

**High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia:
Assessing gender-specific infantile Sprague Dawley outcomes on selected markers of
cardiovascular, haematological and cognitive function**

By

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PREFACE

Foetal programming, occurring during embryonic development, plays a critical role in shaping lifelong health outcomes. Among the various maternal health conditions that can influence foetal development, and infantile outcomes, pregestational prediabetes (PP), a precursor for pregestational type 2 diabetes (pT2D), has emerged as a significant risk factor for preeclampsia (PE), a hypertensive disorder during pregnancy. Prediabetes, characterized by intermediate hyperglycaemia, often precedes type 2 diabetes mellitus and shares many of its early complications. Despite being an asymptomatic state, prediabetes poses substantial risks to maternal and foetal health, necessitating comprehensive research into its effects. In our laboratory a high-fat, high-carbohydrate

(HFHC) diet-induced prediabetic rat model that replicates the modern high calorie, sedentary lifestyle which is prevalent in the 21st century. This rat model was used in this thesis to investigate a series of studies that delves into the multifaceted impacts of PP as a risk factor for preeclampsia on infantile outcomes, focusing on three critical physiological systems: the heart the blood, and the brain. By exploring selected markers within these systems, we aim to elucidate the mechanisms through which maternal prediabetes influences foetal health and development. This series of studies underscores the critical importance of managing PP in pregnant women to prevent the onset and progression of pT2D and PE, thereby safeguarding infant health. The findings from these studies are expected to contribute significantly to the body of knowledge on maternal-foetal health, offering potential pathways for early detection, prevention, and intervention strategies to mitigate the risks and improve outcomes for both mothers and their children. The experimental work described in this thesis was conducted at the University of Kwa- Zulu Natal, Westville Campus, Durban, South Africa, under the supervision of Dr Phikelelani Ngubane and co-supervised by Prof Andile Khathi and Prof Ntethelelo Sibiya.

DECLARATION

I, **Nombuso Duduzile Xulu** hereby declare that the thesis entitled:

“High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cardiovascular, haematological and cognitive function.” is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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PLAGIARISM DECLARATION

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

DOCTORATE DEGREE IN MEDICAL SCIENCES 2024

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.
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LIST OF PUBLICATIONS

The publications are divided as follows: A) Publications emanating from this dissertation, B) Manuscripts from this dissertation under review, and C) Other manuscripts written during PhD.

A) Publications emanating from this dissertation

1. **Xulu N.D, Khathi A, Sibiya N.H and Ngubane P.S. High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of haematological function**, is accepted for publishing in the *Journal of Diabetes, Metabolic Syndrome and Obesity* (ISSN: 1178-7007) and has been formatted according to the journal's guidelines for authors <https://www.dovepress.com/author-guidelines/manuscript-structure>.

B) Manuscripts from this dissertation under review

1. **Xulu N.D, Khathi A, Sibiya N.H and Ngubane P.S (2024). High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cardiovascular function.** *American Journal of Physiology-Heart and Circulatory Physiology* (REF: H-00835-2024)
2. **Xulu N.D, Khathi A, Sibiya N.H and Ngubane P.S (2024). High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cognitive function.** *Heliyon* (REF: HELIYON-D-24-44053R1)

C) Other manuscripts written during PhD

1. Dayanand Y, Pather R , **Xulu N.D**, Booysen I, Sibiya N, Khathi A, Ngubane P. **Exploring the Biological Effects of Anti-Diabetic Vanadium Compounds in the Liver, Heart and Brain.** *Journal of Diabetes, Metabolic Syndrome and Obesity*.
<https://doi.org/10.2147/DMSO.S417700>
2. Ludidi A, Siboto A, Nkosi A, **Xulu N.D**, Ngubane P.S and Khathi A. **High-fat, high-carbohydrate diet-induced prediabetes preconception in Sprague-Dawley rats as a risk factor for the development of preeclampsia: assessing changes in placental metabolic insults.** *Frontiers in Nutrition*.
<https://doi.org/10.3389/fnut.2023.1241785>
3. 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*.
<https://doi.org/10.3390/ijms25105431>.
4. Ngema M, **Xulu N.D**, Ngubane P.S and Khathi A. **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.** *Journal of Biomedicines*.
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LIST OF PRESENTATIONS

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DEDICATION

This work is dedicated to Jesus, God Almighty, and my wonderful, supportive parents.

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LIST OF ABBREVIATIONS

ADHD	Attention Deficit Disorder
AKT	Protein Kinase B
ANOVA	Analysis of Variance
AREC	Animal Research Ethics Committee
ASD	Autism Spectrum Disorder
BDNF	Brain-Derived Neurotrophic Factor
BRU	Biomedical Research Unit
cDNA	Complementary Deoxyribonucleic Acid

cTnI	Cardiac Troponin I
CI	Cephalization Index
COL I	Collagen I
CON	Normal Control
ELISA	Enzyme-Linked Immunoassay
EPO	Erythropoietin
ERE	Oestrogen Response Elements
FGFR	Fibroblast Growth Factor Receptor
FGF2	Fibroblast Growth Factor-2
FPG	Fasting Plasma Glucose
GAPDH	Glyceraldehyde-3-Phosphate
G-CSF	Granulocyte Colony-Stimulating Factor
GND	Gestational Day
GPx	Glutathione Peroxidase
Hgb	Haemoglobin
HFHC	High-Fat High-Carbohydrate
HIFs	Hypoxia-Inducible Factors
HIF- α	Hypoxia-Inducible Factors
IUGR	Growth Restriction
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
L-NAME	L-NG-Nitro Argine Methyl Ester
MAP	Mean Arterial Pressure
MAPK	Mitogen-Activated Protein Kinase
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Cell Volume
MDA	Malondialdehyde
mRNA	Messenger Ribonucleic Acid
mTOR	Mechanistic Target of Rapamycin
MWM	Morris Water Maze
NE	Neutrophil
NF- κ B	Nuclear Factor-Kappa B
OGTT	Oral Glucose Tolerance Test
PCON	Pups Born From Normal Control
PCR	Polymerase Chain Reaction

PE	Preeclampsia
PND	Post-Natal Day
PP	Pregestational Prediabetes
PPD	Pups Born From HFHC Diet-Induced Prediabetic Dams
PPE	Pups Born From L-NAME-Induced Preeclamptic Dams
pT2D	Pregestational Type 2 Diabetes
PLT	Platelet
PU	Protein Urea
RBC	Red Blood Cell
Ras-MAPK	Rat Sarcoma-Mitogen-Activated Protein Kinase
RDW	Red Blood Cell Distribution Width
ROS	Reactive Oxygen Species
SEM	Means $\pm$ Standard Error of Mean
sFlt-1	Soluble Fms-Like Tyrosine Kinase 1
SOD	Superoxide Dismutase
TCs	Cholesterol
TGs	Triglycerides
TGF- β	Transforming Growth Factor- β
TNF- α	Tumour Necrosis Factor-Alpha
TrkB	Tropomyosin Receptor Kinase
UKZN	University of KwaZulu-Natal
WBC	White Blood Cell
WHO	World Health Organization

STUDY OUTLINE

The current thesis is presented in manuscript format, consisting of 6 sections viz. chapter 1: introduction chapter 2: literature review, chapter 3: methodology, chapter 4: prologue, abstract and manuscript 1, chapter 5: prologue, abstract and manuscript 1, chapter 6: prologue, abstract and manuscript 1, chapter 7: synthesis and appendices. Chapter 1 contains the introduction of the study. Chapter 2 gives a brief background and literature review of the current knowledge reported as well as current gaps in the field. It further shows how the study aims to fill in the gaps. Chapter 3 contains the comprehensive methodology of the study. Chapter 4 contains the first research study in manuscript format that seeks to investigate the effects of diet-induced prediabetes on gender-specific cardiovascular outcomes in Sprague Dawley rat offspring. This work is authored by N.D. Xulu under the supervision of Dr P.S. Ngubane, Prof A. Khathi and N.H. Sibiyi, and has been submitted for publication in the *American Journal of Physiology-Heart and Circulatory Physiology* and formatted according to journal's guidelines to authors. Chapter 5 is the second research study in the manuscript format that sought to investigate the effects of diet-induced prediabetes on gender-specific haematological outcomes in Sprague Dawley rat offspring. The manuscript is authored by N.D. Xulu under the supervision of Dr P.S. Ngubane, Prof A. Khathi and N.H. Sibiyi has been accepted for publication in *Journal of Diabetes, Metabolic Syndrome and Obesity*. Chapter 6 is the third research study in manuscript format that sought to investigate the effects of diet-induced prediabetes on gender-specific cognitive outcomes in Sprague Dawley rat offspring. The manuscript is authored by N.D. Xulu under the supervision of Dr P.S. Ngubane, Prof A. Khathi and N.H. Sibiyi and has been submitted for publication in *Heliyon* and formatted according to the journal's guidelines to authors. Chapter 7 is the synthesis of the study with the aim of linking the three studies and illustrating how they filled the gaps in knowledge as well as the appendices which consist of the ethical clearance certificate, journal acceptance email, journal's guideline to authors and HFHC diet composition.

ABSTRACT

Introduction

Pregestational prediabetes (PP), a precursor to type 2 diabetes and a risk factor for preeclampsia, profoundly affects the intrauterine environment, impairing cardiovascular, haematological, and cognitive development in offspring. This study investigates the impact of a high-fat, high-carbohydrate (HFHC) diet-induced prediabetic model on gender-specific infantile cardiovascular, haematological, and cognitive outcomes in Sprague-Dawley rat offspring.

