regimens, particularly in managing oral hypoglycemic agents to prevent hypoglycemia [120]. Additionally, VLCDs can pose long-term risks of complications such as micronutrient deficiencies [121]. The 5:2 diet has been associated with significantly lower compliance rates. It has been indicated that this diet often results in significant overcompensation during non-fasting days [4].

6.3. Time-Restricted Feeding

TRF has been documented to exhibit a high adherence rate among participants [122]. This regimen has been shown to positively influence fasting glucose, glucose tolerance, and HbA1c levels in T2DM [123]. The glycemic impacts can be achieved through the Circadian Timing System [124]. Several studies have proven that aligning with the circadian timing system plays a role in positive outcomes upon TRF adherence [125–128]. Research involving both humans and animals suggests that early TRF is favored as the superior regimen over late TRF

[125,128]. Early TRF has been shown to enhance insulin sensitivity, promote weight loss and fat oxidation, and help manage glycemic levels [128]. Different types of intermittent fasting regimens are shown in Table 2.

Table 2. Summary of Different Types of Intermittent Fasting Regimens, Their Positive Effects, and Adverse Effects on Metabolic Disorders.

IF regimen	Description	Positive effects	Adverse effects
5:2 fasting diet (32-34)	Involves a 5-day non-fasting period and a 2-day fasting period.	Improvements in weight, HbA1c, lipids, fasting glucose, and quality of life	Fasting elevated the occurrence of hypoglycemia even with reduced medication. Fasting may lead to over-compensation during non-fasting days.
Alternate day fasting (35-37)	Involves alternating between a 24-hour fasting period, during which individuals consume less than 25% of their usual energy needs, and a 24-hour eating period, where they can eat normally.	Decrease in body weight, waist circumference, systolic blood pressure, and fasting plasma glucose.	Fatigue, Headaches
16h:8h TRF (21, 38)	Adhering to 16 hours of abstinence from food and 8 hours of food intake within 24 hours	Heightens insulin sensitivity and fat oxidation and decreases body weight, fat profile, and inflammation	Hunger and irritability Palpitations, dizziness, headache, abdominal pain, mood change, vomiting, and hypoglycemia
14h:10h TRF (21, 39, 40)	Adhering to 14 hours of abstinence from food and 10 hours of food intake within 24 hours	Reduced body weight, improved HbA1c, enhanced body composition, lowered blood pressure, and decreased lipids associated with cardiovascular disease.	Disrupted Social Eating Patterns Palpitations, dizziness, headache, abdominal pain, mood change, vomiting, and hypoglycemia

7. Prediabetes

Despite the possibility of correcting prediabetes to normal glucose regulation, it nonetheless imposes a strain [134]. Prediabetes can be characterized by elevated blood glucose levels that do not meet the diagnostic criteria for diabetes mellitus [135]. Prediabetes has an asymptomatic characteristic, and this makes it hard to diagnose [136]. The prevalence of prediabetes is increasing year after year, with 5% to 10% of prediabetic people advancing to fatal T2DM and its related complications [31,136,137]. The diagnostic criteria of prediabetes include FG, IGT, and HbA1c. However, the WHO does not recognize HbA1c as a diagnostic criterion for prediabetes. It has been shown that the ADA identifies individuals with an IFG of 5.6–6.9 mmol/L, an IGT of 7.8–11.0 mmol/L, and a HbA1c of 5.7–6.4% as prediabetic [138]. The rising prevalence of prediabetes is causing significant concern. Recent research suggests that the global prevalence of prediabetes is expected to exceed 400 million individuals by 2045 [139].

Research has demonstrated that insulin resistance in adipose tissue contributes to the onset of hyperglycemia and associated complications [140–143]. Insulin resistance in adipose tissue stimulates the increased release of free fatty acids into the bloodstream, facilitating ectopic fat storage [141]. This process induces insulin resistance in the liver and skeletal muscles, culminating in metabolic issues such as elevated glycemic levels, abnor- mal lipid levels, hypertension, metabolic syndrome, and NAFLD [140–142]. Decreased physical fitness has been linked to increased levels of free fatty acids, reduced insulin clearance, diminished insulin sensitivity in muscles, slightly elevated triglycerides, and decreased levels of HDL cholesterol [142]. Additionally, research indicates that prediabetic individuals with insulin resistance face double the risk of cardiovascular disease compared to prediabetic individuals who do not have insulin resistance [144]. Prompt detection of prediabetes and its associated complications is vital for mitigating the risks it poses to individuals.

HOMA-IR

The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) represents a diagnostic tool used in clinical settings to evaluate the resistance of insulin, calculated as HOMA-IR = (Fasting Insulin (mU/L) × Fasting Glucose (mmol/L))/22.5 [145]. Pre- diabetic individuals have insulin resistance in their hepatocytes, fatty tissues, or skeletal muscles [146].

8. Prediabetes Management

Irrespective of the current criteria employed for prediabetes diagnosis, the presence of IR, obesity, and either IFG, IGT, or both, still poses the risk of progressing to T2DM [21,22,144,147]. Various measures have been utilized to decrease prediabetes prevalence and progression to T2DM. Primary treatments for prediabetes include a combination of lifestyle changes such as weight loss and increased physical activity, as well as the use of medications such as metformin [148]. Lifestyle changes have demonstrated effectiveness in reducing the risk of developing T2DM, even with less intensive interventions [149].

8.1. Biguanides (Metformin)

Metformin is a medication used for managing both prediabetes and T2DM [83]. Interestingly, research indicates that metformin has beneficial effects on glucose measures such as FG, GT, and HbA1c levels [150,151]. However, using metformin alone is less effective than combining it with other antidiabetic medications or lifestyle changes [151,152]. Furthermore, metformin use is associated with adverse effects such as lactic acidosis, vomiting, and diarrhea [150].

8.2. Lifestyle Modification

A lack of physical activity and obesity significantly contribute to the advancement of T2DM [153]. Physical activity involves the body's movement through the contraction of skeletal muscles, which leads to an elevation in energy expenditure [154]. Increased physical activity, such as exercise interventions, has been implemented to alleviate prediabetes [100]. The exercise interventions have improved IFG, IR, IGT, HbA1c levels, and weight loss. It is associated with muscle insulin sensitivity [155]. An increase in insulin sensitivity is facilitated

by the movement of several GLUT4 transporters to the cell membrane in response to a submaximal insulin stimulus [155], thereby promoting glucose tolerance and reducing glucose levels in the bloodstream [100]. However, short exercise interventions have been reported to fail to alter HDL-C levels [156]. A multivariate analysis found that the duration of exercise per session is a key predictor of changes in HDL cholesterol levels [157]. Additionally, the efficacy of exercise intervention in raising HDL-C levels has been associated with lower BMI or higher total cholesterol levels [157,158]. Variations in blood glucose levels could be affected by the type of physical activity engaged in, and specific exercise modalities might not be viable options for individuals who are overweight or obese [159]. Therefore, overweight or obese prediabetic individuals need to prioritize weight loss as a preliminary step to enhance their HDL-C levels.

Weight loss strategies often involve dietary interventions, which typically entail reducing calorie intake to manage body weight and address other clinical factors [160]. Very low-calorie restriction has been linked to improvements in beta-cell function, leading to the restoration of the first phase of insulin secretion in prediabetic individuals [161]. Studies have reported a reduction in fasting glucose levels and HbA1c, weight loss, increased fasting insulin levels, and improved Homeostatic Model Assessment of Beta-cell Function (HOMA-β) [161,162]. Nevertheless, elevated fasting ghrelin levels have been correlated with an increased risk of weight regain following weight loss [163]. Interestingly, achieving positive metabolic outcomes through intermittent fasting may not necessarily require weight loss [164].

8.3. Intermittent fasting

8.3.1. Alternate-Day Fasting

Recent research has emphasized the potential of IF as an alternative approach for addressing metabolic factors associated with prediabetes [52,104,164]. The literature has shown the efficacy and safety of ADF, the 5:2 fasting diet, and TRF in managing prediabetes [165,166]. Although Ingersen and colleagues reported a lack of significant changes in insulin sensitivity or secretion, other studies have suggested that ADF has the potential to decrease body weight, lower fasting insulin levels, improve IFG, reduce postprandial hyperglycemia, and decrease levels of HbA1c [34,113,167].

8.3.2. The 5:2 Fasting Diet

The 5:2 fasting diet has demonstrated notable efficacy in diminishing body weight, improving insulin sensitivity, and lowering both IFG and HbA1c over a 12-week intervention period [168]. Despite achieving favorable results, the effectiveness of fasting methods in enhancing metabolic factors might fluctuate depending on the duration of fasting or the specific fasting strategies employed [52,166]. Fernanda et al. revealed that long-term intermittent feeding led to glucose intolerance while maintaining insulin receptor phosphorylation. It significantly increased insulin receptor nitration in both intra-abdominal adipose tissue and muscle, a modification linked to receptor inactivation [169]. Hence, this study aims to highlight the benefits of the IF regimen, which may serve as the standard alternative approach for prediabetes.

8.3.3. Time-Restricted Feeding

A commonly followed approach to TRF involves fasting for 16 h and consuming meals within an 8 h window each day (16/8) [170]. Alternatively, individuals may choose to fast for 14 h and consume meals within a 10 h window daily (14/10), or they may opt for a 20 h fasting period followed by a 4 h window for food consumption (20/4) [132,171]. Clinical studies have been drawn to these approaches due to their effects in regulating T2DM and its associated complications in short-term studies [12,172]. A systematic review revealed that the 16/8 and 14/10 fasting methods exhibit similar efficacy in weight loss [173].

Research has indicated the effectiveness of adhering to a 14/10 fasting regimen in controlling glycemic levels despite it not affecting insulin sensitivity in T2DM [37,174]. TRF has been demonstrated to increase insulin sensitivity, decrease blood glucose levels, reduce fasting insulin, reduce HbA1c levels, and enhance glucose tolerance by stimulating beta-cell responsiveness [12,37,132,164,172]. On the contrary, literature has examined the effects of adhering to TRF either early or late in the day. The literature has observed that consuming meals late in the day leads to a suppression of resting energy expenditure, reduced fasting carbohydrate oxidation, and impaired glucose tolerance [175,176]. Meal timing plays a role in weight loss therapy, with delayed lunch consumption being associated with less weight loss compared to eating earlier, regardless of adhering to a hypocaloric diet [177]. Research consistently shows that consuming meals earlier in the day (8 a.m. to 7 p.m.) is notably more effective in reducing body weight, fasting glucose levels, and insulin resistance, enhancing insulin response, and decreasing ghrelin levels compared to eating later in the day (12 p.m. to 11 p.m.) [177–179]. Thus, early TRF has superior effects on metabolic factors compared to late TRF. Below is Table 3 summarizing the various types of IF regimens and their effects on prediabetes.

Table 3. Summary of Different Types of Intermittent Fasting Regimens, Their Positive Effects, and Adverse Effects on Prediabetes.

IF regimen	Description	Positive effects	Adverse effects
5:2 fasting diet (41, 42)	Involves a 5-day non-fasting period and a 2-day fasting period.	Improvements in body weight, HbA1c, lipids, fasting glucose, and appetite score	Increased hunger
Alternate day fasting (43-45)	Alternating 24 hours fasting and 24 hours eating period	Improved insulin sensitivity, decreased body weight, lower fasting insulin levels, improved IFG, reduced postprandial	Increased hunger, vomiting, redox imbalance, and glucose intolerance

		hyperglycemia, and decreased levels of HbA1c	
Time-restricted feeding (46, 47)	Fasting and consuming meals within a limited timeframe of 24 hours.	Reduce FG, HbA1c, and postprandial glucose	Dizziness, hunger, nausea

9. Conclusions

The available anti-diabetic medications have demonstrated the potential to improve T2DM and prediabetes. However, significant drawbacks have surfaced, creating an opportunity for alternative approaches. Numerous studies have examined the effects of intermittent fasting on T2DM. However, research on the relationship between intermittent fasting and prediabetes is still scarce. Intermittent fasting has been proven beneficial in short-term studies, highlighting its beneficial impacts on metabolic factors. These effects have been investigated in metabolic disorders such as obesity and T2DM. Nevertheless, the precise mechanisms through which IF modulates glycemic levels and its enduring influence on gene expression, such as GLUT 4 and IRS1, remain unclear. Additionally, the impact of intermittent fasting on glycemic markers and identifying an optimal IF regimen for prediabetes management is yet to be fully elucidated. Therefore, there is a need for comprehensive, long-term investigations to assess the role of IF in glycemic regulation and its associated gene expression, including examining key regulators such as GLUT 4 and IRS1.

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BRIDGE

The first manuscript highlighted the therapeutic potential of various intermittent fasting regimens in managing T2DM and prediabetes. Intermittent fasting approaches include alternate-day fasting (24 hours of fasting followed by 24 hours of eating), time-restricted fasting (e.g., fasting for 14 hours with a 10- hour eating window), and the 5:2 diet (fasting for two days per week and eating normally on the remaining five days). Short-term studies have demonstrated the effectiveness of these regimens in addressing T2DM and prediabetes-related disorders. The literature also reported potential effects of the 14:10 time-restricted fasting regimen on T2DM. Further, this review identified a gap in research specifically focused on the 14:10 protocol in the context of prediabetes. The 14:10 regimen will alleviate prediabetes-associated glycemic dysregulation. This hypothesis led to an investigation into the effects of the 14:10 time-restricted feeding regimen on markers of glucose homeostasis in diet-induced prediabetic rats.

CHAPTER 3: ORIGINAL RESEARCH MANUSCRIPT

PROLOGUE

The findings presented in Chapter 2 highlight reports from the literature on the potential adverse effects of antidiabetic drugs, which have prompted the exploration of intermittent fasting as an alternative strategy for managing T2DM and prediabetes. Studies have demonstrated the impact of adherence to various intermittent fasting regimens on T2DM, including alternate-day fasting (24 hours of fasting followed by 24 hours of eating), time-restricted fasting, for example, fasting for 14 hours with a 10-hour eating window, and the 5:2 diet; fasting for two days per week and eating normally on the remaining five days. Research has shown that alternate-day fasting, the 5:2 diet, and, most notably, the 16:8 time-restricted fasting regimens, have the potential to alleviate T2DM and prediabetes. Since the onset of T2DM complications is often preceded by the prediabetic state, managing prediabetes is crucial, as studies suggest it can be reversed to a normal metabolic state. Prediabetes is characterized by elevated glycemic levels that are higher than normal but below the diagnostic threshold for T2DM. Despite these insights, research specifically investigating the relationship between the TRF regimen and prediabetes remains limited. To address this gap, the original research manuscript explores the effects of the 14:10 TRF regimen on markers of glucose homeostasis in diet-induced prediabetic rats.

The manuscript in Chapter 3 is titled "**The Effect of the 14:10-Hour Time-Restricted Feeding (TRF) Regimen on Selected Markers of Glucose Homeostasis in Diet-Induced Prediabetic Male Sprague Dawley Rats**" and is authored by S. Msane, A. Khathi, and A. Sosibo.

The manuscript is published **https://doi.org/10.3390/nu17020292** in the journal Nutrients and has been formatted according to the journal's guidelines for authors**. See Appendix 4.** This journal is accredited by the Department of Higher Education and Training South Africa and appears in the Scopus accredited list (2025).

Candidate Contribution: S. Msane was responsible for study conceptualization, study design, data curation, first draft writing, and manuscript editing.

The Effect of the 14:10-Hour Time-Restricted Feeding (TRF) Regimen on Selected Markers of Glucose Homeostasis in Diet-Induced Prediabetic Male Sprague Dawley Rats

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Abstract:

Background: Prediabetes is a condition that often precedes the onset of type 2 diabetes mellitus(T2DM). Literature evidence indicates that prediabetes is reversible, making it an important therapeutic target for preventing the progression to T2DM. Several studies have investigated intermittent fasting as a possible method to manage or treat prediabetes. **Objectives:** This study evaluated the impact of a 14:10-hour time-restricted feeding (TRF) regimen on leptin concentration, insulin sensitivity and selected markers associated insulin signalling pathway and glucose homeostasis in diet-induced prediabetic rats. **Methods**: Twenty-four male Sprague Dawley rats were obtained and randomly divided into two dietary groups: Group 1 (n=6) received a standard diet and water, while group 2 (n=18) was provided a high-fat, high-carbohydrate (HFHC) diet supplemented with 15% fructose for a period of 20 weeks to induce prediabetes. After confirming prediabetes, an intermittent fasting (IF) regimen was assigned to the rats while also having untreated and metformin-treated prediabetic rats serving as controls. **Results**: Both IF and HFHC-Met groups yielded significantly lower blood glucose, leptin and BMI results compared to the prediabetic group. The IF group yielded significantly lower insulin, HOMA-IR and HbA1C than both controls. **Conclusions**: The study showed the potential of IF in alleviating prediabetes-induced dysregulation of glucose homeostasis and therefore warrants further investigations into its use in the management of prediabetes.

Keywords: intermittent fasting; prediabetes; insulin signaling pathway; glucose regulation

1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by increased blood glucose concentrations due to insulin resistance and a relative deficiency in insulin production (1). Globally, the prevalence of diabetes is increasing at an alarming rate, with nearly half a billion individuals currently affected (2). This number is expected to grow by 25% in 2030 and 51% in 2045 (2). Although various treatments for T2DM are available, none have been shown to completely reverse the condition. The onset of T2DM is commonly preceded by a state known as prediabetes (3). Prediabetes is a metabolic state characterized by impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) that have not reached the diagnostic criteria for T2DM (3). Despite the World Health Organization not including HbA1c as a criterion, the American Diabetes Association (ADA), on the other hand, defines prediabetes as having an IFG level between 5.6–6.9 mmol/L, an IGT range of 7.8–11.0 mmol/L and an HbA1c level between 5.7–6.4% (4). A recent study indicated that the global prevalence of prediabetes is projected to surpass 400 million people by 2045 (5). Studies have shown that prediabetes is reversible and has thus become a therapeutic target to prevent the onset of T2DM (6-8). Primary strategies for managing prediabetes include increased physical activity, dietary modifications, the administration of metformin as well as intermittent fasting (9).

Intermittent fasting (IF) is a dietary regimen that involves cycling between periods of food intake and extended intervals of calorie restriction (10). Common IF regimens encompass alternate-day fasting, the 5:2 fasting protocol, and time-restricted feeding (TRF) (11,12). TRF is an IF protocol with a specified time of prolonged fasting practiced by adhering to 14 hr of abstinence from food and 10 hr of food intake within 24 hrs (10). The 14:10 hour IF protocol has been shown to decrease HbA1c levels, fasting blood glucose, and body weight (-3.15%), and enhance lipid profiles in obese T2DM patients (13, 14). IF has shown potential benefits for individuals with T2DM: including decreases in fasting insulin levels, insulin resistance, IGT, triacylglycerol levels, fasting glucose levels, and improvements in insulin sensitivity (13-17). However, there is limited knowledge regarding the impact of IF on glucose regulation in individuals with prediabetes and an insufficient understanding of its role within the insulin signaling pathway in the long term.

Research indicates the widespread use of animal models to study the metabolic changes associated with both T2DM and prediabetes as well as the effects of various intervention strategies to manage these conditions (18, 19). Our laboratory employs a diet-induced animal model of prediabetes which has been shown to mimic the human condition (20). Using this model, the study sought to investigate the effects of a 14:10-hour TRF regimen on glucose regulation in prediabetic male Sprague Dawley rats.

