

**Pregestational Prediabetes Induces Maternal Hypothalamic-Pituitary-Adrenal (HPA) Axis
Dysregulation and Results in Adverse Foetal Outcomes**

By

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PREFACE

Foetal programming, occurring during embryonic and foetal development, plays a critical role in shaping lifelong health. Maternal dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis during pregnancy, which can occur in cases of type 2 diabetes mellitus (T2DM) or maternal stress, has been identified as a contributing factor to the foetal programming and intrauterine growth restriction (IUGR). These changes *in utero* have been associated with a greater risk of developing obesity, hypertension, cardiovascular diseases and T2DM later in adulthood. However, prior to the onset of T2DM, there exists an asymptomatic state called pre-diabetes that is characterised by intermediate hyperglycaemia. Despite being asymptomatic, pre-diabetes has been linked to early complications akin to those seen in T2DM. Previous studies using a diet-induced animal model of prediabetes have shown that there is dysregulation of the HPA axis under non-stressed conditions during the prediabetic state. However, no research has been conducted to investigate the impact of pre-existing maternal prediabetes on the HPA axis and foetal outcomes in pregnancy. Using this diet-induced pre-diabetic animal model, investigations were conducted to explore the effects of pregestational prediabetes on maternal HPA axis function and its influence on offspring HPA axis development, along with assessments of glucose handling in offspring. The experimental work described in this dissertation was conducted at the University of Kwa-Zulu Natal, Westville Campus, Durban, South Africa. All work was conducted under the supervision of Dr P.S. Ngubane and co-supervised by Prof A. Khathi and Ms N.D. Xulu.

DECLARATION

I, Ms Mathuli Ngema declare.

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institutions for purposes of obtaining an academic qualification, whether by myself or any other party.
2. The dissertation is my own investing, and work from other researchers is rephrased and referenced, or if their exact words are used, they are quoted and referenced.
3. Dr P.S. Ngubane, Prof A. Khathi and Miss N.D. Xulu supervised the research done in this study.

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Co-Supervisor: Miss Nombuso D Xulu

PLAGIARISM DECLARATION

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

MASTER'S DEGREE IN MEDICAL SCIENCES 2024

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DEDICATION

This work is dedicated to myself and my mother, who was diagnosed with T2DM at 45 and likely had prediabetes at 34, suggesting a strong possibility that I am a prediabetic offspring.

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I would like to express my sincere gratitude to Dr Ngubane for his invaluable guidance and unwavering support throughout the duration of this project.

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To my mother and brother, thank you for your support.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ADA	American Diabetes Association
AREC	Animal Research Ethics Committee
BBB	Blood-brain barrier
BRU	Biomedical Research Unit
CRH	Corticotrophic releasing hormone
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
ELISA	Enzyme-linked immunosorbent assay
GADPH	Glyceridealdehyde-3-phosphate dehydrogenase
GC	Glucocorticoids
GND	Gestational day
GR	Glucocorticoid receptor

HbA1c	Glycated hemoglobin A1c
11 β -HSD2	11 β -hydroxysteroid dehydrogenase type-2
HFHC	High fat high carbohydrate
HPA	Hypothalamic–adrenal–pituitary
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IUGR	Intrauterine growth restriction
MR	Mineralocorticoid receptor
NCD	Non-communicable diseases
NPD	Non-prediabetes
OGTT	Oral glucose tolerance test
PD	Prediabetes
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SEM	Standard error of mean
RNA	Ribonucleic acid
T2DM	Type 2 diabetes mellitus
T1DM	Type 1 diabetes mellitus

UKZN	University of KwaZulu-Natal
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PUBLICATIONS

Published

Ngema M, Xulu N.D, Ngubane P.S and Khathi A. Pregestational Prediabetes Induces Maternal Hypothalamic–Pituitary–Adrenal (HPA) Axis Dysregulation and Results in Adverse Foetal Outcomes. International Journal of Molecular Sciences, 2024. **25**(10): p. 5431. <https://doi.org/10.3390/ijms25105431>. **See chapter 3**

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STUDY OUTLINE

This dissertation is presented in a manuscript format consisting of four chapters: chapter 1: Research proposal; chapter 2: prologue, abstract and manuscript 1; chapter 3: prologue, abstract and manuscript 2; chapter 4: synthesis and appendices. Chapter 1 comprises an introduction to the study, the basis of the study, aims, research questions, objectives and hypothesis. Chapter 2 of this dissertation presents a review manuscript format that is adopted from the literature review titled A Review of foetal development in pregnancies with maternal type 2 diabetes mellitus (T2DM)-associated hypothalamic-pituitary-adrenal (HPA) axis dysregulation: Possible links to pregestational prediabetes. This work is authored by Ms M Ngema, supervised by Dr. P.S. Ngubane and co-supervised by Prof A. Khathi and Ms N.D Xulu. Furthermore, this manuscript has been published in the Journal of Biomedicine and has been formatted according to the journal's guidelines for authors. Chapter 3 of this dissertation presents a second study written in the original manuscript format that sought to investigate the effects of pregestational prediabetes on maternal HPA axis function and postnatal offspring development. This work is authored by Ms M Ngema, supervised by Dr. P.S. Ngubane and co-supervised by Prof A. Khathi and Ms N.D Xulu. The manuscript is published in the International Journal of Molecular Sciences and has been formatted according to the journal's guidelines for authors. Chapter 4 constitutes the synthesis, conclusion and future recommendations of the study as well as the appendices.

ABSTRACT

Background

Foetal programming refers to the concept that maternal insults have the ability to disrupt the normal pattern of foetal development, permanently changing the body's structure, physiology and metabolism, thereby predisposing it to chronic diseases in later life. During pregnancy, maternal HPA (hypothalamic-pituitary-adrenal) axis activity increases due to placental corticotropin-releasing hormone (CRH), leading to higher glucocorticoid (GCs) levels, though within normal ranges. The maternal glucocorticoid influences foetal system development, yet the foetal HPA axis is sensitive to excess glucocorticoids, especially in cases of maternal stress, synthetic glucocorticoids or pregestational diabetes pregnancies. Excess glucocorticoids can irreversibly reprogram the foetal HPA axis, causing persistent changes such as increased bioavailability of GC in basal levels observed from juvenile to adulthood. Alterations such as the programmed HPA axis have been associated with increasing risks of metabolic disorders like hypertension, T2DM, obesity, cardiovascular diseases, and mental health disorders. Prediabetes, often a precursor to T2DM, is marked by asymptomatic hyperglycaemia and is linked to high-caloric diets. Research using high saturated fat, high refined carbohydrate diet-induced prediabetic models indicates that T2DM complications, including myocardial injury, renal dysfunction, hormonal dysregulation and dysregulation in the function of the HPA axis, begin during prediabetic stages. However, there is a lack of research in the existing body of literature that clarifies the influence of maternal prediabetes on the HPA axis during pregnancy and its effects on foetal outcomes. Hence, the present study aimed to investigate the effects of pregestational prediabetes on maternal HPA axis function and postnatal offspring development.

Methods

Female Sprague Dawley rats (n=12) were randomly assigned to one of the two groups (n=6, per group): pre-diabetic (PD) and non-pre-diabetic (NPD). Prediabetes was confirmed through fasting blood glucose and glucose tolerance tests. After PD induction, mating occurred, and pregnancy was confirmed. Post-gestation, 36 male pups from PD and NPD groups were collected. Blood from dams was collected on the weaning day to measure insulin, glycated haemoglobin (HbA1c), and the homeostatic model assessment for insulin resistance, insulin sensitivity and beta cell function as well as the adrenocorticotrophic hormone (ACTH) and corticosterone concentrations. In addition, the pups were divided into three independent experimental weeks, week 3, week 6 and week 16, with 6 pups in each group assigned to either the NPD or PD on the day of weaning. Pups were evaluated at weeks 3, 6, and 16, with body weight, fasting glucose, and glucose tolerance tests conducted. Terminal investigations on each experimental week included measuring plasma ACTH, corticosterone, insulin

via ELISA and gene expressions for glucocorticoid and mineralocorticoid receptors, in pups' hippocampus via polymerase chain reaction (PCR).

Results and Discussion

The high-fat, high-carbohydrate diet caused moderate hyperglycaemia, elevated plasma insulin, HbA1c, and insulin resistance in the PD group. Pregnant PD rats showed higher ACTH and corticosterone levels, indicating HPA axis dysregulation when compared to the NPD group. Pups from the PD group had elevated ACTH and corticosterone, altered GR and MR gene expressions, and increased adrenal gland weights compared to the NPD group. These findings suggest constant HPA axis activation without external stress, indicating alterations at the hypothalamus, pituitary, or adrenal gland levels, which may have been due to exposure to excess maternal corticosterone *in utero*. PD pups also had lower body weight, glucose intolerance, and insulin resistance, suggesting that maternal corticosterone affected foetal growth and glucose metabolism pathways when compared to the NPD group.

Conclusion

The findings suggest that pregestational prediabetes is associated with dysregulated HPA axis function, and it is positively associated with pregestational diabetes and maternal stress pregnancy complications. The foetal adverse outcomes such as HPA axis hyperactivity, reduced growth development and glucose handling disturbances relative to foetal programming might also begin in prediabetic pregnancy.

CHAPTER 1: INTRODUCTION

1. Background

Foetal programming is the permanent response of an organism to a stimulus or insult during a critical period of development [1]. In simple terms, this definition suggests that when a fetus or neonate is exposed to an unusual environment during a rapid phase of growth, the resulting adaptive responses may become permanently fixed and irreversible maladaptation may result in detriment to organ function or longevity, increasing risks to chronic diseases in adulthood [2, 3]. Organs that develop early during foetal life, such as the heart, liver, and brain, are most susceptible to foetal programming [4-6]. During early stages of gestation, when foetal systems have not yet developed, the fetus depends on maternal glucocorticoids (GC), a precursor of the hypothalamic-pituitary-adrenal (HPA) axis, for development and organ formation, including the brain, notably foetal HPA axis [7, 8]. Maternal HPA axis activity increases during pregnancy influenced by a positive feedback loop where maternal glucocorticoid stimulates placental corticotropin-releasing hormone (CRH) synthesis, ultimately resulting in higher glucocorticoid levels however, maintained throughout pregnancy [9, 10].

On the other hand, excess maternal GC, known as cortisol in humans and corticosterone in rodents, has detrimental effects on the developing brain and on the adult brain's neurological function [11, 12]. Obesity, type 2 diabetes mellitus (T2DM) and insulin resistance are associated with secretory increases and metabolic changes in levels of glucocorticoid, as well as tissue-specific reactions to glucocorticoid [13]. A study showed that Individuals with T2DM have sleHPA hyperactivity with elevated basal plasma cortisol levels associated with hypertension [14]. Approximately 87.5% of pregnancies complicated by diabetes are estimated to be due to gestational diabetes, with 7.5% being due to type 1 diabetes mellitus (T2DM) and the remaining 5% being due to T2DM [15]. As a result, changes in cortisol levels during pregestational diabetes remain sparse [15]. Studies indicate that increased maternal cortisol and corticosterone levels, associated with diabetic pregnancy, poor diet, and stress, correlate with insulin resistance and can readily pass through the placenta to the fetus [16-22]. While macrosomia is a common outcome, pregestational diabetes can also lead to intrauterine growth restriction (IUGR) or small for gestational age [23, 24]. One of the mechanisms proposed for IUGR involves maternal microangiopathy—damage to small blood vessels—which is associated with longstanding diabetes [25, 26]. Microangiopathy, hyperglycemia and insulin resistance can impair the placenta's ability to supply adequate nutrients and oxygen to the fetus known as placental insufficiency, leading to growth restriction, which studies connect to higher chances of metabolic disorders later in life, such as T2DM [25-30]. However, the exact mechanism by which maternal diabetes is associated with small gestational age/ IUGR is not completely understood, as it is a multifaceted disease [31]. Research on maternal stress, which shares similarities with Type 2 Diabetes Mellitus, links elevated maternal cortisol and heightened HPA axis activity to adverse effects on foetal development,

particularly impacting the foetal HPA axis and often resulting in small-for-gestational-age or growth-restricted offspring [32-36].

Youth onset of T2DM continues to increase worldwide, and pregnancy in women with T2DM is a substantial clinical problem [37]. To date, reports of the epidemiology and natural history of secondary complications, specifically HPA axis with type 2 diabetes in pregnancy, have been scarce. Yet, we must begin to understand the extent of the coming challenge, especially with the recently found increased prevalence of prediabetes, an early-onset T2DM precursor [38]. T2DM is often preceded by prediabetes, a state of intermediate hyperglycemia with glycaemic indices above normal but below the T2DM diagnosis threshold [39]. However, many individuals progress from the pre-diabetic stage to overt T2DM, experiencing associated complications unknowingly due to the often-asymptomatic nature of early-stage T2DM [40, 41]. In 2019, the International Diabetes Federation estimated the worldwide prevalence of prediabetes to be 373.9 million and approximately 453.8 million people will have prediabetes by 2030 [42, 43]. Studies have shown that the onset of complications associated with T2DM, such as myocardial injury, renal dysfunction, hormonal dysregulation, increased intestinal permeability and dysregulation in the function of the HPA axis, begin during prediabetic [44-49]. This is particularly concerning as pre-diabetes is frequently diagnosed between the ages of 25 and 45, coinciding with the reproductive years for many women [50-52]. Therefore, this prompts the question of whether prediabetes in pregnancy could have an impact on the maternal HPA axis and affect the developing foetal brain. Hence, this study sought to investigate the effects of pregestational prediabetes on maternal HPA axis function and its effects on postnatal offspring development.

Bases of the study

Studies show that pregestational diabetes may impact foetal HPA axis development and contribute to growth restrictions, posing greater risks for metabolic disorders later in life [53-56]. Therefore, it is imperative to investigate the implications of pre-existing prediabetic states during pregnancy on maternal HPA axis function and subsequent foetal HPA axis development and function. Understanding these dynamics may shed light on whether such changes can manifest prior to overt T2DM. By discerning the effects of maternal HPA axis function in prediabetic pregnancies on foetal outcomes and HPA axis development, we may raise awareness about the risks associated with prediabetic pregnancies and implement strategies to prevent or manage processes that could lead to similar complications observed in T2DM pregnancies. This study will use a previous protocol of a high-fat, high-carbohydrate and 15 % fructose diet to induce intermediate hyperglycaemia in rats, inducing pre-diabetes prior to pregnancy. Consequently, this investigation will be conducted in response to the lack of studies addressing the effects of pregestational pre-diabetes on HPA axis function and its influence on offspring HPA axis development. The selected markers for investigation in this study were based on existing

literature and their involvement in maternal-foetal HPA axis interactions. The study will consist of three phases: A, B & C. Phase A will be the induction of prediabetes in female Sprague Dawley rats. In Phase B, the prediabetic rats will be mated for pregnancy and give birth. In Phase C, the male pups will be collected and assigned to 3 developmental milestones (3, 6, and 16 weeks). The 3 developmental stages of weeks 3, 6 and 16 are known as juvenile, adolescence and emerging adulthood compared to humans.

2. Aim

- To investigate the effects of pregestational prediabetes on maternal HPA axis function and its effects on postnatal offspring development

3. Research Questions

- 1) What are the effects of diet-induced prediabetes on HPA axis function in pregnancy?
- 2) Will the offspring born from dams with prediabetes show changes in HPA axis function?
- 3) What impact will maternal prediabetes have on foetal development?

4. Objectives

- To assess changes in glucose homeostasis in the dams by measuring fasting blood glucose, the oral glucose tolerance test and glycated haemoglobin concentration in the blood to diagnose prediabetes.
- To measure ACTH and corticosterone concentration in the dams to determine HPA axis function.
- To measure the body weights of the pups born from non-prediabetic and prediabetic dams during the developmental stages
- To assess the changes in glucose homeostasis in the pups born from non-prediabetic and prediabetic dams by measuring fasting blood glucose, the oral glucose tolerance test and glycated haemoglobin
- To measure ACTH and corticosterone concentrations in pups born from non-prediabetic and prediabetic dams during the developmental stages
- To determine the expression of hippocampal GR and MR in pups born from non-prediabetic and prediabetic dams during the developmental stages to evaluate negative feedback regulation in non-stressed conditions.

5. Hypothesis

- The prolonged uncontrolled prediabetes will cause changes in the maternal HPA axis in pregnancy and impair foetal development.

6. Laboratory methods

- The detailed laboratory methods utilised in this study are comprehensively described in the corresponding manuscript included in the dissertation.

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CHAPTER 2: LITERATURE REVIEW

PROLOGUE

Foetal programming may predispose people to certain adult diseases and is an important matter of concern in the current age as most young adults are developing type 2 diabetes mellitus (T2DM), cardiovascular diseases and other metabolic diseases. Maternal diabetic pregnancies and maternal stress have been proposed to contribute to growth restriction or small for gestational age, which studies connect to higher chances of metabolic disorders later in life, such as T2DM, known as foetal programming. Prenatal glucocorticoid exposure and maternal diabetes alter the development of the hypothalamic region, including foetal HPA function. These programmed changes in HPA function increase the risks of metabolic disorders in adults. However, the onset of T2DM is preceded by a prediabetic state characterised by intermediate hyperglycaemia. T2DM complications, including dysregulation in the HPA axis and risk factors for preeclampsia, among others, have been shown to begin in prediabetic animal models. Research has been done on maternal pregestational diabetes and maternal stress-associated changes in foetal outcomes. However, the potential link between maternal pregestational diabetes and associated changes in pregestational prediabetes in HPA axis function and impact on the foetus has not yet been explored. Therefore, chapter 2 was conducted to review the existing literature on T2DM in pregnancy links to foetal programming with changes in their HPA axis, as well as possible links to pregestational prediabetes.

The literature review was converted into a review manuscript titled “**A review of foetal development in pregnancies with maternal type 2 diabetes mellitus (T2DM)-associated hypothalamic-pituitary-adrenal (HPA) axis dysregulation: Possible links to pregestational prediabetes**” and is authored by M. Ngema, N.D. Xulu, P.S. Ngubane and A Khathi.

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A review of foetal development in pregnancies with maternal type 2 diabetes mellitus (T2DM)-associated hypothalamic-pituitary-adrenal (HPA) axis dysregulation: Possible links to pregestational prediabetes.

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Abstract

Research has identified foetal risk factors for adult diseases, forming the basis for the Developmental Origins of Health and Disease (DOHaD) hypothesis. DOHaD suggests that maternal insults during pregnancy cause structural and functional changes in foetal organs, increasing the risk of chronic diseases like type 2 diabetes mellitus (T2DM) in adulthood. Few studies show that Individuals with T2DM have HPA hyperactivity with elevated basal plasma cortisol levels, and in diabetic pregnancy is associated with hypertension. However, the mechanisms by which maternal diabetes cortisol levels impact the fetus are few. However, many pregestational diabetes studies have associated alterations in foetal growth, which are often associated with altered HPA axis development in maternal stress pregnancy. The programmed HPA axis is linked to increased risks of hypertension, cardiovascular diseases and mental disorders in adulthood. With the global rise in T2DM, particularly among young adults of reproductive age, it is crucial to prevent its onset. T2DM is often preceded by a prediabetic state, a condition that does not show any symptoms, causing many individuals to unknowingly progress to T2DM. Studying prediabetes is essential as it is a reversible stage that may help prevent T2DM-related pregnancy complications. Existing literature focuses on HPA axis dysregulation in T2DM pregnancies and its link to foetal programming. However, the effects of prediabetes on HPA axis function, specifically glucocorticoid in pregnancy and foetal outcomes, are not well understood. This review consolidates research on T2DM during pregnancy, its impact on foetal programming via the HPA axis and possible links with pregestational prediabetes.