Methodology

Pups were born from dams with HFHC diet-induced prediabetes and L-NAME-induced preeclampsia (n=72) were divided into three groups: control (PCON), prediabetic (PPD), and preeclamptic (PPE). Offspring from each maternal group were divided into 2 subgroups. One subgroup was sacrificed at postnatal day 21 (n=36) for biomarker analysis which included BDNF and FGF2, while the second was retained until week 5 (n=36) for behavioural testing followed again by biomarker analysis specifically BDNF and FGF2. Cardiovascular parameters included mean arterial pressure (MAP), cardiac troponin I (cTnI), collagen I (COL I), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx). Cardiac tissue histology assessed cardiomyocyte size. Haematological evaluations included red blood cell markers [erythropoietin (EPO), red blood cell (RBC) count, haemoglobin (Hgb)], white blood cell markers [white blood cell count (WBC), granulocyte colony stimulating factor (G-CSF)], and platelet markers (platelet count). Neurodevelopmental assessments measured brain-derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF2), and inflammatory cytokines (IL-6, IL-1 β , TNF- α). Cognitive performance was evaluated using the Morris Water Maze (MWM) in the remaining pups (n=36) at five weeks.

Results

Cardiovascular biomarkers such as cTnI, COL I, and MAP were significantly elevated in PPD and PPE groups compared to PCON, with PPD showing the highest levels. Antioxidant enzymes (SOD, GPx) were significantly lower in PPD. Histological analysis revealed cardiomyocyte enlargement, particularly in PPD. In the haematological assessments, red blood cell indices were elevated in the PPD group, whereas haemoglobin concentration was reduced. White blood cell markers were higher in PPD, indicating inflammation. Platelet markers were altered in PPD, reflecting haemostatic disruption. Neurodevelopmental markers, including reduced BDNF and FGF2, were observed in PPD and PPE, alongside increased inflammatory cytokines. Male offspring showed more pronounced deficits than female offspring. Cognitive impairments assessed via MWM were more severe in PPD, correlating with altered hippocampal gene expression.

Conclusion

In this study, the murine/rat model showed that PP exacerbates foetal dysfunction via intermediate hyperglycaemia, oxidative stress and inflammation highlighting the need for early, gender-specific

interventions to mitigate long-term impacts in prediabetic pregnancies.

CHAPTER 1: INTRODUCTION

Maternal metabolic health during pregnancy is a critical determinant of foetal development and lifelong health outcomes (1). In the first trimester, maternal spiral arteries are not yet fully remodelled by invading extravillous trophoblasts, resulting in limited maternal blood flow to the intervillous space and thus maintaining a low oxygen tension of approximately 2–3% O₂, compared to 8–10% in later gestation (1,2). This hypoxic state plays a beneficial role by stabilising hypoxia-inducible factors (HIFs), particularly HIF-1 α , which regulate the transcription of genes responsible for angiogenesis (such as vascular endothelial growth factor, VEGF), erythropoiesis, and glycolytic metabolism (3,4). These adaptations support trophoblast proliferation, placental vascularisation, and protection of the embryo from oxidative stress that could occur if high oxygen exposure were introduced prematurely (5). Therefore, early pregnancy hypoxia can be termed as regulated/controlled, as it acts as a protective and adaptive mechanism essential for normal placental morphogenesis and foetal development. Prediabetes, a precursor to type 2 diabetes, is characterized by intermediate hyperglycaemia and insulin resistance, which elevate the risk of PE and thus maternal and infant morbidity and mortality (6, 7). Given the increasing global prevalence of prediabetes among women of reproductive age, understanding the gender-specific impacts of maternal prediabetes on offspring is essential to inform strategies for mitigating intergenerational health risks (8-10). However, despite these insights, the impact of pregestational prediabetes (PP) on gender-specific outcomes in offspring remains under-explored (11, 12).

Our study employs a high-fat, high-carbohydrate (HFHC) diet to induce prediabetes in a rat model, which closely mimics the dietary patterns associated with modern human lifestyles (12). In South Africa, socioeconomic factors play a critical role in shaping dietary habits and metabolic health (13). The HFHC diet mirrors the prevalence of cheap, calorie-dense, and nutrient-poor foods such as pap, vetkoek, fried chicken, and processed meats, which are staples for many low-income households (13-15). Limited financial resources and high unemployment rates (estimated at approximately 32.6% in 2024) often force families to prioritize affordability over nutritional value (15, 16). The burden of low-income jobs with extended working hours and limited benefits exacerbates these issues, leaving little time for physical activity or meal planning (16). Unhealthy dietary practices and sedentary lifestyles create a vicious cycle of metabolic dysfunction that predisposes individuals to prediabetes and T2D, especially in urban and peri-urban communities (17). Moreover, teenage pregnancy is a significant concern in South Africa, with over 150,000 girls aged 10 to 19 reported pregnant in the 2022/2023 period (18). Due to these high rates of teenage pregnancy, the risk of developing complications such as PE and pT2D is elevated in young mothers (17-19). Given that prediabetes is a precursor to these disorders, understanding its progression and impact during pregnancy is imperative (6, 7, 20). This highlights the need for research into prediabetes to inform interventions and policies that address these

interconnected health and socioeconomic challenges (20, 21).

Furthermore, the importance of gender-specific studies has been emphasized by organizations such as the World Health Organization (WHO) and the National Institutes of Health (NIH), as males and females exhibit distinct susceptibilities to metabolic and inflammatory changes during foetal development (22, 23). These differences highlight the need for targeted interventions that address the unique physiological needs of each gender (22, 23). Additionally, South Africa's commitment to achieving sustainable development goals prioritizes maternal and foetal health as a cornerstone for improving population health outcomes (24). By examining the effects of PP on foetal development, this study aligns with both global and national efforts to enhance maternal health, reduce perinatal complications, and inform strategies for early intervention (25). Thus, the aim of this study is to investigate high-fat, high-carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia, assessing gender-specific infantile outcomes on cardiovascular, haematological, and cognitive function in Sprague-Dawley rats. This research aims to elucidate the mechanisms underlying these developmental disparities and therefore add to the scientific pool of knowledge that seeks to improve maternal and foetal health outcomes globally (24, 25).

1.2 Aim:

1.3 The aim of this study was to investigate high-fat, high-carbohydrate (HFHC) diet-induced pregestational prediabetes as a maternal risk factor for the development of a preeclampsia-like phenotype, and to determine its subsequent effects on gender-specific infantile cardiovascular, haematological, and cognitive outcomes in Sprague-Dawley rats

1.4 . Research Questions:

1.4.1 What are the effects of HFHC diet-induced prediabetes on gender-specific differences in infantile cardiovascular function?

1.4.2 What are the effects of HFHC diet-induced prediabetes on gender-specific differences in infantile haematological function?

1.4.3 What are the effects of HFHC diet-induced prediabetes on gender-specific differences in infantile cognitive function?

1.5 Objectives:

The objectives for each of the respective research questions stated in 1.3.1; 1.3.2 and 1.3.3 are stated below. The objectives for research question 1.3.1 are addressed under manuscript 1, while the objectives for 1.3.2 and 1.3.3 are addressed in manuscript 2 and manuscript 3 respectively

1.5.1 Manuscript 1 objectives

- To induce prediabetes in female Sprague-Dawley rats using a high-fat, high-carbohydrate diet.
- To induce preeclampsia in female Sprague-Dawley rats using L-NAME.
- To assess changes in glucose homeostasis in the dams by measuring fasting blood glucose, the oral glucose tolerance test in the blood to diagnose prediabetes.
- To assess changes in blood pressure of the dams by measuring mean arterial pressure and protein urea concentration in the urine to diagnose preeclampsia.

- To measure HIF- α in the placenta to assess placenta hypoxia
- To measure changes to the heart-to-body weight ratio of male and female offspring born from mothers with prediabetes
- To measure changes to the mean arterial pressure (MAP) and plasma lipid profile (TG and TC) of male and female offspring born from mothers with prediabetes
- To measure changes to the cardiac injury marker cardiac troponin I (cTnI) in plasma of male and female offspring born from mothers with prediabetes
- To measure changes to the oxidative stress markers (MDA, SOD and GPx) in plasma of male and female offspring born from mothers with prediabetes
- To measure changes to Left cardiac ventricular histology of male and female offspring born from mothers with prediabetes

L-NAME Rational

1.5.2 Manuscript 2 objectives

- To measure changes to the haematological profile (RBC count, Hgb, MCV, MCH, MCHC, WBC count, neutrophil count and platelet count) in whole blood of male and female offspring born from mothers with prediabetes

1.5.3 Manuscript 3 objectives

- To measure changes to the brain-to-body weight ratio of male and female offspring born from mothers with prediabetes
- To measure changes to the cephalization index of male and female offspring born from mothers with prediabetes
- To measure changes to neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and fibroblast growth factor-2 (FGF2) in the hippocampus of male and female offspring born from mothers with prediabetes
- To measure changes to pro-inflammatory markers such as interleukin-6 (IL-6), tumour necrosis alpha (TNF- α) and interleukin-1 beta (IL-1 β) in the hippocampus of male and female offspring born from mothers with prediabetes

1.6 Hypotheses:

1.6.1 Offspring of prediabetic mothers will exhibit significant alterations in cardiovascular, haematological, and cognitive function, with gender-specific differences in the extent of these alterations.

1.6.2 Male offspring will show more pronounced cardiovascular, haematological and cognitive function impairments as a result of maternal prediabetes and preeclampsia.

1.7 Null hypotheses

1.7.1 Offspring of prediabetic mothers will not exhibit significant alterations in cardiovascular, haematological, and cognitive function, with gender-specific differences in the extent of these

alterations.