2. Materials and Methods

2.1. Animals and Housing

All animal experimentation was approved by the Animal Research Ethics Committee (AREC) of the University of Kwa-Zulu Natal. The authors, the Biomedical Research Unit staff, and the veterinarian were informed about the group assignments at various stages of the experiment. Three-week-old male Sprague-Dawley rats bred and housed in the Biomedical Research Unit (BRU) (ethics no. AREC/00006223/2023(00022008) of the University of Kwa-Zulu Natal were used in the study. The rats were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), carbon dioxide (CO_2) content (< 5000 ppm.), relative humidity (55 ± 5%), and illumination (12 h light/dark cycle, lights on at 07h00). The noise level was maintained at less than the approved 65 decibels. The rats were allowed access to food and fluids *ad libitum*. The rats acclimatized to their new environment for 1 week while consuming standard rat chow and tap water before the induction of prediabetes by exposure to a well-established experimental diet (HFHC) shown in Supplementary Table 1.

2.2. Prediabetes Induction

Twenty-four male Sprague Dawley rats were obtained and randomly divided into two dietary groups: group 1 and group 2. Experimental pre-diabetes was induced in these rats following a previously established protocol (21) . Group 1 (n=6) received a standard diet and water, while group 2 (n=18) was provided with a high-fat, high-carbohydrate (HFHC) diet supplemented with 15% fructose solution as drinking fluid for a period of 20 weeks. After this period, the American Diabetes Association's criteria for pre-diabetes were applied to all rats. Rats were classified as pre-diabetic if they exhibited FBGlevels between 5.6 and 6.9 mmol/L and 2-hour glucose levels between 7.1 and 11.1 mmol/L in an oral glucose tolerance test (OGTT). The rats falling below these thresholds were considered non-pre-diabetic.

To calculate the sample size for this study, we utilized G power software, which is capable of accommodating both distribution-based and design-based modes as well as the minimum number of rats to perform statistical analysis. The calculations from Algorithm 1 determined that at least 12 rats are required to perform the statistical analysis. In alignment with ethical considerations, we opted to adhere to the minimum of 6 per group. The input and output are displayed as follows:

Algorithm 1: A priori power analysis for a one-way ANOVA with fixed effects, calculating the required sample size. The analysis is based on the specified input parameters: effect size, significance level, desired power, and number of groups.

F tests - ANOVA: Fixed effects, omnibus, one-way

Analysis: A priori: Compute required sample size

Input: Effect size f = 1.335799

α err prob = *0.05*

Power (1-β err prob) = *0.80*

Number of groups = *4*

Output: *Noncentrality parameter λ* = *21.4123076*

Critical F = *4.0661806*

Numerator df = *3*

Denominator df = *8*

Total sample size = *12*

Actual power = *0.8681756*

2.3. Oral glucose tolerance response

An oral glucose tolerance test was performed on all rats to assess their glucose tolerance response. This test was completed after carbohydrate loading, following a well-established laboratory technique (22). After fasting for 12 hours, glucose levels were determined at time 0. Then, a monosaccharide syrup was administered orally using an 18-gauge gavage needle that is 38 mm long and curved, with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). The glucose concentration was determined by collecting blood using the tail-prick method (23) and measuring glucose concentrations using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). Glucose levels were subsequently assessed at 15-, 30-, 60-, and 120 minutes following carbohydrate loading.

2.4. Intermittent Fasting Protocol

After measuring glucose levels, an IF protocol was introduced to prediabetic male Sprague Dawley rats. Group 2 was split into three subgroups: A, B and C. Group A (n=6) continued with an HFHC diet supplemented with 15% fructose solution as drinking fluid the entire experimental period (12 weeks). Group B (n=6) followed a well-established IF protocol (11), where food was removed at 17:00 in the evening and returned at 07:00 the next morning, for 5 days each week over a 12-week period. During the eating window, the Sprague Dawley rats were provided with an HFHC diet and 15% fructose solution as drinking fluid. However, during the fasting period, they were only given water. Group C (n=6) was introduced to Metformin. This group continued with HFHC diet supplemented with 15% fructose drinking solution and was treated with an oral dose of metformin (500 mg/kg, Sigma-Aldrich, St Louis, Missouri, USA every third day for 12 weeks. Every 4 weeks, body weight, oral glucose response, and fasting glucose were measured for all groups. Furthermore, body mass index was calculated using the formula: BMI = weight (g)/height (cm^2).

During the 12-week treatment period, calorie intake was measured every four weeks in all the experimental groups. Calorie intake was calculated using the following formulas: For the HFHC diet, Calorie intake = (Total calorie intake for HFHC diet × Amount of food consumed) / 100. For the standard diet, Calorie intake = (Total calorie intake for standard diet × Amount of food consumed) / 100. See Appendix 5 for food composition and

overall caloric contribution from fats, proteins, and carbohydrates for both the HFHC diet and the standard diet.

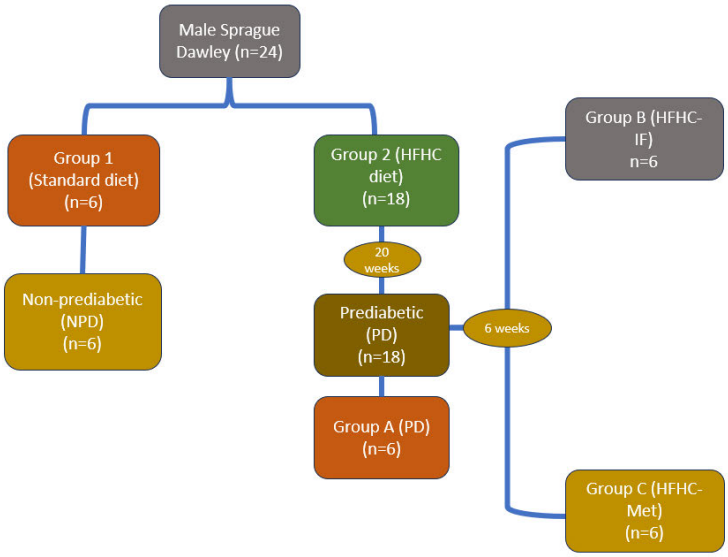


Figure 1. Experimental protocol showing prediabetes induction, intermittent fasting and HFHC-Met implementation on male Sprague Dawley rats. ADA criteria was used to identify prediabetes.

2.5. Blood collection and tissue harvesting

All rats were anaesthetized using Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodepoort, South Africa) in a gas anesthetic chamber (Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa), with a 3-minute inhalation period. Blood was drawn via cardiac puncture and placed into individual pre-cooled heparinized containers. The blood samples were then centrifuged to separate the plasma (Eppendorf centrifuge 5403, Germany) at 4 °C, 503 g for 15 minutes. Following this, the skeletal muscle and the liver were snap-frozen in liquid nitrogen before storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until biochemical analysis.

2.6. Biochemical analysis

Plasma insulin, leptin, and glycated hemoglobin concentrations were measured using their respective sandwich and rat competitive-ELISA kits (Elabscience Biotechnology Co., Ltd, Wuhan, China) according to the manufacturer's instructions. Five wells were prepared for standard points and one well for the blank. 50µL of standard dilutions, blank, and samples were added to the respective wells, followed by 50µL of Detection Reagent A to each well immediately. The plate was gently shaken and covered with a Plate sealer before incubating for 1 hour at 37°C. Detection Reagent A, which may appear cloudy, was warmed to room temperature and mixed until uniform. The solution was aspirated, and each well was washed with 350µL of 1X Wash Solution, repeated 3 times. After

washing, 100µL of Detection Reagent B working solution was added to each well and incubated for 30 minutes at 37°C, then washed 5 times as before. 90µL of Substrate Solution was added, incubated for 10-20 minutes at 37°C, protected from light. The liquid turned blue, and 50µL of Stop Solution was added, turning the liquid yellow. The plate was tapped gently to mix, ensuring no bubbles or fingerprints, and the microplate reader was used for measurement at 450nm.

2.7. Expression of insulin signaling pathway receptors via Real-Time- PCR

The liver and skeletal muscle tissues were collected and homogenized and total RNA was isolated using a ReliaPrep simplyRNA Tissue Miniprep System (Promega, USA) according to the manufacturer's protocol. A Nanodrop 2000 (Thermo Scientific, Roche, South Africa) was used to determine the purity and concentration of RNA. A purity ratio (A260/A280) of 1.7–2.1 was considered acceptable for conversion of RNA to cDNA. Following the manufacturer's instructions (GoTaq® 2-Step RT- qPCR System as a cDNA synthesis kit, Promega, USA). Total RNA was reverse transcribed to cDNA. To perform the PCR amplification on the Applied Biosystem Quantstudio®5 RT PCR system (Thermo-Scientific, Applied Biosystem Quantstudio®5, South Africa), the Go Taq® qPCR Master Mix was used. Primer sequences (Metabion, Germany) used in this study are listed in Table 1 below. PCR was performed using the following cycling conditions: an initial Go Taq DNA Polymerase activation 1 cycle at 95 ℃ for 2 minutes, a denaturation 40 cycles at 95 ℃ for 15 seconds, and annealing and extension 40 cycles at 60 ℃ for 1 minute. Melting curve analysis was performed at 95 ℃ for 30 s, 65 ℃ for 20 s, and 95 ℃ at a ramp rate of 0.05 ℃/s and a continuous fluorescence measurement, followed by a final cooling step at 40 ℃ for 60 s. The RT-qPCR results were analyzed using the $2^{-\Delta\Delta Cq}$ comparative method relative to the control groups (24). The housekeeping gene used in this study was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1. List of primers used in the study.

Sequence name	Sequence
GLUT 4 F	ATCAACGCCCCACAGAAAGT
GLUT 4 R	CCTGCCTACCCAGCCAAGT
Akt F	ATGGACTTCCGGTCAGGTTCA
Akt R	GCCCTTGCCCAGTAGCTTCA
MTORC1 F	TGCAGCCTGACCAATGATGTG
MTORC1 R	CTTGTGTCCGGCAGCATCATC
IRS1 F	GCCAATCTTCATCCAGTTGC
IRS1 R	CATCGTGAAGAAGGCATAGG
IRS2 F	CTACCCACTGAGCCCAAGAG
IRS2 R	CCAGGGATGAAGCAGGACTA
PI3K F	ACTGAGATGGAGACACGGAAC
PI3K R	GCATCCAAGGGTCCAGTTAGTG

2.8. Glycogen Assay

Glycogen levels in the liver and skeletal muscle tissues were analyzed using a standard laboratory protocol (25, 26). Muscle and liver samples were weighed and heated with 2 mL of 30% potassium hydroxide (KOH) at 100

°C for 30 minutes separately. To halt the reaction, 0.194 mL of 10% disodium sulfite was immediately added to the mixture. After cooling, glycogen precipitate formed. A 200 µL aliquot of the cooled mixture was mixed with 200 µL of 95% ethanol. The glycogen precipitate was pelleted, washed, and dissolved in 1 mL of water. Subsequently, 4 mL of anthrone solution (0.5 g in 250 mL sulfuric acid) was added, and the mixture was boiled for 10 minutes. After cooling, absorbance was measured at 620 nm using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Germany). Glycogen concentrations were determined using a glycogen standard curve.

2.9. Statistical analysis

All data are expressed as means ± standard error of means (SEM). GraphPad Prism Instant Software (version 8.00, GraphPad Software, San Diego, California, (USA) was used for statistical analysis. All terminal data was analyzed using the normality and lognormality test and a one-way ANOVA test to assess differences between control and experimental groups. Values of $p < 0.05$ were considered statistically significantly different between the compared groups.

3. Results

3.1. Calorie intake

The 24hr calorie intake was calculated in all experimental groups during the 12-week treatment period. The results (Table 2) showed significantly ($p < 0.05$) higher calorie intake in the prediabetic (PD) control group in comparison to the non-prediabetic (NPD) group. However, the 14:10 TRF-treated group showed significantly ($p < 0.05$) lower calorie intake in weeks 0, 4, 8 and 12 in comparison to the PD control. The HFHC-Met group similarly exhibited significantly ($p< 0.05$) lower calorie intake in weeks 0,4, 8 and 12 compared to the PD control.

Table 2. Effect of the 14:10-hour TRF regimen on 24hr calorie intake in non-prediabetic and prediabetic rats during the 12-week experimental period. Δ= p value <0.05 denotes comparison with NPD and β =p value <0.05 denotes a comparison with PD. The data is represented as means± SEM.

Groups	Week 0	Week 4		Week 8		Week 12	
	Caloric intake (kcal/g)	Caloric intake (kcal/g)	% change	Caloric intake (kcal/g)	% change	Caloric intake (kcal/g)	% change
NPD	55.1±2.8	58.2±6.5	+5.6	61.3±5.9	+5.3	65.7±5.9	+7.2
PD	98.4±6.9$^{\Delta}$	110±10.6$^{\Delta}$	+11.8	112.3±10.1$^{\Delta}$	+2.1	121.8±6.9$^{\Delta}$	+8.5

IF	91.3±3.3$^{\beta}$	91.4±6.6$^{\beta}$	+0.1	93.2±12.5$^{\beta}$	+2.0	108.4±12.0 $^{\beta}$	+16.3
HFHC-Met	93.2±12.5$^{\beta}$	98.9±6.9$^{\beta}$	+6.1	102.6±11.7 $^{\beta}$	+3.7	112.1±6.7$^{\beta}$	+9.3

3.2. Bodyweight change

The average body weight and the percentage changes in body weight were measured and calculated during the 12-week treatment period. The results (Table 3) showed significantly ($p < 0.05$) higher body weight in the PD control group in comparison to the NPD group. However, the 14:10 TRF-treated group showed significantly ($p < 0.05$) lower body weights in comparison to the PD control. The HFHC-Met group exhibited significantly ($p < 0.05$ similar with th 14:10-hour TRF compared to the PD control.

Table 3. Effect of the 14:10-hour TRF regimen on the average body weight and the percentage changes in body weight in prediabetic rats during the 12-week experiment. Δ= p value <0.05 denotes comparison with NPD and β =p value <0.05 denotes a comparison with PD. The data is represented as means± SEM.

Groups	**Week 0**	**Week 4**		**Week 8**		**Week 12**	
	Body weight (g)	**Body weight (g)**	**% change**	**Body weight (g)**	**% change**	**Body weight (g)**	**% change**
NPD	401±10.5	425 ±12.6	+23	489±17.0	+27	503±16.7	+28
PD	603±47.7 $^{\Delta}$	759±82.8 $^{\Delta}$	+26	786±97.0 $^{\Delta}$	+26	831±102.5 $^{\Delta}$	+28
IF	594 ±7.8$^{\Delta}$	613±7.5 $^{\beta}$	+25	632 ±7.2$^{\beta}$	+25	658±7.3 $^{\beta}$	+26
HFHC-Met	542 ±10.4$^{\Delta}$	525 ±21.8 $^{\beta}$	+23	570±29.4 $^{\beta}$	+25	608 ±29.0$^{\beta}$	+27

3.3. Body mass index

The BMI was calculated in all experimental groups during the 12-week treatment period. The results (Table 4) showed significantly ($p < 0.05$) higher BMI in the PD control group in comparison to the NPD group. However, the 14:10 TRF-treated group showed significantly ($p < 0.05$) lower body mass index in comparison to the PD control. The HFHC-Met group exhibited significantly ($p < 0.05$) improved body mass index results compared to the PD control.

Table 4. Effect of the 14:10-hour TRF regimen on the body mass index (BMI) in prediabetic rats during the 12-

week experiment**. The** values are expressed as mean ± SEM. Δ = p value <0.05 denotes comparison with NPD and β =p value <0.05 denotes a comparison with PD. Values are depicted as means ±SEM.

	BMI (g/cm^2)	BMI (g/cm^2)	BMI (g/cm^2)	BMI (g/cm^2)
Groups	Week 0	Week 4	Week 8	Week 12
NPD	4.3±0.2	4.4±0.1	4.9±0.1	5.2±1.5
PD	4.8±0.1	6.8±0.4 $^{\Delta}$	7.0±0.4$^{\Delta}$	7.7±0.4 $^{\Delta}$
IF	4.7±0.3	4.8±0.2 $^{\beta}$	5.1±0.2 $^{\beta}$	6.0±0.12 $^{\beta}$
HFHC-Met	4.6±0.4	4.7±0.1 $^{\beta}$	4.8±0.2 $^{\beta}$	5.3±0.09 $^{\beta}$

3.4. 2-hour oral glucose tolerance tests

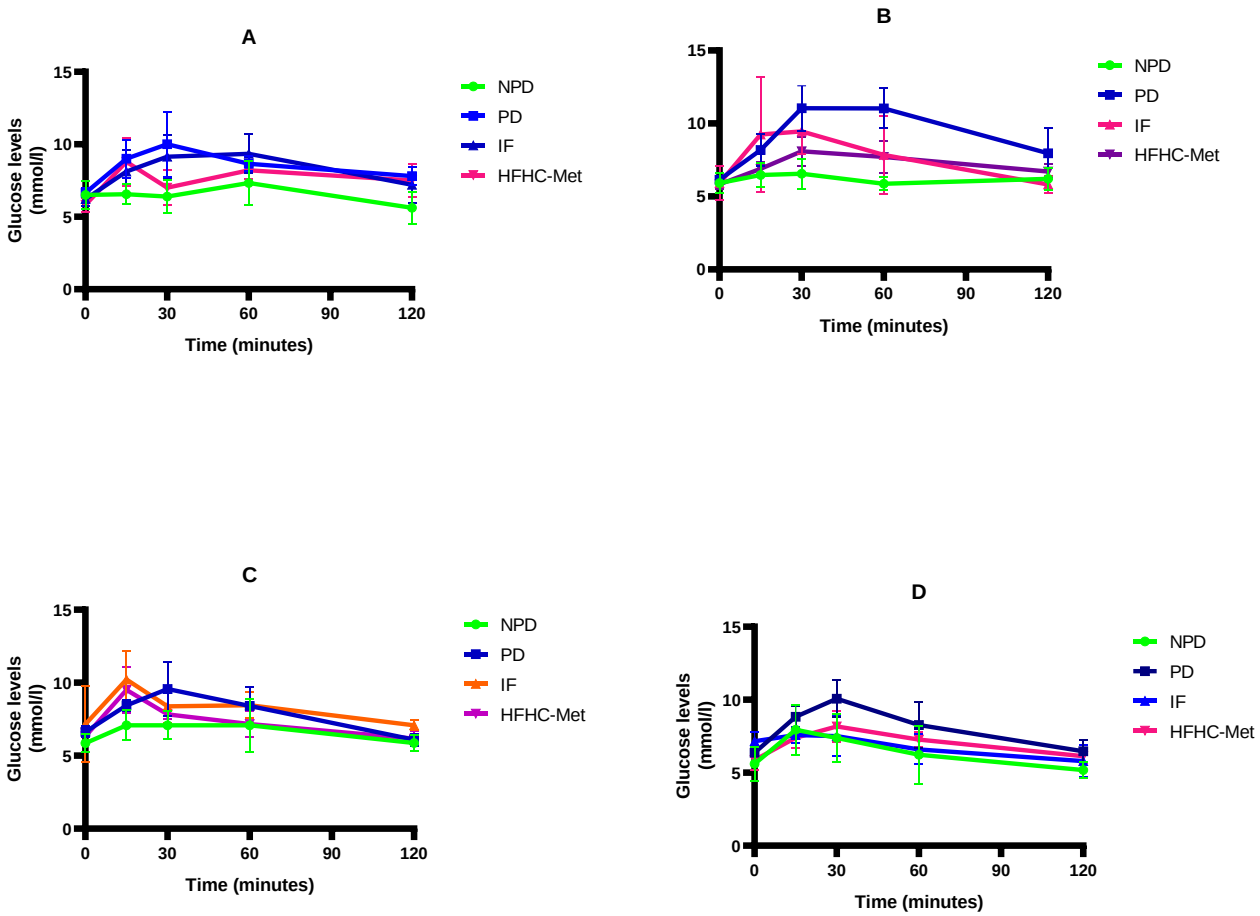


Figure 2. Effect of the 14:10-hour TRF regimen on the 2-hour oral glucose tolerance test in prediabetic rats during the 12-week experiment. Δ = p value <0.05 denotes comparison with NPD and β =p value <0.05 denotes a comparison with PD.