Keywords: type 2 diabetes mellitus; prediabetes; pregnancy; Maternal HPA axis; foetal HPA axis; programming; foetal development; placenta; glucocorticoids; metabolic diseases.

1. Introduction

Foetal programming occurs during embryonic and foetal development, a vital stage during which tissues and organs are formed [1-3]. Many environmental cues, such as excess glucocorticoid exposure in utero, can contribute to various changes that include changes in molecular biological functions, such as receptor cell density or sensitivity, as well as alterations in metabolism or responses to physiological stressors [4, 5]. The global rise in T2DM among young adults and increasing frequency in women of gestational age is concerning due to the potential for these effects can be passed on to future generations via either the maternal or paternal lineage [6, 7]. Type 2 Diabetes mellitus (T2DM) exerts metabolic stress on cells, and it provokes a chronic increase in the long-term activity of the hypothalamus-pituitary–adrenocortical (HPA) axis associated with high cortisol and ACTH levels, perhaps thereby contributing to insulin resistance and hypertension [8, 9]. Research on diabetes in pregnancy has shown elevated maternal cortisol levels, which are linked to insulin resistance [10-13]. However, changes in cortisol levels during diabetic pregnancy remain unclear as most studies on cortisol levels and the relationship with foetal changes in pregnancy focus on postpartum children [13, 14]. Research indicates that maternal high-fat diet and maternal stress in animals have been reported to lead to increased maternal levels of corticosterone, which can readily pass through the placenta to the fetus [9, 15].

Glucocorticoids (GC), such as cortisol in humans and corticosterone in rodents, are well known for their role in glucose homeostasis in adult life [16]. The HPA axis regulates GC production through a feedback loop involving glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) [16]. During pregnancy, this feedback mechanism ensures that GC levels are kept within a range that supports pregnancy while avoiding the adverse effects of hypercortisolism [17]. GC in pregnancy has also been shown to be essential in foetal maturation [5, 18]. However, foetal GC load is usually regulated by 11 β -hydroxysteroid dehydrogenase type-2 (11 β -HSD2), a placental enzyme that inactivates GCs [19, 20]. Increased maternal GC levels observed in maternal stress and attenuating 11 β -HSD2 expression—potentially increase foetal exposure to GCs, slowing foetal growth and altering the gestational period [21-23]. Although the weights of infants of diabetic mothers generally are skewed into the upper range, intrauterine growth restriction (IUGR), commonly diagnosed as low birth weight, occurs with concerning frequency in diabetic women, especially those with underlying hypertension, uncontrolled blood glucose levels and vascular diseases [24-29]. Excessive glucocorticoid exposure in utero goes as far as altering the set-point and development of the offspring's HPA axis that alternately reprograms the HPA axis, thus compromising its function after birth [30-32]. In addition, excess maternal or foetal corticosterone causes the downregulation of foetal GR and MR and impairs the feedback regulation of the HPA axis in both infancy and adulthood [33, 34]. Even though studies on pregestational diabetes are limited Cross-sectional research has also indicated a connection between lower birth weights, elevated cortisol levels, catch-up growth in the neonatal period and adult obesity, which may be an

indication of unfavourable adaptive responses till birth [35, 36]. The association between low birth weight was first reported as a several-fold increase in the incidence of glucose intolerance and T2DM in adult men compared with those born with normal birth weight [37, 38]. Furthermore, studies demonstrate that low birth weight has been associated with high risks of other non-communicable diseases (NCDs) such as hypertension, cardiovascular diseases and mental disorders in adulthood, correlating with the concept of Developmental Origins of Health and Disease (DOHaD) hypothesis [39, 40]. The DOHaD, which emerged as a broadening of the “Barker hypothesis” and was named for epidemiologist David Barker, explains the scenario in which in-utero maternal insults cause structural and functional alterations in foetal organs, extending postnatal life and increasing susceptibility to chronic disease in adulthood [41, 42].

Prediabetes is characterised by impaired glucose metabolism with glucose concentration above the optimal value but still below the diagnostic levels for T2DM [43]. In 2019, the prevalence of prediabetes was 373.9 million with 15.3 % undiagnosed and it is expected to increase to 453.8 million by 2030 parallel with increasing T2DM prevalence [44, 45]. Studies show that prediabetes precedes T2DM and have been suggested that the onset of complications associated with T2DM begins during the prediabetic state including myocardial injury, renal dysfunction, hormonal dysfunction and dysregulation in HPA axis function among others [46-49]. Literature primarily reports alterations that pregestational diabetes with complex commodities may play a role in foetal programming, while the changes in maternal pregestational prediabetes influence HPA axis function and foetal outcomes have not yet been explored. Therefore, this review consolidates research on T2DM during pregnancy, its impact on foetal programming via the HPA axis, and possible links with pregestational prediabetes. The following section describes foetal programming, theories, and associated diseases.

2. Foetal programming

According to Barker, the early life environment affects foetal growth and adds to disease susceptibility [50, 51]. The developing baby adapts to an insult in utero, leading to long-term changes in form, physiology and metabolism that are beneficial for survival [41, 42]. Gluckman discovered that mismatches between early and later life circumstances might cause maladaptive alterations that raise the risk of a variety of cardiometabolic and psychiatric disorders as well as vulnerability factors pertaining to the phenomena known as foetal programming [52, 53]. Foetal programming occurs when the normal pattern of foetal development is disrupted by an abnormal stimulus or ‘insult’ applied at a critical point in in-utero development [54-56]. According to the evidence for the Developmental Origins of Health and Disease (DOHaD) hypothesis, the antenatal period is a particularly vulnerable period of development in which exposure to adverse environments, such as glucocorticoid exposure, can have

long-term or permanent effects on the offspring's health trajectory [57, 58]. Studies have shown that the maternal HPA axis is crucial during foetal development [59, 60]. However, maternal dysregulation in the HPA axis during pregnancy or before pregnancy has been shown to exert its programming effect, especially in the brain, notably the HPA axis [61-63]. The following section details the physiological role of the maternal HPA axis in pregnancy and its role in foetal development.

3. Role of the maternal hypothalamic-pituitary-adrenal (HPA) axis in pregnancy

The hypothalamic-pituitary-adrenal axis is a complex system of neuroendocrine pathways and feedback loops that function to maintain physiological homeostasis through a synthesis of glucocorticoids (GCs). Active GCs known as cortisol in humans and corticosterone in rodents [64]. The maternal HPA axis adapts during pregnancy and regulates stress-related deleterious effects on the mother and offspring [65, 66]. A non-diabetic pregnancy is a state of hyperactivity of the HPA axis and of hypercortisolism, especially towards late gestation [67-69]. The increased cortisol in late gestation is regulated by the placenta, an important source of secreting corticotropin-releasing hormone (CRH), which further enters the maternal pituitary gland via the hypophyseal portal circulation and enhances adrenocorticotropin (ACTH) synthesis and secretion into the peripheral circulation (Figure 1) [70, 71]. ACTH increases glucocorticoid synthesis and secretion through the adrenal cortex in the kidney into the bloodstream in the course of pregnancy [72, 73]. GC levels influence the hypothalamic CRH in a negative feedback loop, while the placental CRH is strongly stimulated by GC in a mechanism of a positive feedback loop [74, 75]. In addition, studies show that high GC in pregnancy also play a primary role in regulating fuel homeostasis. After the uptake of free cortisol from the circulation, cortisol increases the availability of potential fuel substrates by mobilisation of glucose, free fatty acids and amino acids through enhancing hepatic gluconeogenesis and glycogenolysis [76, 77]. Hence, research shows that GC contributes to insulin resistance, which is necessary to ensure that an adequate amount of glucose reaches the fetus for its growth and development [78].

Research shows that there are two types of corticosteroid receptors in the brain namely glucocorticoid (GR) and mineralocorticoid (MR) receptors, involved in the feedback regulation of the HPA axis [17, 79]. The proper functioning of these receptors ensures that glucocorticoid levels remain within a range that supports pregnancy without causing undue stress to the mother or the fetus [80]. In the brain, MR binds to endogenous glucocorticoid with a higher affinity than GR and at basal concentrations of cortisol and corticosterone, MR is occupied while the GR remains largely unoccupied [81, 82]. During times of elevated plasma glucocorticoid levels, such as during stress, increased occupation of GR helps reduce the release of CRH and ACTH, ultimately lowering glucocorticoid production and thereby regulating the function of the HPA axis. [83, 84]. In pregnancy, MR mRNA expression in the hippocampus is unaltered and GR gene expression is only modestly increased which promotes negative feedback, maintaining HPA axis activity (Figure 1) hence, the diurnal secretion of cortisol is maintained

throughout pregnancy [18, 85]. Furthermore, studies show that late pregnancy (the last week in the rat) is associated with a substantial reduction in HPA axis responses to both psychological and physical stressors in several species [85-87]. This adaptation is considered to buffer the impact of stress by reducing foetal exposure to maternal glucocorticoid, thus minimising the risk of detrimental glucocorticoid programming [18, 88].

Furthermore, studies show that glucocorticoids are lipophilic and can readily pass through the placental barrier by simple diffusion [89, 90]. While glucocorticoid's most well-known function is to stimulate differentiation and functional development of the lungs, glucocorticoids also play crucial roles in the development of several other organ systems including the HPA axis [59, 60]. However, 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) acts as a barrier enzyme to control the passage of glucocorticoid from the mother to the fetus and protects the fetus from the much higher maternal levels of glucocorticoid [91, 92]. This enzyme is found on both placental sides of the syncytiotrophoblast [92, 93]. It metabolises active glucocorticoids (cortisol in humans and corticosterone in rats) into inactive glucocorticoids, thereby shielding the fetus against excessive glucocorticoid exposure from the mother shown in (Figure 1) [93, 94]. Although the placenta metabolises a significant proportion of cortisol (80-90% during gestation), excess cortisol may reach the fetus and the 'barrier' can be further weakened by maternal high maternal glucocorticoid or placental dysfunction, which is commonly caused by increased oxidative stress resulting in hypoxia, allowing increased transfer of glucocorticoids from mother to fetus [18, 62, 95]. The following section describes T2DM, prevalences, pre-existing dysregulation in HPA axis function maternal diabetes associated with foetal programming of the HPA axis and associated diseases in adulthood.

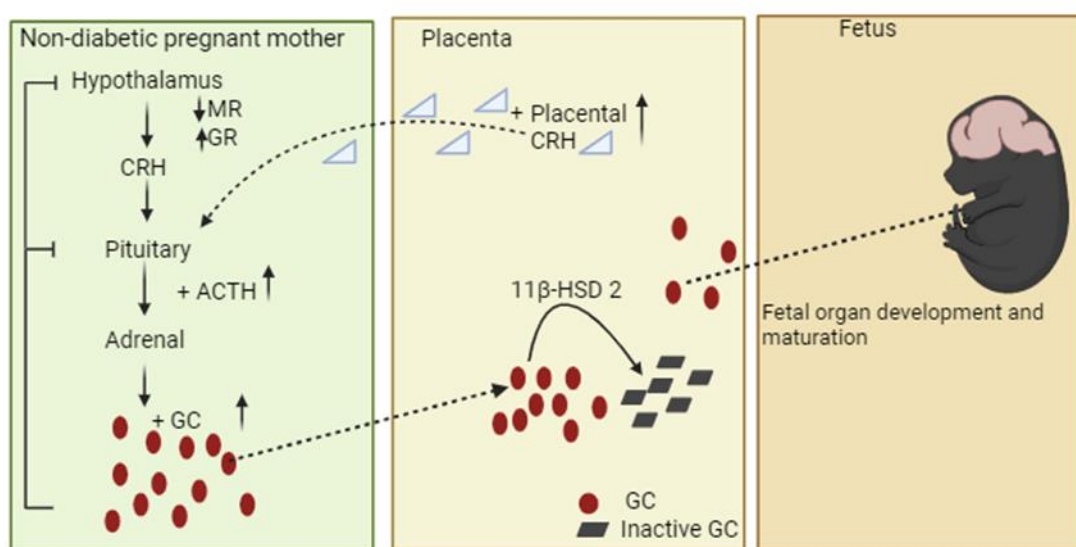


Figure 1: A schematic presentation of maternal HPA axis and GC signalling between mother, placenta

and fetus. Glucocorticoids tightly control HPA axis activity through glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in the pituitary and hypothalamus to inhibit CRH release, ACTH and its own secretion [91, 96]. In pregnancy, the placenta secretes large quantities of CRH into the maternal bloodstream as the pregnancy progresses which promotes the production of GC [18, 85]. Increased Placental CRH secretion and GC also increase GR, promoting negative feedback and therefore, maintaining the HPA axis activity in pregnancy. Nevertheless, the fetus is shielded from excess maternal GC exposure by the increased activity of 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2). The limited amount of GC that crosses the placenta into the foetal compartment is crucial for foetal organ development, including the brain notably the foetal HPA axis and lung maturation.

4. Changes in the HPA axis in T2DM pregnancies associated with foetal programming.

T2DM accounts for 90-95% of all diagnosed diabetes mellitus (DM) cases and is regarded as a complicated and multifaceted illness caused by a mix of genetic and environmental risk factors [97]. T2DM is characterised by insulin resistance and inadequate β -cell responsiveness to glucose stimulation [98]. Globally, the International Diabetes Federation (IDF) estimated that by 2045, 693 million are expected to have T2DM aged 18-99 years [99, 100]. The swift urbanisation marked by the uptake of unhealthy, calorie-rich diets and sedentary lifestyles has played a role in the progressively rising prevalence of T2DM, particularly among females compared to males and the prevalence rises with age [101, 102]. Studies reported that T2DM patients have elevated basal ACTH levels and high cortisol level [10, 103, 104]. In this regard, the activity of the HPA axis is enhanced in diabetic patients [8, 104]. A study estimated that 87.5% of diabetes-affected pregnancies are attributed to gestational diabetes, 7.5% to type 1 diabetes mellitus (T1DM), and the remaining 5% to type 2 diabetes mellitus (T2DM) [105]. Nonetheless, a study associated with diabetes in pregnancy reported that there are increased maternal levels of cortisol, which correlates with insulin resistance [10-12, 106]. In addition, studies on a maternal unhealthy diet and maternal stress have been reported to lead to increased maternal levels of corticosterone, which can readily pass through the placenta to the fetus [15]. However, changes in cortisol effects during pregestational diabetes remain limited. However, studies have reviewed that dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is hypothesized to serve as a primary mechanism through which life course stress influences maternal-foetal health in the prenatal period and subsequent offspring development [33, 107, 108]. Research indicates that pregnant women with T2DM may have comparable patterns to those observed in maternal obesity, depressed and stressed pregnant women [109, 110]. These patterns involve prolonged activation and dysregulated function of the HPA axis with elevated glucocorticoid levels [109-111]. A study discovered a correlation between maternal

diabetes accompanied by elevated cortisol levels and alterations in the development of the brain's cortical neuroendocrine system by reducing the number of hippocampal neurons [112].

The pathophysiology of foetal growth in the context of T2DM pregnancy is intricate and multifaceted [113, 114]. However, the complications of diabetes affecting the mother and fetus are well-known [115-117]. Although macrosomia is frequently observed in T2DM, pregestational diabetes can also result in intrauterine growth restriction (IUGR) or small-for-gestational-age outcomes [24, 25]. The term intrauterine growth restriction (IUGR) refers to neonates whose birth weight and length fall below the 10th percentile for their gestational age [118, 119]. IUGR is a common antenatal diagnosis; nevertheless, some of these fetuses, particularly those who were not checked during pregnancy, may be discovered only after birth [120, 121]. The primary diagnostic criteria of IUGR include low birth weights (LWs), a surrogate marker of an adverse intrauterine environment and subsequent cardio-metabolic disease and mental health problems [122, 123]. Approximately 20% of pregnant women with diabetes experience gestational hypertension and/or preeclampsia [124]. The individuals most susceptible to these conditions are those who have pre-existing microvascular complications such as microangiopathy, hypertension, or hyperglycemic, which also contribute to endothelial dysfunction-which is associated with longstanding diabetes [24-29, 124, 125]. These complications have been shown to induce a reduction in trophoblast proliferation, delaying placental growth and development, particularly in the first few weeks of gestation [126]. This mechanism suggests a dysregulation of trophoblast invasion by the diabetic environment, leading to decreased placental perfusion and reduced nutrient supply, known as placental dysfunction [127].

Studies show that placental dysfunction is associated with relatively low placental 11 β -HSD2 activity, therefore increasing active maternal GC transfer to the fetus's bloodstream [128, 129]. In addition, studies show that foetal excess maternal GC exposure relative to early increases in the foetal GC concentration also triggers tissue differentiation and reduces accretion in the fetus [5]. As a result, the overall rate of maturation and growth declines as GC concentrations rise in the fetus toward term and in response to adverse intrauterine conditions, resulting in growth-retarded fetuses recognised as IUGR [130, 131]. Previous studies show that glucocorticoid excess exposure in maternal stress during foetal development programs has specific effects on the brain, notably the HPA axis [132, 133]. This exposure changes its development, sensitivity, and activity in utero, relatively stressing its growth as the HPA axis begins to develop during the embryonic stage and continues to mature throughout pregnancy [33, 134]. As a result, studies show that prenatal glucocorticoid exposure permanently increases basal plasma corticosterone levels in adult rats [135, 136]. This was because the density of both types of corticosteroid receptors, GRs and MRs, are permanently reduced in the hippocampus, changes anticipated to attenuate HPA axis feedback sensitivity [135-137].

On the other hand, the brain is heavily reliant on glucose for energy and mammals have redundant systems for controlling glucose production [138]. As a result, it is possible that altered hypothalamus function may promote dysregulation of peripheral glucose metabolism, leading to insulin resistance or T2DM in adulthood [139]. Research conducted by Hales et al. revealed a several-fold higher incidence of glucose intolerance and type 2 diabetes in adult men who were born with low birth weight as opposed to those who were born with normal birth weight, which established the first link between low birth weight and the development of T2DM [37]. A study in rats showed that the smallest fetuses with the largest placentas had lower placental 11 β -HSD2 activity and were projected to have the highest adult blood pressures [19, 140]. Heightened HPA axis activity, particularly with increased ACTH and high plasma glucocorticoid levels, is seen in children and adults who were born underweight [141, 142]. In addition, previous research has indicated that infants born with lower birth weights undergo catch-up growth within the initial two years of life [143, 144]. This process is seen as a means to offset their genetically predetermined growth patterns [144]. Catch-up growth is also observed in other aspects of growth, such as changes in body weight and body composition [145]. As per the theory of DOHaD, the rapid catch-up growth experienced by low birthweight infants in their early years is associated with various metabolic conditions such as obesity, hypertension, cardiovascular diseases, metabolic syndrome and endothelial dysfunction later in adulthood [146, 147]. Men were found to be more likely to develop cardiovascular disorders than women born with IUGR due to hormonal differences, as men had lower levels of oestrogen, which has protective effects on the circulatory system and may contribute to women's decreased risk of cardiovascular disease [148, 149]. A prior study discovered that a combination of placental weight and birth weight predicts blood pressure and hypertension risk in men and women around the age of 50 [141, 150]. People who were babies with large placentas had the highest blood pressure and a higher risk of hypertension [151]. Both The Barker and DOHaD hypotheses support these theories.