1.7.2 Male offspring will not show more pronounced cardiovascular, haematological and cognitive impairments as a result of maternal prediabetes and preeclampsia.

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CHAPTER 2: LITERATURE REVIEW

High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cardiovascular, haematological and cognitive function

2. Background

Foetal programming refers to the process by which conditions during pregnancy shape the development and health of the foetus, influencing outcomes into adulthood (1). In healthy pregnancies, the maternal environment supports optimal growth, ensuring that the foetal circulatory system, heart, and brain develop without restriction (2). The foetal heart undergoes precise structural and functional changes that enable it to meet the needs of a rapidly growing body, while the brain, fueled by a well-regulated blood supply, undergoes crucial neurodevelopmental stages (3, 4). Adequate blood flow, oxygenation, and nutrient transfer across the placenta are essential for maintaining foetal growth within regulated limits (5). However, deviations from this norm, such as those seen in pregnancies complicated by conditions like preeclampsia and diabetes, can have lasting consequences (6, 7).

In pregestational type 2 diabetes (pT2D) foetal programming is significantly influenced, disrupting the development of the blood, heart, and brain (6-8). Maternal hyperglycaemia and insulin resistance lead to altered vascular function, increased oxidative stress, and systemic inflammation, which negatively impact foetal haematological, cardiovascular, and neurological systems (7, 8). These pathogenic mechanisms are closely linked to the development of preeclampsia, where endothelial dysfunction and maternal inflammation are central aetiological mechanisms (6-8). Therefore, pT2D enhances the risk of developing preeclampsia by exacerbating vascular and inflammatory dysregulation, which are key pathological processes in both conditions (6, 7). Preeclampsia (PE), characterized by hypertension and organ dysfunction, often leads to intrauterine growth restriction (IUGR) and small-for-gestational-age (SGA) infants due to poor placental perfusion and hypoxia (6). Similarly, in pT2D, excessive glucose transfer to the foetus induces hyperinsulinaemia, macrosomia, and, paradoxically, IUGR, which is driven by placental dysfunction, hypoxia and impaired nutrient exchange (7, 8). The complications to inflammation and oxidative stress regulation in both conditions set the stage for long-term haematological, cardiovascular and neurodevelopmental issues through shared pathways of placental insufficiency and altered nutrient exchange (6-8).

Moreover, type 2 diabetes is often preceded by prediabetes, a condition characterized by impaired glucose tolerance and insulin resistance, which progressively escalates to full-blown type 2 diabetes if left unmanaged (9). Prediabetes not only serves as a precursor to pT2D but also increases the risk of developing PE (10). As such, prediabetes heightens the likelihood of maternal and foetal morbidity and mortality by contributing to the pathophysiology of both pT2D and PE, which are associated with significant health complications during pregnancy (9, 10). Furthermore, the rising global prevalence of

prediabetes among women of childbearing age highlights the importance of understanding how maternal metabolic health can influence foetal development (11). Therefore, studies investigating the mechanisms of prediabetes during pregnancy and its impact on maternal and foetal development are crucial (10, 11). Understanding prediabetes in pregnancy can help identify ways to prevent its onset, detect early markers of foetal developmental dysfunction, and provide insights into potential interventions to reduce its progression (11, 12). By targeting prediabetes, we can lower the risk and prevalence of pT2D and PE, which are both associated with adverse pregnancy outcomes (11, 12). Early identification and management of prediabetes may lead to improved maternal health, reduced foetal complications, and better long-term health outcomes for both mother and child, ultimately reducing the incidence of type 2 diabetes in pregnancy and related complications such as PE and gestational hypertension (11-13). Moreover, it is crucial to consider gender-specific outcomes in offspring when studying disorders of pregnancy (14). Males and females exhibit distinct physiological responses to metabolic, oxidative and inflammatory changes in pregnancy due to differences in genetic, hormonal and metabolic influences (14). These variations can cause disturbances to haematological, cardiovascular and cognitive function in infants (15). Therefore, this study aims to investigate high fat high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of haematological, cardiovascular, and cognitive function.

2.1 Foetal programming and development

Foetal programming refers to the process by which environmental factors during pregnancy influence foetal development and can have lasting effects on the offspring's physiology and health throughout their life (18). This process is shaped by maternal factors such as nutrition, stress, and hormonal changes, which interact with the foetal genome to influence developmental trajectories. Understanding foetal programming mechanisms and foetal development is essential for identifying potential interventions to mitigate adverse outcomes in offspring (1, 18). This literature review will explore the impact of foetal programming on haematological, cardiovascular, and neurological systems, examining early-life influences that may shape gender-specific blood, heart, and brain function in offspring.

2.2 Healthy Pregnancy

A healthy pregnancy is essential for setting the foundation for a child's health, growth, and development throughout life (1, 2). From the moment of conception, a complex series of events unfolds to support the developing foetus, and each step is carefully regulated to meet the growing demands of foetal development (1, 2). Central to this process is the formation of the placenta, an organ that provides the developing foetus with oxygen, nutrients, and a stable environment (1-3). The early stages of pregnancy occur in a low-oxygen state, and this controlled hypoxia plays an important role by activating hypoxia-inducible factor 1-alpha (HIF-1 α) (1-4). This protein responds to low oxygen levels and triggers genes

responsible for placental growth, particularly trophoblast invasion, which is when placental cells embed into the uterine wall and remodel the maternal arteries to increase blood flow to the developing foetus (4). Inflammatory markers also contribute to placental development, but they do so in an intricately regulated manner (4). Pro-inflammatory cytokines like IL-6 and TNF- α play critical roles during the initial stages by supporting trophoblast invasion and vascular remodelling. However, as pregnancy progresses, there's a shift toward an anti-inflammatory environment to maintain placental function and avoid potential immune rejection (4,5). Furthermore, angiogenic factors like vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) further ensure that the placenta develops a strong vascular network, which is essential for oxygen and nutrient exchange (4, 5). Together, HIF-1 α , inflammatory markers, and angiogenic factors work in concert to create a placental environment that can adapt to the foetus's increasing demands as it grows. The health and function of the placenta are directly linked to foetal development (1-4). An optimal placental environment allows for proper blood cell lineage formation, as the developing foetus needs a steady oxygen and nutrient supply to produce red and white blood cells critical for immune function and oxygen transport (1-4). In turn, these resources enable robust neurogenesis and synaptogenesis in the foetal brain, laying the groundwork for cognitive development and mental resilience (1-5). Adequate oxygen and nutrient supply also support cardiac development, guiding the formation of the heart's chambers, valves, and vascular structures to sustain the infant post-birth (2-5). Figure 1 below summarises the events of pregnancy and foetal development.

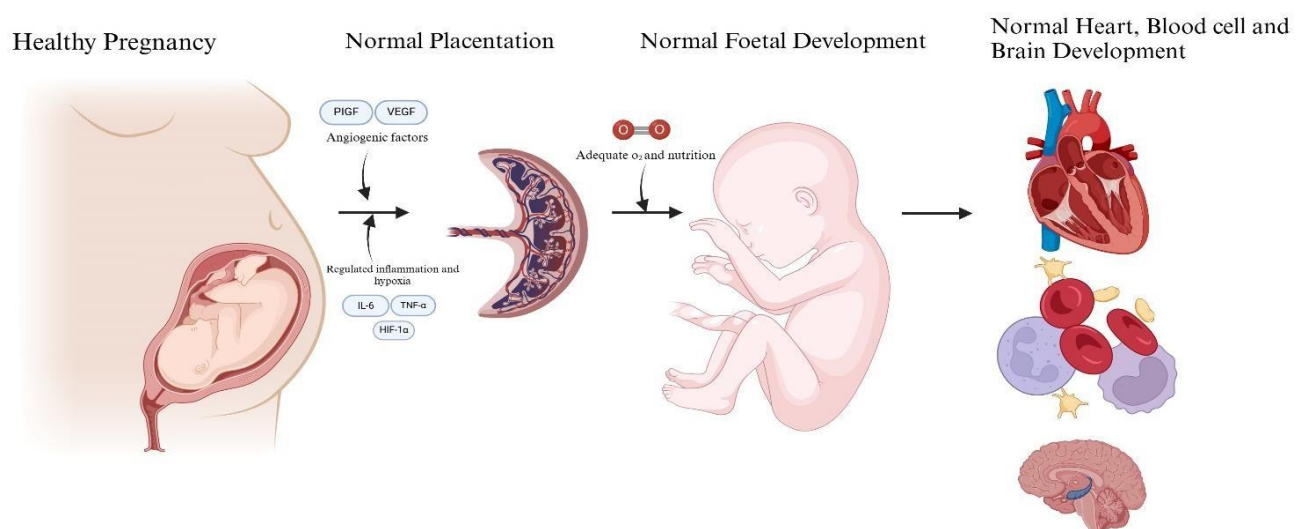


Figure 1: The diagram illustrates the normal processes of foetal heart, blood, and brain development in a healthy pregnancy. Proper placental function ensures adequate nutrient and oxygen delivery to the foetus, supporting optimal growth and development. In the heart, this results in normal cardiomyocyte differentiation, vascularization, and formation of the heart chambers. Blood development is characterized by regulated erythropoiesis, with healthy differentiation of haematopoietic stem cells into mature blood cells. In the brain, neurogenesis, synaptogenesis, and myelination occur without disruption, ensuring proper brain structure and function. The diagram emphasizes the coordinated

interactions between these systems for overall foetal development. Adapted from Öztürk and Türker. (2021) (1)

2.2.1 Cardiovascular development

Cardiovascular development during foetal programming is a complex process involving coordinated interactions between genetic regulation, maternal-placental function, and foetal demands (25). The placenta-to-body weight ratio is an important indicator of placental efficiency in transferring oxygen and nutrients from the mother to the growing foetus (26). A well-functioning placenta ensures adequate foetal perfusion, which directly influences cardiac development (26). If placental growth is insufficient, this ratio becomes skewed, potentially leading to intrauterine growth restriction (IUGR) and suboptimal heart development (26). The heart-to-body weight ratio reflects the proportionate growth of the foetal heart relative to overall body size, which is critical for maintaining cardiac output as foetal oxygen demands increase (27). During normal development, cardiomyocytes proliferate and differentiate under the influence of growth factors such as insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) (27, 28). These growth factors ensure the proper organization and functionality of cardiac muscle tissue (28).