The 2-hour oral glucose tolerance tests (OGTT) were measured in all experimental groups during weeks 0, 4, 8, and 12 of the treatment periods. The results (Table 5, Figure 2) showed significantly ($p < 0.05$) higher OGTT in the PD control group in comparison to the NPD group. However, in week 12 of treatment, the 14:10 TRF-treated group showed significantly ($p < 0.05$) improved glucose tolerance in comparison to the PD control. The HFHC- Met group also exhibited significantly ($p < 0.05$) improved glucose tolerance results compared to the PD control.

Table 5. Effect of the 14:10-hour TRF regimen on the 2-hour oral glucose tolerance test in prediabetic rats during the 12-week experiment. The data are represented as areas under the curve (AUC). Δ = p value <0.05 denotes comparison with NPD and β =p value <0.05 denotes a comparison with PD. Values are depicted as Means± SEM

Groups	Week 0	Week 4	Week 8	Week 12
NPD	789.5 ±65.0	738.8±33.6	804.3±66.4	763.4±76.7
PD	1033± 47.8$^{\Delta}$	1152 ±74.6$^{\Delta}$	952.9 ±55.5$^{\Delta}$	972.8±61.0$^{\Delta}$
IF	1011± 66.0$^{\Delta}$	923.1±104.7$^{\beta}$	988.7±43.0$^{\Delta}$	807.2±53.5$^{\beta}$
HFHC-Met	927.3±47.9$^{\Delta}$	877.0±43.8$^{\beta}$	873.8±37.3$^{\beta}$	849.8±29.6$^{\beta}$

3.5. Fasting glucose, plasma insulin, HOMA-IR, and blood HbA1c

The FG, insulin levels, homeostatic insulin resistance, and glycated hemoglobin were measured in all experimental groups during the 12-week treatment period. The results (Table 6) showed significantly ($p < 0.05$) higher FG and HbA1c in the PD control group in comparison to the NPD group. However, in week 12 of treatment, the 14:10 TRF-treated group showed significantly ($p < 0.05$) improved insulin concentration, HOMA- IR, and HbA1c in comparison to the PD control. The HFHC-Met group also exhibited significantly ($p < 0.05$) improved HbA1c results compared to the PD control.

Table 6. Effect of the 14:10-hour TRF regimen on the fasting glucose (FG), insulin levels, homeostatic insulin resistance, and glycated hemoglobin in prediabetic rats at week 12 of the experimental period. The values are expressed as Mean ± SEM. Δ = p value <0.05 denotes comparison with NPD and β =p value <0.05 denotes a comparison with PD.

Groups	Fasting glucose (mmol/L)	Insulin (μU/mL)	HOMA IR	HbA1c (%)
NPD	5.4±0.2	1.1±0.2	0.3±0.05	5.5±0.6
PD	6.4±0.1	1.9±0.5	0.5±0.1	8.2±1.1$^{\Delta}$
IF	6.0±0.3	0.8±0.2 $^{\beta}$	0.2±0.05 $^{\beta}$	6.0±0.5 $^{\beta}$
HFHC-Met	5.9±0.4	1.8±0.5	0.5±0.1	6.7±0.6$^{\beta}$

3.6. Plasma leptin

The plasma leptin concentrations were measured in all experimental groups during the 12-week treatment period. The results (Figure 3) showed significantly ($p < 0.05$) higher leptin concentration in the PD control group in comparison to the NPD group. However, the 14:10 TRF-treated group showed significantly ($p < 0.05$) improved leptin concentration in comparison to the PD control. The HFHC-Met group also exhibited significantly ($p < 0.05$) improved leptin concentration results compared to the PD control.

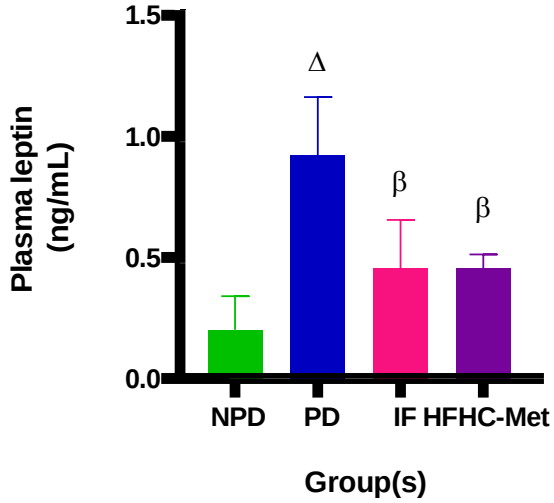


Figure 3. Effect of the 14:10-hour TRF regimen on the plasma leptin concentration in prediabetic rats at week 12 of the experimental. Data are presented as mean values. Δ = p value <0.05 denotes comparison with NPD and β =p value <0.05 denotes a comparison with PD.

3.7. Expression of IRS1, IRS2, Akt, PI3K, mTORC1 and GLUT 4

The expressions of IRS1, IRS2, Akt, PI3K, mTORC1 and GLUT 4 were measured all experimental groups during the 12-week treatment period. The results (Figure 4) showed significantly ($p < 0.05$) higher IRS1, IRS2, Akt, PI3K, and mTORC1 in the PD control group in comparison to the NPD group. However, the 14:10 TRF-treated group showed significantly ($p < 0.05$) improved IRS1, IRS2, Akt, PI3K, mTORC1, and GLUT4 in comparison to the PD control. The HFHC-Met group also exhibited significantly ($p < 0.05$) improved IRS1, Akt, PI3K, mTORC1, and GLUT4 results compared to the PD control. Interestingly, the HFHC-Met group also demonstrated significantly higher IRS2 compared to the PD control group.

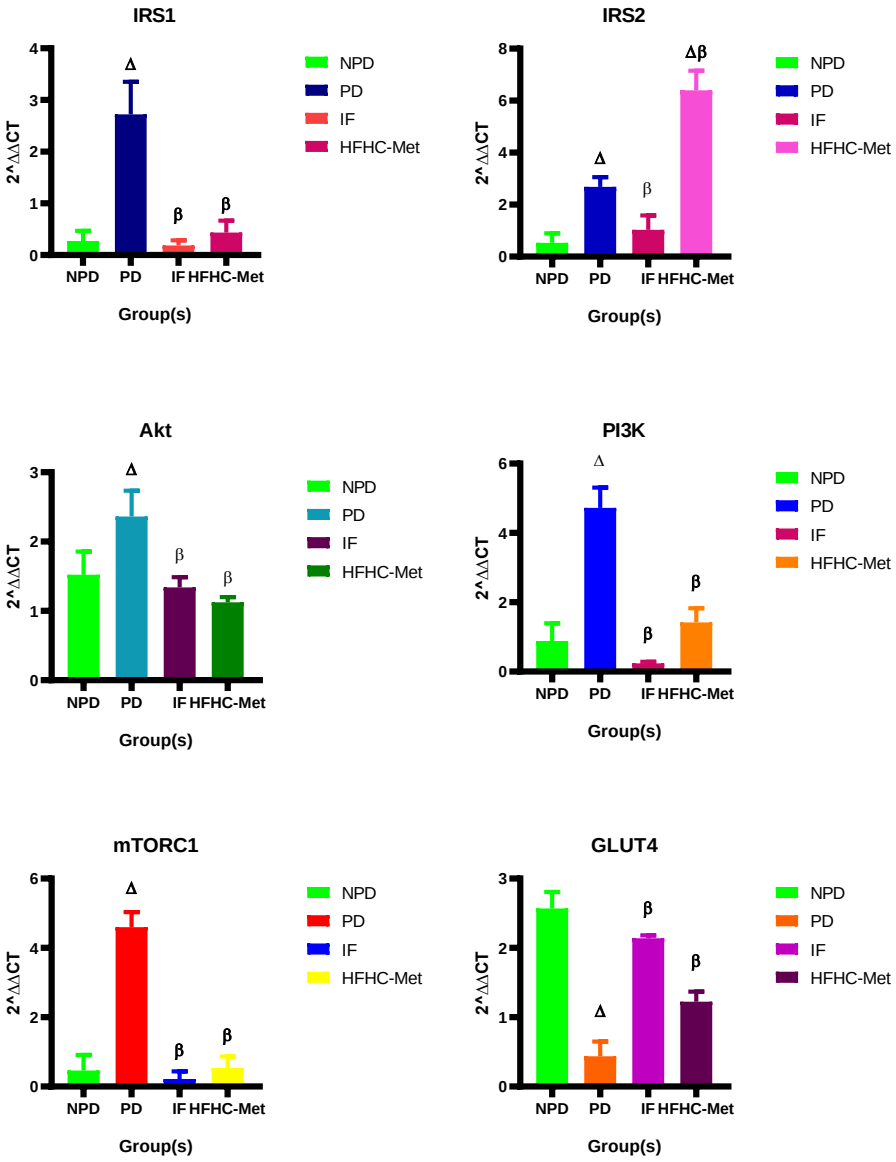
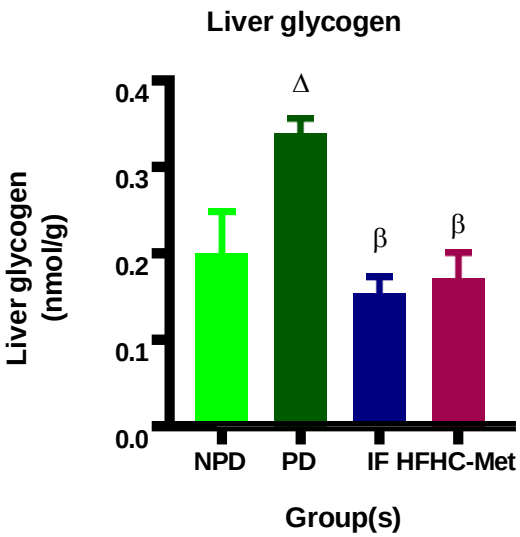


Figure 4. Effect of the 14:10-hour TRF regimen on the IRS1, IRS2, Akt, PI3K, mTORC1, and GLUT 4 in prediabetic rats at week 12 of the experimental Δ = p value <0.05 denotes comparison with NPD and β =p value

<0.05 denotes a comparison with PD.

3.8. Glycogen concentration in liver and skeletal muscle

The glycogen concentrations were measured in the liver and skeletal muscles in all experimental groups during the 12-week treatment period. The results (Figure 5) showed significantly ($p < 0.05$) higher liver glycogen concentration in the PD control group in comparison to the NPD group. Further, the 14:10 TRF-treated group showed substantially lower liver glycogen concentration in comparison to the PD control. Furthermore, the HFHC-Met group exhibited significantly ($p < 0.05$) lower glycogen concentration compared to the PD control. Interestingly, the opposite was reported in skeletal muscle glycogen concentration.


Liver glycogen
Liver glycogen (nmol/g)
0.4
0.3
0.2
0.1
0.0
Δ
β
β
NPD
PD
IF
HFHC-Met
Group(s)

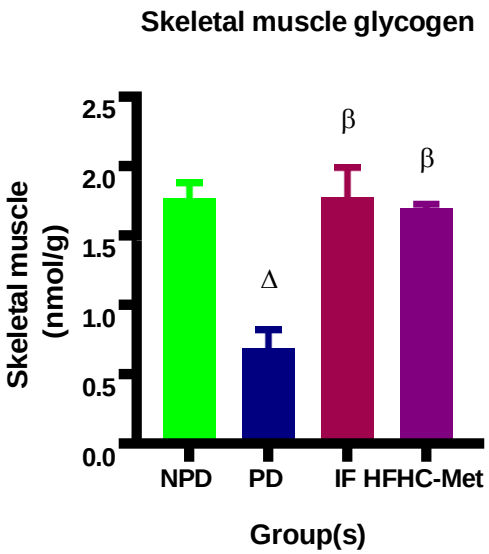

Skeletal muscle glycogen
Skeletal muscle (nmol/g)
2.5
2.0
1.5
1.0
0.5
0.0
β
β
Δ
NPD
PD
IF
HFHC-Met
Group(s)

Figure 5. Effect of the 14:10-hour TRF regimen on the glycogen concentration on liver and skeletal tissues in prediabetic rats at week 12 of the experimental Δ = p value <0.05 denotes comparison with NPD and β =p value <0.05 denotes a comparison with PD.

4. Discussion

Type 2 diabetes mellitus remains a major factor contributing to global mortality and morbidity rates (2). It is a chronic hyperglycaemic condition triggered by a preceding loss of β-cell insulin secretion and insulin resistance (1). The onset of T2DM is often preceded by prediabetes, a condition characterized by elevated blood glucose levels that do not meet the diagnostic criteria for diabetes mellitus (27). Despite the World Health Organization not including HbA1c as a criterion, the ADA, on the other hand, defines prediabetes as having an IFG level between 5.6–6.9 mmol/L, an IGT range of 7.8–11.0 mmol/L and an HbA1c level between 5.7–6.4% (4). Research indicates that prediabetes is reversible, making it a key focus for preventing the progression of T2DM. Primary strategies for managing prediabetes include enhancing physical activity, adopting dietary changes, and using metformin (6). Despite the availability of these management strategies, prediabetes remains a significant challenge. Recent studies have indicated that intermittent fasting has shown promising effects on the management of T2DM. Intermittent fasting refers to various dietary approaches where individuals alternate extended periods of normal calorie consumption with significantly reduced or no calorie intake intervals (28, 29). The timing of fasting and feeding periods varies among different IF protocols, such as the 5:2 diet, alternate-day fasting, and time-restricted feeding (12, 28, 30). The 14:10 hour IF protocol has been shown to decrease HbA1c levels, fasting blood glucose effectively, and body weight, and enhance lipid profiles in obese T2DM patients (11, 12). However, the impact of intermittent fasting on glucose regulation in individuals with prediabetes remains poorly understood, and its long-term role in the insulin signaling pathway is not yet well-defined. This study employed a diet-induced prediabetes animal model, which closely mimics the human condition, to investigate the effects of a 14:10-hour TRF regimen on glucose regulation in prediabetic male Sprague Dawley rats.

This study specifically investigated the effects of a 14:10-hour time-restricted feeding regimen on leptin levels, insulin sensitivity, and various markers related to the insulin signaling pathway, including IRS1, IRS2, Akt, PI3K, mTORC1, GLUT4, and glucose homeostasis in diet-induced prediabetic rats. Significant changes were observed in calorie intake, body weight, leptin concentrations, OGTT, HbA1c, insulin levels, HOMA-IR, IRS1, IRS2, Akt, PI3K, mTORC1, GLUT4, and glycogen concentration compared to the untreated prediabetic group. However, no significant differences were found in fasting glucose, and OGTT at weeks 0 and 8.

Under normal conditions, body weight reflects a balance between energy intake and expenditure (31, 32). When

fat stores are adequate, leptin signals the body to increase energy expenditure and reduce energy intake (33, 34). However, an energy imbalance occurs in the prediabetic state, leading to increased body weight, BMI, and leptin resistance (33-35). Our findings align with existing literature, showing significantly higher body weight, BMI, and leptin concentrations in the prediabetic group than in the non-prediabetic group throughout the experimental period, despite a minimal body weight percental change of ±0.3%. Studies suggest that the prediabetic group experiences higher energy intake relative to expenditure due to increased leptin levels
(36). Interestingly, the 14:10-hour TRF group exhibited significantly lower body weight, BMI and leptin levels than the prediabetic group. This suggests that fasting periods may help restore energy balance by activating leptin sensitivity, promoting reduced caloric intake (37, 38). A study revealed that the IF can activate the insulin signaling pathway regardless of unchanged body weight (39). Additionally, the HFHC-Met group showed notably lower body weight, BMI, and leptin concentrations than the prediabetic group. Growing evidence indicates that metformin-induced weight loss is driven by its effects on hypothalamic appetite-regulation centres including increasing leptin sensitivity, changes in the gut microbiome, and the reversal of aging-related factors (40). This study proposes that the body weight and BMI changes observed in both the 14:10-hour TRF and HFHC-Met groups were driven by increased leptin sensitivity, which further contributed to reduced calorie intake.

Impaired glucose tolerance along with IFG and elevated glycated hemoglobin (HbA1c), are recognized as contributing factors to moderate insulin resistance in insulin-dependent tissues (41). Under normal conditions, an increase in blood glucose levels following meals prompts insulin release, which promotes glycogenesis (42). However, in the prediabetic state, insulin fails to regulate blood glucose, leading to impaired blood glucose and glucose intolerance (43). Our findings are consistent with prior research, showing significantly higher blood glucose levels in the prediabetic group than in the non-prediabetic group throughout the experimental period. Interestingly, after one week of the 14:10-hour TRF regimen intervention, the 2-hour OGGT showed significantly lower results in the 14:10-hour TRF group compared to the prediabetic group. In contrast, fasting glucose levels showed no significant difference between the 14:10-hour TRF and prediabetic groups. However, HbA1c indicated significantly lower glycemia in the IF group compared to the prediabetic group. This suggests that the 14:10-hour TRF regimen effectively regulates glycaemic metabolism by activating the insulin signaling pathway, thereby promoting glucose uptake through GLUT4 and reducing liver glucose release (39, 44). A study demonstrated that improvements in glucose metabolism under the IF regimen were mainly attributed to the suppression of hepatic glucose output, mediated by the degradation of the liver glucocorticoid receptor and its downstream transcription factor, Kruppel-like factor 9 (44). Additionally, the HFHC-Met group showed improved 2-hour oral glucose tolerance, and HbA1c results compared to the prediabetic group. Metformin has been shown to regulate glucose metabolism by inhibiting gluconeogenesis. Additionally, it activates AMPK, which improves insulin sensitivity by modulating fat metabolism and promotes the translocation and expression of GLUT4 (45-47).

Insulin plays a critical role in stabilizing blood glucose levels. In the normal state, insulin is released in response to elevated blood glucose levels to regulate glucose concentrations (48, 49). However, in the prediabetic state, elevated glucose levels trigger the release of large amounts of insulin, eventually causing cells to become resistant to its effects (50, 51). Our findings align with these studies, revealing significantly higher insulin concentration in the prediabetic group compared to the non-prediabetic group. It indicates the presence of insulin insensitivity. Studies have demonstrated that the presence of insulin insensitivity leads to disruption of the insulin signaling pathway and the failure of GLUT4 translocation to cells, which hinders glucose uptake (52, 53). Interestingly, at the end of the 12-week experimental period, the 14:10-hour TRF group demonstrated significantly lower insulin concentration and HOMA-IR results than the prediabetic group. It indicates that 14:10-hour TRF aids in controlling hyperglycemia by maintaining insulin sensitivity. Research has demonstrated that 14:10 hour TRF decreases the body's energy intake, resulting in reduced insulin production and elevated AMPK levels, which may contribute to improved insulin sensitivity and glucose homeostasis (39, 43, 54, 55). This has been shown to occur in the fasted state, enabling the body to utilize and manage blood glucose. Furthermore, in the current study, the HFHC-Met group exhibited no significant differences in insulin levels and HOMA-IR compared to the prediabetic control group. It may be because the metformin-treated group remained on the HFHC diet throughout the study. It is recommended that to obtain the maximal efficacy of metformin, it must be combined with lifestyle modifications such as dietary intervention may enhance its efficacy (56, 57). However, there is reported poor patient compliance as patients tend to have an over-reliance on metformin and neglect lifestyle modifications.