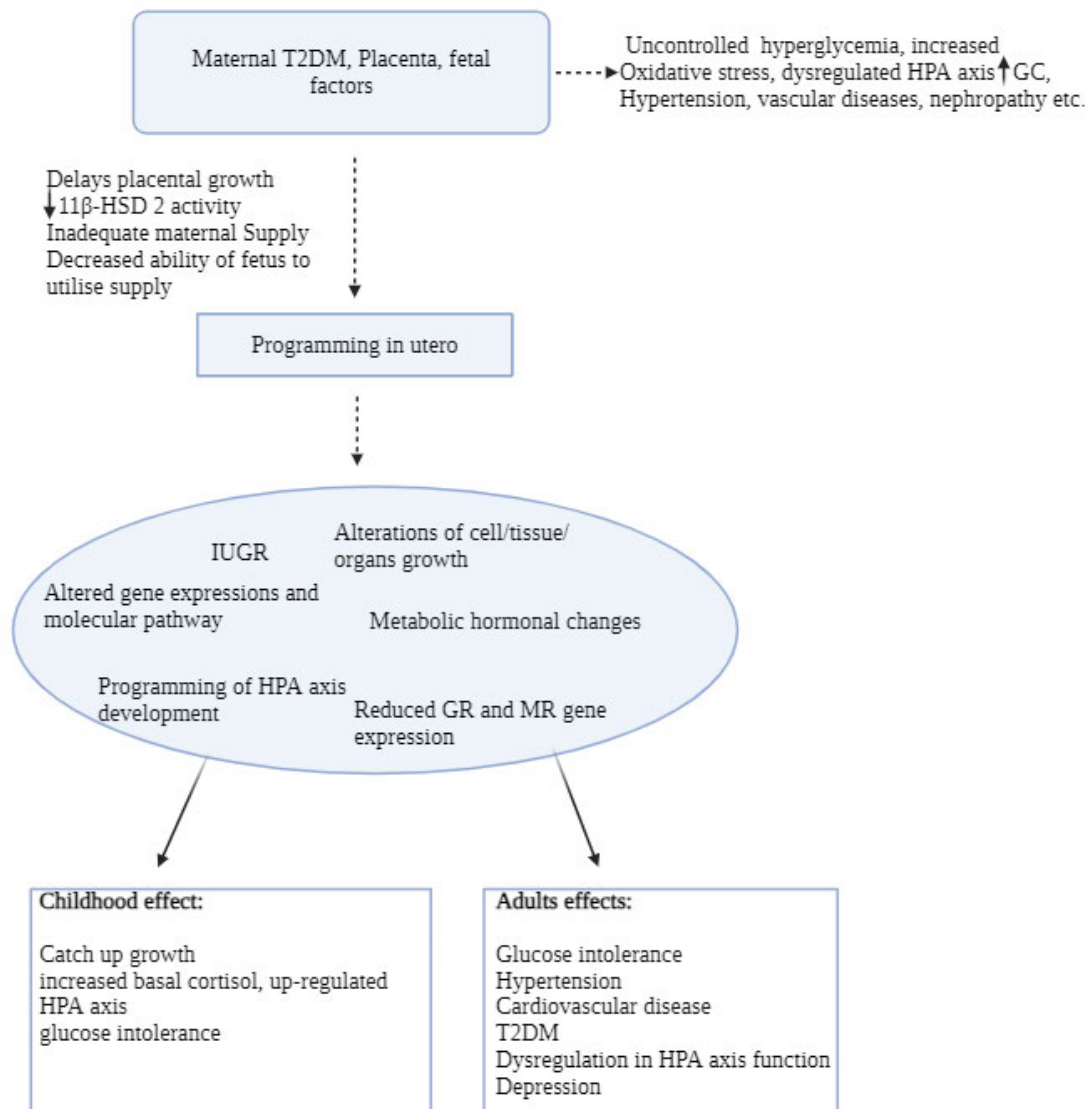


Figure 2: The schematic diagram presents the summary of maternal T2DM pregnancy complications leading to foetal consequences *in utero* that persist to adulthood.

5. Prediabetes

Prediabetes is a condition in which blood glucose levels are abnormally high but do not meet the diagnostic criteria for type 2 diabetes mellitus (T2DM) [152]. Prediabetes can be identified by at least two of these characteristics: impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or high glycated haemoglobin A1c (HbA1c) [153]. IFG patients have significant hepatic insulin resistance with normal skeletal muscle values and poor glucose suppression, which causes hyperglycemia during

fasting due to impaired insulin secretion or reduced sensitivity of β -cells to glucose stimulation [154]. IGT mainly impacts muscle insulin resistance, with minimal effects on liver insulin sensitivity. The reduced glucose absorption observed in individuals with impaired glucose tolerance (IGT) contributes to postprandial hyperglycemia [155, 156]. This is primarily due to pancreatic β -cell dysfunction, resulting in inadequate secretion of insulin to counteract elevated glucose levels and stimulate a response in insulin-targeted peripheral tissues [157, 158]. Lastly, IFG Individuals have a poor early insulin response during the oral glucose tolerance test (OGTT) but improve insulin secretion during the second phase, which is one of the reasons why prediabetes is frequently undetectable [159]. As a result, the American Diabetes Association (ADA) recommendations were changed in 2003 to identify patients with prediabetes based on the following values: Fasting plasma glucose levels range from 5.6 mmol/L to 6.9 mmol/L, whereas IGT values recorded after OGTT range from 7.8 mmol/L to 11.0 mmol/L [160, 161]. Glycated haemoglobin (HbA1c) levels between 5.7% and 6.4% are used as an additional diagnostic criterion for prediabetes [162, 163]. Prior to the diagnosis of pre-diabetes, there is a presence of insulin resistance and malfunctioning of pancreatic β -cells [157, 158]. Studies show that a diet high in saturated fats, high in carbohydrates or high in fructose contributes to the development of intermediate hyperglycemia [164, 165]. In addition, studies also show that these caloric foods lead to elevated triglycerides and increased release of free fatty acid (FFA) from adipocytes into circulation, which is accompanied by decreased FFA uptake by adipocytes in insulin-dependent tissues promoting insulin resistance and dyslipidemia [166, 167]. These actions result in increased circulating FFA levels and FFA flux to the liver, which stimulates increased production and secretion of atherogenic very-low-density lipoprotein and small dense low-density lipoprotein particles and reducing high-density lipoprotein cholesterol (HDL-C) levels, increasing the risk of microvascular and macrovascular diseases [166, 168]. In addition, in the condition of insulin resistance, normal levels of insulin in the blood would be unable to elicit a reaction in the peripheral tissues that are targeted by insulin due to a decrease in the number of insulin receptors on the surface of cells, including muscle cells [169, 170]. With fewer receptors available, the cells become less responsive to insulin, reducing their ability to take up glucose [98]. Consequently, the β -cells of the pancreas react by producing additional insulin to counterbalance the increased glucose levels [171]. When the β -cells fail to secrete sufficient insulin to counteract insulin resistance, the blood glucose levels commence fluctuating, leading to intermediate hyperglycemia and hyperinsulinemia, leading to further alterations in β -cell function [172].

5.1 Prevalence of prediabetes

The prevalence of prediabetes has grown worldwide and in 2019, the International Diabetes Federation estimated the worldwide prevalence of prediabetes to be 373.9 million, with 15.3 % undiagnosed according to studies [44, 45]. It is also projected that by 2030, approximately 453.8 million people will have prediabetes [173, 174]. The prevalence of prediabetes is anticipated to increase to 8.3% of the

global adult population, equivalent to an estimated 587 million individuals by the year 2045 [175]. Studies show that prediabetes is frequently undetected due to its often-asymptomatic nature in its early stages; hence, most humans tend to unknowingly bypass the prediabetes stage to overt T2DM [163, 176]. In addition, studies show that the increase in prediabetes prevalence is due to rapid urbanisation, increasingly sedentary lifestyles and unhealthy eating habits [177, 178]. As a result, a retrospective study in a rapidly urbanising area such as Durban, South Africa, indicated that 68% of the individuals are prediabetic in the sample population between the ages of 20-45 years, with 51.0 % of the study population being women [179]. This suggests that women of childbearing age are also affected by the global rise in prediabetes [180]. Furthermore, studies show that people with prediabetes have a 2-fold increased likelihood of developing T2DM [181, 182]. Moreover, studies show that complications of T2DM are already evident in some people with prediabetes; these complications include myocardial injury, renal dysfunction, hormonal dysfunction and dysregulation in HPA axis function among others [46-49].

5.1. HPA axis function in Prediabetes

Animal models have been observed to mirror human disease conditions, making them extensively utilised for studying physiological systems and human disease states [183, 184]. A high-fat high carbohydrate and 15% fructose diet-induced animal model of prediabetes has been found to mimic the human condition [46]. In addition, this animal model showed dysregulation in the functioning of the HPA axis in the prediabetic state as shown by elevated basal corticosterone and impaired regulation of their glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in male prediabetic rats animals [47]. At present, there have been no investigations to show whether this phenomenon also exists in female prediabetic rats. Further, if present, this raised the question if maternal basal corticosterone and ACTH levels in prediabetic dams may impact foetal HPA axis development.

6. Possible links of prediabetes with T2DM pregnancies in association with the HPA axis.

Type 2 diabetes mellitus (T2DM) and prediabetes share significant metabolic and physiological similarities [158, 185]. T2DM and prediabetes induce metabolic stress in cells, leading to a sustained elevation of cortisol, ACTH and hyperactivity of the HPA axis associated with exacerbated hyperglycemia and insulin resistance [8, 104]. The maternal cortisol in diabetic pregnancy is high corroborating with maternal stress, however, its impact is not fully understood [10-12, 105, 186, 187]. Conversely, research shows that pre-existing metabolic disorders associated with T2DM, such as hypertension and maternal microangiopathy during pregnancy, decrease trophoblast proliferation [124, 125]. This delays placental growth and its ability to supply the fetus with enough nutrients and oxygen, resulting in foetal hypoxia and inadequate nutrition supply [126]. Decreased placental function is linked to placental dysfunction and relatively low activity of placental 11 β -HSD2 [129, 188]. These

complications have been associated with increasing the vulnerability of the fetus during this period to unwanted programming effects such as increased transfer of active maternal cortisol to the foetal compartments [129, 188]. Early maternal GC exposure to the fetus has been associated with alterations to the balance of both GR and MR development in the foetal brain, leading to changes in gene expression patterns and neural circuit formation evidenced by low GR and MR expression in offspring after birth, even in both basal and stressed animals [135]. Moreover, studies showed that excessive GC exposure during critical periods of development could program the foetal HPA axis to be hyper-responsive beginning in utero, persisting later in life, such as prolonged increases in ACTH and GC seen in adulthood, potentially predisposing the offspring to diseases such as depression, cardiometabolic or T2DM [61]. Furthermore, studies suggest pregestational diabetes is also associated with reduced placental function during pregnancy is associated with reduced foetal growth, contributing to intrauterine growth restriction (IUGR), commonly diagnosed at birth as low birth weight [130, 131]. Low birth weight offspring has been associated with HPA axis hyperactivity, glucose intolerance, hypertension, obesity and greater risks for developing depression, anxiety, T2DM and cardiovascular diseases in adulthood, especially when there was catch-up growth in the first 2 years supported by DOHaD hypotheses [57, 58].

There are various possible causes that contribute to IUGR or an unfavourable hostile environment during pregnancy including preeclampsia and hypertensive disorders that have also been associated with greater risk in T2DM pregnancies [124]. Prediabetes, which often precedes the onset of T2DM, has been shown by various studies to be the genesis of complications associated with T2DM, including myocardial injury, renal dysfunction, hormonal dysfunction and dysregulation in HPA axis function among others [46-49]. A recent study by Ludidi and colleagues showed that prediabetes is a risk factor for developing pre-eclampsia similar to T2DM pregnancy [189]. In addition, since prediabetes often goes undiagnosed, this suggests that there is a population of people unaware of their elevated risk of developing hypertension and preeclampsia [189]. Therefore, the presence of prediabetes in pregnancy might increase the likelihood of IUGR and impaired glucose tolerance in offspring. With the increasing prevalence of prediabetes, especially in women of childbearing age, there is an increased possibility of pre-gestational, gestational and foetal outcome consequences. However, there have been no studies that have looked at how maternal prediabetes affects the HPA axis along with foetal outcomes. Conducting preliminary investigations, generating hypotheses, carrying out invasive procedures, collecting tissue samples and understanding the fundamental mechanism of pregnancy-related disorders would be ethically or practically challenging in humans. As a result, most primary studies are recommended to begin with animal models; hence, this study recommends that future studies focus on pre-existing prediabetes in pregnancy, detailing its effects on maternal HPA axis function and its influences on foetal HPA axis development and offspring development in Sprague Dawley rats.

Table 1: Summarized possible links of prediabetes with T2DM pregnancies in association with the HPA axis.

Prediabetes	T2DM
There is dysregulation in the functioning of the HPA axis associated with elevated basal corticosterone and ACTH in animals. Complications are noted over-stressed and non-stressed prediabetes progression [47]	Metabolic stress on cells leads to constant over-activity of the HPA axis associated with high cortisol and ACTH. Complications are noted in poorly controlled T2DM [8, 9, 103, 104]
The literature predominantly documents prediabetes in animals as a high-factor risk for pre-eclampsia and increased placental oxidative stress [189]. Therefore, the occurrence of prediabetes during pregnancy may increase the likelihood of exacerbated maternal dysregulation in HPA axis function, leading to adverse foetal consequences.	Pregestational diabetes is documented to have high cortisol, although the mechanism is still unknown. However, high cortisol levels in maternal stress and high caloric diet cortisol prominently affect foetal development. Diabetes is associated with microangiopathy in pregnancy, increased risks of pre-eclampsia, IUGR or small for gestational age, which is associated with poor intrauterine development, including HPA axis and overall increased risks of developing T2DM in adulthood [9-12, 15, 17, 24-29, 60, 106,136, 186,187, 190-193].
Nevertheless, no research has been conducted to investigate the impact of maternal prediabetes on the HPA axis in relation to foetal outcomes in pregnancy.	

5. Conclusion

This review paper highlighted the significant impact of dysregulation of the maternal HPA axis during pregnancy, particularly elevated glucocorticoid levels, on foetal growth and programming, with potential implications for HPA axis development in the fetus seen in T2DM. It discussed the possible links between prediabetes and T2DM pregnancies relative to impaired HPA axis function. Women with type 2 diabetes represent high-risk groups during pregnancy. As the incidence of diagnosed diabetes and prediabetes continues to increase, especially at young ages, the number of women with diabetes or prediabetes in pregnancy may also continue to increase. However, further research is needed to

understand the effects of pregestational prediabetes on the maternal HPA axis and its impact on foetal outcomes. This could further underscore the importance of continued investigation into the complex interplay between maternal metabolic health, HPA axis regulation and foetal development to inform clinical management and improve pregnancy outcomes.

Graphical Abstract

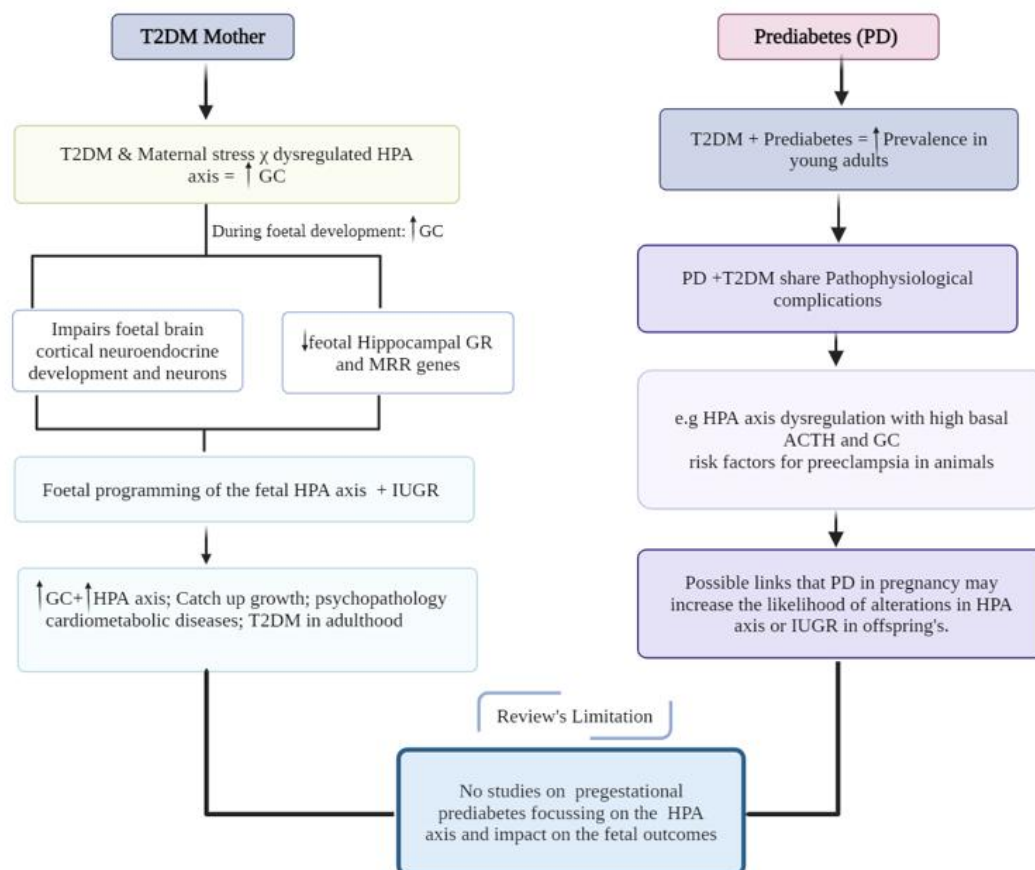


Figure 3: This abstract synthesizes the key points and provides a visual representation of the main concepts discussed in the text regarding the impact of T2DM and maternal stress on foetal programming and long-term health outcomes.

Author Contributions

Conceptualization, M.N., A.K., N.D. X and P.S. N; writing—original draft preparation, M.N.; writing—review and editing, A.K. and P.S. N; visualization, M.N. and A.K.; supervision, A.K., N.D. X and P.S. N. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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BRIDGE

The first manuscript illustrated that pregestational diabetes and maternal stress impact foetal development and contribute to growth restrictions, posing greater risks for metabolic disorders later in life from an underlying root of glucocorticoid excess exposure. Considering that prediabetes is associated with dysfunction in the HPA axis and reported as a risk factor for preeclampsia, similarly with T2DM complication. This suggests that many individuals progress from the pre-diabetic stage to overt T2DM, experiencing associated complications unknowingly due to the often-asymptomatic nature

of early-stage T2DM. In addition, this suggests that there is a population of people unaware of their elevated risk of developing hypertension and preeclampsia, especially in young adults at their childbearing ages. Therefore, the occurrence of prediabetes during pregnancy may increase the likelihood of exacerbated maternal dysregulation in HPA axis function, leading to foetal adverse consequences a potential link that may increase the likelihood of alteration in the HPA axis, foetal growth disturbances and impaired glucose tolerance in offspring evident after birth. This hypothesis led to the second study which sought to investigate the effects of pregestational prediabetes on maternal HPA axis function and postnatal offspring development.