Triglycerides (TG) and cholesterol (TC) are vital substrates for foetal energy metabolism and are necessary for the formation of cell membranes, including those of cardiomyocytes (29). In a healthy pregnancy, the placenta regulates the transfer of lipids to the foetus, providing sufficient substrates for cardiac energy production, membrane biosynthesis, and signalling pathways that regulate cardiomyocyte growth (30). Cardiac troponin I (cTnI) is a key protein involved in the contractile function of the heart (31). In normal foetal development, low plasma levels of cTnI suggest that cardiomyocyte integrity is maintained, with no signs of ischemia or myocardial stress (32). Troponin I regulate calcium-mediated interactions between actin and myosin filaments, which are essential for myocardial contraction (32). Proper troponin expression is critical for ensuring effective cardiac output and maintaining systemic circulation during foetal development (32).

Oxidative stress plays an essential role in the developing heart as it acts as a signalling mechanism during cardiogenesis (23, 24). Foetal cardiomyocytes are particularly vulnerable to oxidative stress due to their high metabolic activity (33). Reactive oxygen species (ROS) regulate key pathways involved in cardiomyocyte differentiation, proliferation, and maturation. ROS activate transcription factors such as HIF-1 α , which helps modulate angiogenesis and vascular development in the heart (33). Moreover, oxidative stress supports mitochondrial biogenesis and the metabolic transition of cardiomyocytes from glycolysis to oxidative phosphorylation, which is a crucial adaptation for postnatal life (33). However, excessive oxidative stress can disrupt these processes, leading to structural and functional cardiac defects (33). Therefore, antioxidants, superoxide dismutase (SOD) and glutathione peroxidase (GPx), mitigate ROS-induced damage, preventing lipid peroxidation and maintaining the structural integrity of the cardiac membranes (23, 24). The balance between oxidative stress and antioxidant defense ensures that the developing myocardium can sustain its high energy demands without succumbing to oxidative

injury (23, 24). Histological examination of the foetal Left ventricle reveals the structural maturation of cardiomyocytes, supported by the synthesis of collagen I (34). Collagen I is a major component of the extracellular matrix (ECM), and its regulated expression is critical for maintaining myocardial structure and function (34). In healthy foetal development, maintained collagen deposition supports the mechanical stability of the heart without leading to fibrosis, allowing for appropriate ventricular remodelling as the heart grows and adapts to increasing circulatory demands (34).

Furthermore, when considering cardiac development, male offspring are prone to a greater degree of risk to physiological hypertrophy due to their higher metabolic requirements unlike their female counterparts (44). This results in a proportionally higher heart-to-body weight ratio compared to female offspring, which may present with less pronounced hypertrophic changes in future (44, 45). The mechanisms underlying this hypertrophy involve the activation of signalling pathways such as the phosphoinositide 3-kinase (PI3K)/Akt pathway, which promotes cell growth and survival in response to increased metabolic demands (46). Additionally, male offspring may have higher circulating levels of insulin-like growth factor 1 (IGF-1), a key regulator of cardiac growth, further promoting cardiac hypertrophy (47). Despite these differences, both genders show overall healthy cardiac function, characterized by normal growth patterns and the absence of structural abnormalities, due in part to the effects of maternal factors such as adequate nutrition and physical activity (44). These maternal influences help to maintain optimal cardiac development through improved placental perfusion and nutrient delivery (44). Furthermore, the heart, as the first organ to form, is initially dependent on maternal oxygenation and nutrient provision in the first trimester for its development and function (25). However, as pregnancy progresses into the second trimester, the foetus establishes an autonomous haematopoietic system, with the heart subsequently relying on this independent circulatory network to fulfil its metabolic demands (25).

2.2.2 Haematological development

During a healthy pregnancy, erythropoiesis is initiated early in foetal development, driven by the release of erythropoietin (EPO) from the foetal liver and later the kidneys, in response to hypoxia-inducible factors (HIFs) that sense oxygen levels (19). EPO stimulates the proliferation and differentiation of erythroid progenitor cells in the foetal bone marrow, leading to the production of red blood cells (RBCs) (19, 20). The RBC count directly correlates with the oxygen-carrying capacity of foetal blood, a critical factor for ensuring that oxygen is delivered efficiently to all tissues, which is especially important given the rapidly growing and metabolically demanding organs such as the brain and heart (19, 20). Haemoglobin (Hgb), the primary oxygen-carrying molecule in RBCs, is essential for this process. The synthesis of haemoglobin is regulated by both foetal iron availability and oxygen tension, ensuring that each RBC carries sufficient haemoglobin to maximize oxygen delivery (19, 20). Mean cell volume

(MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) are all tightly regulated by erythropoietic activity, ensuring that RBCs are of the appropriate size and haemoglobin content, thereby optimizing their function (19, 20).

Red blood cell distribution width (RDW) reflects the variability in RBC size, and a low RDW in healthy pregnancies indicates uniform erythropoiesis, minimizing potential inefficiencies in oxygen delivery (21). The tight regulation of EPO ensures a balance between the production of RBCs and oxygen demand, which is crucial for avoiding excessive erythropoiesis, which could lead to hyperviscosity and compromised microcirculation (19-21). Granulocyte colony-stimulating factor (G-CSF) supports foetal haematopoiesis, particularly the production of neutrophils, which are crucial for immune defence postnatally (22). This factor also contributes to the maturation of the foetal immune system, ensuring the foetus is prepared to respond to infections after birth (22).

Oxidative stress is another critical aspect of foetal development (23). Oxidative stress regulates foetal erythropoiesis by promoting erythroid progenitor proliferation and erythropoietin production under controlled conditions (23). It influences haematopoietic stem cell differentiation into various blood cell lineages and supports the foetal-to-adult haemoglobin switch through transcriptional regulation (23). Malondialdehyde (MDA), a byproduct of lipid peroxidation, serves as a marker for oxidative damage (23). However, regulated levels of MDA play a signalling role in foetal blood cell development by influencing cellular pathways involved in haematopoiesis and erythropoiesis (23, 24). MDA contributes to membrane remodelling, ensuring proper differentiation and maturation of haematopoietic cells, it can also activate antioxidant defences in foetal blood cells, enhancing their resilience to oxidative stress thus aiding in blood cellular development (23, 24). However, excessive MDA may cause oxidative damage, disrupting red and immune cell development leading to anaemia or immune deficiencies (23, 24). In such cases antioxidant systems such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) are upregulated to counteract oxidative stress (23). These enzymes neutralize reactive oxygen species (ROS), thereby preventing cellular damage in erythroid and other developing cells (24). This balance between ROS production and antioxidant defence is vital for maintaining the integrity of the foetal circulatory system, as excess oxidative stress could impair erythropoiesis and damage foetal tissues (23, 24)

Moreover, in terms of haematological parameters, male offspring often exhibit higher red blood cell counts, and haemoglobin levels compared to females, reflecting the greater oxygen demands associated with male growth rates (42). The increased erythropoiesis is typically well-regulated, allowing for effective oxygen transport without the pathological changes seen in compromised pregnancies. Furthermore, the differences in RBC production may be linked to hormonal influences, as higher testosterone concentrations in males have been shown to enhance erythropoiesis through mechanisms involving EPO signalling pathways (42). Female offspring, while generally having lower overall erythropoietic markers, may benefit from more stable iron metabolism, facilitated by higher hepcidin

concentrations, which regulates iron homeostasis and prevents excessive iron absorption (42, 43). Female offspring, while generally having lower overall erythropoietic markers, may benefit from more stable iron metabolism, which supports healthy foetal development (42, 43). This careful balance supports healthy foetal development, allowing both genders to thrive within the optimal ranges of blood markers (16, 17). The newly established circulatory system plays a crucial role in the development of the neurological system, as the brain, similar to the heart, relies on a constant supply of oxygen and nutrients for its growth and function (19, 35).

2.2.3 Neurological development

The brain is one of the most metabolically active organs during foetal development, and its growth is highly dependent on a well-regulated supply of nutrients, oxygen, and neurotrophic factors (35). The brain-to-body weight ratio and cephalization index are key indicators of the prioritization of brain development relative to other organs (36). The cephalization index (CI) measures the relative size of the brain compared to the body, reflecting the degree of brain growth during foetal development (36). A higher CI indicates more advanced brain development, particularly in regions critical for cognitive and motor functions (36). Deviations from normal CI values can signal developmental disorders, and maternal health factors can influence the CI, affecting foetal brain growth and future cognitive outcomes (36, 37). In healthy pregnancies, these ratios reflect the allocation of a significant proportion of foetal resources to the brain, supporting neurogenesis, synaptogenesis, and myelination (37). Adequate placental function and foetal circulation are essential for delivering oxygen and glucose required for neuronal metabolism and development (37).

Brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2) are critical for neural development (38). BDNF promotes neuronal differentiation, synaptic plasticity, and survival, particularly in regions such as the hippocampus, which is involved in memory formation and cognitive function (38). FGF2 stimulates the proliferation of neural progenitor cells and supports neurogenesis (38). In a healthy pregnancy, plasma BDNF and FGF2 concentrations, along with their gene expression in hippocampal tissue, are upregulated, ensuring that neurodevelopmental processes proceed without interruption (38). These neurotrophic factors play crucial roles in the maturation of neural circuits, which are essential for postnatal cognitive and behavioural function (38). The inflammatory milieu in the developing brain is carefully regulated to prevent neurodevelopmental impairments (39). Maintained levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) in both plasma and brain tissue are indicative of a controlled immune environment (40). Excessive inflammation during foetal development can disrupt neurogenesis and lead to long-term cognitive deficits (40, 41). In healthy pregnancies, anti-inflammatory mechanisms, including maternofoetal hormones, regulatory cytokines and immune-modulating factors, keep inflammation in check, preserving the integrity of the developing neural networks (41).

Neurologically, male offspring often demonstrate more rapid brain growth during the second and third trimesters, which may be linked to increased levels of neurotrophic factors like brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2) (48,49). BDNF plays a crucial role in promoting neuronal differentiation, synaptic plasticity, and survival, while FGF2 supports the proliferation and differentiation of neural progenitor cells (50). The expression of these neurotrophic factors is influenced by maternal factors such as diet and overall health, which can impact placental function and foetal nutrient delivery (51). This growth is crucial for neurogenesis and synapse formation, thus supporting cognitive development (52). Female offspring, while exhibiting slightly slower growth trajectories, often show more efficient synaptic pruning processes, mediated by signalling pathways like the mammalian target of rapamycin (mTOR) pathway, which is involved in regulating cell growth and metabolism (53). This efficient pruning can lead to more refined neural connections by the time of birth, allowing for optimal cognitive development in both genders (54). Both genders benefit from a healthy intrauterine environment, but the differential growth patterns highlight the importance of considering gender-specific strategies to optimize foetal health outcomes throughout pregnancy (55). Understanding these variations can inform prenatal care approaches to support healthy development in both male and female offspring (56). As observed in a healthy pregnancy, the placenta supports proper maternal and foetal development through balanced metabolic and endocrine functions (1, 2). However, in preeclampsia, these processes become disrupted, leading to altered metabolic, haematological, and cardiovascular outcomes that impact both the mother and foetus (6).

2.3 Preeclampsia

Preeclampsia is a complex, multifactorial disorder of pregnancy characterized by hypertension and proteinuria after 20 weeks of gestation (6). It begins with improper remodelling of the uterine spiral arteries in early pregnancy (6, 10). This failure to properly convert the arteries into high-capacity vessels creates a high-resistance and low-flow placental environment that promotes hypoxia (6, 10). The hypoxic state results in the upregulation of hypoxia-inducible factors (HIFs), like HIF-1 α , driving a cascade of pro-inflammatory and oxidative stress signals (6, 10). The key inflammatory cytokines, including tumour necrosis factor-alpha (TNF- α) and interleukins IL-6 and IL-1 β , are released, along with anti-angiogenic factors like soluble fms-like tyrosine kinase-1 (sFlt-1) and endoglin (sEng), disrupting the normal function of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (6). These disruptions impair placental angiogenesis, compromising the placental villous structure, particularly damaging the syncytiotrophoblast microvilli responsible for nutrient and gaseous exchange between the mother and the foetus. (10). This impaired placental environment restricts nutrient and oxygen delivery, impacting foetal haematopoiesis, heart and brain development (6, 10). Consequently, these adverse prenatal conditions establish a high-risk foundation for cardiovascular, haematological, and cognitive complications in the offspring, with potential lifelong implications (6, 10). Figure 2 below summaries the events of pregnancy and foetal development in preeclampsia.

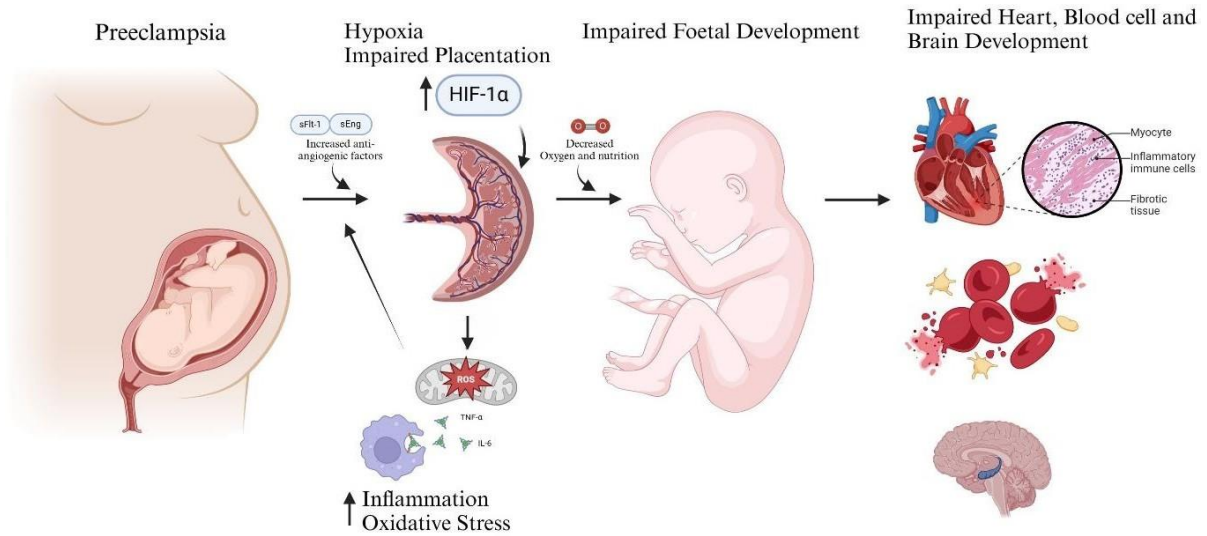


Figure 2: The diagram illustrates the interconnected impacts of preeclampsia on foetal heart, blood, and brain development. Placental dysfunction, characterized by impaired spiral artery remodelling, oxidative stress, and hypoxia, serves as the central driver of these disruptions. In the heart, these conditions result in cardiomyocyte hypertrophy, fibrosis, and altered vascularization. In blood development, they impair erythropoiesis and promote an inflammatory cytokine environment, leading to dysregulated haematopoiesis. HIF-1 α represents a *key regulatory marker*, not an exclusive placental mediator. For the brain, hypoxia and inflammation disrupt neurogenesis, synaptogenesis, and blood-brain barrier integrity, potentially causing long-term neurodevelopmental deficits.

Adapted from Odegård et al. (2000) (6) and Ludidi et al. (2023) (10)

2.3.1 Cardiovascular development in preeclampsia

The cardiovascular system of the foetus is severely impacted by the maternal pathology of preeclampsia (71). The placenta-to-body weight ratio is typically abnormal due to placental insufficiency, which reduces the efficient transfer of nutrients and oxygen to the foetus (72). This impaired placental function directly affects foetal heart development (73). The heart-to-body weight ratio may become dysregulated as the foetal heart compensates for chronic hypoxia and increased haemodynamic stress (74). The foetal heart undergoes hypertrophy as cardiomyocytes enlarge in response to the increased demand for cardiac output to maintain oxygenation, a process that can lead to long-term cardiac remodelling and dysfunction (75). In cases of severe preeclampsia, foetal hearts may show signs of right ventricular hypertrophy or diastolic dysfunction during the infantile stage (76). This results from the increased afterload imposed by placental insufficiency and hypoxia (77).

Triglyceride (TG) and cholesterol (TC) metabolism in preeclampsia is also disrupted (78). The placenta's inability to properly regulate lipid transfer leads to altered lipid levels in the foetal circulation (79). Abnormal TG and TC levels may impair the development of cardiomyocyte membranes and

energy metabolism, further contributing to cardiac dysfunction (80). Cardiac troponin I (cTnI), a marker of myocardial injury, is often elevated in foetuses exposed to preeclampsia (81). This suggests that the foetal heart experiences ischaemic stress, likely due to the combination of hypoxia and oxidative stress (82). Elevated cTnI indicates compromised cardiomyocyte integrity and function, which may lead to long-term cardiovascular complications for the foetus (83).

The oxidative stress associated with preeclampsia is particularly detrimental to the developing heart (84). Malondialdehyde (MDA) levels increase as ROS accumulate, causing lipid peroxidation in cardiac cells which disrupts foetal cardiac development by impairing cardiomyocyte differentiation, proliferation, and contractile protein expression (85). Furthermore, oxidative stress promotes cardiac fibrosis by activating fibroblasts and increasing extracellular matrix production, which stiffens the cardiac tissue (85). It also damages calcium-handling proteins and mitochondrial function, leading to reduced cardiac contractility and energy deficits (85). Abnormal ROS signalling interferes with angiogenesis and coronary vessel development, compounding oxygen and nutrient deficiencies in the foetal heart (85). Although antioxidant enzymes such as SOD and GPx are upregulated in response to oxidative stress, these defences are overwhelmed in the preeclamptic environment (86). These changes compromise the structural and functional integrity of the heart and may predispose the foetus to cardiovascular disease later in life (87). Histological analysis of the Left ventricle in infants exposed to preeclampsia often shows disorganized cardiomyocytes, increased collagen deposition, and myocardial fibrosis (88). The gene expression of collagen I is upregulated in response to the inflammatory and hypoxic environment, promoting excessive extracellular matrix deposition and cardiac stiffness (89). These changes increase the risk of cardiovascular diseases later in life for offspring of preeclamptic pregnancies (90). Figure 3 below summaries the events of foetal cardiovascular development in preeclampsia.