Insulin binds to its receptor on the surface of insulin-responsive cells, triggering the receptor's activation. The activated insulin receptor then recruits insulin receptor substrate 1 (IRS1) and IRS2, which are phosphorylated at specific tyrosine sites (59-61). These phosphorylated IRS proteins act as docking platforms for downstream signaling molecules. One such molecule, PI3K, binds to the phosphorylated IRS1/2 and becomes activated. PI3K then converts phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the cell membrane (62, 63). PIP3 facilitates the activation of Akt (protein kinase B) by recruiting it and enabling phosphorylation by phosphoinositide-dependent kinase-1 (PDK1). Akt plays a pivotal role in regulating insulin's metabolic functions (63). It activates the mechanistic target of rapamycin complex 1 (mTORC1) by inhibiting the Tuberous Sclerosis Complexes (TSC1/TSC2), which normally restrains mTORC1 (63, 64). The activation of mTORC1 supports protein synthesis, cell growth, and glucose metabolism. Additionally, Akt promotes the movement of glucose transporter 4 (GLUT 4) vesicles to the cell membrane, enabling glucose uptake into the cell (65, 66). In the prediabetic state, cells develop a reduced sensitivity to insulin due to impairments in IRS1/2 signaling and associated downstream pathways (53). To compensate, the pancreas increases insulin secretion, which intensifies feedback inhibition and places additional strain on β-cells (67, 68). Interestingly, unlike the prediabetic state, 14:10-hour TRF effectively restored the insulin signaling pathway by regulating IRS1/2, Akt,

PI3K, and mTORC1 while promoting GLUT4 activity. Similarly, studies have shown that the IF regimen can effectively restore the insulin signaling pathway (39, 43, 54, 64, 66, 69-72). Additionally, this study revealed a significant decrease in IRS1, Akt, PI3K, and mTORC1 and a significantly increased GLUT 4 activity. This is in line with a study demonstrating that metformin in the presence of insulin directly stimulates IRS-1 and-2 to activate Akt via PI3K (73).

Glycogen levels in the liver and skeletal muscle were evaluated throughout the 12-week experimental period. Under normal physiological conditions, insulin enhances glucose uptake in the liver and activates glycogen synthase, promoting glycogen storage (74, 75). In the liver, glucose transport is mediated by GLUT 2, a bidirectional transporter that facilitates glucose movement in and out of cells based on the concentration gradient (76). In conditions such as insulin resistance or hyperglycemia, GLUT 2 facilitates an increased influx of glucose into hepatocytes (77, 78). Since GLUT 2 operates along the concentration gradient, elevated blood glucose levels drive higher glucose uptake into the liver (77). In this study, liver glycogen levels were significantly higher in the prediabetic group compared to the non-prediabetic group, likely due to compensatory mechanisms in response to insulin resistance. Notably, the IF group displayed a marked reduction in glycogen levels compared to the prediabetic group, aligning with findings from other studies highlighting the effectiveness of an 14:10-hour TRF regimen in regulating glucose storage (79, 80). Similarly, the HFHC-Met group showed results comparable to those of the prediabetic group.

In contrast, skeletal muscle glycogen levels were significantly lower in the prediabetic group compared to the non-prediabetic group, likely reflecting impaired insulin-mediated glucose uptake associated with insulin resistance (81, 82). However, the 14:10-hour TRF group exhibited a notable recovery in muscle glycogen levels, suggesting improved insulin sensitivity and glucose uptake, consistent with evidence supporting the metabolic benefits of the IF (83-84). Additionally, the HFHC-Met group also demonstrated significantly increased skeletal muscle glycogen levels. The glycogen findings are in line with data on body weight, leptin, OGTT, insulin, HOMA-IR, IRS1, IRS2, PI3K, Akt, mTORC1, and GLUT 4 across all groups.

5. Limitations and Future Studies

Although the study demonstrated improvements in markers related to the insulin signaling pathway, a significant limitation was the lack of measurement of proteins in actual tissue to validate whether the gene expression changes identified by PCR correspond to protein-level alterations. Due to limited funding at this time, these could not be done but these will be addressed in future studies by using Western Blotting and immunohistochemistry to support the PCR findings. This study showed significant effects on glycogen formation in the livers and skeletal

muscle of the 14:10 TRF -treated diet-induced prediabetic rats. This suggests that this intervention may have effects on the overall physiology of these tissues therefore future studies need to investigate the changes in the markers associated with the physiology of these tissues. Furthermore, future studies could also look at other organ systems such as the cardiovascular and renal systems as these are greatly affected by changes in glucose homeostasis.

6. Conclusions

In this study, the results showed that chronic ingestion of HFHCdiet results in prediabetes. As expected, the untreated prediabetic group exhibited glucose dysregulation. However, despite being maintained on an HFHC diet, introducing an IF regimen effectively mitigated glucose-related dysregulation. The benefits of IF were highlighted by significant improvements in GLUT4 activity, accompanied by reductions in body weight, leptin and insulin levels and HOMA-IR. These changes aid in enhancing insulin sensitivity, ensuring the proper function of IRS1 and IRS2. These proteins then effectively transmitted signals to PI3K, Akt, and mTORC1, facilitating GLUT4 translocation and promoting glucose uptake into cells. Taken together, the study showed the potential of IF to alleviate prediabetes-related dysregulation of glucose homeostasis and therefore warrants further investigations into its use in the management of prediabetes. Although the diet-induced animal model of prediabetes has been shown to closely replicate the human condition, we cannot conclude that the effects of IF would be identical in humans, as factors like genetic makeup and dietary habits may influence the outcomes. Additionally, we recommend conducting further research to explore its application in humans.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Composition of the HFHC diet; Table S2: Nutritional value of the HFHC diet; Table S3: The overall caloric contribution from fats, proteins, and carbohydrates in a high-fat high-carbohydrate diet; Table S4: The overall caloric contribution from fats, proteins, and carbohydrates in a standard diet; Table S5: Composition of fats, proteins, and carbohydrates of the standard diet.

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Abbreviations.

ADA	American Diabetes Association
AREC	Animal Research Ethics Committee
Akt	Protein kinase B
AUC	Area Under the Curve
BRU	Biomedical Research Unit
ELISA	Enzyme-linked immunosorbent assay
BMI	Body Mass Index
HbA1C	Glycated hemoglobin
FG	Fasting glucose
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
OGTT	Oral glucose tolerance test
GLUT4	Glucose transporter 4
IRS1	Insulin Receptor Substrate 1
IRS2	Insulin Receptor Substrate 2
PI3K	Phosphoinositide 3-kinase
R	Reverse
F	Forward
mTORC 1	Mechanistic Target of Rapamycin Complex 1
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
IF	Intermittent fasting
HFHC	High fat high carbohydrate
TRF	time-restricted feeding
T2DM	Type 2 diabetes mellitus
Met	Metformin
SEM	Standard Error of Mean
NPD	non-prediabetic
PD	Prediabetes
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
RT-qPCR	Real-time quantitative polymerase chain reaction
cDNA	complementary Deoxyribonucleic acid
RNA	Ribonucleic acid

WHO	World Health Organization
GLUT2	Glucose transporter 2

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CHAPTER 4: SYNTHESIS AND CONCLUSION

1. Synthesis

Prediabetes is a metabolic state characterized by glycemic levels that are above normal but below the threshold for a diagnosis of T2DM (1). The World Health Organization and the American Diabetes Association (ADA) both define prediabetes as having an impaired fasting glucose (IFG) level of 5.6–6.9 mmol/L and having an impaired glucose tolerance (IGT) range of 7.8–11.0 mmol/L. The ADA has an additional criterion of glycated hemoglobin (HbA1c) levels between 5.7–6.4% (2). A recent study indicated that the global prevalence of prediabetes will exceed 400 million individuals by the year 2045 (3). Prediabetes has also been linked to the onset of leptin and insulin resistance, elevated HbA1c levels, obesity, intermediate hyperglycemia, moderate glucose intolerance and impaired fasting glucose (4,5) While the effects of 14:10 time-restricted feeding (TRF) on T2DM are well-known, no study has yet examined the impact of this regimen on glycaemic regulation during the prediabetic state

Examining the metabolic changes associated with prediabetes in humans is challenging because such research often involves invasive procedures (6). Therefore, animal models such as the HFHC diet-induced animal model used in this study, are frequently utilized to explore these metabolic alterations and assess the effectiveness of various intervention strategies for managing prediabetes as this animal model has been shown to mimic the human condition (7-9). While several studies have looked at the effects of other pharmacological alternatives to managing prediabetes, no study has yet examined the impact of 14:10 TRF on glycaemic regulation during the prediabetic state (10-12). Hence, using this model, the current study sought to investigate the effects of a 14:10-hour TRF regimen on markers associated with glycaemic control in diet-induced prediabetic male Sprague Dawley rats.

The first manuscript of this study reviewed the potential therapeutic effects of various intermittent fasting regimens on T2DM and prediabetic conditions. It emphasized that antidiabetic drugs can cause adverse effects, and poor adherence to these medications often diminishes their effectiveness, highlighting the need for alternative approaches such as IF (12-17). The review discussed the impact of IF regimens, including alternate-day fasting, the 5:2 fasting diet, and time-restricted feeding, on both conditions (18). For T2DM, these regimens were found to improve dysregulation associated with the condition, though they may cause side effects like fatigue and headaches (19-23). Similarly, in prediabetes, IF regimens showed improvements in metabolic dysregulation but with potential adverse effects such as vomiting and redox imbalance (23, 24). The review also identified a gap in the current knowledge regarding the effects of the 14:10 TRF regimen on prediabetes, though existing studies

suggest its potential benefits for individuals with T2DM.

This prompted the examination of the impact of a 14:10-hour TRF regimen on calorie intake, body weight, leptin levels, and markers associated with glycemic regulation including HbA1c levels, fasting blood glucose levels, and 2hr oral glucose tolerance test. The study produced results consistent with the literature for a 14:10-hour TRF schedule, showing a notable reduction in calorie intake, leptin, and insulin levels, as well as key markers related to glycemic regulation, including HbA1c levels, fasting blood glucose levels, and the 2-hour oral glucose tolerance test (12-23). Additionally, markers associated with insulin sensitivity and the expression of specific proteins involved in insulin were assessed (25, 26) signalling including IRS1, IRS2, PI3K, Akt, mTORC1 and GLUT4 in diet- induced prediabetic male Sprague Dawley rats.

Body weight, calorie intake, fasting blood glucose levels and glucose tolerance were monitored every four weeks throughout the treatment period. At the end of the experimental period, terminal blood HbA1c, plasma leptin and insulin levels, liver and skeletal muscle glycogen content were determined using ELISA following the manufacturer's protocols. HOMA-IR was calculated as described by or using the equation HOMA-IR = (Fasting Insulin (mU/L)×Fasting Glucose (mmol/L))/22.5 (27), while the BMI was calculated using the formula BMI= weight(g)/height(cm^2) (28). The expressions of IRS1, IRS2, PI3K, Akt, mTORC1, and GLUT4 was analyzed using RT-qPCR. The results demonstrated a significantly lower calorie intake, body weight, BMI, liver glycogen, and leptin concentration in the 14:10 TRF regimen group compared to the prediabetic group. These findings correlate with the existing literature showing that the prediabetic group is at higher risk of obesity (29).

The14:10 TRF group showed significantly improved HbA1c levels and glucose tolerance compared to the prediabetic group, indicating enhanced insulin sensitivity. This was further corroborated by the lower insulin and HOMA-IR results in 14:10 TRF, which aligned with these findings. These findings also align with the previous studies that showed that the IF regimen can alleviate glycaemic dysregulation and insulin insensitivity (30-35). Additionally, the insulin signaling pathway was restored in the 14:10 hour TRF group than in the prediabetic group, along with increased skeletal muscle glycogen, suggesting enhanced insulin sensitivity. This study has for the first time demonstrated that the 14:10 TRF regimen mediates beneficial effects in a prediabetic state by reducing body weight, reducing leptin and insulin levels, improving glycemic control, and enhancing the regulation of the insulin signaling pathway.

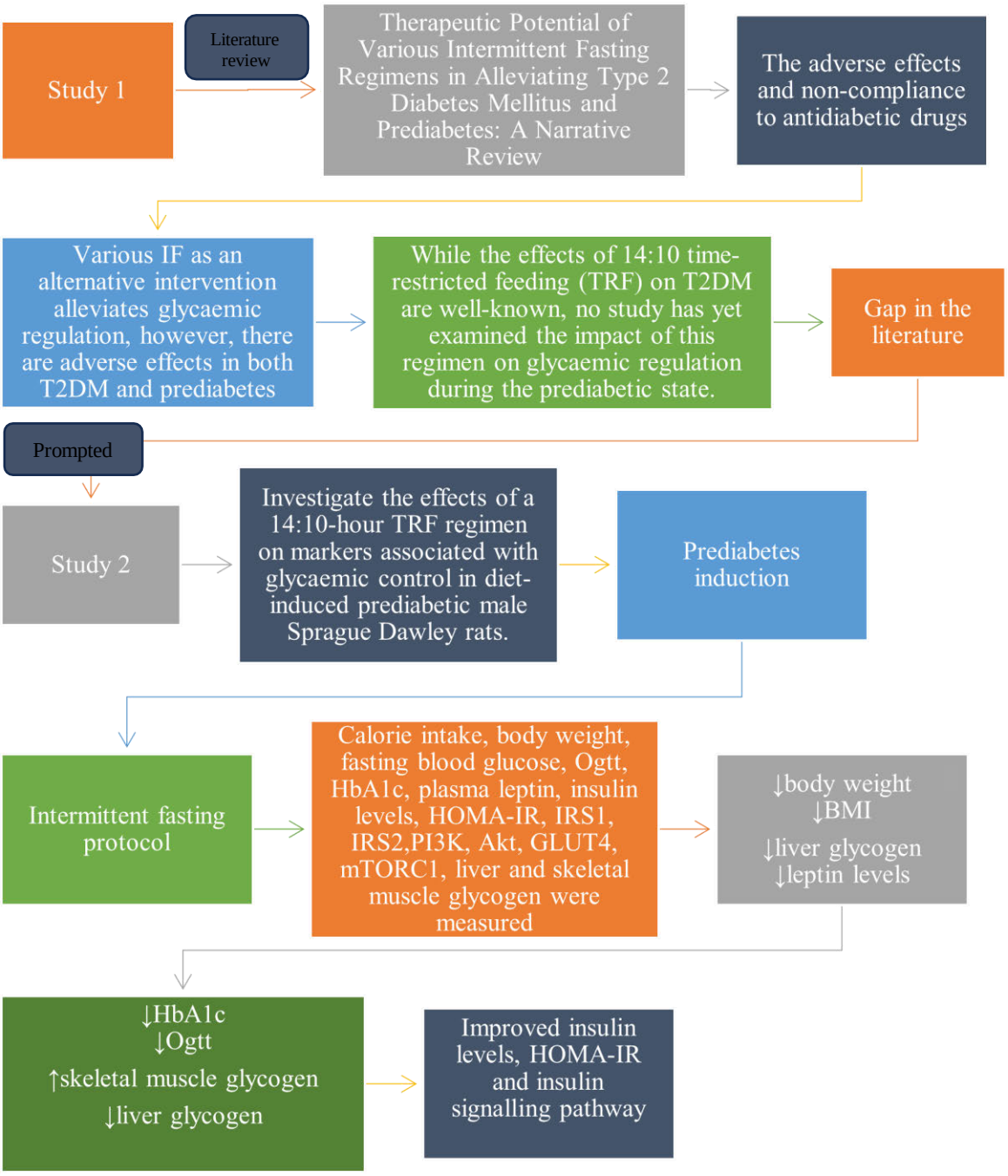

Figure 1: Diagram illustrating the main findings from the dissertation on the evaluation of the effects of the 14:10 TRF on markers associated with glucose homeostasis in diet-induced prediabetic rats.

2. Conclusion

Following the review that highlighted the potential of the 14:10 protocol for managing T2DM, the results of the second manuscript demonstrated that the introduction of 14:10 regimen effectively alleviated the glucose-related dysregulation observed in diet-induced prediabetes. The benefits of IF were evident through significant improvements in GLUT4 activity, along with reductions in body weight, leptin and insulin levels, and HOMA-IR. These changes contributed to enhanced insulin sensitivity and proper function of IRS1 and IRS2, which then effectively transmitted signals to PI3K, Akt, and mTORC1, facilitating GLUT4 translocation and promoting glucose uptake into cells. This study, for the first time collectively demonstrated its effectiveness in the prediabetic state by reducing body weight, leptin, insulin levels, and HOMA-IR improving glycemic control. Further, enhancing the regulation of the insulin signaling pathway.

3. Shortfalls and Future Studies

Although the study demonstrated improvements in markers related to the insulin signaling pathway, a significant limitation was the lack of measurement of proteins in actual tissue to validate whether the gene expression changes identified by PCR correspond to protein-level alterations. Future studies must do further assessments using Western Blotting and immuno-histochemistry to support the PCR findings. This suggests that this intervention may have positive effects on the overall physiology of these tissues therefore future studies need to investigate the changes in the markers associated with the physiology of these tissues. Furthermore, future studies could also look at other organ systems such as cardiovascular and renal systems as these are greatly affected by changes in glucose homeostasis. Another study suggestion involves TRF and fasting-mimicking diets (FMD) as they are widely regarded as promising primary strategies or first-line interventions for preventing or reducing the risk T2DM. Conducting a comparative study on the effects of a 14:10-hour TRF regimen versus FMD could provide valuable insights for future research.

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APPENDICES

APPENDIX 1: ETHICAL CLEARANCE

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

20 March 2024

New Application	
Protocol Reference number:	AREC/00006223/2023(00022008)
Project title:	**Evaluating the cardioprotective effect of metformin therapy and lifestyle intervention in diet-induced prediabetic rats: Novel mechanisms of metformin.**
Main Applicant and School:	Sandiswa Malinga (218033812) School of Laboratory Medicine and Medical Sciences

Dear Ms Malinga,

Regarding your revised application received on 12 March 2024, the UKZN Animal Research Ethics Committee (AREC) has accepted the documents submitted, and **FULL APPROVAL** for the protocol is now granted, effective 20 March 2024.

About your approved protocol, please note:

1. All correspondence with the AREC about this protocol must be accompanied by the full reference number.
2. Any alterations to the approved protocol (e.g. title, location, methods, research team) must be reviewed and approved through an official amendment application, via the RIG system, prior to its implementation.
3. Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered veterinarian/para-veterinarian or SAVC authorized person, and their details must be logged with the AREC if it changes during the approval period.
4. The AREC may actively monitor research, ask further questions, request additional information, or require modification on welfare grounds. If animal welfare is compromised in any way, the AREC may suspend the study/withdraw approval.
5. The research team must declare any adverse or serious adverse event(s) to the AREC via the RIG system as soon as possible but no later than **48 hours** after the event.
6. Research data should be securely stored in the discipline/school for a period of 5 years.
7. The approval is valid for one year from the date of issue. If the project is continuing, a renewal application must be submitted to the AREC at least **30 days before the expiration date if a timeline extension is applied for**, and **60-90 days ahead of the expiration date if amendments are included in the renewal application**. If work is completed at the end of the approval period, a close-out report must be submitted. Renewals/close-out reports are submitted via the RIG system.