CHAPTER 3: MANUSCRIPT 2

PROLOGUE

Dysregulation in HPA axis function in adults impacts neural function and the presence of excess glucocorticoid exposure *in utero* also acts to program and alter the foetal HPA axis development and function shown in T2DM and maternal stress pregnancies. Offsprings exposed to excess glucocorticoid *in utero* have been associated with hypercortisolaemia states, down-regulated GR and MR genes

alternately programmed HPA axis seen in adulthood. HPA axis programming has been linked to greater risks for glucose intolerance, insulin resistance, obesity, hypertension, cardiovascular diseases, T2DM, anxiety and depression, among others, from adolescence to adulthood. In the previous study in Chapter 2, we established possible links that prediabetes in pregnancy may increase the likelihood of exacerbated maternal dysregulation in HPA axis function, leading to foetal adverse consequences. Prediabetes, an intermediary state between normoglycaemia and T2DM, has been reported to be caused by a diet high in saturated fats and refined carbohydrates. The onset of T2DM-associated complications has been documented to begin in the prediabetic state including dysregulation in HPA axis function. However, no studies have yet shown the influence of pre-existing prediabetes during pregnancy on the maternal–foetal HPA axis interaction. Therefore, the second manuscript explores the effects of pregestational prediabetes on maternal HPA axis function and postnatal offspring development in animals.

The manuscript in Chapter 3 is titled “**Pregestational Prediabetes Induces Maternal Hypothalamic-Pituitary-Adrenal (HPA) Axis Dysregulation and Results in Adverse Foetal Outcomes**” and is authored by M. Ngema, N.D. Xulu, P.S. Ngubane and A. Khathi.

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Pregestational Prediabetes Induces Maternal Hypothalamic-Pituitary-Adrenal (HPA) Axis Dysregulation and Results in Adverse Foetal Outcomes

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Abstract

Pregestational diabetes has been shown to result in foetal programming, leading to adverse foetal outcomes. T2DM is preceded by prediabetes and shares similar pathophysiological complications. However, no studies have investigated the effects of maternal prediabetes on foetal HPA axis function and postnatal offspring development. Hence, this study investigated the effects of pregestational prediabetes on maternal HPA axis function and postnatal offspring development. Pre-diabetic (PD) and non-pre-diabetic (NPD) female Sprague Dawley rats were mated with non-prediabetic males. After gestation, male pups born from the PD and NPD groups were collected. Markers of HPA axis function, adrenocorticotropin hormone (ACTH) and corticosterone, were measured in all dams and pups. Glucose tolerance, insulin and expression of mineralocorticoid (MR) and glucocorticoid (GR) receptors were

further measured in all pups at birth and their developmental milestones. The results demonstrated increased basal concentrations of ACTH and corticosterone in the dams from the PD group by comparison to NPD. Furthermore, the results show an increase in basal ACTH and corticosterone concentrations, disturbed MR and GR gene expression and glucose intolerance accompanied by insulin resistance assessed via the Homeostasis Model Assessment (HOMA) indices in the pups born from the PD group compared to the NPD group in all developmental milestones. These observations reveal that pregestational prediabetes is associated with maternal dysregulation of the HPA axis, impacting offspring HPA axis development along with impaired glucose handling.

Keywords: maternal hypothalamic–pituitary–adrenal (HPA) axis; foetal HPA axis; pregestational prediabetes; pups

1. Introduction

Foetal programming is a process whereby a stimulus or insult at a critical period in development results in permanent adaptation of the organism's structure or physiology [1-3]. Evidence in foetal programming studies has shown that foetal overexposure to endogenous glucocorticoids may underpin the link between early life events and later disease [4-7]. It is proposed that dysregulation of the hypothalamic-pituitary–adrenal (HPA) axis determines foetal exposure to stress hormones, influencing foetal development and programming the foetal HPA axis [8-12].

In humans, the physiologically active glucocorticoid (GC) is cortisol, whereas in rodents, it is corticosterone [13, 14]. During pregnancy, the maternal HPA axis experiences significant changes, with the placenta secreting corticotropin-releasing hormone (CRH), which further elevates adrenocorticotropin hormone (ACTH) and cortisol levels [15-17]. This creates a positive feedback loop where maternal cortisol stimulates placental CRH synthesis, ultimately resulting in higher

glucocorticoid levels [4, 18]. In addition, previous research has reported that despite the increasing circulating levels of glucocorticoid, the diurnal secretion of corticosterone is maintained throughout pregnancy [19-21]. A study showed that Individuals with T2DM have HPA hyperactivity with elevated basal plasma cortisol and ACTH levels associated with hypertension [22]. Research shows that higher maternal levels of cortisol, linked to diabetes in pregnancy, inadequate nutrition, and stress, are associated with insulin resistance and can easily cross the placenta to reach the fetus; however, its impact on the off-spring in diabetic pregnancy is not clearly understood [23-29]. Additionally, research has indicated that T2DM shares similarities with maternal stress conditions, such as metabolic stress during pregnancy, including the persistent activation and dysregulated function of the HPA axis with elevated glucocorticoid levels [30-35]. Rapid economic development, urbanisation, sedentary lifestyles, and a Westernised diet have led to a rising burden of 453.8 million by 2030 adults living with T2DM, resulting in an increasing proportion of pregnancies complicated by diabetes [36, 37].

Given that T2DM is a complicated and multifaceted disease caused by a mix of genetic and environmental risk factors, it is considered a stressor to the human body [38-42]. Studies show that excessive levels of maternal can overwhelm the enzymatic barriers that effectively prevent excessive foetal exposure to maternal GCs, therefore exposing foetuses to excess glucocorticoids [5, 34, 35, 43]. Studies have shown that pregnancies affected by T2DM in conjunction with uncontrolled hyperglycaemia, increased oxidative stress, hypertension, vascular diseases, and increased glucocorticoids, among other complications, exhibit intrauterine growth restriction (IUGR), often manifested as a low birth weight [44-47]. Studies have also shown that excessive foetal exposure to GCs is associated with the downregulation of foetal GR and MR and impairment of the feedback regulation of the HPA axis in both infancy and adulthood [5, 13, 48, 49]. Cross-sectional research has indicated a connection between low birth weight and disrupted functioning of the HPA axis, leading to elevated levels of GC in adulthood [10, 50-52]. In addition, the association between low birth weight and the development of T2DM was first reported in studies by Hales et al., who demonstrated a several-fold increase in the incidence of glucose intolerance and T2DM in adult men who were born with low birth weight compared with those born with normal birth weight [53, 54]. Additionally, research suggests that individuals born with a low birth weight are often associated with catch-up growth with an increased risk for various non-communicable diseases (NCDs) such as hypertension, cardiovascular diseases, and mental disorders in adulthood, aligning with the developmental origins of health and disease (DOHaD) hypothesis [52, 55-60].

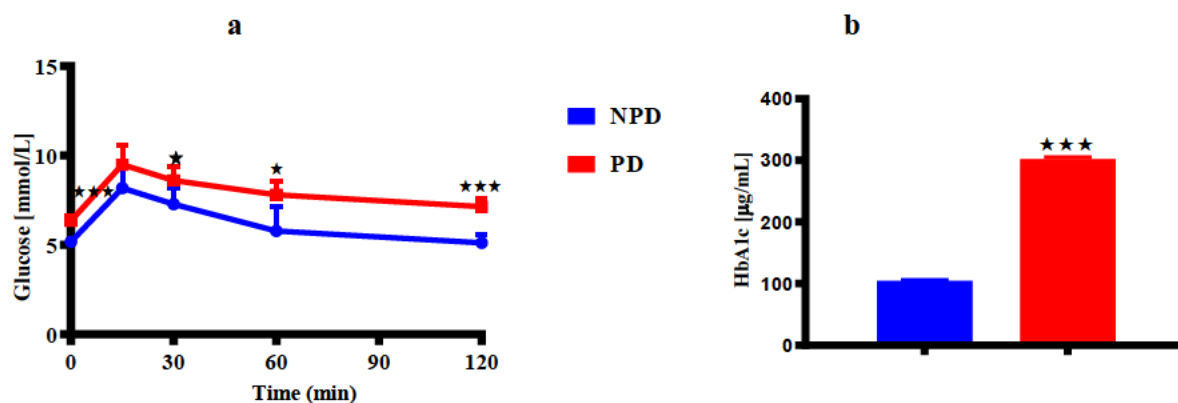
Furthermore, due to the transgenerational risk and foetal complications associated with diabetic pregnancies and the rising frequency of diabetes in young adults [61-68], researchers have redirected their focus towards the concept that intrauterine periods may be one of the contributing factors to an increased risk of metabolic diseases in adults [62-66]. However, studies have shown that T2DM is often

preceded by an early-onset condition known as prediabetes [69-71]. Prediabetes is a condition in which blood glucose concentrations are higher than normal but do not meet the diagnostic criteria for T2DM [72-74]. Studies show that prediabetes is predicted to affect 453.8 million people by 2030 and usually 5–10% progress to T2DM each year due to asymptomatic characteristics [36, 75]. A diet-induced animal model for prediabetes was established in our laboratory and it was found to mimic the human condition [76-78]. In addition, this animal model showed similarities in pathophysiology with T2DM, including dysregulation in the HPA axis associated with increased basal corticosterone and impaired regulation of their GR and MR in male animals [79]. This raised the question of whether the difficulties associated with maternal stress and preexisting T2DM pregnancies and foetal programming of foetal HPA axis are also present during prediabetes and whether basal corticosterone and ACTH levels in prediabetic dams may impact foetal HPA axis development. Therefore, using this animal model, the study sought to investigate the effects of pregestational prediabetes on maternal HPA axis function and its effects on postnatal offspring development.

2. Results

2.1. Dams' Oral Glucose Tolerance (OGT) Response, Glycated Haemoglobin (HbA1c) Concentrations and Area under the curve (AUC) values

The OGT (Figure 1a) and HbA1c concentrations (Figure 1b) and AUC values in the non-prediabetic (NPD) female group (n = 6) and prediabetic (PD) female group (n = 6) at 36 weeks are shown in Figure 1. In the OGTT (Figure 1a), the blood glucose concentration is significantly higher in the PD group at time 0 when compared to the NPD group. The blood glucose concentration in the PD group remained significantly higher when compared to the NPD group throughout the 2 h test. The HbA1c concentration (Figure 1b) was significantly higher in the PD group when compared to the NPD group. Moreover, the AUC values of the PD group showed no significance when compared to the NPD group.



The area under curve Values [mmol/L/min]

NPD 85.25 ± 12.73

PD 127.3 ± 27.93

Figure 1. The dams' OGTT (a) and HbA1c concentrations (b) and AUC values of NPD 85.25 ± 12.73 and PD 127.3 ± 27.93 in the NPD and PD groups ($n = 6$ per group) at 36 weeks. Repeated measures ANOVA and Dunnett's post-hoc Test was used for OGTT analysis, while the Student T-test was used for HbA1c analysis, and the values were expressed as mean $\pm$ SEM. $\star p < 0.011$; $\star\star\star p < 0.001$ denotes comparison with NPD. The blue bar and line graph represent the NPD group and the red bar and line group represent the PD group.

2.2. Dams' Hypothalamic–Pituitary–Adrenal (HPA) Axis Components

The dams' ACTH (Figure 2a) and corticosterone (Figure 2b) concentrations in the non-prediabetic (NPD) female group ($n = 6$) and prediabetic (PD) female group ($n = 6$) at 21 days postpartum. The ACTH concentration (Figure 2a) was significantly higher in the PD group when compared to the NPD group. Similarly, the corticosterone concentration (Figure 2b) was significantly higher in the PD group when compared to the NPD group.

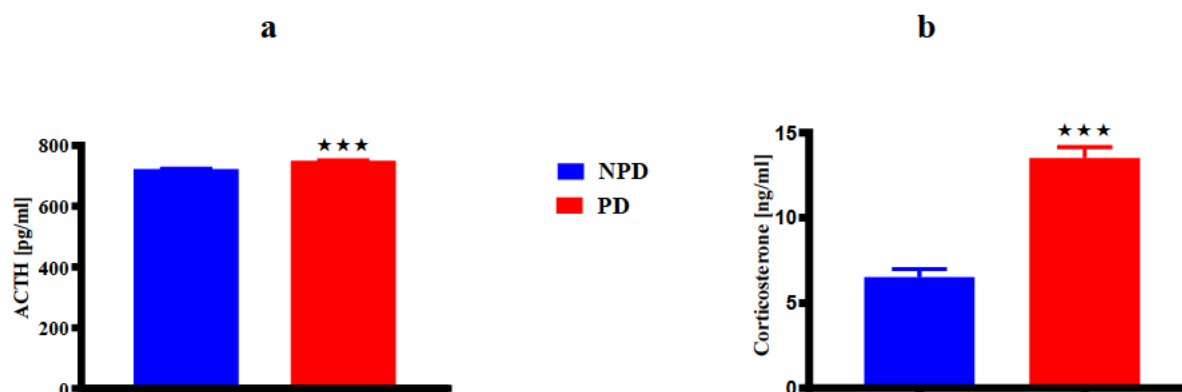


Figure 2. The dams' ACTH (a) and corticosterone (b) concentrations in the NPD and PD groups (n = 6 per group) at 21 days postpartum. Student T test was used for analysis and the values were expressed as mean $\pm$ SEM. *** $p < 0.001$ denotes a comparison with NPD. The blue bar represents the NPD group and the red bar represents the PD group.

2.3. The Dams' Homeostasis Model Assessment (HOMA)-IR, HOMA-S% and HOMA- β Indices and Plasma Adrenocorticotrophic Hormone (ACTH) and Corticosterone Concentrations

The dams' glucose handling was measured by assessing insulin sensitivity and beta-cell function through the insulin resistance (HOMA-IR), insulin sensitivity (HOMA-S%) and Beta-cell secretory capacity (HOMA- β %) indices, along with plasma ACTH and corticosterone concentrations. Table 1 shows the HOMA-IR index assessment for glucose handling in the non-pre-diabetic (NPD) and pre-diabetic (PD) female groups (n = 6 per group) with measurements of plasma ACTH and corticosterone concentrations. The HOMA-IR value for NPD was within the insulin-sensitive range (<1.0), while the PD group had a significantly higher HOMA-IR value compared to the PD, which was in the range of significant insulin resistance. HOMA-S percentage was significantly lower in the PD group in comparison to the NPD group, while the HOMA- β percentage of the PD group was significantly higher compared to the NPD group. The results further showed that there was an increase in plasma ACTH and corticosterone concentrations in the PD group compared to the NPD.

Table 1. HOMA-IR, HOMA-S% and HOMA- β indices and ACTH and corticosterone plasma concentrations in the non-pre-diabetic (NPD) and pre-diabetic (PD) female groups (n = 6, per group).

Groups (n = 6)	Glucose (mmol/L)	Insulin (pmol/L)	HOMA-IR	HOMA-S Values (%)	HOMA- β Values (%)	Plasma ACTH (pg/mL)	Plasma Corticosterone (ng/mL)
NPD	5.18 $\pm$ 0.09	43.97 $\pm$ 0.08	0.84 $\pm$ 0.00	120.8 $\pm$ 0.20	80.10 $\pm$ 0.22	722.3 $\pm$ 0.88	6.50 $\pm$ 0.19
PD	6.35 $\pm$ 0.12 ***	158.60 $\pm$ 0.45	3.06 $\pm$ 0.00 α	32.70 $\pm$ 0.26	126.6 $\pm$ 0.24	750.0 $\pm$ 0.68 ***	13.51 $\pm$ 0.26 ***

Student T test was used for HOMA-IR, HOMA-S% and HOMA- β indices and ACTH and corticosterone plasma concentrations analysis and the values were expressed as mean $\pm$ SEM. α $p < 0.001$ *** $p < 0.001$ denotes a comparison with NPD. The HOMA-IR represented as α denotes significant insulin resistance.

2.4. Pups Hypothalamic–Pituitary–Adrenal (HPA) Axis Components

The ACTH (Figure 3a) and corticosterone (Figure 3b) concentrations of the pups born from the non-prediabetic (NPD) pregnant female group ($n = 6$ per week) and pups born from the prediabetic (PD) pregnant female group ($n = 6$ per week) at weeks 3, 6 and 16 of the experimental periods. The ACTH concentration (Figure 3a) post-hoc test revealed that there was a significant increase in the PD groups when compared to the NPD groups throughout the experimental weeks. Similarly, the corticosterone concentration (Figure 3b) post-hoc test revealed that there was a significant increase in the PD groups when compared to the NPD groups throughout the experimental weeks.

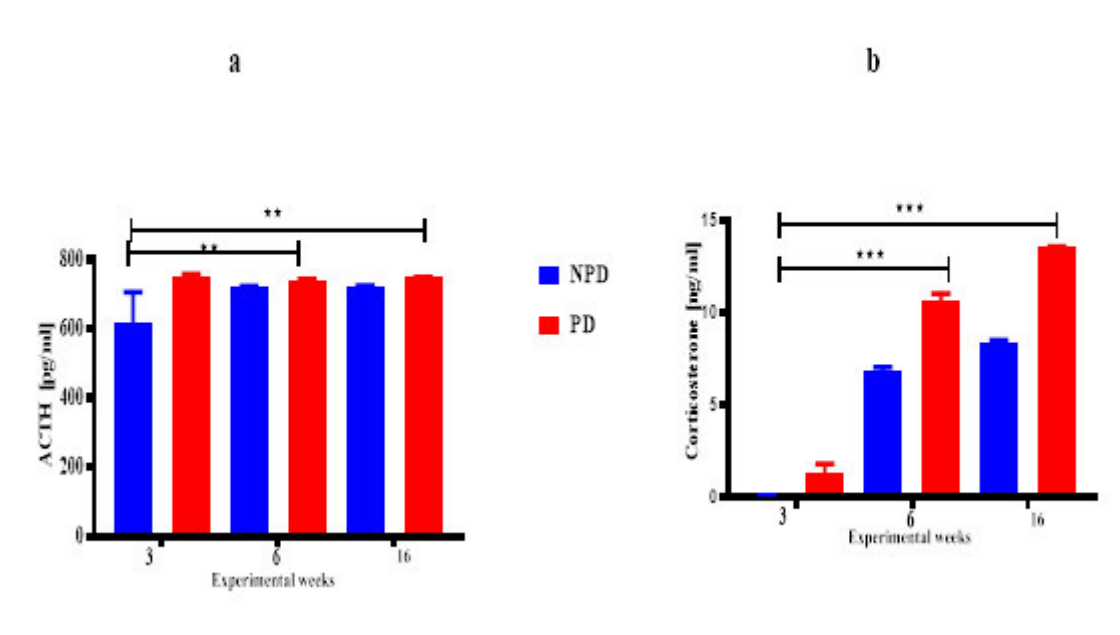


Figure 3. The ACTH, (a) and corticosterone (b) concentrations of the pups born from the NPD group ($n = 6$ per week) and PD group ($n = 6$ per week) at weeks 3, 6 and 16 of the experimental periods. Repeated measures ANOVA and Dunnett's post-hoc Test was used for ACTH and corticosterone analysis and the values were expressed as mean $\pm$ SEM. ** $p < 0.0022$; *** $p < 0.001$ denotes comparison with NPD. The blue bar represents the NPD group and the red bar represents the PD group.