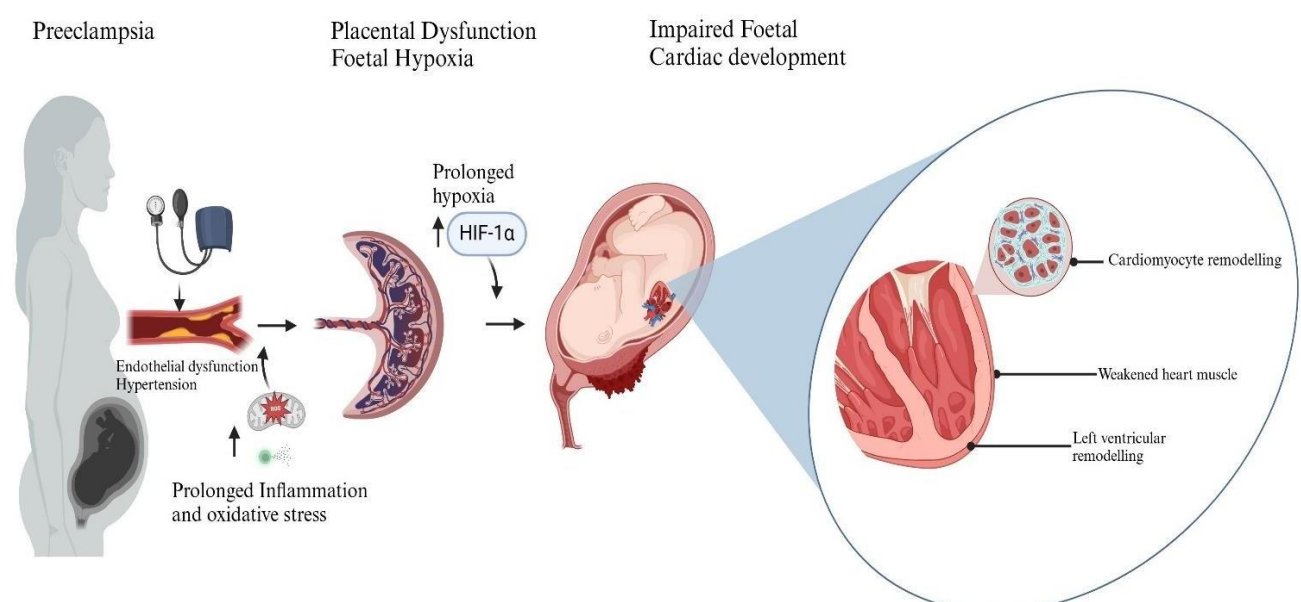


Figure 3: The diagram illustrates the key stages of foetal heart development in the context of a preeclamptic pregnancy. It highlights the impact of impaired placental function, including reduced nutrient and oxygen supply, which leads to foetal hypoxia and oxidative stress. These conditions influence cardiomyocyte development, causing alterations such as hypertrophy, fibrosis, and changes in myocardial vascularization.

Adapted from Wojezakowski et al. (2021) (72) and Ludidi et al. (2023) (10)

Cardiac development also displays gender-specific patterns in the context of preeclampsia (72). Male offspring often demonstrate increased susceptibility to cardiac hypertrophy due to the heightened stress imposed by the intrauterine environment characterized by maternal hypertension and placental dysfunction (73). This results in abnormal heart-to-body weight ratios and higher concentrations of cardiac injury markers such as troponin I (74). Furthermore, the activation of pathways such as the renin-angiotensin system (RAS) plays a significant role in increased susceptibility to cardiac hypertrophy as elevated angiotensin II levels lead to vasoconstriction and increased cardiac workload (75). Moreover, male offspring are more likely to experience oxidative stress, which can exacerbate cardiac injury and contribute to pathological remodelling of cardiac tissue (76). In contrast, female offspring, while still affected by preeclampsia, may show less severe hypertrophic changes, potentially due to protective effects from oestrogen, which has been shown to promote vasodilation and improve placental blood flow, thus alleviating some stress on the foetal heart (77).

2.3.2 Haematological development in preeclampsia

Preeclampsia disrupts normal erythropoiesis and the regulation of blood markers during foetal development, leading to complications that impair oxygen transport and foetal growth (57). In preeclampsia, placental dysfunction results in chronic foetal hypoxia, which stimulates an overproduction of erythropoietin (EPO) as the foetal body attempts to compensate for reduced oxygen availability (58). This leads to an elevated red blood cell (RBC) count, but these RBCs are often dysfunctional (59). Haemoglobin (Hgb) levels may be altered, with abnormal oxygen-binding capacity due to changes in foetal iron metabolism and placental insufficiency (60). Mean cell volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) become dysregulated, reflecting the production of larger or smaller RBCs with altered haemoglobin content (61). These alterations contribute to inefficient oxygen delivery, exacerbating foetal hypoxia and potentially leading to intrauterine growth restriction (IUGR) (62).

The red blood cell distribution width (RDW) is often increased in preeclampsia, indicating a higher variability in RBC size and a disruption in uniform erythropoiesis (63). This variation further compromises oxygen delivery to foetal tissues, aggravating the metabolic challenges faced by the developing organs (64). Granulocyte colony-stimulating factor (G-CSF) levels are elevated in response

to inflammation and oxidative stress, as preeclampsia is characterized by heightened immune activation (65). While G-CSF supports foetal immune development, the inflammatory environment associated with preeclampsia can lead to immune dysregulation, which may impair foetal immune system maturation (66).

Oxidative stress is significantly elevated in preeclampsia, with higher levels of malondialdehyde (MDA) reflecting lipid peroxidation and damage to foetal tissues (67). Oxidative stress arises primarily from the placenta due to impaired placental perfusion and ischemia-reperfusion injury (67). Insufficient trophoblast invasion leads to poor remodelling of spiral arteries, causing hypoxia-reoxygenation cycles that generate excessive reactive oxygen species (ROS) (68). Additionally, mitochondrial dysfunction in placental cells and the release of pro-inflammatory cytokines exacerbate oxidative stress, further disrupting the placental environment (67, 68). The ROS, overwhelm the foetal antioxidant systems, despite the upregulation of superoxide dismutase (SOD) and glutathione peroxidase (GPx) (68). These antioxidant defences are insufficient to counteract the oxidative damage, contributing to endothelial dysfunction and impaired vascular development in the foetus (69). This imbalance between oxidative stress and antioxidant defence leads to damage in erythroid progenitor cells and other developing tissues, compromising foetal growth and overall health (70). Figure 4 below summaries the events of foetal haematological development in preeclampsia.

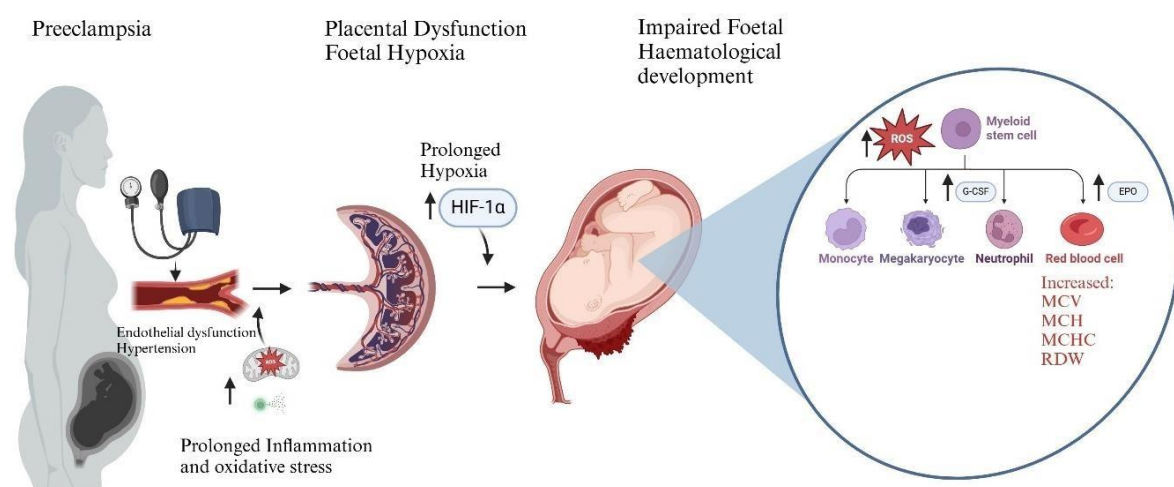


Figure 4: The diagram illustrates the key stages of foetal blood development under the influence of preeclamptic conditions. The image highlights the aberrant physiological processes occurring in the placenta, including impaired spiral artery remodelling, reduced oxygen transfer, and increased oxidative stress. These factors contribute to altered haematopoiesis, characterized by dysregulated erythropoiesis which leads to potential shifts in foetal haemoglobin levels. The figure also depicts inflammatory cytokine infiltration and endothelial dysfunction, both of which exacerbate disruptions in normal foetal blood development.

Adapted from Duplaa et al. (2013) (57), Qui et al. (2015) (61) and Masoumi et al. (2019) (58)

Male offspring in preeclampsia tend to experience greater haematological changes, often characterized by higher incidences of foetal anaemia and polycythaemia (19, 65). This is linked to the increased demand for erythropoiesis in response to placental insufficiency and maternal hypertension (66). The underlying mechanism involves heightened levels of foetal erythropoietin (EPO), which stimulates red blood cell production in the bone marrow (67). Male offspring may have a more pronounced response to maternal hypoxia due to preeclampsia, resulting in increased EPO production (68). Additionally, the inflammatory environment associated with preeclampsia may disrupt iron metabolism and lead to oxidative stress, further compromising normal erythropoiesis (69). In contrast, female offspring may exhibit a more balanced erythropoietic response, potentially due to differences in how oestrogen modulates erythropoiesis and iron homeostasis (70). Oestrogen influences the regulation of hepcidin, a key hormone in iron metabolism, thereby supporting stable iron levels and reducing the risk of anaemia in females (71).