I take this opportunity to wish you all the best with your study.

Yours faithfully

[redacted]

Dr Dalene Vosloo, PhD, Pr. Sci. Nat.
Chair: Animal Research Ethics Committee
/kr

cc Supervisor: Andile Khathi, Lindokuhle Mabuza Mashaba
cc BRU Manager: Dr Jaca

The UKZN AREC is registered with the SA National Health Research Council (Reg. No. AREC-200111-006; Expires on 31/12/2027) in accordance with the National Health Act (No 61 of 2006) and conforms to the guidelines of the Department of Health (DOH2015) and SANS10386:2021. The UKZN AREC has an active US NIH OLAW assurance (Reg. No. F18-00371; Expires on 31/12/2027).

Animal Research Ethics Committee (AREC)
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APPENDIX 2: PUBLICATION 1

nutrients

MDPI

Review

Therapeutic Potential of Various Intermittent Fasting Regimens in Alleviating Type 2 Diabetes Mellitus and Prediabetes: A Narrative Review

Sthembiso Msane, Andile Khathi * and Aubrey Sosibo *

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Abstract: Intermittent fasting has drawn significant interest in the clinical research community due to its potential to address metabolic complications such as obesity and type 2 diabetes mellitus. Various intermittent fasting regimens include alternate-day fasting (24 h of fasting followed by 24 h of eating), time-restricted fasting (fasting for 14 h and eating within a 10 h window), and the 5:2 diet (fasting for two days and eating normally for the other five days). Intermittent fasting is associated with a reduced risk of type 2 diabetes mellitus-related complications and can slow their progression. The increasing global prevalence of type 2 diabetes mellitus highlights the importance of early management. Since prediabetes is a precursor to type 2 diabetes mellitus, understanding its progression is essential. However, the long-term effects of intermittent fasting on prediabetes are not yet well understood. Therefore, this review aims to comprehensively compile existing knowledge on the therapeutic effects of intermittent fasting in managing type 2 diabetes mellitus and prediabetes.

Keywords: intermittent fasting; type 2 diabetes mellitus; prediabetes; HbA1c; glucose tolerance

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1. Introduction

Intermittent fasting (IF) is a broad term describing several eating regimens in which individuals alternate long periods of normal calorie intake with periods of minimal or no energy intake [1]. In 1935, McCay elucidated the correlation between calorie restriction and lifespan or longevity [2]. Since then, studies have extensively investigated calorie restriction, evolving into the practice of intermittent fasting [1,3–5]. Research findings have highlighted the use of intermittent fasting and its efficacy in metabolic-related disorders [1,3–6]. Several intermittent fasting protocols have been recognized for their capacity to mitigate metabolic disorders [7–15].

The IF protocols include dietary approaches that involve alternating eating periods with either fasting by restricting calorie intake or zero calorie intake during the fasting period [6]. The timing of fasting and feeding periods varies among different IF protocols, such as the 5:2 diet, alternate-day fasting, and time-restricted feeding [16]. The 5:2 fasting diet is a dietary regimen where individuals eat without restrictions for five days, followed by two days per week during which they consume a very-low-calorie diet (fewer than 800 calories per day) [17]. Alternate day fasting (ADF) involves alternating between a 24 h fasting period, during which individuals consume less than 25% of their usual energy needs, and a 24 h eating period, where they can eat normally [15]. Time-restricted feeding (TRF) is an IF protocol with a specified time of prolonged fasting practiced by adhering to 16 h of abstinence from food and 8 h of food intake within 24 h [6]. IF has gained popularity in body weight management and alleviating metabolic-related disorders [18,19]. Therefore, this review aims to synthesize existing knowledge on the therapeutic effects of intermittent fasting on the management of T2DM and prediabetes, providing a critical overview of its current state of understanding. This review will begin by examining the impact of various

APPENDIX 3: PUBLICATION 2

nutrients

MDPI

Article

The Effect of the 14:10-Hour Time-Restricted Feeding (TRF) Regimen on Selected Markers of Glucose Homeostasis in Diet-Induced Prediabetic Male Sprague Dawley Rats

Sthembiso Msane, Andile Khathi * and Aubrey Mbulelo Sosibo

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Abstract: Background: Prediabetes is a condition that often precedes the onset of type 2 diabetes mellitus (T2DM). Literature evidence indicates that prediabetes is reversible, making it an important therapeutic target for preventing the progression to T2DM. Several studies have investigated intermittent fasting as a possible method to manage or treat prediabetes. Objectives: This study evaluated the impact of a 14:10-hour time-restricted feeding (TRF) regimen on leptin concentration, insulin sensitivity and selected markers associated with the insulin signalling pathway and glucose homeostasis in diet-induced prediabetic rats. Methods: Twenty-four male Sprague Dawley rats were obtained and randomly divided into two dietary groups: group 1 (n = 6) received a standard diet and water, while group 2 (n = 18) was provided a high-fat, high-carbohydrate (HFHC) diet supplemented with 15% fructose for a period of 20 weeks to induce prediabetes. After confirming prediabetes, an intermittent fasting (IF) regimen was assigned to the rats while also having untreated and metformin-treated prediabetic rats serving as controls. Results: Both IF and HFHC-Met groups yield significantly lower blood glucose, leptin and BMI results compared to the prediabetic group. The IF group yielded significantly lower insulin, HOMA-IR and HbA1C than both controls. Conclusions: The study showed the potential of IF in alleviating prediabetes-induced dysregulation of glucose homeostasis and therefore warrants further investigations into its use in the management of prediabetes.

Keywords: intermittent fasting; prediabetes; insulin signalling pathway; glucose regulation

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1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by increased blood glucose concentrations due to insulin resistance and a relative deficiency in insulin production [1]. Globally, the prevalence of diabetes is rising at an alarming rate, with nearly half a billion individuals currently affected [2]. This number is expected to grow by 25% in 2030 and 51% in 2045 [2]. Although various treatments for T2DM are available, none have been shown to completely reverse the condition. The onset of T2DM is commonly preceded by a state known as prediabetes [3].

Prediabetes is a metabolic state characterized by impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) that have not reached the diagnostic criteria for T2DM [3]. Despite the World Health Organization not including HbA1c as a criterion, the American Diabetes Association (ADA), on the other hand, defines prediabetes as having an IFG level between 5.6–6.9 mmol/L, an IGT range of 7.8–11.0 mmol/L and an HbA1c level between

APPENDIX 4: JOURNAL GUIDELINES

MDPI Style Guide Second edition
MDPI, St Basel Anlage 66, 4052 Basel, Switzerland Content

1. Introduction

Welcome to the MDPI style guide. Its purpose is to offer guidance and advice to authors intending to publish in an MDPI journal. Topics covered include formatting and conventions specific to MDPI and some tips for how to improve clarity and writing style.

This is a guide, not a set of instructions. English is a flexible language and most of its rules can be broken under the right circumstances. Our aim is to communicate the latest

scholarly findings in a way that is accessible and readable. Most of the guidelines here are to aid clarity and precision. Where rigidly following this guide does not achieve that goal, exceptions can be made.

We do not expect authors to have strictly followed all of these guidelines when they submit their paper. Preparing a manuscript for publication is a key task of a publisher and includes applying the house style. Our editors will not reject a manuscript where the authors do not add a space before a unit of measurement or use the wrong tense to describe their experiment—at MDPI, we pride ourselves on providing a comprehensive production service prior to publication. Authors may benefit from reading and applying the conventions given here, though, as improving clarity and removing ambiguity can increase the chance of passing peer review.

This style guide is organized according to the sections of a research article. It begins with the front matter, which includes the title, article type, author information, and abstract. It continues with the main text, where the majority of advice on writing style can be found. There is a chapter on the presentation of mathematical content, followed by one on representations of data—including how to assemble tables and figures. Next comes information about the back matter, which includes various declarations by the authors and advice on writing the bibliography. Finally, there is a short section on publication ethics and how to revise and resubmit a paper.

1. Front Matter

The front matter covers parts of the article that usually appear at the top of the first page and give general information, including the title, abstract, journal name, and information about the authors. The format is standardized and much of it is added and formatted by the publisher.

1.1. Article Types

All articles are assigned a type, depending on the content of the article. This is useful to readers, informing them of the style of content to expect (original research, review, communication, etc.) and for indexing services when applying filters to search results. This section details the most common article types, although is not exhaustive. Editors have the final say on which type should be assigned to a published article.

1.1.1. Article

These are original research manuscripts. The work should report scientifically sound experiments and provide a substantial amount of new information. The article should include the most recent and relevant references in the field. The structure should include an Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, and Conclusions (optional) sections, with a suggested minimum word count of 4000 words. Please refer to the journal webpages for specific instructions and templates.

1.1.2. Brief Report

Brief reports are short, observational studies that report preliminary results or a short complete study or protocol. Brief reports usually contain two figures and/or a table; however, the Materials and Methods sections should be detailed to ensure reproducibility of the presented work. The structure is similar to that of an article, and there is a suggested minimum word count of 2500 words.

1.1.3. Case Report

Common in medical journals, case reports present detailed information on the symptoms, signs, diagnosis, treatment (including all types of interventions), and outcomes of an individual patient. They usually describe new or uncommon conditions that serve to enhance medical care or highlight diagnostic approaches. The structure of case reports differs from articles and includes an Abstract, Keywords, Introduction, Detailed Case Description, Discussion, and Conclusions, with a suggested minimum word count of 2500 words. Special care should be taken when submitting case reports to ensure that appropriate permission for

publication has been obtained from patients featuring in the paper. A sample blank consent form can be found on the "Instructions for Authors" pages of the relevant journals. Please refer to the journal websites for more information, because not all MDPI journals publish case reports.

1.1.4. Communication

Communications are short articles that present groundbreaking preliminary results or significant findings that are part of a larger study over multiple years. They can also include cutting-edge methods or experiments, and the development of new technology or materials. The structure is similar to an article and there is a suggested minimum word count of 2000 words.

1.1.5. Conference Report

Conference reports are records of the events of a conference, seminar, or meeting. They should provide a comprehensive overview of a meeting or session, along with relevant background information for the reader. The structure should contain Abstract, Keywords, Introduction, Conference Sections, and Concluding Remarks, with a suggested minimum word count of 2500 words. They can also include all accepted meeting abstracts.

1.1.6. Editorial

These are non-peer-reviewed texts used to announce the launch of a new journal, a new section, a new Editor-in-Chief, a Special Issue, or an invited editorial. The main text should provide a brief introduction of the purpose and aim of the Editorial—to present the new journal, close the Special Issue, report on a pressing topic, etc. Editorials should not include unpublished or original data, although must provide a Conflict of Interest statement. Editorials prepared for the launch of new journals may also include a short biography of the Editor-in- Chief.

1.1.7. Essay

Essays are an article type commonly used in humanities and social sciences to present provocative arguments aimed to stimulate the readers' re-thinking of certain issues. The structure is similar to that of a review, with a suggested minimum word count of 4000 words. Arguments should be supported by relevant references.

1.1.8. Hypothesis

Hypothesis articles introduce a new hypothesis or theory, or a novel interpretation of that theory. They should provide: (1) a novel interpretation of recent data or findings in a specific area of investigation; (2) an accurate presentation of previously posed hypotheses or theories; (3) the hypothesis presented which should be testable in the framework of current knowledge; and (4) the possible inclusion of original data as well as personal insights and opinions. If new data are presented, the structure should follow that of an article. If no new data are included, the structure can be more flexible, but should still include an Abstract, Keywords, Introduction, Relevant sections, and Concluding Remarks, with a suggested minimum word count of 4000 words.

1.1.9. Opinion

Opinions are short articles that reflect the author's viewpoints on a particular subject, technique, or recent findings. They should highlight the strengths and weaknesses of the topic presented in the opinion. The structure is similar to a review; however, they are significantly shorter and focused on the author's view rather than a comprehensive, critical review. The suggested minimum word count is 2000 words.

1.1.10. Perspective

Perspectives are usually an invited type of article that showcase current developments in a specific field. Emphasis is placed on future directions of the field and on the personal assessment of the author. Comments should be situated in the context of existing literature from the previous 3 years. The structure is similar to a review, with a suggested minimum word

count of 3500 words.

1.1.11. Project Report

Project reports are short and/or rapid announcements of project results and implications. They should include a research strategy or approach, the activities, technologies, and details of the project undertaken, conclusions, and recommendations for the future direction of work in the field. The structure is similar to an article, but permits a higher degree of flexibility. The suggested minimum word count is 3500 words.

1.1.12. Protocol

Protocols provide a detailed step-by-step description of a method. They should be proven to be robust and reproducible and should accompany a previously published article that uses this method. Any materials and equipment used should be explicitly listed. Conditions, quantities, concentrations, etc., should be given. Critical timepoints and steps, as well as warnings, should be emphasized in the text. The structure should include an Abstract, Keywords, Introduction, Experimental Design, Materials and Equipment, Detailed Procedure, and Expected Results, with a suggested minimum word count of 4000 words.

1.1.13. Registered Report

Registered reports are scientific articles which are peer reviewed before the research is performed and the data are collected. The ideas that meet high scientific standards, such as rigor, soundness, significant importance, and implications for the scientific community are then provisionally accepted for publication before data collection starts. Detailed guidelines for registered reports can be accessed on the journals' Instructions webpage.

1.1.14. Technical Note

Technical notes are brief articles focused on a new technique, method, or procedure. These should describe important modifications or unique applications for the described method. Technical notes can also be used for describing a new software tool or computational method. The structure should include an Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, and Conclusions, with a suggested minimum word count of 3500 words.

1.1.15. Review

Reviews offer a comprehensive analysis of the existing literature within a field of study, identifying current gaps or problems. They should be critical and constructive and provide recommendations for future research. No new, unpublished data should be presented. The structure can include an Abstract, Keywords, Introduction, Relevant Sections, Discussion, Conclusions, and Future Directions, with a suggested minimum word count of 4000 words.

1.1.16. Book Review

Book reviews are short literary criticisms analyzing the content, style, and merit of a recently published book. Full book details should be provided at the beginning of the article. The structure should only include an Introduction and be a discussion of critical points with no sections or conclusions, with a suggested minimum word count of 500 words.

1.1.17. Systematic Review

Systematic review articles present a detailed investigation of previous research on a given topic that use clearly defined search parameters and methods to identify, categorize, analyze, and report aggregated evidence on a specific topic. The structure is similar to a review, with a suggested minimum word count of 4000 words; however, they should include a Methods section.

Systematic reviews should strictly follow the PRISMA checklist (**http://prisma-statement.org/PRISMAStatement/Checklist**) and include a completed PRISMA flow diagram as part of the main text or Supplementary Materials. Templates for the flow diagram can be downloaded from the PRISMA website. We strongly encourage authors to register their detailed protocols before data extraction commences, in a public registry such as PROSPERO

(**https://www.crd.york.ac.uk/prospero/**). Authors must include a statement about following the PRISMA guidelines and registration information (if available) in the Methods section.

1.1.18. Abstract and Proceeding Paper

These types of articles contain peer reviewed research output from conferences and can be submitted to one of MDPI's proceedings journals: **https://www.mdpi.com/about/proceedings**.

Abstracts could be a short single paragraph summarizing the main topic and findings presented at the conference, or the extension of a typical abstract that contains a moderately detailed account of the work. They should be submitted to a conference in advance and provide details in support of a presentation made at the conference. The main text usually has no sections, but may include tables, figures, and references. The length should not exceed four pages.

Proceeding papers report new evidence or conclusions, and are expanded versions of work presented in a conference presentation. Conference proceedings can be incomplete findings that report on an idea, technique, or important results, thus providing readers with a brief overview of recent work or specific projects of significant interest. The structure is similar to a standard research article, and should include sections such as an Introduction, Methods, Results, Conclusions, etc. It is recommended that the length should not exceed eight pages.

All published items will be assigned a digital object identifier (DOI) and be citable, and posters, videos, or PPT presentations can be published together as the Supplementary Materials.

For updating published papers, please see the descriptions for Corrections, Retractions, Comments and Replies, and Expressions of Concern online at ***Research and Publication Ethics***.

1.2. Article Titles

There are few rules about the titles of submitted papers; however, there are some points that authors should keep in mind. The title conveys the main topic of the research and normally includes the principal results. It should be concise, descriptive, and grammatically correct. Periods should be avoided; instead, authors can use commas, colons, or en dashes. Italics should only be used where they are required for specific nomenclature (such as species names or journal titles) but should not be used for emphasis.

We recommend that authors keep their audience in mind and try to appeal to as broad a readership as possible. Therefore, avoid abbreviations and jargon that those outside of your field may not understand. Creative and original titles can be used, but make sure they do not sacrifice clarity in an effort to be eye-catching. Running titles should be avoided.

Some article types, including Corrections, Retractions, and Expression of Concern, have specific formats for the title that must be followed. We also strongly recommend this format for Comments and Reply, although authors may submit an alternative title, which will be used at the discretion of the editorial office. An example of the standard format is as follows:

Correction: Nasonova et al. Linking Regional Winter Sea Ice Thickness and Surface Roughness to Spring Melt Pond Fraction on Landfast Arctic Sea Ice. *Remote Sens.* 2018, *10*, 37

For titles of Comments, the following is an example of the standard format without a comment title:

Comment on Tanmoy et al. CRISPR-Cas Diversity in Clinical Salmonella enterica Serovar Typhi Isolates from South Asian Countries. Genes 2020, 11, 1365

Titles of book reviews should have the following format:

Book Review: *Microbiology in Dairy Processing: Challenges and Opportunities*; Poltronieri, P., Ed.; IFT Press Series: Wiley-Blackwell, UK, 2017; ISBN: 978-1-119-11480-2

1.3. Author Names and Affiliations

In order to identify who wrote the paper and contributed to the work, author names and affiliations are displayed at the beginning of a paper. More details about qualification for authorship and author roles are given in the section on author contributions.

It is very important that author names and affiliations are correct. Incorrect information can mean a lack of proper attribution or incorrect citation and can even lead to problems with promotion or funding.

The publisher attempts to verify the authors' identities and where necessary will make contact with the authors to confirm details. Misrepresenting affiliations is extremely serious and may constitute fraud.

Below are some important points about author names:

- Author names should be written in full (the last name must be the full name), with capitalized initials and in the order "Firstname Lastname".
- Middle names can be abbreviated, and a dot should be added after the abbreviated name, e.g., "Mark N. Breckels".
- A normal space is required between initial letters, e.g., "Fernanda C. G. Barbosa".
- The author name format in a paper needs to be consistent, especially for the authors in the same country. For example, Chinese authors can use the name format "Xiaoming Wang" or "Xiao-Ming Wang", and the name format should be as uniform as possible.
- A group or team name can be the author name. If provided the member list, add a note for the group/team name in the Authorship section to indicate that complete authors are listed in the Acknowledgments, Appendix or Supplementary Materials, e.g., "on behalf of the ELANS Study Group [†]. [†] Collaborators/Membership of the group/team Name is provided in the Supplementary Materials".
- To avoid additional checks, please indicate any authors that only have a single name.
- We strongly recommend that authors use the suggested standard name format, but for any special cases, please indicate it during proofreading.
- In the article citation on the left column of the first page, the author name will be formatted as "Lastname, F.M.". In this place, please write the last name accurately.
- If an author name contains II, III, IV, 2nd, 3rd, etc., there should be no comma between II, III, IV, 2nd, 3rd, etc. and the author names (e.g., Charles J. Smith III). If there is an author name with Jr. or Sr., there should be a comma between Jr., Sr., and author names (e.g., Teodoro Fajardo, Jr.).
- Any titles (Prof., Dr., Mr., Ms., etc.) or Academic suffixes (MD, MSc., BSc., etc.) should be avoided.