2.5. Pups Hippocampal Glucocorticoid Receptors (GR) and Mineralocorticoid Receptors (MR)

The hippocampal GR (Figure 4a) and MR (Figure 4b) gene expressions were measured in non-stressed pups born from the non-prediabetic (NPD) pregnant female group ($n = 6$ per week) and prediabetic (PD) pregnant female group ($n = 6$ per week) at weeks 3, 6 and 16 of the experimental periods. The post-hoc test revealed a significant half-fold decrease in GR (Figure 4a) gene expression relative between week 3 and week 6 of the PD group when compared to the NPD groups. Whereas the post-hoc test revealed a no significant in GR (Figure 4a) gene expression relative in the PD group when compared

to the NPD group. In addition, the post-hoc test revealed a significant two-fold increase in MR (Figure 4b) gene expression relative between week 3 and week 6 of the PD group when to the NPD groups. While the post-hoc test revealed a no significant in MR (Figure 4b) gene expression relative in the PD group when compared to the NPD group

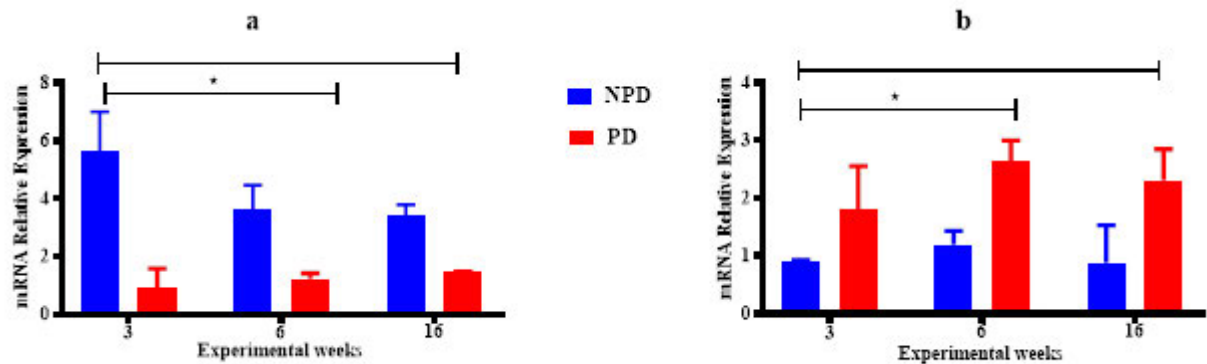


Figure 4. The hippocampal GR (a) and MR (b) gene expressions of the pups born from the NPD group (n = 6 per week) and PD group (n = 6 per week) at weeks 3, 6 and 16 of the experimental periods. Repeated measures ANOVA and Dunnett's post-hoc Test was used for GR and MR analysis and the values were expressed as mean ± SEM. * $p < 0.011$ denotes comparison with NPD. The blue bar represents the NPD group and the red bar represents the PD group.

2.6. Pups Adrenal Gland Weight

The adrenal gland weight of the pups born from the non-prediabetic (NPD) pregnant female group (n = 6 per week) and pups born from the prediabetic pregnant (PD) female group (n = 6 per week) at weeks 3, 6 and 16 of the experimental periods. The post-hoc test revealed a significant increase in the adrenal weight in the PD groups when compared to the NPD groups throughout the experimental weeks (Figure 5).

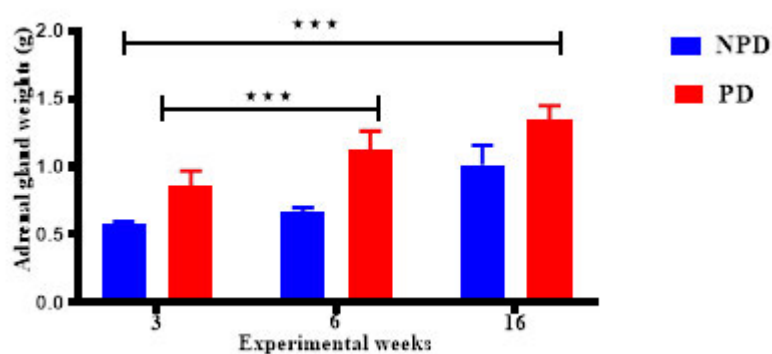


Figure 5. The adrenal gland weight of the pups born from the NPD group (n = 6 per week) and PD group (n = 6 per week) at weeks 3, 6 and 16 of the experimental periods. Repeated measures ANOVA and Dunnett's post-hoc Test was used for adrenal gland weights analysis and the values were expressed as mean $\pm$ SEM. *** $p < 0.001$ denotes comparison with NPD. The blue bar represents the NPD group and the red bar represents the PD group.

2.7. Pups Bodyweights

The body weights of the pups born from the non-prediabetic (NPD) pregnant female group (n = 6) and pups born from the prediabetic (PD) pregnant female group (n = 6) on day 7 (Figure 6a) and at weeks 3, 6 and 16 (Figure 6b) of the experimental periods. On day 7 (Figure 6a), the body weight of the PD group was significantly lower when compared to the NPD group. The post-hoc test revealed no significant between week 3 (Figure 6b) and week 6 body weight in the PD group when compared to the NPD group. However, the post-hoc test revealed a significant increase in body weight between week 3 (Figure 6b) and week 16 (Figure 6b) of the PD group when compared to the NPD group.

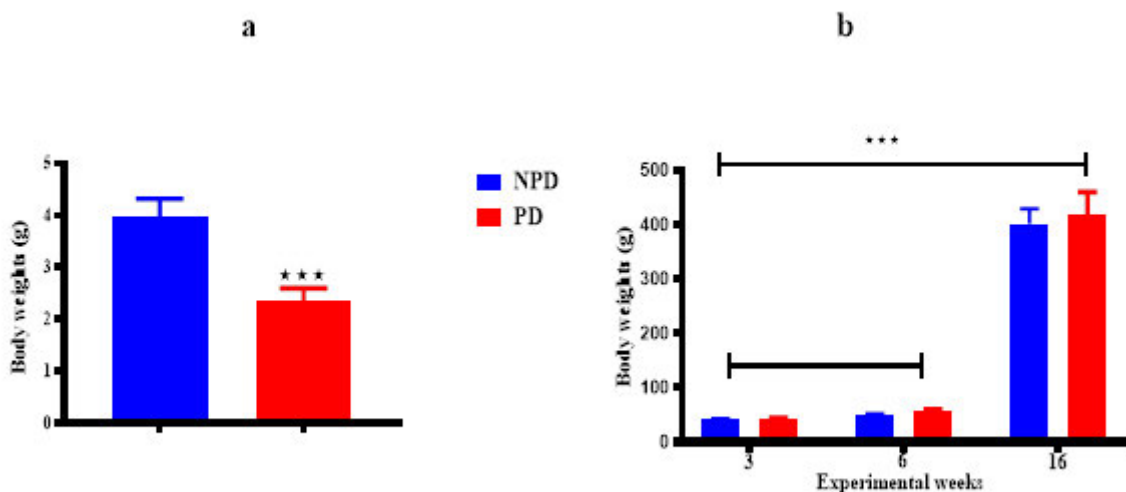


Figure 6. The body weights of the pups born from the NPD group and PD group on day 7 (a) and at weeks 3, 6 and 16 (b). Student T test was used for body weight (a) analysis and Repeated measures ANOVA and Dunnett's post-hoc Test was used for the body weights (b) analysis and the values were expressed as mean $\pm$ SEM. *** $p < 0.001$ denotes comparison with NPD. The blue bar represents the NPD group and the red bar represents the PD group.

2.8. Pups Oral Glucose Tolerance (OGT) Response

The OGT of pups born from non-prediabetic (NPD) pregnant female group ($n = 6$ per week) and prediabetic (PD) pregnant female group ($n = 6$ per week) at weeks 3, 6 and 16 of the experimental periods. The OGTT blood glucose concentration was significantly higher in the PD groups at time 0 when compared to the NPD groups in all experimental weeks. The blood glucose concentration in the PD groups remained significantly higher throughout the 2-hr test when compared to the NPD groups in all experimental weeks (Figure 7).

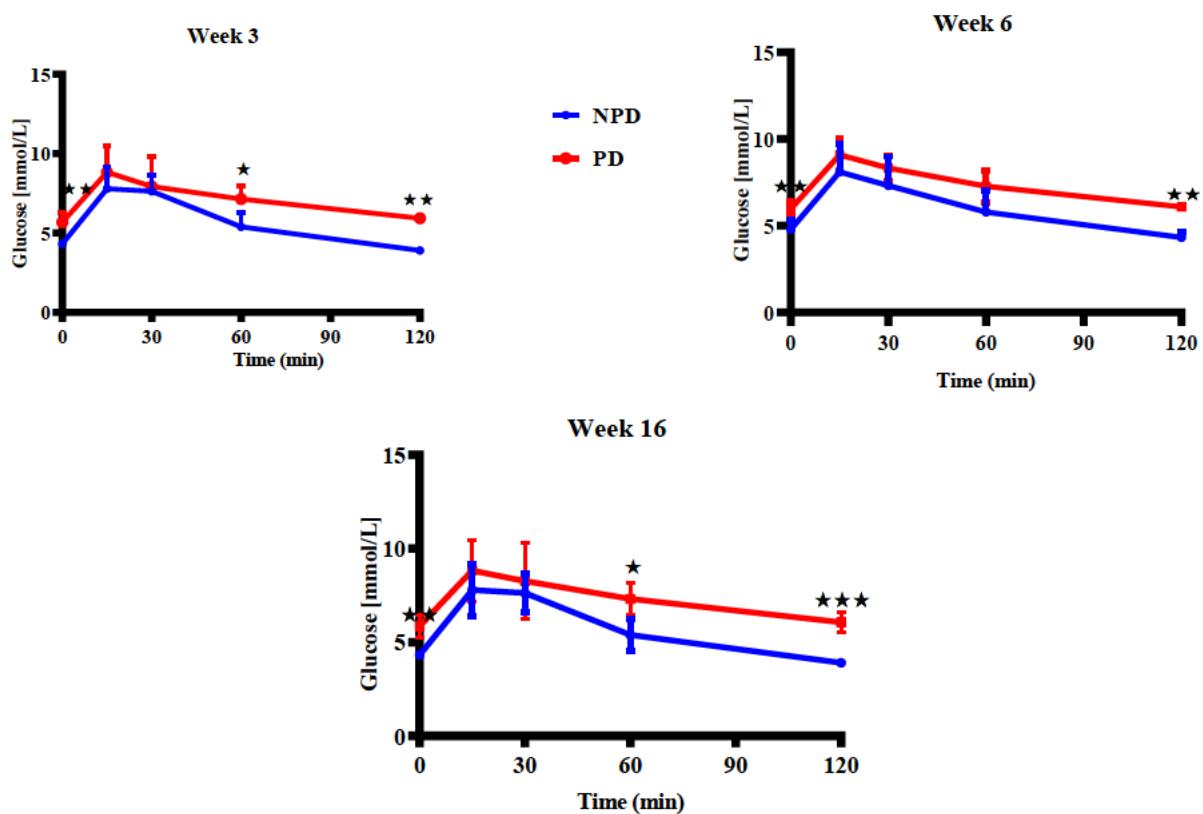


Figure 7. The OGTT of the pups born from the NPD group ($n = 6$ per week) and PD group ($n = 6$ per week) at weeks 3, 6 and 16 of the experimental periods. Repeated measures ANOVA and Dunnett's post-hoc Test was used for analysis and the values were expressed as mean $\pm$ SEM. * $p < 0.0144$; ** $p < 0.0022$; *** $p < 0.001$ denotes comparison with NPD. The blue line graph represents the NPD group and the red line graph represents the PD group.

2.9 The Pups' Area Under the Curve (AUC) values

Table 2 shows Area under curve (AUC) values measured in pups born from non-prediabetic (NPD) pregnant female group ($n = 6$ per week) and prediabetic (PD) pregnant female group ($n = 6$ per week) at weeks 3, 6, and 16 of the experimental periods. The AUC at week 3 showed no significant in pups born from the PD group when compared to the pups born from the NPD group. Whereas in week 6 and

16 the AUC showed a significant increase in pups born from the PD group when compared to the pups born from the NPD group.

Table 2: AUC values in the pups born from the non-pre-diabetic (NPD) female and pre-diabetic (PD) female group (n = 6, per group) at weeks 3, 6, and 16.

Groups (n=6)		Area under the curve Values [mmol/L/min]
Week 3	NPD	79.50 ± 10.60
	PD	91.00 ± 16.75
Week 6	NPD	61.04 ± 4.87
	PD	87.00 ± 10.49 *
Week 16	NPD	75.63 ± 9.14
	PD	120.8 ± 9.44 **

Student T-test was used for AUC analysis and the values were expressed as mean ± SEM. * p < 0.01; **p < 0.0022 denotes comparison with NPD.

2.10. The Pups' HOMA-IR, HOMA-S and HOMA-β Indices and Plasma Adrenocorticotrophic Hormone (ACTH) and Corticosterone Concentrations

The pup's glucose handling was measured by assessing insulin sensitivity and beta-cell function through the HOMA-IR, HOMA-S and HOMA-β indices, along with plasma ACTH and corticosterone concentration at weeks 3, 6 and 16. Table 3 shows the HOMA-IR index assessment for glucose handling in the pups born from non-pre-diabetic (NPD) and pre-diabetic (PD) female groups (n = 6 per group) with measurements of plasma ACTH and corticosterone concentration. HOMA-IR value for NPD was within the insulin-sensitive range (<1.0) except at week 16, while the pups born from the PD group had a significantly higher HOMA-IR value, which was in the range of significant insulin resistance. The HOMA-S percentage was significantly lower in the pups of the PD group in comparison to the pups of the NPD group while the HOMA-β percentage in the pups born from the PD group was significantly higher compared to the pups born from the NPD group except for week 16. The results further showed that there was an increase in plasma ACTH and corticosterone concentrations in the pups born from the PD group compared to the pups born from the NPD group.

Table 3. HOMA-IR, HOMA-S and HOMA-β indices and ACTH and corticosterone plasma concentrations in the pups born from the non-pre-diabetic (NPD) female and pre-diabetic (PD) female group (n = 6, per group) at weeks 3, 6 and 16.

Groups (n = 6)		Glucose (mmol/L)	Insulin (pmol/L)	HOMA-IR	HOMA-S Values (%)	HOMA- β Values (%)	Plasma ACTH (pg/mL)	Plasma Corticosterone (ng/mL)
Week 3	NPD	4.3 $\pm$ 0.04	45.89 $\pm$ 8.91	0.83 $\pm$ 0.05	121.0 $\pm$ 2.67	116.2 $\pm$ 0.51	616.20 $\pm$ 40.21	0.05 $\pm$ 0.01
	PD	5.7 $\pm$ 0.24 **	132.80 $\pm$ 10.80	2.51 $\pm$ 0.05 α	39.80 $\pm$ 0.56	137.1 $\pm$ 0.32	751.00 $\pm$ 2.36 *	1.30 $\pm$ 0.21 ***
Week 6	NPD	4.7 $\pm$ 0.23	53.51 $\pm$ 5.13	0.98 $\pm$ 0.02	101.6 $\pm$ 0.38	107.7 $\pm$ 0.28	720.80 $\pm$ 1.07	6.85 $\pm$ 0.07
	PD	5.9 $\pm$ 0.42 **	196.70 $\pm$ 11.91	3.69 $\pm$ 0.05 α	27.10 $\pm$ 0.07	168.9 $\pm$ 0.79	741.20 $\pm$ 1.07 ***	10.65 $\pm$ 0.15 ***
Week 16	NPD	4.3 $\pm$ 0.04	94.60 $\pm$ 25.18	1.68 $\pm$ 0.03 α	59.50 $\pm$ 0.26	188.6 $\pm$ 0.30	722.10 $\pm$ 0.83	8.30 $\pm$ 0.05
	PD	6.1 $\pm$ 0.17 **	233.40 $\pm$ 23.36	4.37 $\pm$ 0.04 α	22.90 $\pm$ 0.26	179.1 $\pm$ 0.70	748.00 $\pm$ 0.44 ***	13.50 $\pm$ 0.01 ***

Student T-test was used for HOMA-IR, HOMA-S and HOMA- β indices and ACTH and corticosterone plasma concentrations analysis, and the values were expressed as mean $\pm$ SEM. α $p < 0.001$ * $p < 0.01$; *** $p < 0.001$ denotes a comparison with NPD. The HOMA-IR represented as α denotes significant insulin resistance.

3. Discussion

Foetal programming, a response to adverse foetal conditions, leads to lasting adaptations altering organ growth, physiology, and metabolism, thus increasing adult disease risk [80, 81]. Excessive GC exposure in utero, often due to maternal HPA axis dysregulation, has been shown to link early events with later diseases such as hypertension, cardiovascular diseases, T2DM, and mental disorders [5, 82, 83]. During normal pregnancy, maternal HPA axis undergoes significant changes, yet diurnal GC secretion remains maintained [9, 84]. There are studies suggesting that T2DM exhibits resemblances to maternal stress conditions during pregnancy, such as dysregulated HPA axis with increased levels of GC [30-33]. Foetal exposure to excess maternal GCs causes growth re-restriction and programmed life-long changes in HPA axis activity, which increases the risk of developing T2DM and cardiometabolic diseases in adult life [49, 84, 85]. Several studies have suggested that the onset of complications associated with T2DM begins during the prediabetic state [69-71]. An experiment in our lab established a di-et-induced prediabetic animal model and showed similarities with humans, including dysregulation in the function of HPA axis [79]. However, no studies have yet shown the influence of pre-existing prediabetes during pregnancy on the maternal–foetal HPA axis interaction. Therefore, this study aimed to investigate the effects of pregestational prediabetes on maternal HPA axis function and its effects on postnatal offspring development.

In non-diabetic individuals, glucose homeostasis is tightly regulated with fasting plasma glucose (FPG) maintained at 3.9–5.6 mmol/L and postprandial glucose level of less than 7.8 mmol/L [86]. In the postprandial state, elevated blood glucose concentration stimulates pancreatic beta β cells to produce adequate insulin enough to clear glucose from the bloodstream through the insulin signalling pathway [87, 88]. Studies show that during T2DM, the insulin signalling pathway is disturbed primarily due to chronic hyperglycaemia, which then contributes to pancreatic β -cell dysfunction and insulin resistance [89, 90]. Studies show that T2DM is also associated with IGF and IGT and elevated HbA1c $\geq 6.5\%$ [91]. Studies show that prediabetes can be diagnosed by at least two of these characteristics: impaired fasting glucose (IFG) (5.5–6.9 mmol/L), impaired glucose tolerance (IGT) (7.8–11.0 mmol/L) and elevated glycated haemoglobin A1c (HbA1c) (5.7–6.4%) [92, 93]. In the present study, there was a significant increase in the fasting plasma glucose concentration before glucose loading and a failure of blood glucose concentration post-glucose load to return to baseline following a 2 h OGT test in the PD group, suggesting the presence of IGF and IGT in Figure 1a. In the present study, the PD group had a significant increase in HbA1c concentration whereas AUC values showed no significant when compared to the NPD group in Figure 1b. The results align with previous research indicating that elevated plasma glucose concentrations, also seen in PD and T2DM, result in non-enzymatic glycation of haemoglobin [76, 94, 95]. This glycation process occurs throughout the entire 120-day lifespan of red blood cells through an Amadori reaction, forming a stable and irreversible ketoamine linkage [76].