2.3.3 Neurodevelopmental impairments in preeclampsia

Brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2), critical for supporting neuronal survival, differentiation, and synaptic plasticity, are dysregulated in preeclampsia (48-52). Plasma levels and hippocampal gene expression of these neurotrophic factors are reduced, compromising the growth and connectivity of neural circuits (51, 52). BDNF is particularly sensitive to oxidative stress and inflammation, both of which are elevated in preeclampsia (48-52). The reduction in BDNF impairs hippocampal development, which is associated with long-term cognitive deficits and memory impairments (48-52). Similarly, FGF2 dysregulation impairs the proliferation of neural progenitor cells, further contributing to abnormal brain development (51). The inflammatory environment of preeclampsia exacerbates neurodevelopmental challenges (48-52). Elevated pro-inflammatory cytokines levels such as interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) in both plasma and brain tissue indicate a state of chronic neuroinflammation (48, 49). This inflammatory response can disrupt the blood-brain barrier and lead to neuronal injury, contributing to adverse neurodevelopmental outcomes (48-50). IL-6, in particular, has been implicated in altered neuronal differentiation and synapse formation, potentially leading to long-term cognitive and behavioural issues (48-50). Oxidative stress in the brain is another critical factor in preeclampsia-associated neurodevelopmental impairment (48-50). Increased MDA levels in foetal brain tissue reflect heightened lipid peroxidation and oxidative damage to neurons and glial cells (48-50). This oxidative stress contributes to neuronal apoptosis, synaptic loss, and impaired myelination, all of which are critical for proper brain function (48-52). The imbalance between oxidative stress and antioxidant defence mechanisms leads to neurodevelopmental delays and increases the risk of cognitive disorders later in life for offspring exposed to preeclampsia (48-50). Figure 5 below summaries the events of foetal neurological development in preeclampsia.

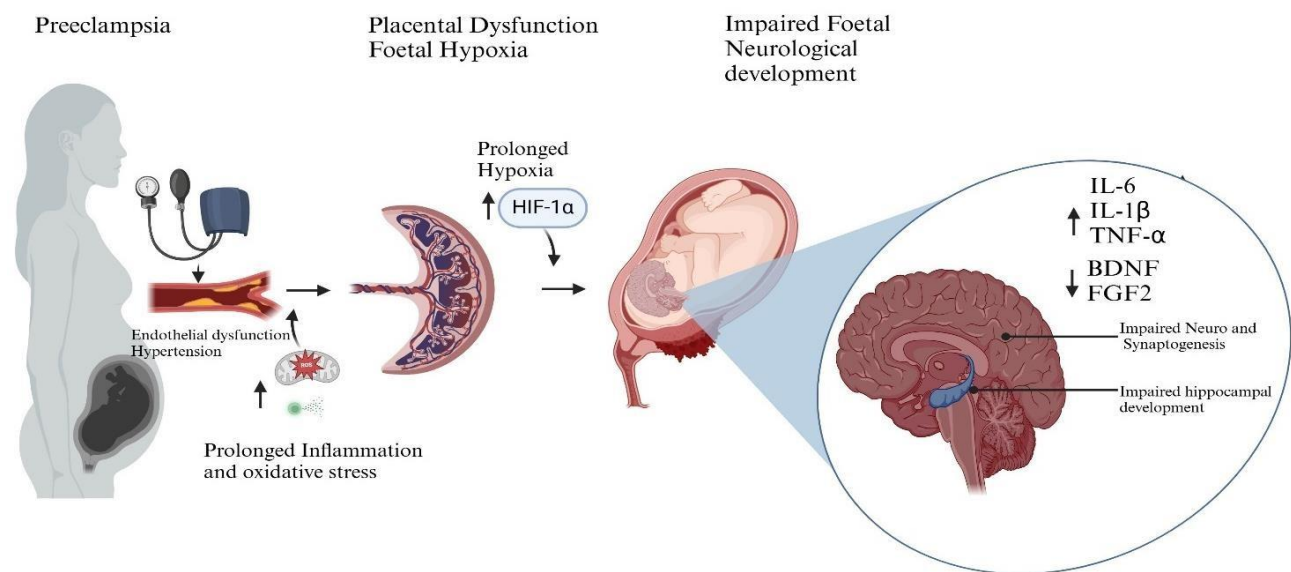


Figure 5: The diagram depicts the stages of foetal brain development affected by preeclamptic conditions. It highlights the impact of placental dysfunction, including reduced oxygenation and nutrient delivery, leading to foetal hypoxia and oxidative stress. These adverse conditions disrupt neurogenesis, synaptogenesis, and myelination, contributing to impaired brain structure and function. The figure also illustrates elevated inflammatory cytokines and endothelial dysfunction, which exacerbate blood-brain barrier permeability and neuronal damage. Key regions affected, such as the hippocampus and cortex, are emphasized, indicating potential long-term neurodevelopmental consequences.

Adapted from D’Souza et al. (2014) (50), Liu et al. (2016) (48), Gumusoglu et al. (2020) (51) and González-Rojas et al. (2024) (49)

In terms of cognitive impact, male offspring are often more vulnerable to the adverse effects of the inflammatory and oxidative stress environment created by preeclampsia (78). This can lead to reduced levels of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2), impairing neurogenesis and increasing the risk of cognitive deficits (79). The inflammatory cytokines present in the preeclamptic environment, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF- α), can interfere with the signalling pathways responsible for neurotrophic factor production, further compromising brain development in male offspring (80). In contrast, female offspring may exhibit slightly better neuroprotective responses, benefiting from different inflammatory profiles and a more robust antioxidant defence system, which helps to mitigate oxidative damage (81). However, both genders are at risk for long-term neurodevelopmental issues,

emphasizing the need for tailored interventions that address these gender-specific vulnerabilities in the management of preeclampsia (82). The aetiology of preeclampsia mirrors that of pT2D, with both conditions involving underlying oxidative stress, vascular dysfunction, and inflammation. As pT2D is a risk factor for PE, it will have significant effects on the cardiovascular, haematological, and neurological systems, influencing both maternal and foetal health outcomes

2.4 Pregestational type 2 diabetes mellitus

Diabetes during pregnancy may present as either gestational diabetes mellitus (GDM), which develops during pregnancy and typically resolves postpartum, or pregestational type 2 diabetes mellitus (pT2D), which is present prior to conception and persists throughout gestation (7,8, 107). While both conditions expose the developing foetus to a hyperglycaemic intrauterine environment, pT2D is associated with earlier and more sustained metabolic disruption, particularly during critical windows of placental and foetal development (7, 8). In pregnancies complicated by pT2D, the early intrauterine environment that shapes foetal development is often disrupted, introducing challenges to the long-term health of the child (7, 8). Pre-existing maternal diabetes leads to elevated blood glucose levels, which can affect placental development and compromise the foetus's access to oxygen and nutrients (7, 8). Hyperglycaemia, along with insulin resistance, create an inflammatory and oxidative environment that disrupts the balance of molecular signals vital for placental function (7, 8). As a result, the precise processes required for healthy foetal growth, from blood cell formation to brain and heart development, are often altered (7, 8). Furthermore, in pT2D, hypoxia-inducible factor 1-alpha (HIF-1 α) and inflammatory marker function is dysregulated due to the high-glucose environment (7, 8). While HIF- 1 α would typically respond to early low-oxygen conditions by promoting placental growth, in diabetic pregnancies, its activity is often disrupted by oxidative stress, leading to impaired placental invasion and spiral artery remodelling (7, 8). This can result in insufficient blood flow to the foetus, as the spiral arteries fail to remodel effectively, which affects the oxygen and nutrient supply the foetus receives (7, 8). At the same time, pro-inflammatory cytokines, such as IL-6 and TNF- α , are excessively elevated, creating an inflammatory environment that can lead to placental dysfunction, further compromising foetal growth (7, 8). Limited oxygen and nutrients impair foetal haematopoiesis, leading to imbalances in red and white blood cells which reduces the foetus's ability to manage oxygen and immune functions effectively (7, 8). Reduced oxygenation also hinders neurogenesis and synaptogenesis, the processes that underpin brain development, potentially affecting cognitive outcomes and neural resilience in adulthood. Cardiac development is similarly affected as the foetal heart undergoes structural adaptations in response to low oxygen (7, 8). This leads to altered cardiac morphology and function, raising the risk of cardiovascular complications post-birth (7, 8). Thus, in pT2D pregnancies, the disruptions to placental health create a cascade of challenges for the developing blood, brain, and heart systems of the foetus, highlighting the critical importance of stable maternal glucose levels for a healthy pregnancy and foetal development (7, 8). Figure 6 below summaries the events of pregnancy and foetal development in pregestational type 2

diabetes.