Affiliations should be those that the authors had at the time the work was carried out. The main role of the affiliations is, as far as possible, to unambiguously identify the authors. Please pay attention to the following important points about affiliations:

- The necessary composition of affiliations is institution, city post/Zip code, and country/region, e.g., "Department of Materials Science and Engineering, Pusan National University, Busan 46241, Republic of Korea".
- If the address is a university, it should usually have department/school/faculty/campus as well. Note that the address information should be sorted from subordinate to superior; e.g., the department should be put before the university, and they should be separated by commas.
- For the USA and Canada, the state/province (abbreviated) must be provided, and the zip code should be added after state/province, e.g., "Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA".
- Except for the USA and Canada, it is not recommended to add state/province

information. If provided, it should be abbreviated as much as possible, placed before the country name and after the city name, and separated by a comma. Whether or not there is state/province information needs to be consistent within the same article. City and country name should be in English.

- The post code should be put before (for European countries except for the UK) or after (for other countries including the UK) the city, except for the USA, Canada, and Australia where the zip code or postal code is put after the state or province abbreviation. The post code can be omitted or replaced by P.O. Box if the countries/regions do not use a postal code.
- Please use "Independent Researcher" as the authors' affiliation when they do not have any affiliated institutes (e.g., "Independent Researcher, 08036 Barcelona, Spain").
- Authors may also add a current address as a note in the front matter, but the current address should not be the same as any address in affiliations.
- Content words in affiliations in English need to be capitalized.
- Duplicate affiliation information should be merged in one item; multiple affiliations/addresses cannot be listed in one item.
- The same university title, city, postcode, province/state (with or without an abbreviation), and country information should be consistent with the same format used in each item.
- We strongly recommend that authors use the suggested standard affiliation format, but for any special cases, please indicate it during proofreading.

We strongly recommend that authors have an ORCID account (see orcid.org), which is a unique identifier for scholarly researchers. Your ORCID can be added in the submission system and will be included in the final version of the paper with an icon linking to your online ORCID profile.

We also strongly recommend that authors have a SciProfiles account (see https://sciprofiles.com/), which is a social network for researchers and scholars. It will help you find relevant publications and conferences and keep you updated with the latest events in your network. Your SciProfiles can be added in the submission system and will be shown on the paper's homepage with an icon (before each author name) linking to your online SciProfiles.

1.4. Abstracts

The abstract contains a summary of the entire paper and can be up to 200 words long with only one paragraph. It must not contain any images or tables (although a graphical abstract may also be submitted). Do not included running title, website links, equations, figures (or other graphical elements), tables, or structures that require display on a line separate from the text.

Authors should follow the style of a structured abstract, which is based on the IMRAD structure of a paper but without using headings. In other words, give a background and motivation to the paper, a brief description of the methods, the principal results, and then conclusions or interpretations. Some journals in the medical field may require subheadings within the abstract; you may refer to the instructions for authors to see if this is required. Abstracts without headings should consist of a single paragraph.

Abstracts must be self-contained: they are often displayed and read independently of the rest of the paper. This means that any abbreviations used must be defined in the abstract, and no reference can be made to the bibliography or any figures. Citations to previously published papers are not required in abstracts.

The abstract, along with the main title, is the first part of your paper that a reader will see. It should give them a good overview of all the major aspects of the work carried out. It should not be thought of as a sales pitch to encourage readers to download and read the full article, although including some motivation is a good idea. Instead, you should focus on making

it informative and comprehensive. A well-written abstract will mean that someone who goes on to read the full article will already have a good idea of the content and will be able to focus on the parts they are most interested in.

1.5. Graphical Abstract

A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it. The detailed requirements for a GA are listed below.

- The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, or TIFF.
- Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu, or Calibri. Make sure the reader can easily read the smallest font size of a character, number, or symbol.
- The minimum required size for the GA is 560 pixels × 1100 pixels (height × width). When submitting larger images, the size should be of high quality in order to be easily reproducible.
- Avoid large blank space in the GA. There should be a proper distance between the actual content of the picture and the margins.
- The GA should not be exactly the same as Figures in the paper or just a simple superposition of several subfigures.
- The GA should not be a simple combination of the Abstract part and a Picture (even just a Figure from the main text). Long blocks of text should be avoided in the GA.

1.6. Digital Object Identifiers

A Digital Object Identifier (DOI) is a unique number registered through a central organization, usually CrossRef for journal articles. Its role is to act as a persistent identifier, meaning that if the URL of an article changes, the DOI can still be used to find its most recent location. The DOI is defined by the publisher.

We recommend using the DOI (expressed as a URL) when citing articles as it will help readers to quickly locate the cited work. Any article can be located from the DOI by prefixing it with **https://doi.org/**, e.g., **https://doi.org/10.3390/s10100001**.

1.7. Pagination

In addition to the DOI, MDPI also issues pagination for articles. This includes several numbers or series of letters that identify where and when the paper was published:

- **ISSN:** A code that uniquely identifies serial works, such as academic journals. Each journal has a unique ISSN.
- **Volume, Issue:** These numbers originated from when journals were physically printed. Typically, journals publish one volume per year with issues on a biannual, quarterly, monthly, or semi-monthly basis. Electronic journals still often use these, and they are useful for identifying when a paper was published.
- **Page range or article number:** These identify the specific article in an issue. The page number typically starts with 1 at the beginning of a volume. Many electronic journals have switched to article numbers, which assigns a single number to the entire paper.

Except for the ISSN, these numbers occur in citations, e.g., *Sensors* **2013**, *13*(6), 6910–6935. Note, however, that the MDPI reference style omits the issue number (see the section on references).

1.8. Copyright Statement

This part does not need to be edited by the authors and has a standard wording. Copyright of the manuscript is not transferred from the authors to MDPI, meaning that those who produce the work retain ownership. Sometimes, authors are not legally entitled to own the

work. In these cases, it should first be verified whether this applies in Switzerland, where MDPI is registered. If so, the authors should inform the editorial office about the correct copyright owner.

The license determines how the work can be used after publication. MDPI articles are published using a creative commons CC BY license, meaning that the work may be reused—either in full or in part—without restriction, provided that the original source is acknowledged. In practice, this means that anyone using the article must cite it and thus give recognition to the authors. The terms of this license are what makes the articles open access. A different open access license may only be used in exceptional circumstances and must be approved at the submission step by the editorial office.

2. Structure and Formatting

The next few sections cover the main text of an article, which is written almost entirely by the authors. For research articles, this is where details of the experiments and results are presented. The main text may be supplemented by additional documents or sections, such as appendixes and supplementary material. Accession numbers, URLs, or DOI URLs can be used to refer to data or code hosted on other websites.

2.1. Overall Structure

Research articles have a standard structure, which is set out in the instructions for authors of the journal and the journal template. The majority of journals use a so-called IMRAD structure, meaning that the sections are Introduction, Materials and Methods, Results, and Discussions. Some journals require a Conclusions section at the end, and others have the Materials and Methods section after the Results and Discussions. Authors may choose to have Results and Discussions as one or two sections.

Review articles, essays, and other article types usually have a different structure, which is often more flexible. There should, however, still be a logical pattern. We recommend that the structure of an article is still considered, so the paper firstly presents a motivation for the work, followed by relevant data and previous work, and gives conclusions at the end. For systematic reviews, the structure should more closely follow that of a research article, with the methods describing how literature was chosen for inclusion.

Figures, tables, and schemes should appear in the text shortly after the first time they are cited. Where possible, they should be in the same section as the citation. It is not necessary to add them at the top or bottom of a page, and they should not break paragraphs. However, authors do not need to strictly follow these rules, and the production team will determine the most appropriate placement of figures. Note that there may be some adjustments in figure placement between author proofreading and final publication. Further details about adding these elements are given below.

2.2. Paragraph Content and Structure

There are no specific requirements from MDPI regarding the structure of paragraphs, but they should follow conventions for English writing. Paragraphs should contain and develop a single theme. They should be self-contained, which means, for example, that you should not use pronouns (it, he, she, they) referring to previous paragraphs.

A recommended structure for paragraphs is first to introduce the main idea, then give further relevant details, and finally to give interpretations or conclusions. This structure gives clarity to readers: if the idea contained in a paragraph is not clear from the start, there is more chance for misinterpretation. In some subjects, particularly in the humanities, an alternative structure may produce a particular effect on the reader that the author is trying to create; however, we recommend that care be taken to ensure that the message is as clear as possible.

2.3. Headings and Sections

For research articles, the headings are defined. For other types of paper, the authors have more flexibility to choose the headings. You may use up to three levels of

headings/subheadings. Section headings are numbered, with first-level headings as, e.g., 1.; second-level heading as, e.g., 1.2.; and third-level headings as, e.g., 1.2.3. (as in this guide). Any headings used in a fourth level may simply appear as a paragraph with no indentation. In this case, though, we recommend reassessing the section definitions to see whether only three levels could be used. Headings without numbers may also be used to introduce a series of different cases. See the mathematics section for certain special environments with their own heading styles (e.g., Theorem, Proposition, and Proof).

Headings are written using title case, which means that the first letter of all words is capitalized with the exception of short words, including articles (a, the, etc.), and all prepositions (before, after, through, under, etc.). Pronouns (he, she, it, etc.) should be capitalized, as should prepositions used in compound words (e.g., set-up). Capitalize each component of compound words if the component would be capitalized when standing alone (e.g., Half-Life and Cross-Link).

Italicized species names should not be capitalized (e.g., in *Escherichia coli*). The first word of the title and the first word after a colon or em dash should be capitalized regardless of the previous rules.

2.4. Formatting, Fonts, and Symbols

When writing symbols, use common, standard fonts where possible. If you are using a template in Microsoft Word, ensure that the font is correctly set for all text, especially when copying and pasting text from a different document. The format painter tool can help.

Avoid using fonts such as symbol, wingdings, or webdings. Authors should also avoid adding symbols as pictures, as this can lead to difficulties in formatting the final version. If there is a symbol you have difficulty in adding, leave a comment in the text so that the production team can take note.

For LaTeX, we recommend using an editor that includes a good list of symbols in the menus and a spell checker. This increases accuracy in writing and decreases the need to memorize many different codes. Table 1 contains LaTeX codes for a few especially useful symbols.

Table 1. Commonly used LATEX symbols.

Symbol Name	Symbol	LATEX Code
En dash	–	--
Em dash	—	---
Micro/mu	µ	\upmu (from the upgreek package)
Degree symbol	°	$^{\circ}$
New line	(n/a)	\\

2.5. Abbreviations

Most abbreviated phrases should be written in full the first time that they are used, with the abbreviation in brackets, for example, “small angle X-ray scattering (SAXS)”. Some very common abbreviations do not need to be defined—some of these are universal and others depend on your intended audience. Below are a few common abbreviations that usually do not need defining. Non-standard abbreviations for phrases that are commonly used throughout an article can be defined, but avoid redefining abbreviations that already have a more common meaning. Words used in abbreviations do not need to be capitalized, even if the abbreviation is capitalized.

Note that the abstract, main text, and figure/table/scheme captions are treated separately for abbreviations. This means that you need to define the abbreviation the first time you use it in each part—you may have to define the same abbreviation three separate times. The reason

for this is that they are often displayed in isolation; for example, indexing services usually only display the abstract and you can browse figures without the main text via the journal website.

Table 2. Common abbreviations that do not need defining in the text. This list is not exhaustive, and you may still choose to define these abbreviations for clarity.

Abbreviation	Meaning
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
CI	confidence interval
CNS	central nervous system
COSY	correlation spectroscopy
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESR	electro-spin resonance
FTIR	Fourier-transform infrared spectroscopy
GDP	gross domestic product
GFP	green fluorescent protein
GIS	geographic information system
GLC	gas–liquid chromatography
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
HPLC/MS	high performance liquid chromatography/mass spectrometry
IR	infrared
LC–MS	Liquid chromatography–mass spectrometry
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
MEMS	micro-electro-mechanical systems
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
PCR, RT-PCR, qPCR	polymerase chain reaction, real-time PCR, quantitative PCR
pKa	negative base-10 logarithm of the acid dissociation constant of a solution

R&D	research and development
RGB	red green blue
RNA	ribonucleic acid
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TOCSY	total correlated spectroscopy
UV-VIS	ultraviolet-visible
v/v, w/v, etc.	volume per volume, weight per volume, etc.

The following Latin abbreviations may also be used in text: etc. (et cetera), to indicate that a list is incomplete; i.e., (id est), meaning "in other words" to add clarification to a phrase; and e.g., (exempli gratia), meaning "for the sake of example" to introduce a list of examples. Note that it is not necessary to use, "e.g." and "etc." in the same list. Confusion between "i.e." and "e.g." is common—if your list of examples is complete, then use "i.e." but if there are additional cases not mentioned, then use "e.g." Both "e.g." and "i.e." should be followed by a comma and do not need to be italicized.

Do not abbreviate "also known as" to "aka" or use an ampersand (&) instead of "and"—they should be written in full.

2.6. Italics

Authors may use italics for emphasis at their discretion. Be careful that there is no confusion, especially in disciplines where italicization is used for another purpose, such as in mathematical symbols or gene names.

Foreign words do not need to be highlighted or italicized, including Greek/Latin terms, such as i.e., e.g., etc., et al., vs., ca., cf., in vivo, ex vivo, in situ, ex situ, in vitro, in utero, ad hoc, in silico, ab initio, vice versa, and via. Authors may choose use italics for purposes of emphasis or where a term is being defined. Journal and book titles should always be written in italics, e.g., *International Journal of Environmental Research and Public Health*.

Italics must be used for the genus and species when using Latin names of organisms. The first time the name is used it should be spelled out in full, but for further uses, the genus can be abbreviated to the first letter. Note that the species is always written without a capital letter, including when it appears in a title; the genus name should always be capitalized. Similarly, italics should be used for gene names (but not for the corresponding proteins). Examples:

Escherichia coli is a common bacterium ... *E. coli* was used in this article.

Lac1 is the gene that encodes for the Lac1 protein.

2.7. Bold Font

Bold font should generally be avoided. If you wish to add emphasis, italics are preferred. Bold font is used in specific contexts, including figure captions and subtitles. In chemistry, bold numbers can be used to refer to molecules defined in Schemes.

2.8. Quotations

Any text taken from previous work, whether published or not, should be clearly indicated. The recommended way to do this is as a quotation accompanied by a citation and bibliography entry. Quotations should appear in double quotation marks (" . . . "). Long quotations may appear as a block quotation: a separate paragraph set off from the rest of the text with an indent on both sides. The exact formatting will be completed by a layout editor during production.

2.9. Notes

Most scientific journals from MDPI do not allow notes. Check the instructions for authors of the journal to see the specific policy of a journal.

Where notes are permitted, they may be used to add additional explanatory notes to text. All notes will be shown in the Notes section, which is before the References section. Information essential to understanding the text should not be added to notes. They can be used to add additional sources, explain the background to a particular point, reinforce ideas, or clarify intended meaning. Notes should not be used as a replacement for a bibliography, since citations included only in notes will not be detected and counted by indexing services.

2.10. Lists, Itemized Lists, and Bullet Points

Most lists can be included as inline text; however, authors may decide that the information is more clearly represented using bullet points. If there is a specific order to the list, a numbered list may be used. A descriptive list may also be used, in which each item begins with an emphasized word or short phrase.

For inline lists, items should be separated by commas. An exception is where one or more items contains a comma, in which case semicolons can be used. Do not use commas for lists of two items. Serial (or Oxford) commas are recommended; however, they may be omitted if done so consistently, especially when not using American English for spellings.

For itemized lists, introduce the list with a colon, add a semicolon at the end of each item, and a period at the end of the last item. Alternatively, periods may be used at the end of each item. Always capitalize the first word of each item.

Examples of lists. Using bullets:

- The first;
- The second;
- The third.

A numbered list:

The first;
The second;
The third.

A descriptive list:

Item 1 the first;
Item 2 the second;
Item 3 the third.

Note that lists that mention that only a few examples are given do not need to end with ‘etc.’, ‘and so on’, or similar text. Doing so means that you have indicated twice that the list is incomplete. For example:

Examples include red, white, and green varieties. Popular varieties are red, white, green, etc.

2.11. Patents

Authors may declare any patents related to the published work, either those pending or already obtained. The aim of this section is to create a better link between research articles and new inventions to which they have contributed. This section is not obligatory, and there is no penalty for not declaring patents, but in most cases authors benefit from adding any relevant information here.

When declaring patents, please include the patent number and title so that any interested readers can access the full details.

We strongly recommend against submitting papers for publication before patents have been granted, since publication can compromise the patent application process. Published

papers will not be removed from journals in order for patent applications to be filed.

3. Grammar and Tenses

3.1. Tenses

In scientific writing, the language around what is already known and what remains unknown needs to be precise. This relates to the passage of time and hence the use of tenses. The following looks at how tenses are used in each part of a research paper.

3.1.1. Introduction

In the introduction, current problems and past work are typically discussed, along with a description of what the paper presents. The authors should use the present tense to describe outstanding problems:

"The increase in the number of RF electromagnetic sources is associated with a growing concern about potential harmful health effects of human exposure to RF radiation."

Former work can be in the past or present tense:

"These uncertainties are due to the directivity of the body-worn antennas [5], or body shadowing in which the body shields part of the EM fields."

"In [4] it was shown that the location of PEMs contributes to the uncertainty of their measurements and results in an underestimation of the incident electric fields."

New results being presented by the authors should be in the present tense (not future):

"The designed antennas and the frequency bands of the BWDM are summarized in Section 2.2. Section 2.3 presents the design of the receiver nodes."

3.1.2. Methods

Methods should typically be presented in the simple past tense:

"The multi-antenna measurement system consisted of 22 autonomously working measurement units, for 11 different frequency bands, connected to a common serial bus system."

An exception is where methods are described in the form of instructions or an algorithm. These are most often used in theoretical papers:

"The following describes the calibration procedure. First, place the subject (Sb-1) on a rotational platform in the far field of a transmitting horn antenna (TX) in an anechoic chamber."

The present perfect should be avoided:

"The subject has been placed on a rotational platform . . . "

3.1.3. Results and Discussion

This is the section where tenses are most often mixed and there is more flexibility/ambiguity about the correct tense to use. As a rule, established facts should use the present tense; however, difficulty arises when a single result is presented as establishing a fact. Authors may write the same phrase in different ways:

"The results show that commercial PEMs underestimate the actual incident power densities by a factor of 1.6 to 20.6."

"In our study, commercial PEMs underestimated the actual incident power densities by a factor of 1.6 to 20.6."

"Commercial PEMs underestimate the actual incident power densities by a factor of 1.6 to 20.6."

The first example uses the present tense because the results are fixed and will not change in the future. The second uses the past tense, much like the methods section, to describe what happened during the experiments. The third is a bolder statement that generalizes the results of the paper to all commercial PEMs.