These findings in our results indicate that the levels of glucose in the blood and the length of time that red blood cells are exposed to glucose are responsible for the production of HbA_{1c}. This suggests that glucose utilisation in insulin-dependent peripheral tissues such as skeletal muscles is decreased, suggesting there is some insulin resistance in the tissues [96, 97]. In this study, insulin sensitivity and beta cell function were assessed using the Homeostasis Model assessment (HOMA), showing insulin resistance (HOMA-IR), reduced insulin sensitivity (HOMA-S), and increased beta cell function (HOMA of β -cell function) in the PD group in Table 1. Studies show that increased pancreatic β -cell function is associated with the production and increased secretion of insulin as a compensatory mechanism to a high blood glucose concentration circulating in the peripheral tissues [98]. The coexistence of IGF and IGT, elevated HbA_{1c} levels, insulin resistance, reduced insulin sensitivity, and increased beta cell function in our PD group indicated the induction of prediabetes at 36 weeks. A prior investigation conducted in our lab revealed that male animals developed prediabetes after 20 weeks of being fed a diet high in fat and carbohydrates [76]. Our current study extends this timeframe by an additional 16 weeks. A previous study has attributed this to several physiological disparities, including genetic and hormonal differences such as progesterone and oestrogen, that have been shown to exert protective effects that may have delayed the induction of prediabetes in females [99].

During pregnancy, the regulation of the maternal HPA axis undergoes dramatic changes, such as regulating stress response and maintaining homeostasis for both the mother and the developing foetus [100, 101]. The HPA axis controls the diurnal secretion of GCs, which play a crucial role in foetal development [9, 19]. Physiological active glucocorticoid is known as cortisol in humans and corticosterone in rats [13, 14]. Studies show that elevated cortisol levels during pregnancy enhance the inhibition of insulin activity, leading to decreased insulin sensitivity and increased insulin resistance in skeletal muscle by reducing glucose utilisation [102, 103]. Studies show that patients and animals with diabetes commonly have both elevated basal ACTH and high levels of GCs due to the altered regulation of the HPA axis [22, 104-106]. In addition, according to a previous study, diabetic pregnant women exhibit higher cortisol concentrations [24-29, 107]. In addition, according to a previous study, diabetic pregnant women exhibit higher cortisol concentrations [24-29,107]. Studies show that T2DM in pregnancy shows similar findings seen among maternal obesity, depressed and stressed pregnant women with prolonged activation and dysregulated function of the HPA axis with elevated glucocorticoid levels [108, 109]. Studies show that pregnancy in women with T2DM worsens, especially in those who already have other complications such as uncontrolled hyperglycaemia, hypertension, or vascular diseases [110, 111]. In the present study, we evaluated HPA axis activity by measuring two components of the HPA axis under basal non-stressful conditions and found that both plasma ACTH levels and corticosterone concentrations were significantly increased in the PD pregnant female group when compared to the NPD pregnant female group in Figure 2. Diabetic polyneuropathy is associated with a persistent increase in the basal activity of the HPA axis with maintenance of the

circadian variation for ACTH and cortisol. The PD group ACTH concentrations in our study are higher than in previous studies. This may have been due to prolonged hyperglycemia in prediabetes, which can impair glucocorticoid receptor sensitivity, particularly in the pituitary. This means that even though ACTH is produced at higher levels, the adrenal glands might not respond as efficiently to produce cortisol. These results corroborated a previous study that found these high cortisol levels in pregestational diabetes [24,25]. In addition, the combinational increase of both corticosterone and ACTH deduced hyperactivity in the HPA axis in pregestational prediabetes, which correlated with maternal stress pregnancies [108, 109].

During non-diabetic pregnancy, the placenta CRH, which initiates a complex feed-forward loop [112-114]. Placental CRH activates the maternal HPA axis, resulting in the production of cortisol. At the same time, maternal cortisol increases the synthesis of placental CRH, leading to an increase in cortisol levels, which is a vicious cycle [15-17]. In addition, during development, foetuses need glucocorticoids for various aspects of brain development and at late gestational lung maturation [115, 116]. However, approximately 5–10% of cortisol must only pass the placenta to the foetus, and this is due to the partial placental protective barrier 11 β -Hydroxysteroid Dehydrogenase 2 (11 β -HSD 2) [117]. Studies in diabetic pregnancy, similarly to maternal obesity, de-pressed, and stressed pregnancy, report that high maternal cortisol is associated with the dysregulation of placental CRH, which results in a vicious cycle of high maternal cortisol [109, 118, 119]. Studies show that high maternal cortisol in pregnancy can cross the placenta by overwhelming the placental barrier into the foetal compartment which may directly affect foetal development [120-122]. Furthermore, studies show that exposure to adverse maternal cues, such as high glucocorticoids during critical developmental periods, has been shown to increase the risk of altered HPA axis function, stress-related conditions such as depression, cardiometabolic diseases and T2DM later in life [123-126]. The above is in line with the foetal programming hypothesis. The exposure to glucocorticoid in utero is thought to compromise foetal brain development, specifically the prefrontal cortex, hippocampus, and amygdala, brain areas associated with regulating the HPA axis [83, 127, 128]. In addition, research indicates that excessive cortisol exposure during early gestation triggers an early shift from tissue accretion to differentiation, thereby reducing foetal growth in various vital organs such as the brain, heart, liver, kidney, and adrenal glands [129-131]. This process often leads to the clinical manifestation of intrauterine growth restriction (IUGR), characterised by the development of growth-retarded foetuses [132, 133]. The diagnosis of IUGR is as-signed to infants with a birth weight below the 10th percentile for gestational age [134, 135]. In diabetic pregnancy, IUGR is observed most in patients with vasculopathy (retinal, renal, or chronic hypertension) [46, 54, 136, 137]. The association between a low birth weight and elevated plasma cortisol concentrations, hypertension, cardio-vascular diseases, T2DM, and mental disorders has been documented by epidemiological studies [138, 139]. However, the impact of maternal dysregulated HPA axis function in pregestational prediabetes and its influence on foetal HPA axis and

postnatal offspring development has not yet been explored. Therefore, this leads to the next phase of the study.

The foetal pituitary matures first, with foetal HPA activity beginning at mid-gestation [19, 140]. The actions of the foetal HPA axis are essential in foetal development, maturation, and homeostasis and eventually prepare for the survival of the neonate [140, 141]. After birth, the HPA axis is able to regulate responses to adverse conditions, acting on the metabolism of carbohydrates, proteins and lipids and participating in anti-inflammatory effects and suppression of the immune response [142, 143]. A study found that maternal diabetes with high cortisol levels is linked to changes in the development of the brain's cortical, neuroendocrine system by reducing the number of hippocampal neurons [144]. This is accompanied by disruptions in the foetal HPA axis, resulting in structural changes such as cortical thinning, enlarged amygdala, and impaired development of the foetal cerebellum [145, 146]. The modifications in brain structures caused by cortisol have been consistently linked to impairments in both cognitive and emotional development, as well as basal high cortisol levels in children [144-146]. However, the exact molecular and cellular mechanism by which diabetes during pregnancy affects the development of the brain is still unknown [147]. However, previous studies conducted in maternal stress pregnancy show that GC exposure alters hippocampal glucocorticoid receptor density and sensitivity, permanently altering the set-point and HPA axis regulation [148, 149]. Studies show that these are observed from the neonatal, prepubertal and post-pubertal periods and appear to persist through to adulthood with increased fasting cortisol levels associated with upregulated HPA axis function [150-153]. Studies show that there is increased adrenocortical function in children in the juvenile period who were exposed to GC *in utero* with increased fasting cortisol concentrations in adults [154, 155]. The upregulation of postnatal HPA function in other species may reflect changes in the HPA axis at the level of the hypothalamus, pituitary or adrenal gland itself [156, 157].

Therefore, with the maternal dysregulation in HPA axis function associated with high corticosterone concentration in pregnancy in our study and the associated consequences shown in the literature. In the present study, we evaluated ACTH and corticosterone concentration components of the HPA axis of the pups born from the PD group and found a significant increase in all developmental stages when compared to pups born from the NPD group in Figure 3. These results indeed correlate with literature that offspring born of maternal diabetes and/or maternal stress exhibit increased corticosterone in animals [158-160]. However, previous studies have shown that a prolonged and continual increase in glucocorticoids travels to the brain, where constant, elevated corticosterone levels in the highly regulated brain cause constant activation of the HPA axis, thus leading to HPA axis hyperactivity, especially in non-stressed conditions [161, 162]. Therefore, the prolonged increase in ACTH and corticosterone concentrations in all developmental stages in our study may have resulted in HPA axis hyperactivity, as our animals were not stressed.

In addition, HPA axis activity is modulated by feedback regulation of glucocorticoids exerted by two different types of receptors, namely the MR and the GR [162, 163]. The relationship between MR and GR is also critical to negative feedback as the two receptors act co-ordinately to reduce corticosterone secretion by inhibiting ACTH secretion following exposure to stress and maintaining homeostatic balance at rest [164, 165]. This balance is critical for the normal function of the HPA axis [166]. However, excess glucocorticoid exposure to maternal stress has been shown to reduce the number of both glucocorticoid and mineralocorticoid receptors in the hippocampus, subsequently altering the set point of the foetal HPA axis, which is evident after birth [35, 127, 133]. Hence, the present study evaluated hippocampal GR and MR gene expressions. In this study, the pups born from the PD group had significant decreased GR gene expression between week 3 and week 6. Similarly, MR gene expression had significantly increased between week 3 and week 6, when compared to pups born from the NPD group in Figure 4. The decreased GR expression correlates with the previous study and supports that maternal increased glucocorticoid overexposure in our study may have instantly decreased the expression of GR in the hippocampus, accompanied by the elevation of basal glucocorticoids thus, programming effects on the HPA axis often involve the alteration of GR gene expression in regions of the brain that regulate stress response [187]. However, the increase in MR gene expression contrasted with other studies that show a decrease in its expression. Studies show that excess glucocorticoid exposure during utero can lead to changes in DNA methylation patterns, histone modification, and microRNA expression that influence gene expression patterns [48, 167]. These epigenetic changes may specifically upregulate MR gene expression and downregulate GR expression as a regulatory response to cope with the persistently elevated levels of glucocorticoid in utero and even after birth [50, 168]. Therefore, the imbalances of GR and MR gene expression in our result may have been due to compensatory mechanisms that occurred during utero and persisted after birth to emerging adulthood. Our study supports the hypothesis that high maternal corticosterone or cortisol during pregnancy exposes the foetus to excess glucocorticoids, which promotes persistent changes in the HPA axis function during development, as evidenced by increased ACTH and corticosterone and imbalances in GR and MR gene expressions. Furthermore, studies show that the adrenal glands may undergo adrenocortical hypertrophy as a result of increased production of corticosterone, as seen in this study [169, 170]. This excessive corticosterone secretion has been shown to lead to enlargement of the adrenal glands over time, which correlates with the observed increased adrenal gland weight in pups born from the PD group compared to the NPD group in all the developmental stages in Figure 5.

Moreover, other studies have provided further mechanistic insight into HPA axis programming [171-174]. A study showed that in the white population, some studies showed maternal cortisol level is a predicted factor for foetal birth weight [175, 176]. A study in diabetic pregnant rats demonstrated a foetal growth retardation/IUGR evident as low birth weight [177]. The findings of this study were also similar to those of depressed and stressed pregnant women. According to previous studies, elevated

activity of the HPA axis, specifically characterised by higher levels of ACTH and high levels of glucocorticoids in the blood, is observed in both children and adults who were born with a low birth weight [139, 178-180]. In addition, previous studies have shown that about 30% of all infants with a low birth weight show catch-up growth during the first 2 years of life, and this is to compensate for their genetically determined growth trajectory [181-183]. The detrimental effects of catch-up growth in humans have been associated with the development of glucose intolerance, insulin resistance, T2DM, hypertension, and cardiovascular disease in adulthood [184-186]. In the present study, the pups born from the PD group had a significantly lower body weight when compared to pups born from the NPD group Figure 6a. The results corroborated previous findings that showed that high maternal glucocorticoid was also observed in the study, which disturbed foetus development and reduced foetal growth, which is evident in lower body weight. However, in this study, the pups born from the PD group had no significant change in body weight at week 3, known as the juvenile period, corresponding to starting 2 years of human age in rats and at emerging adults' periods, whereas at the emerging adulthood period week 16, there was a significant increase in body weight when compared week 3 to pups born from NPD group (see Figure 6b). Therefore, we deduced that the body weight in the developmental stages showed an absence of catch-up growth even though there was an increase in body weight between week 3 and week 16. Studies show that the absence of catch-up growth may play an important role in protecting the animals from adverse metabolic outcomes in the long term and preventing the catch-up growth deterioration effect [188].

Moreover, studies show that elevated maternal GC levels during pregnancy influence both the programming of the foetal HPA axis and metabolic pathways such as glucose metabolism [9, 50]. This programming can lead to alterations in the development of tissues, such as skeletal muscle involved in glucose homeostasis, as a result of foetal programming during the period of growth restriction also observed in diabetic pregnancies [189, 190]. In addition, studies show that offspring born to mothers with diabetes exhibited a greater prevalence of IGT. A study shows that the incidence of IGT increased from 9.4% to 17.4% at juvenile age in children who were born to diabetic mothers [191]. Several studies show that low-birth-weight offspring have been associated with glucose intolerance, insulin resistance, and T2DM in adulthood [59, 192]. Hence, in our study, we evaluated glucose tolerance in the pups. In the present study, there was impaired fasting glucose as there was a significant gradual increase in blood glucose concentration in the PD group of all developmental stages in Figure 7. There was also evidence of impaired glucose tolerance in the PD group as the blood glucose concentrations remained higher and a failure of blood glucose concentration to return to baseline after the 2-hour test in all developmental stages. In addition, the Area under the curve values in pups born from the PD group showed no significant at week 3 while showed increase significant at week 6 and 16 when compared to pups born from the ND group shown in Table 2. These results may have suggested that there was an increased glucose intolerance correlating with higher glucose concentrations in week 3 and 6 [193].

Glucocorticoids have been shown to inhibit pancreatic- β cells from secreting insulin directly, impair insulin-mediated glucose uptake, and interfere in the insulin signalling cascade in peripheral tissues such as skeletal muscle, thus inducing diabetogenic effects and insulin resistance [194, 195]. However, studies show that non-diabetic individuals have the ability to counteract the insulin resistance caused by glucocorticoids by either enhancing pancreatic β -cell activity or increasing insulin secretion and sensitivity [196, 197]. In the present study, we assessed insulin resistance, pancreatic β -cell and insulin sensitivity, and our results demonstrated insulin resistance, increased pancreatic β -cell, and reduced insulin sensitivity in pups born from the PD group when compared to the NPD group in Table 3. As a result, the observed increased corticosterone, insulin resistance, increased pancreatic β -cell, and reduced insulin sensitivity alongside IGT implies that in the present study, the compensatory mechanism failed to counteract glucocorticoid-induced insulin resistance, resulting in hyperglycaemia. Interestingly, in the NPD group, at week 16, we observed an early-onset insulin resistance with elevated pancreatic β -cell consistent with high corticosterone concentration compared to the other weeks. Studies show that normal glucose-tolerant offspring may have insulin resistance involvement and may be attributable to higher body weight [63]. Therefore, we speculate that the observed onset of insulin resistance in the normal group may be due to the gradual increased body weight and lack of physical activity associated with the increased corticosterone when compared to the other groups. Furthermore, according to previous studies, men born with low birth weight had a several-fold-higher incidence of glucose intolerance and type 2 diabetes in adulthood as opposed to those who were born with a normal birth weight [53, 54]. Therefore, the results in this study show that our lower-body-weight offspring may have a greater risk of developing T2DM in adulthood and other diseases such as metabolic diseases or psychiatry-related disorders.

4. Materials and Methods

4.1 Animals and Housing

All animal experimentation was approved by the Animal Research Ethics Committee (AREC) of the University of Kwa-Zulu Natal (AREC/032/020D). Three-week-old female Sprague Dawley rats were bred and housed in the Biomedical Research Unit (BRU) of the University of Kwa-Zulu Natal and were used in the study. The animals were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), carbon dioxide (CO₂) content (<5000 p.m.), relative humidity ($55 \pm 5\%$) and illumination (12 h light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels approved. The animals were allowed access to food and fluids ad libitum. The animals acclimatised to their new environment for 1 week while consuming standard rat chow and tap water

before the induction of pre-diabetes by exposure to a well-established experimental diet (HFHC). The experimental design was divided into three distinct phases (A, B and C), which are outlined in the following paragraphs.

4.2 Induction of prediabetes

In phase A of the study, female Sprague Dawley rats (150–180 g) were randomly assigned to two diet groups, groups A and B (n = 6 per group). Experimental pre-diabetes was induced in female Sprague–Dawley rats using a protocol previously described [76]. To summarise, group A, labelled as the non-prediabetic group (NPD), was given a standard rat chow diet with normal water for a 36-week period. The animals in group B were given a high-fat, high-carbohydrate (HFHC) diet with 15% fructose added for the same duration of 36 weeks. After a duration of 36 weeks, the diagnostic criteria of the American Diabetes Association (ADA) were applied to identify pre-diabetes in all animals. This involved identifying animals exhibiting pre-diabetic indicators, such as fasting blood glucose levels ranging from 5.6 to 7.1 mmol/L, oral glucose tolerance test (OGTT) 2 h glucose levels between 7.1 and 11.1 mmol/L and glycated haemoglobin (HbA1c) levels between 5.7% and 6.4%, which were used as additional diagnostic criteria for prediabetes. Animals meeting these criteria were categorised as pre-diabetic, while those with measurements below the pre-diabetic thresholds were considered non-pre-diabetic.

4.3 Oral Glucose tolerance test (OGTT)

An oral glucose tolerance test was performed on all animals to assess their glucose tolerance response. This test was completed after carbohydrate loading, following a well-established laboratory technique [198]. After fasting for 16 h, 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 and measuring glucose concentrations using a OneTouch select glucometer (Lifescan, Mosta, Malta, UK) [199]. Glucose levels were subsequently assessed at 15-, 30-, 60- and 120-min following carbohydrate loading.

4.4 Mating

This led to phase B of the study. Prior to mating, all 12 female Sprague–Dawley rats underwent assessment for the proestrus stage through vaginal smear analysis under a microscope. Those in the proestrus stage were permitted to mate with non-prediabetic male Sprague–Dawley rats. Confirmation of pregnancy and assignment of gestational day (GND) 0 were determined the following morning by the presence of a vaginal plaque or vaginal smear containing spermatozoa observed under a microscope [200]. The male rats were then removed from the cage and male rats were returned to their cage after

successful mating [201]. Pregnant rats were then observed until the end of gestation (21 days). This marked the end of phase B of the study.

4.5 Male pups were collected for the study.

Phase C of the study began when the pups were born naturally at gestational day 21 and kept with their dams. However, on day 7, the animals were immediately collected, weighed and immediately returned to the dams. According to previous studies, day 7 represents a neonatal/newborn development period which loosely correlates with similar developmental stages in humans (summarised in Table 5) (202–204). The animals were kept with their mothers for a period of 21 days/3 weeks under standard laboratory conditions (for temperature and humidity) in a 12-day and 12-night cycle. This 21-day/3-week cycle allows rats to undergo crucial stages of development, including growth socialisation and the establishment of basic behaviours [205–207]. Additionally, the regular day/night cycle helps regulate their circadian rhythms, which are important for overall health and well-being (208–210). After 21 days/3 weeks, the animals were weaned and 18 male Sprague Dawley pups born from the non-prediabetic (NPD) female group and 18 male pups born from the prediabetic (PD) female group were collected. Afterwards, the dams were subjected to a period of fasting in order to evaluate the oral glucose tolerance (OGT) test and then euthanised for further investigations. This was considered phase one. In the second part of the investigation, the pups were divided into three independent experimental weeks, namely week 3, week 6 and week 16, with six pups in each group assigned to either the NPD or PD group. These animals were given unlimited access to standard chow and normal drinking water throughout their experimental periods. With regard to the number of weeks chosen in our study, studies show that there are six recognised developmental time periods in rats (211,212). These include neonatal, infantile, juvenile, peripubertal, late pubertal and emerging adulthood, which are recognised at weeks 3, 6 and 16 in our study, respectively. These stages have been shown to roughly correspond to similar developmental stages in humans (213–218). Table 5 provides a concise overview of the developmental phases in humans and rats, allowing for a comparison of their respective ages. In addition, at each of the different time points, 6 animals from each group were fasted for 16 h to assess fasting glucose and OGT performance test. Subsequently, the animals were euthanised for terminal investigations at each subsequent week of investigation.

Table 4. Postnatal developmental stages in rats and humans (219–221).

Developmental Stages in Rat/Human	Rat (Male)	Human
Neonatal/newborn	0-7 days	0-28 days
Infantile/infant	8-20 days (1-2 weeks)	1-23 months
Juvenile/child	21-32 days (3-5 weeks)	2-12 years

Peripubertal/adolescent	33-55 days (5-8 weeks)	12-16 years
Late puberty/adolescent	56-70 days (8-10 weeks)	
Emerging adulthood	70-150 days (10-21 weeks)	18-25 years

Adapted from Barrow et al., 2011 (219), Picut et al., 2015 (2200 and Parker and Picut, 2016) (221).

4.5.1 Summarised Flow Chart

The methodology flow chart illustrates the phase-to-phase methodology employed in the study shown in Figure 8. Rectangle shapes with A, B and C represent phases of the study. Phase A shows the induction of prediabetes in female Sprague Dawley rats. In Phase B, the prediabetic rats were mated, became pregnant and gave birth. In Phase C, the male pups were collected and assigned to 3 developmental milestones (3, 6 and 16 weeks) ([219–221]). In addition, the animals' body weights were evaluated and an oral glucose tolerance test (OGTT) was conducted each week of the experiment prior to euthanising the animals for further analysis.

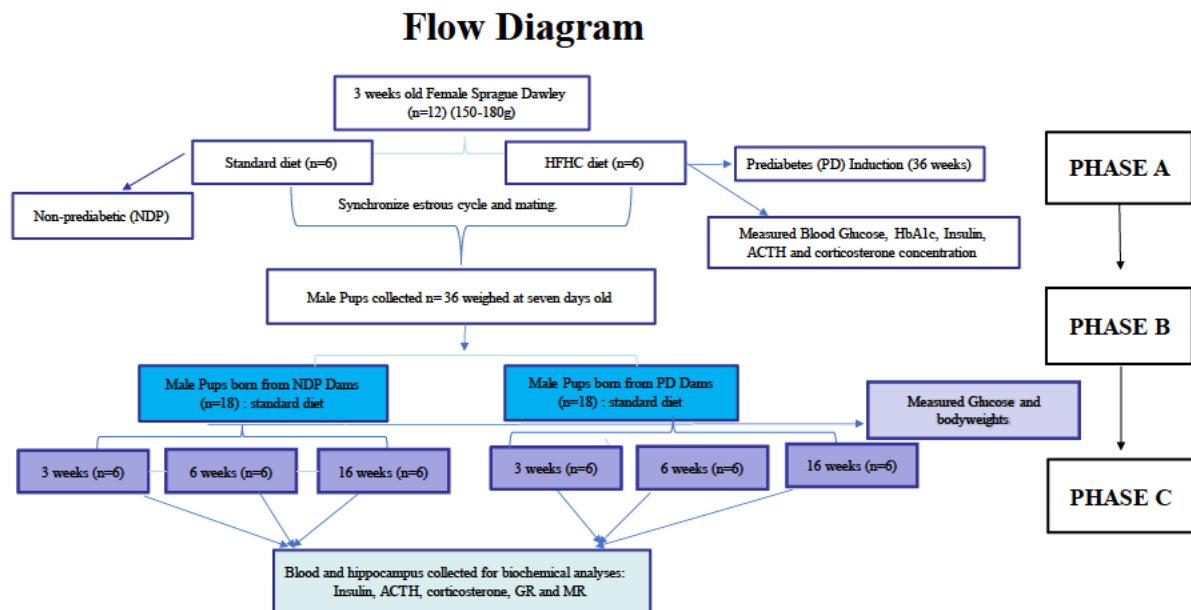


Figure 8. The summarised methodology flow chart in phase segments A, B and C.

4.6. The Homeostasis Model Assessment

At the end of each experimental week (3, 6 and 16), the Homeostasis Model Assessment was utilised to quantify HOMA-IR, HOMA-S and HOMA- β indices. These indices are used to evaluate insulin resistance, insulin sensitivity and beta-cell function capability. The calculations were performed using the HOMA2 Calculator v2.2.3 application [213]. Homeostasis model assessment (HOMA) expresses insulin resistance as a HOMA-IR value. A value of less than 1.0 indicates insulin sensitivity, whereas a

value more than 1.9 indicates early insulin resistance and a value greater than 2.9 indicates significant insulin resistance. Insulin sensitivity is quantified using the HOMA-S% index, where a larger percentage indicates more insulin sensitivity in the individual. Beta-cell secretory ability is quantified as HOMA- β %, with higher values indicating more insulin secretion by beta-cells to regulate blood glucose levels (223).

4.7. Blood collection and tissue harvesting

For blood collection, all animals were sacrificed using the guillotine including the dams and blood was collected into the separated pre-cooled heparinised tubes and was centrifuged (Eppendorf centrifuge 5403, *Hamburg*, Germany) at 4 °C 530× *g* for 15 min. The separated plasma was stored at −80 °C in a Bio Ultra freezer (Snijders Scientific, Holland, *The Netherlands*) for Biochemical analysis. Following blood collection, the hippocampus was removed and placed in pre-cooled Eppendorf containers and snap-frozen in liquid nitrogen before storage in a Bio Ultra freezer (Snijders Scientific, Tilburg, *The Netherlands*) at −80 °C.

4.8. Biochemical analysis

Plasma insulin, adrenocorticotrophic hormone (ACTH) for dams and pups' concentrations and HbA1c concentrations for dams were measured using their respective rat competitive-ELISA kits (Elabscience Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's instructions. The kits included a micro-ELISA plate that was coated with antibodies specific to each of the parameters measured. Standards and samples were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min at 37 °C. This was followed by adding the relevant biotinylated detection antibody (100 μ L). After 60 min incubation at 37 °C, avidin–horseradish peroxidase conjugate (100 μ L) was added to each microplate well. After a further 30 min incubation at 37 °C, the unbound components were washed away using the wash buffer provided. Substrate solution (100 μ L) was added to each microplate well and after 15 min incubation at 37 °C, the stop solution (50 μ L) was added. Optical density was measured using a nano spectrophotometer 47 (BMG Labtech, Ortenburg, Germany) at 450 nm. The concentrations of each parameter in the samples were extrapolated from a standard curve.

Corticosterone concentrations for dams and pups were measured in plasma using different Linco-plex kits (Millipore, Darmstadt, Germany). Metabolic hormone kits (Merck-Millipore, Darmstadt, Germany) were used to quantify plasma and Corticosterone concentrations. Assays were performed according to the manufacturer's instructions. Concentrations were read on Luminex[®] (50 μ L, 50 beads per bead set) using a Luminex[®] 200[™], HTS, FLEXMAP 3D[®], MAGPIX[®] instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA) with xPONENT[®] software (version 3.1.971.0). All samples, quality controls (QCs) and inter-plate control were duplicated. Concentrations of all the analytes in the

QCs were within the expected ranges and the inter-plate variation was below 20%. The data generated were managed using Bio-Plex Manager Software, version 4.1.1.

4.9. Glucocorticoid receptor and mineralocorticoid receptor gene expression via Real-Time-polymerase chain reaction (PCR)

The hippocampus tissues collected at 3 weeks, 6 weeks and 16 weeks were homogenised and total RNA was isolated using a ReliaPrep miRNA Cell and Tissue Miniprep System (Promega, Madison, WI, USA). The purity and concentration of RNA was determined by 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 ROCHE LightCycler96 (Roche, South Africa), the BIO-RAD iTaq Universal SYBR Green I master mix was used. Primer sequences (Metabion, Germany) used in this study are listed in Table 6 below. The Rattus primer sequences were blasted to verify the primer sequence and their accession number. PCR was performed using the following cycling conditions: an initial denaturation cycle at 95 °C for 10 min followed by PCR, which consisted of 45 cycles at 95 °C for 30 s, 65 °C for 30 s and a final elongation at 72 °C for 30 s with a single fluorescence measurement. Melting curve analysis was performed at 95 °C for 30 s, 65 °C for 20 s and 95 °C at a ramp rate of 0.05 °C/s and a continuous fluorescence measurement, followed by a final cooling step at 40 °C for 60 s. The RT-qPCR results were analysed using the $2^{-\Delta\Delta C_q}$ comparative method relative to the control groups. The housekeeping gene used in this study was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 5: List of primers used in the study.

Sequence Name	Sequence
Glucocorticoid receptor (Nr3c1 gene)	Forward: 5'-ACCTCGATGACCAAATGACC-3' Reverse: 5'-AGCAAAGCAGAGCAGGTTTC-3'
Mineralocorticoid receptor (Nr3c2 gene)	Forward: 5'-AAAGGGTAGTGTGTGCAGGG-3' Reverse 5'-GTTCTCCTAGTTCCCGGAGG-3'
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward: 5'-AGTGCCAGCCTCGTCTCATA-3' Reverse 5'-GATGGTGATGGGTTTCCCGT-3'

4.10. Statistical analysis

All data was expressed as means + standard error of means (SEM). A normality test was conducted, after which the data was analysed using GraphPad Prism InStat Software (version 8.00, GraphPad Software, San Diego, California, USA). The unpaired student t-test was used for parametric data and a Mann-Whitney test for non-parametric data. Other terminal parameters were analysed using a repeated measure analysis of variance (ANOVA) followed by a Dunnett's post-hoc Test to determine the statistical differences between the control and experimental groups. Values of $p < 0.05$ were considered significant, and corrected degrees of freedom statistics were reported where applicable.

5. Study Limitations and Future Recommendations

One of the limitations of this study is that we were unable to obtain dam placentas for the measurement of placental CRH and partial protective barrier of a glucocorticoid known as 11β -Hydroxysteroid Dehydrogenase 2 (11β -HSD 2) activity. This limitation arose because rats, like many other mammals, typically consume their placenta immediately after giving birth, a behaviour known as placentophagy. Future studies also need to look at the physiological response to stress in pups born from dams with pregestational prediabetes. We also recommend that future studies use hyperinsulinemic clamps for animals for better detection of insulin sensitivity.

6. Conclusions

In conclusion, the findings of this study showed that pregestational prediabetes may be associated with maternal dysregulated function of the HPA axis, as evidenced by the elevated ACTH and corticosterone concentrations in the dams. The findings of the study further show that maternal dysregulation in the HPA axis alters the set-point and development of the offspring's HPA axis, as evidenced by the elevated ACTH and corticosterone concentrations, impaired GR and MR gene expression and subsequently increased adrenal gland weights in non-stressed conditions in the offspring. In addition, this study shows that maternal HPA axis dysregulation is also associated with reduced foetal growth manifested as lower body weight and lack of catch-up growth during development. In addition, the offspring still exhibited catch-up growth-related detrimental effects such as impaired fasting glucose and glucose intolerance alongside insulin resistance, resembling features seen in T2DM and maternal stress studies. Moreover, offspring from prediabetic pregnancies may be at risk of early-onset prediabetes and mental health disorders associated with HPA axis hyperactivity, hypertension and T2DM in adulthood. As a result, foetal programming might also begin in prediabetic pregnancy. Moreover, due to the challenging nature of diagnosing prediabetes, this study on prediabetes during pregnancy could potentially uncover clinical indicators that are valuable in gaining a deeper understanding of the physiological changes that take place throughout pregnancy and their impact on foetal development. Therefore, monitoring maternal

periconceptional health status and conditions during pregnancy, especially for prediabetic females, may be of great importance.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, **Table S1: Systolic Blood Pressure**

The systolic blood pressure of pups born from the non-prediabetic (NPD) female group (n=6 per group) and pups born from the prediabetic (PD) female group (n=6 per group) at week 3, 6 and 16. The systolic blood pressure in the pups born from the PD group was significantly higher when compared to pups born from the NPD group in all the experimental weeks.

Table S1: Systolic Blood Pressure in the pups born from the non-pre-diabetic (NPD) female and pre-diabetic (PD) female group (n = 6, per group) at week 3, 6 and 16.

Groups (n=6)		Systolic pressure (mmHg)
Week 3	NPD	119.5 ± 0.230
	PD	122.2 ± 0.207 ***
Week 6	NPD	119.7 ± 0.202
	PD	122.4 ± 0.263***
Week 16	NPD	120.6 ± 0.365
	PD	128.7 ± 1.302 ***

Student T test was used for systolic analysis and the values were expressed as mean ± SEM. *** $p < 0.001$ denotes a comparison with NPD

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Conflicts of Interest

The authors declare no conflicts of interest.

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CHAPTER 4: SYNTHESIS AND CONCLUSION

The maternal hypothalamic-pituitary-adrenal (HPA) axis is composed of a cascade of neuroendocrine pathways that maintain physiological homeostasis and play a critical role in foetal development, notably the HPA axis [1, 2]. Homeostasis is primarily achieved through the control of circulating levels of glucocorticoid hormones, known as cortisol in humans or corticosterone in rodents, being the main downstream of glucocorticoid [3, 4]. Studies show that the foetal systems are dependent on maternal cortisol, making excess foetal exposure to maternal cortisol a crucial biological process in the foetal programming of the foetal HPA axis [5, 6]. In addition, evidence is emerging that a substantial part of diabetes susceptibility is acquired early in life, probably owing to foetal programming via epigenetic phenomena [7-11]. As a result, both maternal pregestational diabetes and maternal stress in pregnancies are proposed as contributing factors in foetal programming [12, 13]. For this reason, maternal and early childhood health might, therefore, be crucial to the development of effective prevention strategies.

T2DM is a complex endocrine and metabolic disorder characterised by insulin resistance and chronic hyperglycemia due to impairment in insulin action and/or insulin secretion [14-16]. This condition is regarded as a complicated and multifaceted disease caused by a mix of genetic and environmental risk factors such as over-consumption of high-caloric diets as well as decreased physical activity [17, 18]. The worldwide incidence of diabetes mellitus has been rising consistently for more than half a century, now reaching pandemic proportions and this increase has been associated with a higher number of pregnancies [19, 20]. Before the onset of T2DM, there exists a condition known as prediabetes, characterised by blood glucose concentrations that are higher than normal but do not meet the diagnostic criteria for T2DM and its onset is also due to high-caloric diets and sedentary lifestyles [21, 22]. However, prediabetes often goes unnoticed because it typically does not show any symptoms in its early stages. [23, 24]. As a result, many people unknowingly skip the prediabetes stage and progress directly to overt T2DM. However, there have been no studies to look at the effects this may have on the maternal HPA axis and on foetal development. Hence, in this study, we used a prediabetic-induced diet established and explored an animal model to investigate the effects of pregestational prediabetes on maternal HPA axis function and its effects on postnatal offspring development.

The first manuscript was adopted from the literature review which is focused on the dysregulation of the HPA axis function in maternal T2DM insults inclusive of maternal stress and explored potential links to pregestational prediabetes. The review demonstrated that T2DM complications, including HPA axis dysregulated function, have now been shown to begin in a prediabetic state evident in animal models [25]. The review also demonstrated that individuals with T2DM and in pregestational diabetes have elevated levels of cortisol alongside when compared to non-diabetic pregnant [26-32]. With the understanding that T2DM shares complications with prediabetes, such as dysregulation in HPA axis

function, the review revealed a possible link that pregestational prediabetes may also have an impact on the HPA axis function. However, the research has not yet been explored. In addition, the review demonstrated that there are several mechanisms involved in diabetic and maternal stress pregnancies, such as excess maternal glucocorticoid overwhelming the placental barrier as it is lipophilic and crosses over to the foetal compartment [27, 32-35]. Furthermore, various factors in pregnancy with type 2 diabetes (T2DM) have been demonstrated to worsen during pregnancy [36-38]. These factors include pre-existing heightened activity of the hypothalamic-pituitary-adrenal (HPA) axis, gestational hypertension, vascular diseases, renal disease and maternal microangiopathy, which are all associated with an increased risk of preeclampsia [39-46]. These conditions lead to delayed placental growth and reduced blood flow to the placenta, resulting in impaired placental function during pregnancy [47-53]. Placental dysfunction refers to any impairment or abnormality in the placenta's structure, function, or development during pregnancy [54]. The review further revealed that a recent animal study reported that prediabetes is a risk factor for preeclampsia, similar to diabetic pregnancy [55, 56]. Therefore, since prediabetes often goes undiagnosed, this suggested that there may be a high population of people unaware of their elevated risk of developing preeclampsia, a hypertensive disorder in pregnancy associated with placental dysfunction. Furthermore, placental dysfunction is also linked to a decrease in the activity of placental 11 β -HSD2, which allows excess active maternal glucocorticoids to cross from the maternal to the foetal compartment [57]. Excessive exposure to glucocorticoids during the development of a fetus has been shown to program the foetal HPA axis, permanently altering basal and stress-induced HPA axis activity and regulation in offspring throughout life thus increasing adult disease risk [58-60]. In maternal diabetes, excess cortisol alters the offspring's development of the brain's cortical neuroendocrine system, leading to decreased hippocampal neurons [12].