The second is the best option as it is a clear statement of what happened during the current study. Anything that speculates or extrapolates the results should be clearly differentiated. For example:

"In our study, commercial PEMs underestimated the actual incident power densities by a factor of 1.6 to 20.6. This could imply significant measurement errors where PEMs are used

in an industrial environment."

This phrasing separates the third statement above into two distinct phrases that differentiate the results from the conclusions.

3.1.4. Review Papers

In a review, the writing often jumps rapidly between established facts, the results of studies, and speculation. Paragraphs or sections can be a microcosm of a complete paper, firstly setting out a problem, describing work done, and then making a conclusion about the current state of the field or speculating on the future. Be aware of which tense is appropriate for each statement:

"Smartphone imaging *is used* extensively in remote sensing, for example in aerial photography and grass roots mapping applications . . . It *has been applied* quantitatively, for example in determining 'leaf area index', which *is a measure* of foliage cover [18], and *could offer* powerful tools for tracking longer term trends in sky [19], land cover and vegetation conditions."

There are different writing styles for reviews, such as using references to support a stated fact, written in the present tense:

"LOD is defined as the smallest concentration of an analyte that can be reliably detected, where reliable detection means the sensor response should be different from that of blank/reference [27]."

Alternatively, the authors may describe work done, similar to a methods section:

"A microstrip coupled CSRR has been proposed as a chemical sensor [34]. In this setup, the microstrip line is designed on the top of substrate and CSRR is etched on the bottom ground. Withayachumnankul et al. [33] varied the concentration of water–ethanol solution, and the corresponding S-parameters were measured. To validate the proposed sensing system, the measured complex permittivity values of mixture were compared with the reference ones. This sensor showed four times higher sensitivity compared to their previous work."

It is authors' responsibility to differentiate established facts from speculation through the use of tenses. Use of tenses should be consistent throughout a manuscript in order not to confuse readers.

(Quotations in this section adapted from *Sensors* **2018**, *18*, 272; https://doi.org/10.3390/s18010272, *Sensors* **2018**, *18*, 232; https://doi.org/10.3390/s18010232, and *Sensors* **2018**, *18*, 223; https://doi.org/10.3390/s18010223.)

3.2. Plurals

Plurals need to agree with other parts of the sentence; this is an area where errors often occur, particularly in complex phrases. For example:

"The full implementation of the trained networks are available at"

should be

"The full implementation of the network algorithms is available at"

since the verb agrees with "implementation", not "networks".

The word "data" should be considered to be plural, so write "The data show that . . . " rather than "The data shows that . . . ". The singular form is "datum".

3.3. Different Types of English: US vs. British

In MDPI papers, US, British, or other variations of English can be used; however, authors must be consistent throughout the paper. We recommend that authors use American English unless they live or work in a country that uses a different variation (e.g., the UK, Australia, or Canada).

One of most notable differences between British and US English is the use of -ise instead of -ize as a suffix. Some words are spelled differently; a few examples are

British	**US**

Enquire	Inquire
Travelled	Traveled
Aluminium	Aluminum
Orientated	Oriented

A good spell-checker can help to identify words that are incorrectly spelled and authors should set the proofing language in their writing software to the version of English they wish to use.

3.4. Non-English Words

Articles can include text written in languages other than English provided that a translation is provided. This includes labels used in figures. Note that non-English words or phrases do not need italicizing.

4. Punctuation

Authors should have a good knowledge of standard punctuation. This guide is not intended to be comprehensive and authors should refer to textbooks to ensure the correct use of punctuation. In this section, we highlight aspects that particularly reflect the MDPI style.

4.1. Periods/Full Stops

Periods that end sentences should be followed by a single space. Most abbreviations use periods to indicate where letters have been omitted. Note that "vs." should be followed by a period, except in papers covering law, where the convention is to omit a period.

4.2. Commas

Good use of commas can ensure clarity in your writing. Sometimes, it comes down to personal preference; however, there are some guiding principles that should be applied. See **Section 3.10** for the use of commas in lists.

Commas separate non-restrictive sentence modifiers—a phrase added to a sentence that is not essential to its meaning. Do not add commas for restrictive modifiers. For example:

"Due to a slower than expected process, the experiment continued for an additional five days."

"The experiment continued for an additional five days in one case."

With regard to style, it is usually best to minimize the number of commas used in writing. Since commas separate different ideas, too many commas in a single sentence may be an indication that the structure is too complex. The result will be that readers are confused, especially if the sentence starts on one theme, adds a lengthy subclause for explanation, then goes back to the original theme.

4.3. Hyphens and Dashes

There are four types of dashes used in writing:

- - Hyphen: joins two separate words into a single concept.
- – En dash: a mid-sized dash (longer than a hyphen but shorter than an em dash), shows a link or relationship between two concepts, or a range.
- — Em dash: used to introduce a phrase or subclause that clarifies the previous phrase.
- − Minus sign: used in equations or negative numbers.

When using prefixes and suffixes, hyphens are not required unless omitting them creates ambiguity in the meaning or double letters. Some words are conventionally written with or without hyphens, and for others, multiple forms are in common use. You will not be expected to know all of these and the editorial team will check before publication. A few examples are

Prehistoric, lifelike, anti-inflammatory, and un-ionize.

For compound adjectives, hyphens should typically be used. Two words together used to modify a single noun are termed "unit modifiers". Note that hyphens should also be used in double-barreled names. For example:

Three-dimensional, time-dependent, Parker-Bowles, and grey-green. Chemical names,

however, should not be hyphenated (e.g., sulfuric acid).

En dashes can be used to denote a chemical bond, a range between two numbers, or a relationship between two separate entities. They are also used between the last names of two different people when their names are used for a scientific concept. Some examples:

Carbon–oxygen bond

A time–frequency plot

17–30 m in length

Fabry–Perot

Bose–Einstein

For MDPI papers, em dashes are preferred to colons when introducing phrases that provide clarification or definitions. Spaces should not be included either side of em dashes. For example:

"We measured alignment using linear dichroism—differential absorbance in perpendicular directions."

We recommend using em dashes sparingly to avoid disrupting the flow of sentences.

4.4. Colons and Semicolons

As mentioned above, em dashes are preferred to colons for introducing definitions. Colons may be used to introduce lists or before equations, but not where they separate a verb and its object or a preposition and its object.

Semicolons may be used in lists, as mentioned above. For other uses of semicolons, refer to a grammar book. In general, we recommend using semicolons sparingly and considering whether a period or comma would be more appropriate.

4.5. Apostrophes

Apply the common usage of apostrophes to indicate ownership or contraction of words, although note that most contractions should be written in full ("cannot" instead of "can't", "it is" instead of "it's", etc.). Do not use apostrophes to pluralize abbreviations or numbers, e.g.,

"The results of five PCRs are shown." "This was common practice in the 1960s."

5. Numbers and Mathematical Environments

5.1. Numbers

Numbers should usually be written as digits, with a few exceptions. Where there are five or more digits to the left of the decimal point, use a comma to separate every three digits, e.g., 123,456 or 153,958.9476. As in the previous sentence, numbers 0–9 should be written as words unless they are a measurement, i.e., they are accompanied by a unit. For example:

five trees

5 m from the tree

If a sentence starts with a number, the number should always be written out in full; however, it is often better to reword the sentence. As an example:

. . . and was heated. One hundred and seventeen grams of NaCl was added to the mixture.

However, this could be reworded as:

. . . and was heated. A total of 117 g NaCl was added to the mixture.

5.2. Measurements and Units

When writing about measurements, use a space between a number and its unit. SI or SI-derived units should be used where possible; if you use alternative units, please explain to the editors why it is necessary. Middle dot (always use middle dot as multiple sign in units) or a normal space can be used in units; however, they must be consistent throughout the paper. For example:

3×10^8 m·s^{-1}.

Do not leave a space before a percentage (%) symbol, since the symbol is part of the number and not a unit. The same applies to degree (°) symbols when used for angles, so write

90° but 90 °C.

A space is optional between wt%, mol%, vol%, at% or wt.%, mol.%, vol.%, at.%, but keep the format consistent within a paper. Other formats are not allowed, e.g.,wt %, % wt.

For some common time units and measurement units, it is recommended to use abbreviated units if Arabic numerals are in front of them.

Table 3. Abbreviated SI Fundamental Units.

Units	Abbreviation
second/seconds	s
minute/minutes	min
hour/hours	h
millimeter/millimeters	mm
centimeter/centimeters	cm
meter/meters	m
kilometer/kilometers	km
gram/grams	g
kilogram/kilograms	kg
liter/liters	L

Powers of 10 can be indicated by a prefix to a unit. The following table shows terms that can be used in this way. For example, 1 pm = 1 × 10−12 m.

Table 4. Prefixes to units that indicate powers of 10.

Symbol	Prefix	Power of 10
y	yocto	−24
z	zepto	−21
a	atto	−18
f	femto	−15
p	pico	−12
n	nano	−9
μ	micro	−6
m	milli	−3
c	centi	−2
d	deci	−1
da	decka	1
h	hecta	2
k	kilo	3
M	mega	6

G	giga	9
T	tera	12
P	peta	15
E	exa	18
Z	zetta	21
Y	yotta	24

5.3. Dates and Times

Times should be written using the 24-hour clock with a colon between the hours and minutes, e.g., 12:42. Dates should be written with the format day (as a digit) month (as a word) year (four digits), e.g., 1 January 2001. BC (before Christ) or AD (anno domini) can be added if necessary; CE (Christian era) and BCE (before the Christian era) are also acceptable. Where other calendars are used (e.g., lunar calendars), we recommended including the date using the Gregorian calendar as well.

5.4. Symbols

Mathematical symbols that appear between two numbers should have a space on either side, such as in "a = 2b". Do not leave a space around mathematical operators in subscripts and superscripts, e.g., a_{n+1}, and also do not leave a space around other expressions in subscripts and superscripts, unless doing so would lead to confusion or misreading, e.g., E^{365nm}. Do not leave a space where there is only one number, e.g., "the number of samples in each case was >50". Do not include a space when writing ratios, e.g., 1:100. Decimals need to be completed; e.g., a = .01 should be written as a = 0.01. Use scientific notation, i.e., $a \times 10^b$ rather than aEb or aeb. Leave a space before or after trigonometric function, e.g., cos Θ, cot Θ, sin Θ, tan Θ, sec Θ, csc Θ, etc.

5.5. Equations

You may include appropriate equations in your manuscript. They may be included inline or as a separate paragraph. Non-inline equations may be numbered starting from 1 (do not include a section number), e.g., Equation (1). In the appendixes, all equations should be prefixed with A and in the supplementary information with S, e.g., Equation (A1), Equation (S1). Subequations are not recommended; if necessary, they should be cited, for example, as Equation (1a). Minor or trivial equations do not necessarily need to be numbered, at the discretion of the author. In derivations involving multiple steps, obvious intermediate results may be omitted.

Punctuate equations as part of a regular sentence. For example, if the equation comes at the end of a sentence, a period should be placed immediately after the equation. It is not necessary to always use a colon to end the paragraph before an equation. If the equation is followed by "where . . . " to define the symbols used, "where" should be all lower case and flushed to the margin (without first line indentation) to indicate that it does not begin a new paragraph.

All terms used in an equation should be defined in the text. It is highly recommended to check specifically for this during proofreading before submission, as undefined terms could lead reviewers and editors to misinterpret your meaning. Additionally, be aware of multiply defined symbols, and we recommend using standard notation in the field where it exists (e.g., P for a probability function). The format (italics/non-italics) of each character in an Equation should be consistent with the main text. Symbols used in equations should use italic font, although exceptions will be permitted where there is a convention not to use italics. Words and numbers in equations should not use italic font, for example,

$P(x) = 2a$ if $x > 0$.

The final formatting of equations will be done by MDPI staff. To assist them, take note of any examples in the journal template. In Microsoft Word, make sure your equations can be edited using the standard Word equation editor, rather than appearing as a picture. The content of one equation should be in the same environment (written in plain text or Word equation editor). Formatting is sometimes changed during production, and errors may be introduced if the equation appears only as a figure. LaTeX is convenient for writing equations. Users of LaTeX should try, where possible, to use common packages for introducing symbols, since this will make the production process more straightforward and error-free.

5.6. When to Use Mathematical Environments

Papers that report mathematical proofs have a structure that differs from other kinds of research papers. They usually contain a short motivation and introduction followed by a series of logically argued results (lemmas, proofs, corollaries, etc.) intermingled with some examples, remarks, and definitions. In principle, these environments could be used by authors from any field, but it is recommended only to use them for mathematics, as some readers may not be familiar with the structure. The following environments may be used for mathematical content: Theorem, Lemma, Corollary, Proposition, Characterization, Property, Problem, Example, Examples and Definitions, Hypothesis, Remark, and Definition. Any mathematical environments should be labeled with an Arabic number and numbered sequentially.

The 'Proof' environment may be used for (mathematical) proofs of results. If they immediately follow the result, there is no need to state which result they refer to. If they appear later, the type and number of the result should be referenced, e.g., "Proof of Theorem 3". This can be automated in LaTeX using the \label and \ref commands. Proofs may finish with a square box or "Q.E.D."—the LATEX proof environment automatically adds the former.

Note that the MDPI LATEX class file automatically loads the amsmath and amsthm environments packages, which contain many commonly used symbols. You can see comments in the preamble of the MDPI LATEX template for more details.

6. Figures, Tables, and Data

Figures, tables, and similar items may be added to the text as appropriate. This section details how to use these. Authors are required to make their original data available unless there is a valid reason for not doing so (e.g., related to patient confidentiality). The best way to do this is to publish the data at the same time as, or before, the published article. This may be done alongside the article as an Appendix or Supplementary Material or on a separate platform. In the latter case, we strongly recommend a platform that uses the datacite mechanism (see **https://datacite.org**) to assign a digital object identifier (DOI) to your data.

For figures previously published or tables cited from other publications, the necessary permission must be obtained from the copyright holder. For non-open access journals, this can usually be obtained via an online form or by e-mailing the editorial office. It is the authors' responsibility to obtain the necessary permission.

Any figures, tables, supplementary information, etc., must be cited in the main text of the document, e.g.,

"The data are shown in Table 3."

"This case is depicted in Figure 3d."

Do not abbreviate Table and Figure to Tab. or Fig. The cited object should usually appear shortly after the citation and at the end of a paragraph. The final position of objects in the published PDF file is determined by the MDPI production team and may change between proofreading and publication.

6.1. Figures

Figures are graphics that support the main text. They may show data, an algorithm, a model, an image, or any other pictorial representation. Figures must be clear and readable, and we recommend a minimum resolution of 600 dpi. Any common figure formats may be used,

including (but not limited to) tif, jpg, and png. For CAD and similar formats, a representation as, for example, a png file may be included in the text and the full original file included as supplementary material.

Others notes on figures:

- The order and the citation of each Figure must be in sequence and correct;
- All fonts must be embedded;
- Special characters or icons in an image (e.g., *, **, #, ...) need to have a corresponding explanation (may be added in the image or caption);
- The aspect ratio should be locked;
- All the images should not be duplicated;
- Non-English words are not allowed in the figures unless there is an explanation;
- Some symbols, such as the red/blue wavy lines under the words, which indicate spelling/grammar errors, and the new-line/paragraph sign after the word, should not appear in the image;
- Scale bars and numbers need to be clearly identified;
- The right form of minus sign and en dash must be used; see Section 5.3 for the use of hyphens and dashes;
- e or E to mean "multiplied by the power of 10" is not allowed; please use the correct scientific notation for numbers, e.g., 3.7×10^5 (not 3.7e5 or 3.7E+5);
- For numbers with five or more digits in images, commas should be added; see Section 6.1 for the use of numbers;
- Add 0 before the decimal point;
- The decimal point should always be a dot in numbers;
- The subscripts/subscripts of chemical formulas and the en dash of chemical bonds should be correct;
- Unit format should be correct and keep the consistent format within a paper;
- The special symbols should be the same as those in the caption;
- The integrity of image must be ensured; avoid missing or overlapping text;
- References in the form of "[XX]" are not allowed in the image;
- Except for Retraction of paper, other pictures cannot be watermarked.

Note that the production process may change the type of your file, and all files will be published in tif format. This should not affect the quality of your figure; however, if you notice a decrease in the quality after publication, you should contact the editorial office as soon as possible.

For figures with more than one part, the panels should be labeled a, b, c, d, etc., and each part can be separately cited in the main text. Each part must be individually described in the caption. It is recommended to use label "A, B..." or "a, b..." instead of "left, right, top, bottom" for the subfigures.

Captions are mandatory and are added below figures.

6.2. Tables

Long lists of categorized data may be added as a table. This could be done, for example, where there are many cases with similar information or many numerical data.

Tables will be reformatted to the standard MDPI style prior to publication, and the journal template provides an example. Use of color (including the color of table background and texts) is not recommended in tables but may be accommodated where necessary; if necessary, colors must be described in an image or a caption. Similarly, merged cells may be included but should be used sparingly, and it must be clear which rows/columns correspond to each other. Do not supply tables as images—they must be editable by MDPI staff.

Subtables are not recommended; if necessary, subtables could be numbered by Latin letters with parentheses, e.g., (a), (b), (c), etc., or (A), (B), (C), etc., which should be put before

the table. Additionally, use the format of Table 1a,b in the main text.

A table footnote can be added to explain material referring to the whole table and to specific entries, and it usually comprises one paragraph. A hyphen may be inserted into a table body cell to stand for "None": such an entry does not need further explanation in the table footnote.

Very large tables, or many different tables showing similar cases, may be included in an Appendix or as supplementary data.

Captions are mandatory for tables and are placed above the table.

Others notes on tables:

- The order and the citation of each table must be made in sequence and correct.
- Vertical line, blank row, and columns are not advised.
- Any special characters or icons in table (e.g., *, **, #, ...) need to have a corresponding explanation.

6.3. Boxes

A box is equivalent to a table with a single cell. They are typically used to describe a case study that illustrates and supports some aspect of the main text. Boxes must include a caption, placed above. Each box should be marked by continuous numbers and cited in the main text. Boxs should be editable; do not use uneditable images.

6.4. Schemes

Schemes are common in chemistry to define the synthesis of a chemical. They can be included in a similar manner to figures. Carefully verify that the structures given are correct. It is not usually necessary to include hydrogen molecules in schemes. Captions are mandatory for schemes and are placed below the scheme. Each scheme should be marked by continuous numbers and should be cited in the main text.

6.5. Algorithms

Algorithms are typically used in computing to explain a series of steps performed in a calculation or program. They may simply be included in the main text, but can also be numbered for easier citation. Use of monospace font is common for algorithms but not mandatory. A caption must be included above the algorithm. Each algorithm should be remarked by continuous numbers and should be cited in the main text. Algorithms should be editable; do not use uneditable images.

6.6. Captions

As mentioned above, captions are obligatory and must be placed above or below objects. They should provide a description of the object such that the reader does not need to refer to the main text to fully understand it. For example

is not helpful to readers, whereas

"The four methods used."

is not helpful to readers, whereas

"The four minimization methods used to find the optimum parameters of the Navier–Stokes equation for three microfluidic devices."

is better. Recall that figures and captions sometimes appear online separately from the rest of the article and so must make sense when not accompanied by the main text.

6.7. Copyright Permission in Caption

For previously published figures or tables, the necessary permission must be obtained from the copyright holder, except for publications with the open access license. The copyright permission can usually be obtained via an online form or by e-mailing the copyright holder. It is the authors' responsibility to obtain the necessary permission.