Furthermore, studies have demonstrated that diabetic pregnancy is linked to intrauterine growth restriction (IUGR) [61, 62]. This condition arises from various factors, such as impaired placental function, which compromises and decreases the uteroplacental unit to deliver oxygen and nutrients [63, 64]. Additionally, an excess of glucocorticoids triggers tissue differentiation, leading to reduced accumulation in the fetus and consequently slowing down the growth rate of organs, resulting in IUGR [65]. Offspring born with IUGR diagnosed as low birth weight are associated with high glucocorticoid notably heightened HPA axis function from the neonatal developmental stage to adulthood [66, 67]. Furthermore, both the Barker hypothesis and the DOHaD hypothesis demonstrate that infants with low birth weight resulting from maternal insults are prone to developing glucose intolerance, obesity, hypertension, cardiovascular illnesses, anxiety, depression and T2DM in adulthood, a phenomenon referred to as foetal programming [55, 68-70]. In conclusion, the review showed that with the increasing prevalence of prediabetes, especially in women of childbearing age, there is an increased possibility of pre-gestational, gestational and foetal outcome consequences. Therefore, the presence of prediabetes in pregnancy as a risk factor for preeclampsia might increase the likelihood of alteration in the HPA axis,

IUGR and impaired glucose tolerance in offspring. However, there have been no studies that have looked at prediabetic pregnancies' HPA axis function and changes in adverse foetal outcomes while also exploring their HPA axis function as they develop to adulthood, mimicking human developmental stages.

In our laboratory, a pre-diabetic animal model mimicking the human pre-diabetic state has been developed and used to observe the changes that may occur in pre-diabetes [71]. Therefore, for the second manuscript, we used the diet-induced prediabetic animal model to fulfil the review limitations and investigated maternal HPA axis function in prediabetic pregnancy and the associated changes in HPA axis function in offspring after birth, which may reflect maternal insults during utero as the offspring grows from neonatal to adulthood developmental stages.

This began with the induction of prediabetes in female rats by allowing chronic ingestion of a high-fat, high-carbohydrate diet for 36 weeks. This was confirmed as these rats exhibited glucose intolerance, elevated plasma insulin, elevated HbA1c, elevated HOMA-IR and reduced insulin sensitivity by comparison to the NPD group. After induction of prediabetes, mating and pregnancy were allowed in both the non-prediabetic (NPD) and prediabetic (PD) animals. At the end of pregnancy and on the weaning day, the dams measured plasma ACTH and corticosterone concentrations were measured via ELISA and Multiplex in the PD and NPD groups. The results have shown that the PD pregnant group had significantly higher ACTH and corticosterone concentrations when compared to the NPD pregnant group. These results, for the first time, concurred with the observations made by previous studies, which revealed that diabetic pregnant women exhibit higher cortisol concentration with exacerbated HPA axis dysfunction compared to non-diabetic pregnant women [72]. The elevated plasma ACTH and corticosterone levels may be due to the co-ordination between prolonged increased postprandial blood glucose and insulin resistance exacerbated by the compensatory mechanism failed to counteract glucocorticoid-induced insulin resistance in maternal prediabetes. Furthermore, the increased ACTH and corticosterone may have dysregulated the placental CRH positive feedback due to continuous maternal corticosterone, therefore feeding on the vicious cycle by increasing more of the maternal ACTH, thus elevated corticosterone. In addition, studies on maternal stress-dysregulated HPA axis associated with high basal corticosterone have been shown to overwhelm the partial barrier placental 11 β -HSD2 activity since studies show that it can be weakened by stress relative to high corticosterone [73]. This was an indication that, indeed, the pre-existing prediabetic state in pregnancy maintained the HPA axis dysregulation.

Moreover, as per the first manuscript, high maternal cortisol levels from maternal stress and diabetic pregnancies are linked to changes in the development of the brain's cortical, neuroendocrine system by reducing the number of hippocampal neurons as well as altering hippocampal glucocorticoid receptor

density and sensitivity, permanently altering the set-point and HPA axis regulation [58-60]. This led us to determine the concentrations of ACTH and corticosterone as well as the GR and MR genes involved in HPA axis negative feedback in offspring born from the PD group to elucidate whether offspring HPA axis function is impacted. In this study, plasma ACTH concentrations were measured via ELISA. Corticosterone concentrations were measured via a multiplex, GR and MR gene expressions were evaluated via RT-qPCR for both PD and NPD groups. The results showed that for the first time, PD group offsprings had significantly higher basal ACTH and corticosterone concentrations and dysregulated MR and GR gene expression as early as from the weaning period known as juvenile to emerging adulthood stages. The elevated ACTH and corticosterone concentrations in pups born from the PD group these observations confirm that maternal excess corticosterone in prediabetic pregnancy may have changed the foetal HPA axis development, reduced glucocorticoid sensitivity and led to persistent deficits of neurological function in offspring. In addition, the continuously elevated ACTH and corticosterone levels may have accounted for the impaired negative feedback receptors. The low GR and high MR gene expression would have been classified as normal in this study. However, the hippocampal GR is a typical neuron target sensitive and vulnerable to GC, whose over-activation would insult hippocampal neurons [74, 75]. As a result, the low GR gene expression while corticosterone levels are elevated implied that the GR protective effect of the glucocorticoid surge and lowering HPA axis detrimental effects is impaired. While the increased MR may have been a compensatory mechanism that was adopted from utero to cope with the consistently high levels of glucocorticoid exposure even after birth. Furthermore, an increased MR may have been due to the epigenetic modifications which may have affected the patterns of gene expression in utero persisted after birth [76, 77]. This study, for the first time, shows that there is an MR compensatory mechanism contradicting the other researchers; however, it may have been unsuccessful as the ACTH and corticosterone positively correlating with increased adrenal gland weight continued to rise as the offspring grew. Therefore, the prolonged increase in ACTH, corticosterone concentrations and impaired negative feedback in all developmental stages in our study may have resulted in HPA axis hyperactivity in PD offspring. This implied that pregestational prediabetes HPA axis dysregulation influenced offspring's early life events, evidenced by the persistent, permanent change in the HPA axis.

Glucocorticoid is known to be an important factor in insulin resistance and metabolic syndrome in adults [78, 79]. As a result, glucocorticoid exposure during development not only disturbs HPA axis development but also disturbs systems that regulate metabolic processes including glucose homeostasis, adipose function as well as growth [80-83]. Studies have shown that offspring exposed to GC tend to have lower birth weight due to high glucocorticoid triggering tissue differentiation and reducing accretion in the fetus, therefore reducing the overall rate of maturation and growth [65]. In addition, studies also show that low-birth offsprings tend to rapidly gain weight known as catch-up growth in the first 2 years of life which has been associated with greater risks for hypertension, T2DM development

and cardiovascular diseases [84-86]. This led us to measure rat body weights at various developmental stages, such as newborn (7 days), juvenile (week 3) and adolescent (week 6) to emerging adult (16 weeks).

Indeed, this study observed a low body weight in the newborns born from dams with prediabetes at newborn age. However, there was no catch-up growth in offspring born from the PD group at the juvenile stage (week 3) equivalent to 2 years in humans. This was followed by a no significant in bodyweight between week 3 and week 6 however there was a significant increase between week 3 and week 16 in PD pups. Therefore, we deduced that our study had no catch-up growth due to inconsistency in body weight. However, this inconsistency might have been due to a compensatory mechanism, as high cortisol levels without weight gain could suggest enhanced lipolysis in certain tissues, reducing fat accumulation. In both animal and human studies, birth weight is a crude indicator of antenatal exposures; therefore, low body weight in our study may indicate that, indeed, there was an unfavourable environment in utero and one of the influences may have been the excess maternal corticosterone in the study. Low birth weight is still one of the greatest problems faced by perinatal medicine therefore research is still needed to explore other mechanistic factors that may be involved [87, 88].

Researchers have argued that low birth weight offspring that have no catch-up growth have a diminished risk of metabolic disorders [89]. However, there are studies that have shown that glucocorticoid exposure during development also disturbs the metabolic pathway, such as organs that play a role in glucose metabolism [82, 83]. In addition, studies have shown that a hallmark feature of metabolic syndrome, insulin resistance and resultant hyperinsulinism, is commonly present in hypercortisolaemic states [90, 91]. Interestingly, in this study, the pups born from the PD group showed impaired fasting glucose and glucose intolerance alongside high AUC values, insulin resistance, increased pancreatic β -cell and reduced insulin sensitivity, suggesting that there was impairment in insulin-mediated glucose uptake peripheral tissues such as skeletal muscle, thus inducing diabetogenic effects and insulin resistance. Furthermore, the observed elevated corticosterone, insulin resistance, increased pancreatic β -cell and reduced insulin sensitivity alongside glucose intolerance implies that the compensatory mechanism failed to counteract glucocorticoid-induced insulin resistance, resulting in hyperglycaemia observed in the study. These observations suggest that, indeed, hyperactivity or dysregulation in the HPA axis function is associated with glucose intolerance and insulin resistance. Consequently, elevated levels of corticosterone may have also played a role in the exacerbation of elevated blood glucose levels and the cycle of increased HPA axis activity in this study. Moreover, research indicates that offspring with normal glucose tolerance may exhibit insulin resistance, which could be linked to increased body weight [92]. Remarkably, within the NPD group, we noticed a prompt development of insulin resistance at week 16, accompanied by increased pancreatic β -cell activity, which may have been in line with the increasing levels of insulin and corticosterone compared to the other weeks. We hypothesised that the

observed initiation of insulin resistance in the control group could be attributed to the progressive rise in body weight and sedentary lifestyle linked to the elevated levels of corticosterone and insulin, as compared to the other weeks.

In summary, this study, for the first time, revealed that pregestational prediabetic pregnancy is related to maternal insults including glucocorticoid detrimental effects that contribute to adverse foetal outcomes HPA axis hyperactivity relative to maternal diabetes and stressed pregnancies. Moreover, for the first time, this study was able to compare developmental stages in rats to humans alongside changes in HPA axis function, body weight and glucose handling. Thus, the results of this study indicate that the metabolic abnormalities caused by prediabetes during pregnancy make the offspring more susceptible to developing metabolic diseases in the future.

Conclusion

This Study 2 showed that maternal prediabetes and offspring are associated with constantly activated HPA axis function, which may have been exacerbated by the presence of glucose intolerance and insulin resistance, a hallmark of metabolic disorders. These observations suggest that despite non-stressed conditions, prediabetes in pregnancy alters the foetal HPA axis set points and development that is persistent even after birth correlative to maternal stress and diabetic pregnancies. This study further showed that complications in pregnancy and foetal adverse outcomes such as HPA axis hyperactivity, reduced growth development and glucose handling disturbances relative to foetal programming might also begin in prediabetic pregnancy. Moreover, due to the challenging nature of diagnosing prediabetes, this study on prediabetes during pregnancy could potentially uncover clinical indicators that are valuable in gaining a deeper understanding of the physiological changes that take place throughout pregnancy and their impact on foetal development. This information would be highly valuable for generating new strategies and awareness, ultimately preserving the quality of life for both mother and child.

Future recommendations

We recommend future studies to investigate the placental CRH and the partial protective barrier of a glucocorticoid known as 11 β -HSD 2 activity while implementing techniques and tests that would not disturb the natural benefits of placentophagy. In addition, we recommend that future studies assess the development of rats using a range of tests, such as body length measurement, motor skills and coordination evaluations, cognitive and learning assessments, anxiety and stress tests, muscle strength tests like grip strength, endocrine and metabolic tests. Each of these tests offers valuable insights into various aspects of rat development and using multiple tests together may often provide a more complete understanding of their developmental progress.

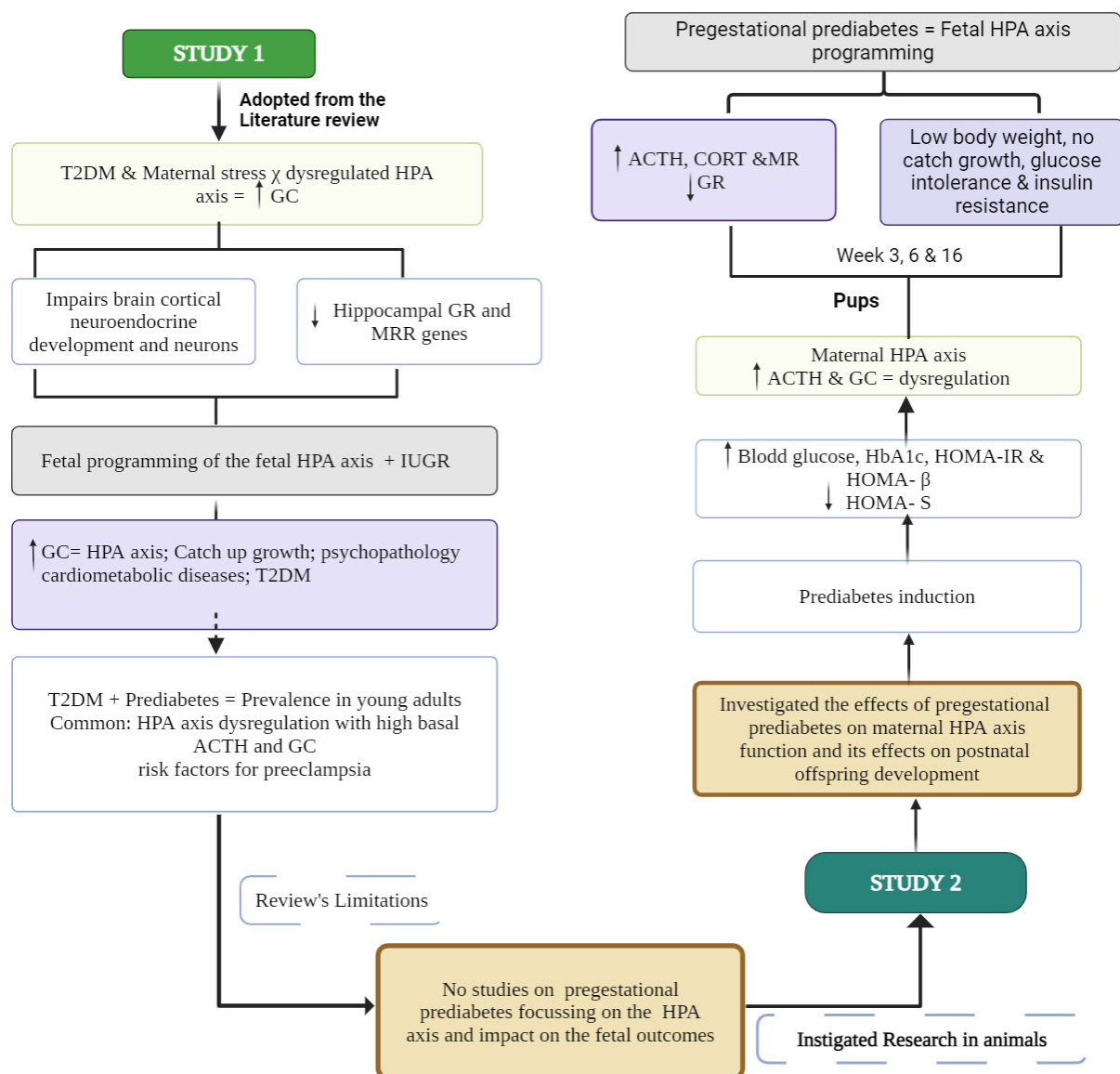


Figure 1: Diagram illustration of pregestational prediabetes HPA axis dysregulation and impacts on the foetal HPA axis and development evident after birth.

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APPENDICES

APPENDIX 1: ETHICAL CLEARANCE



19 April 2022
Ms Nombuso Xulu (215019278)
School of Laboratory Medicine and Medical Sciences
Westville Campus

Dear Ms Xulu,

Protocol reference number: AREC/032/020D

Project title: Effects of Oleanolic Acid (OA) on the developmental and functional changes in the blood, hearts, and brains, of the offspring born from preeclamptic-prediabetic female Sprague Dawley rats.

Full Approval – Research Application

With regard to your report on the number of pups born received on 12 April 2022, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

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

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

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

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

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 15 March 2023.

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

Yours faithfully



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

/kr

cc Supervisor: Dr Phikelelani Ngubane
cc BRU Manager: Dr Jaca

Animal Research Ethics Committee (AREC)

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APPENDIX 2: JOURNAL GUIDELINES

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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.

2. 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.

2.1. *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.

2.1.1. 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.

2.1.2. 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.

2.1.3. 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.

2.1.4. 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.

2.1.5. 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.

2.1.6. 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.

2.1.7. 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.

2.1.8. 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.

2.1.9. 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.

2.1.10. 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.

2.1.11. 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.

2.1.12. 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.

2.1.13. 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.

2.1.14. 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.

2.1.15. 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.

2.1.16. 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.

2.1.17. 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.

2.1.18. 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](#).

2.2. 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:

2.3. 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.

2.4. 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 include 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.

2.5. 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.

2.6. 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>.

2.7. 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).

2.8. 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.

3. 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.

3.1. *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.

3.2. *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.

3.3. 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.

3.4. 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)	\\

3.5. 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	electron 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.

3.6. *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.

3.7. *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.

3.8. *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.

3.9. *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.

3.10. 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.

3.11. *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.

4. Grammar and Tenses

4.1. *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.

4.1.1. 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.”

4.1.2. 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 . . .”

4.1.3. 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.

4.1.4. 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>.)

4.2. 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”.

4.3. 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.

4.4. 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.

5. 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.

5.1. 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.

5.2. 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.

5.3. *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.

5.4. 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.

5.5. 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.”

6. Numbers and Mathematical Environments

6.1. 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.

6.2. 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:

$3 \times 10^8 \text{ m}\cdot\text{s}^{-1}$.

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

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	deka	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

6.3. 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.

6.4. 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., $\cos \Theta$, $\cot \Theta$, $\sin \Theta$, $\tan \Theta$, $\sec \Theta$, $\csc \Theta$, etc.

6.5. 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,

$$P(x) = 2a \text{ if } x > 0.$$

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.

6.6. *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.

7. 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.

7.1. 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.

7.2. 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.

7.3. 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. Boxes should be editable; do not use uneditable images.

7.4. 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.

7.5. 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.

7.6. 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.

7.7. 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:

Format 1 (ACS Style)

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

For example,

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Appendix

References

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

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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).

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8.11.1. 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.

8.11.2. 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.

9. 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.

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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.

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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.

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APPENDIX 3: DIET COMPOSITIONS

Table 1: Composition of the high fats high carbohydrates (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 2: Nutritional value of the high fats high carbohydrates (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 3: Composition of fats, proteins, and carbohydrates of the standard diet.

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

1. Chapman K, Holmes M, Seckl J. 11β -hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action. *Physiological reviews*. 2013;93(3):1139-206.
2. Koorneef LL, Viho EM, Wahl LF, Meijer OC. Do corticosteroid receptor mRNA levels predict the expression of their target genes? *Journal of the Endocrine Society*. 2023;7(2):bvac188.
3. Brunton P. Resetting the dynamic range of hypothalamic-pituitary-adrenal axis stress responses through pregnancy. *Journal of neuroendocrinology*. 2010;22(11):1198-213.
4. Sheng JA, Bales NJ, Myers SA, Bautista AI, Roueifar M, Hale TM, et al. The hypothalamic-pituitary-adrenal axis: development, programming actions of hormones, and maternal-foetal interactions. *Frontiers in behavioral neuroscience*. 2021;14:256.