In MDPI publications, credit lines for art reproduced from previously published work appear at the end of the caption in parentheses in one of two formats:

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Reprinted with permission from Ref. [X]. Copyright Year Copyright Owner's Name.

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Format 1 (ACS Style)

Reprinted from Ref. [X].

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Reprinted from Author Names (Year of Publication).

Adapted from Author Names (Year of Publication).

7. Back Matter

The back matter includes important information that supplements the main text and provides further information and context. Most of the back matter is provided by the authors, but the structures are quite standard.

The sequence of back matter elements in an article is listed below. Although each of them can be optional, very few articles have no reference list of some sort. There is no numeral label for back matter headings:

- Supplementary Materials
- Author Contributions
- Funding
- Institutional Review Board Statement
- Informed Consent Statement
- Data Availability Statement
- Acknowledgments
- Conflicts of Interest/Disclaimer
- Glossary/Nomenclature/Abbreviations
- Appendix
- References

7.1. Supplementary Material

Additional data or information can be included in the supplementary material. Examples of information that can be presented as supplementary material include additional graphs, tables, original datasets, and computer codes. In most cases, authors are free to choose what is included as supplementary material. All materials should be provided in English (except for translations of the manuscript or abstract), and the provided version should be clean,

without tracked changes, highlights, comments or line numbers.

There is also no restriction regarding file type, although we recommend using common, open file types that will remain readable in the future. There may be restrictions on file size for files hosted by MDPI; however, the editorial office will be able to offer alternative options if this is a problem.

There are two ways that supplementary material can be added. It can be submitted to MDPI along with the manuscript, or it can be hosted on a third-party platform and the details can be included in the paper. In the first case, additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. In the latter case, authors should use a repository that uses datacite or an equivalent mechanism to give the files a digital object identifier. The website must also have a policy for data preservation, which reduces the chance that, at some point in the future, the link to the files will no longer work. A personal website, for example, would not be suitable. There is a list of suitable repositories at **https://www.re3data.org**. The link of Externally Hosted Supplementary Files can be listed in the "Supplementary Materials" section, and an access date is necessary.

Supplementary materials must be mentioned in the main text. The citation format of Supplementary Figure, Scheme, Table, Equation, etc., should start with a prefix S (i.e., Figure S1, Equation (S2), Table S1, etc.).

In the Supplementary Materials section of the main text, describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows: Figure S1: title, Table S1: title, etc.

Citations and References in supplementary files are permitted provided that they also appear in the reference list of the main text; if references in individual supplementary files are included in the main text, all of the references should have a citation in the "Supplementary Materials" section (e.g., "References [x,x] are cited in the Supplementary Materials") or in main text.

7.2. Author Contributions

Each author is expected to have made substantial contributions to the conception or design of the work; the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or they must have drafted the work or substantively revised it. In addition, each author must have approved the submitted version (and versions substantially edited by journal staff that involve the author's contribution to the study) and agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.

MDPI uses the CREDiT taxonomy for authorship and a standard wording is given in the journal article template. Further details are available at **https://casrai.org/credit/**, and a brief explanation of each role is available at **https://www.mdpi.com/data/contributor-role-instruction.pdf**.

7.3. Funding

Financial support in preparation of the publication is included in the funding section. Check carefully that the details given are accurate, and use the standard spelling of funding agency names at **https://search.crossref.org/funding**. Any errors may affect your future funding.

7.4. Institutional Review Board Statement

In this section, please add the Institutional Review Board Statement and approval number and approved date for studies involving humans or animals (for more details,

see **https://www.mdpi.com/ethics**). Please note that the Editorial Office might ask you for further information, and it is recommended to upload the approval file to the journal office when submitting the manuscript. You might also add "not applicable" for studies not involving humans or animals to exclude this statement.

7.5. Informed Consent Statement

Any research article describing a study involving humans should contain this statement. Written informed consent for publication must be obtained from participating patients who can be identified (including the patients themselves). Please state "Written informed consent has been obtained from the patient(s) to publish this paper" if applicable. You might also add "Not applicable" for studies not involving humans.

7.6. Data Availability Statement

We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at **https://www.mdpi.com/ethics**.

7.7. Acknowledgments

Acknowledgments are a place to recognize any contributions made to the paper that do not meet the criteria for authorship. This may include technical support, gifts received, or organizational assistance. There are few restrictions on what should be included, with the primary exception that anyone who meets the criteria for authors must be included as an author and not merely acknowledged. Personal acknowledgments (e.g., of family members) are acceptable, and it is recommended to add the full name for them; titles (Dr., Mr., Prof., etc.) should not be used. This section should be kept relatively short.

7.8. Conflict of Interest

MDPI uses the recommendations of the International Committee of Medical Journal Editors with regard to Conflicts of Interest (CoIs) **(http://www.icmje.org/icmje-recommendations.pdf**):

"A conflict of interest exists when professional judgment concerning a primary interest (such as patients' welfare or the validity of research) may be influenced by a secondary interest (such as financial gain). Perceptions of conflict of interest are as important as actual conflicts of interest."

CoIs come in different forms and can affect authors, editors, and publishing staff. Having a CoI does not mean that your paper will not be published; however, omitting them could lead to retraction or at least re-evaluation of your paper. No conflicted third parties should be able to directly influence the results of your research or have a say in the final version. Conflicts of interest where there is a negative effect on the author as a result of the paper's publication should also be declared.

Types of CoIs include:

Direct/indirect: This concerns whether the CoI refers specifically to an author (direct) or one of their associates, such as a close colleague or family member (indirect).

Financial/non-financial: Both of these are important. Financial CoIs concern receiving money from people or organizations with a vested interest in the outcome of the research, holding patents or salaried positions that depend on the research outcomes, or holding shares or other items whose value is dependent on the research. Non-financial CoIs include benefits to groups the author is associated with and reputational benefits.

There are some grey areas about what to disclose as a conflict of interest. If you are unsure, we recommend making a declaration and checking with the editorial office prior to publication. Colleagues may also be able to provide advice. Examples of CoI statements can

be found in the instructions for authors and the journal submission template.

7.9. Glossary/Nomenclature/Abbreviations

This is an optional section defining terms and abbreviations used in the paper. It can be omitted for most papers but may be useful if a large number of novel terms are defined. They can also be used where the author expects the readership to be unfamiliar with many of the terms used, for example, if the paper is multidisciplinary.

7.10. Appendixes

Authors can use Appendixes to add further information to support the results reported in the manuscript. They should be used when including the information in the main text would disrupt the flow for readers or where only a minority of the audience is expected to be interested. Appendixes may include full details of lengthy mathematical proofs, additional figures, further experimental details, or additional data. If the information is very lengthy or in a format that does not work well on a printed page, it may also be included as supplementary material (see above).

Appendixes must be cited in the main text. Note that sections in the Appendix are labeled with capital letters (as opposed to numbers, which are in the main text), e.g., Appendix A, Appendix B. Sub-headings should be listed sequentially with the correct number (e.g., Appendix A.1., Appendix A.2.1, etc.).

Figures, tables, equations, etc. in an Appendix are prefixed with "A" (regardless of the section), and numbering begins from 1 at the beginning of the Appendix (i.e., Figure A1, Figure A2, etc.).

7.11. References

Almost all papers contain a reference list giving details of previous work cited in the manuscript. The purpose of the reference list is to enable others to find works on which the published paper is based.

A citation should be included when what you are writing refers to or is based on previous work. Examples can also be cited. The citation list should contain only references to static content, i.e., something that is not expected to change over time. This includes journal and newspaper articles, patents, and details of specific equipment. Content that does not fulfil these criteria may be listed directly in the main text and might include company websites, or websites to track project development (such as github).

The reference section is highly structured, and different types of references are formatted in a specific way. Full details are available from the instructions for authors page of the journal you are submitting to; however, below are examples of the most common types.

MDPI uses two reference styles, one based on the American Chemical Society (ACS) style and the other following the Chicago style. You should consult the instructions for authors to see which one applies to the journal you are submitting to. Templates for both are available for most common referencing software. Examples of the most common reference types are given in the following two sections.

7.11.1. ACS reference style

Journal article

Fisher, J.A.; Krapf, C.B.E.; Lang, S.C.; Nichols, G.J.; Payenberg, T.H.D. Sedimentology and architecture of the Douglas Creek terminal splay, Lake Eyre, central Australia. *Sedimentology* **2008**, *55*, 1915–1930.

Conference paper

Chum, O.; Philbin, J.; Zisserman, A. Near duplicate image detection: min-Hash and tf-idf weighting. In Proceedings of the 19th British Machine Vision Conference (BMVC 2008), Leeds, UK, 1–4 September 2008; pp. 812–815.

Book with editors

Shaw, P.A.; Bryant, R.G. Playas, pans and salt lakes. In *Arid Zone Geomorphology:*

Process, Form and Change in Drylands; Thomas, D.S.G., Ed.; John Wiley & Sons, Ltd.: Chichester, UK, 2011; pp. 373–401.

Book without editors

McKie, T. A Comparison of Modern Dryland Depositional Systems with the Rotliegend Group in the Netherlands. In *The Permian Rotliegend of The Netherlands*; SEPM Society for Sedimentary Geology: Darlington, UK, 2011; pp. 89–103.

Preprint

Ward, D.W.; Nelson, K.A. Finite Difference Time Domain (FDTD) Simulations of Electromagnetic Wave Propagation Using a Spreadsheet. *arXiv* **2004**, arXiv:physics/0402096. Available online: http://arxiv.org/abs/physics/0402096 (accessed on 13 October 2004).

Thesis

Mäckel, H. Capturing the Spectra of Silicon Solar Cells. Ph.D. Thesis, The Australian National University, Acton, Australia, 2004.

Patent

Sheem, S.K. Low-Cost Fiber Optic Pressure Sensor. U.S. Patent 6,738,537, 18 May 2004.

Company website

Proto Labs Ltd. Protolabs. Available online: https://uploads.protolabs.co.uk/es/PartUpload-MultiPart.aspx?LinkFrom=FC (accessed on 24 April 2017).

Software

Mathematica, version 5.1; software for technical computation; Wolfram Research: Champaign, IL, USA, 2004.

Data set

The Sadtler Standard Spectra: 300 MHz Proton NMR Standards; Bio-Rad, Sadtler Division: Philadelphia, PA, USA, 1994; No. 7640 (1-Chloropentane).

Newspaper

Squires, S. Falling Short on Nutrients. *The Washington Post*, 4 October 2005, p. H1.

Standard

Standard's Number; Standard's Title. Publisher: City, State, Country, Year.

Blog

Matthew, L. FCC Chair Willing to Consecrate XM-Sirius Union. Ars Technica (blog), 16 June 2008. Available online: http://arstechnica.com/news.ars/post/20080616-fcc-chair-willing-to-consecrate-xm-sirius-union.html (accessed on 23 May 2017).

Unpublished work

Unpublished materials intended for publication:

Author 1, A.B.; Author 2, C. Title of Unpublished Work (optional). Correspondence Affiliation, City, State, Country. year, *status (manuscript in preparation; to be submitted)*.

Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name year, phrase indicating stage of publication (submitted; accepted; in press)*.

Unpublished materials not intended for publication:

Author 1, A.B. (Affiliation, City, State, Country); Author 2, C. (Affiliation, City, State, Country). Phase describing the material, year. (phase: Personal communication; Private communication; Unpublished work; etc.)

Presentation

Zhang, Z.; Chen, H.; Zhong, J.; Chen, Y.; Lu, Y. ZnO nanotip-based QCM biosensors. Presented at the IEEE International Frequency Control Symposium and Exposition, Miami, FL, USA, 4–7 June 2006.

7.11.2. Chicago reference style

Journal article

Žilinské, Asta. 2010. Negative and positive effects of foreign direct investment. *Economics and Management* 15: 332–36.

Conference paper

Teplin, Linda A., Gary M. McClelland, Karen M. Abram, and Jason J. Washburn. 2005. Early Violent Death in Delinquent Youth: A Prospective Longitudinal Study. Paper presented at the Annual Meeting of the American Psychology-Law Society, La Jolla, CA, USA, March 1.

Book with editors

Gould, Glenn. 1984. Streisand as Schwarzkopf. In *The Glenn Gould Reade*. Edited by Tim Page. New York: Vintage, pp. 310–12.

Book without editors

Huang, Yongfu. 2011. *Determinants of Financial Development*. London: Palgrave Macmillan UK.

Preprint

Lein, Matthias. 2008. Characterization of Agostic Interactions in Theory and Computation. Preprint, submitted July 10. Available online: http://xxx.lanl.gov/abs/0807.1751 (accessed on 16 July 2017).

Thesis

Choi, Mihwa. 2008. Contesting Imaginaires in Death Rituals during the Northern Song Dynasty. Ph.D. thesis, University of Chicago, Chicago, IL, USA, May 1.

Patent

Kraay, Aart. 1984. Transparency on Foreign Direct Investment. U.S. Patent 3,5871,325, June 26.

Company website

Claessens, Stijn, Daniela Klingebiel, and Sergio L. Schmukler. 2001. FDI and Stock Market Development: Complements or Substitutes? Available online: http://www.iadb.org/WMSFiles/products/research/files/pubS-FDI-4.pdf (accessed on 23 December 2017).

Software

Sony. 2014. *Sony Vegas Trial* (version 13). Minato: Sony.

Data set

The Sadtler Standard Spectra: 300 MHz Proton NMR Standards. 1994. No. 7640 (1-Chloropentane). Philadelphia: Bio-Rad, Sadtler Division.

Newspaper

Weisberg, Michael. 2012. Cross-national studies in crime and justice. *New York Times*, March 3.

Blog

Lasar, Matthew. 2008. FCC Chair Willing to Consecrate XM-Sirius Union. *Ars Technica* (blog), June 16. Available online: http://arstechnica.com/news.ars/post/20080616-fcc-chair-willing-to-consecrate-xm-sirius-union.html (accessed on 23 May 2017).

Unpublished work

Williamson, Oliver E. 2017. The New Institutional Economics: Taking Stock; Looking Ahead. *Published Weekly*, forthcoming.

Presentation

Posthuma, Jonathan. 2015. The God of Material Things. Paper presented at Dordt College Kuyper Scholar's Honor Program, Sioux Center, Iowa, IA, USA, September 28.

Standard

Institute. Year. *Standard Title*. Standard Number. City: Publisher.

8. Publication Ethics

Research and publication ethics is a large topic, and a full discussion is beyond the

scope of this guide. For further information, we recommend consulting local sources such as university ethics committees or libraries or the Committee on Publication Ethics **(https://publicationethics.org**).

Here, are the main points to be aware of when writing and submitting papers:

Authorship: Include all and only authors that qualify for authorship. Avoid "gift authorship" for those that did not contribute, and avoid omitting someone who played a significant role in the work.

Add ethical approval: If your work required ethical approval, add the name of the committee that approved the work and the approval code in the Institutional Review Board Statement and Informed Consent Statement. Additionally, make sure that you have obtained permission to publish from any relevant third parties, such as funders, collaborators, or research subjects.

Plagiarism/copied text: It is considered unethical to present someone else's words or ideas as your own—this is plagiarism. In addition, large amounts of copied text can constitute a copyright infringement. Do not directly copy text from other sources unless it is clearly indicated as such using quotation marks and is correctly cited.

Cite sources appropriately: Related to plagiarism, make sure that citations are made appropriately. Ensure that you have cited all of the relevant work. At the same time, avoid citing work that is outside the scope of the paper. Where reviewers or editors suggest that you add extra citations, you may disagree provided that you can argue why they are not relevant. It is also not necessary to add extra citations to the journal you intend to submit to—this will not make your paper any more or less likely to be accepted for publication.

Ensure that all of your co-authors are aware of the ethical standards expected for academic publishing. Any infringement is considered by publishers as the responsibility of all authors.

9. Revision and Resubmission

This part briefly covers general advice about how to revise and resubmit your manuscript. You will receive notification by email of specific opportunities to revise and resubmit your paper. If you urgently need to submit a new version at some other time during the peer review process, please make a request to the editorial office via email, but it may not always be possible if the paper is with editors or reviewers.

During revision, authors will be asked to prepare point-to-point responses to the reviewers' comments, as well as a new cover letter to the Academic Editor summarizing the changes made and/or any authorship change thats should be highlighted.

Please use the "track changes" feature in Microsoft Word when making revisions. This makes it easier for editors and reviewers to see the changes that have been made. The "compare" function in Word can add tracked changes to the final version by comparing it with an earlier version.

During the proofreading stage, authors will find parts of the text highlighted together with comments that the editorial office would like you to check in the latest version of your manuscript. These could be to verify information that has been added or modified, to check the original meaning of a word/phrase/sentence where it is ambiguous, or to request additional information. Please pay close attention to these parts to ensure that the final published version is as you intended. Common requests are to ask to define abbreviations, to add the city and country of companies from which materials were sourced, to check the author names and affiliations, to check modifications made to the reference list, etc.

For papers written in LaTex, it is not necessary to highlight changes, but there is various document comparison software that can be used to see the differences between different versions of tex files. Check carefully for comments in the tex file, prefixed by %, where authors may need to give feedback.

Reviewer comments are made to improve your work and help to make it acceptable for journal publication. Authors should be able to modify their manuscript to accommodate most comments. Sometimes, however, authors feel that remarks made are not completely fair or misunderstand their work. In this case, you can write a response to the reviewers and editors explaining your point of view. In addition, if a reviewer suggests additional experiments that would take an unreasonably long time, you can also explain the situation. While the reviewers' comments are taken into serious consideration, it is the editor(s) (Editor-in-Chief/Guest Editor/assigned Editorial Board Member) handling the paper who make(s) the final acceptance decision.

If authors have difficulty understanding a request for revision, or re-uploading your document, get in touch with the assistant editor handling your paper via email. Make sure you quote the manuscript ID assigned to your paper in all correspondence.

APPENDIX 5: DIET COMPOSITIONS

Table S1: Composition of the 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

Table S2: Nutritional value of the HFHC diet.

Nutrient	Units	Actual
Dry Matter	g/kg	919.93
Metabolizable Energy	MJ/kg	15.86
Crude Protein	g/kg	151.27
AShreonine	g/kg	4.51
ASIsoluecine	g/kg	5.24
ASLysine	g/kg	6.54
ASMethionine	g/kg	4.86
ASryptophan	g/kg	1.30
ASstidine	g/kg	3.30
ASTSAA	g/kg	6.79
ASValine	g/kg	5.80
Fat	g/kg	250.46
Carbohydrate	g/kg	427.29
Fibre	g/kg	22.08
Ash	g/kg	26.31
Avl Phosphorus	g/kg	1.66
Calcium	g/kg	5.47
Total Phosphorus	g/kg	3.60

Table S3: The overall caloric contribution from fats, proteins, and carbohydrates in an HFHC diet

Carbohydrates	**219.92 kcal/g**
Fats	**323.82 kcal/g**
Proteins	**27.2 kcal/g**
Total	**570.94 kcal/g**

Table S4: The overall caloric contribution from fats, proteins, and carbohydrates in a standard diet.

Carbohydrates	**155.92 kcal/g**
Fats	**134.91 kcal/g**
Proteins	**27.2 kcal/g**
Total	**318.03 kcal/g**

Table S5: Composition of fats, proteins, and carbohydrates of the standard diet.

Fats	**15%**
Proteins	**30%**
Carbohydrates	**35%**
Other components	**20%**