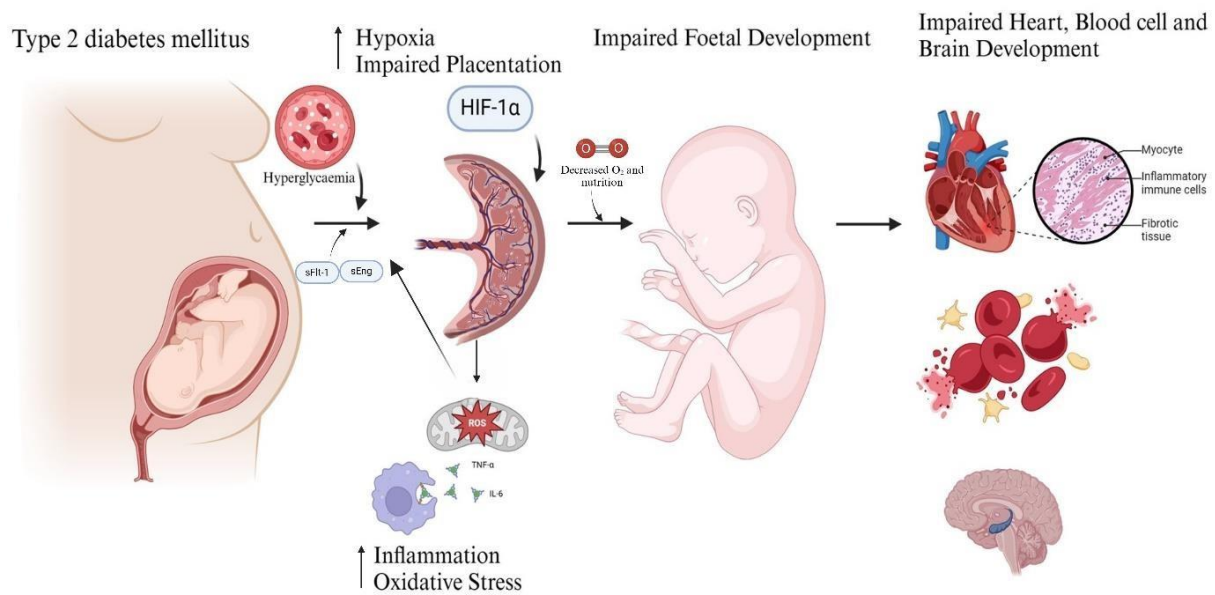


Figure 6: The diagram illustrates the effects of pregestational type 2 diabetes on foetal heart, blood, and brain development. Chronic hyperglycaemia and insulin resistance lead to placental dysfunction, characterized by excessive oxidative stress, inflammation, and altered nutrient transport. In the heart, these conditions disrupt cardiomyocyte development, causing hypertrophy, fibrosis, and abnormal vascularization. Blood development is impaired by erythropoietic dysfunction and hematopoietic dysfunction, driven by oxidative and inflammatory stressors. In the brain, hyperglycaemia-associated hypoxia and increased cytokine levels interfere with neurogenesis, synaptic development, and blood-brain barrier integrity, contributing to potential neurodevelopmental impairments

Adapted from Biachi et al. (2021) (7) and Desoye and Carter. (2022) (8)

2.4.1 Cardiovascular development in pregestational type 2 diabetes

Foetal cardiovascular development is significantly disrupted by maternal hyperglycaemia in pregestational type 2 diabetes (pT2D), with implications for both structural and functional outcomes (8). The placenta-to-body weight ratio is typically abnormal, reflecting placental insufficiency, hyperplasia, and hypertrophy, which impair nutrient and oxygen transfer to the foetus (86-89). This suboptimal placental function forces the foetal heart to adapt to a chronically stressed environment (86-89). The heart-to-body weight ratio is often increased due to cardiac hypertrophy, as foetal cardiomyocytes enlarge in response to the increased metabolic and oxygen demands imposed by hyperinsulinaemia and hypoxia (86-89). This hypertrophy, however, is pathological, predisposing the foetus to long-term cardiac dysfunction (80). Triglyceride (TG) and cholesterol (TC) metabolism is disrupted in foetuses exposed to maternal T2D (86-89). Hyperinsulinaemia stimulates excessive lipid

accumulation, resulting in elevated TG and TC levels in the foetal circulation (86-89). This lipid dysregulation negatively affects cardiomyocyte membrane composition and energy metabolism (86-89). Abnormal lipid handling in the foetus increases the risk of developing cardiovascular diseases later in life, including atherosclerosis and metabolic syndrome (86-89). Cardiac troponin I (cTnI), a marker of myocardial injury, is often elevated in foetal plasma due to the ischaemic stress caused by placental insufficiency and oxidative stress (86-89). Elevated cTnI levels suggest that foetal cardiomyocytes are undergoing ischaemic damage, which may impair contractile function and lead to long-term cardiac complications (86-89). Oxidative stress is a key driver of cardiac dysfunction in fetuses exposed to maternal T2D (86-89). Increased malondialdehyde (MDA) levels indicate lipid peroxidation and damage to foetal cardiomyocytes, which compromises the structural integrity of the foetal heart (86-89). This oxidative damage leads to endothelial dysfunction, impaired vasculogenesis, and abnormal cardiac remodelling (86-89). Histological examination of the foetal Left ventricle often reveals disorganized cardiomyocytes and increased deposition of collagen I, which leads to myocardial stiffness and fibrosis (86-89). This fibrosis impairs the ability of the infant heart to properly expand and contract, increasing the risk of heart failure in the postnatal period (86-89). The gene expression of collagen I is upregulated in response to chronic inflammation and oxidative stress, contributing to excessive extracellular matrix deposition (86-89). The accumulation of collagen in the foetal heart alters its mechanical properties, making it less efficient at maintaining cardiac output (86-89). This structural remodelling of the heart, combined with the oxidative and metabolic stress experienced in utero, predisposes offspring of mothers with T2D to cardiovascular diseases, including hypertension, coronary artery disease, and heart failure (86-89). Figure 7 below summaries the events of foetal cardiovascular development in pregestational type 2 diabetes.

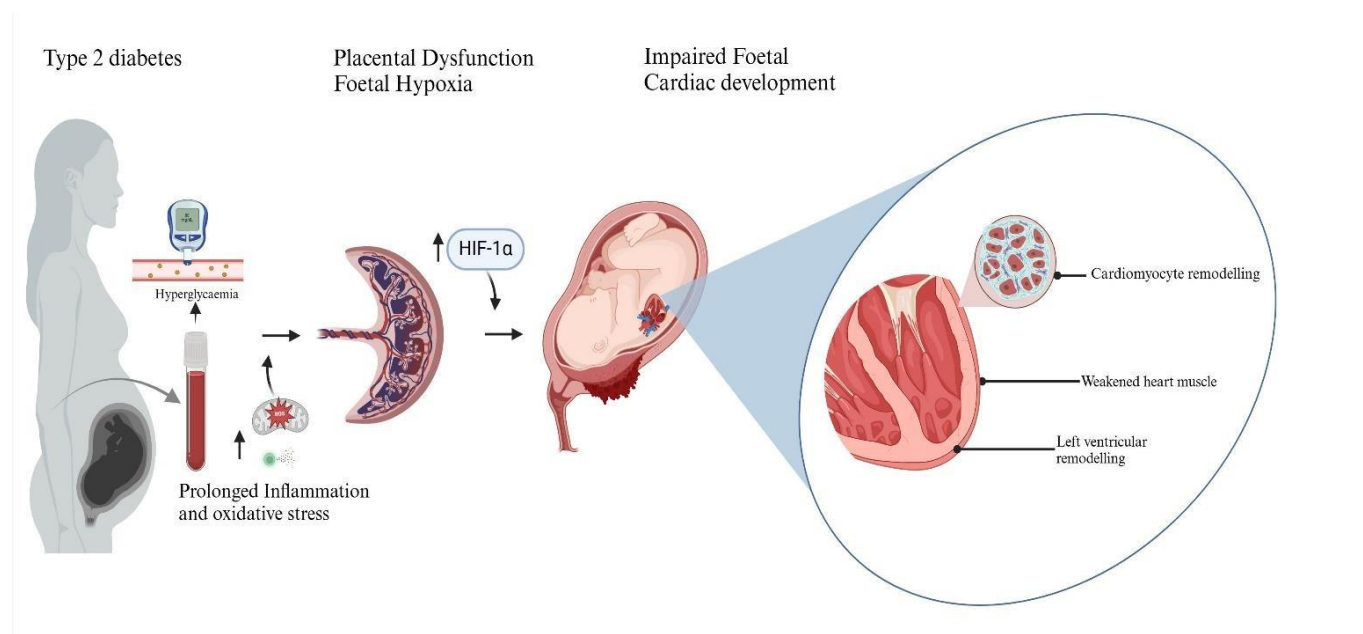


Figure 7: The diagram illustrates the effects of pregestational type 2 diabetes (pT2D) on foetal cardiac

development. Maternal hyperglycaemia, insulin resistance, and associated metabolic dysfunction leads to placental oxidative stress and inflammatory cytokine production, resulting in impaired nutrient and oxygen delivery to the foetus. These conditions disrupt key cardiac developmental processes, including cardiomyocyte proliferation, differentiation, and myocardial vascularization causing structural abnormalities such as cardiomyocyte hypertrophy, fibrosis, and altered heart morphology, potentiating long-term cardiac dysfunction in the offspring.

Adapted from Bhide et al. (2013) (89), Al-Biltagi et al. (2021) (88) and Skovsgaard et al. (2024) (87)

Gender-specific differences in foetal development also manifest in cardiovascular outcomes associated with pregestational type 2 diabetes. Male offspring exposed to maternal T2D are more susceptible to cardiac hypertrophy and increased lipid accumulation in the heart, heightening the risk of future cardiovascular complications such as hypertrophy and fibrosis (3, 17, 28). These changes may be driven by the higher metabolic demands of male foetuses in utero, rendering them more vulnerable to ischaemic stress and oxidative damage (3, 29). In contrast, while still affected, female offspring tend to exhibit milder hypertrophic responses and greater resilience to lipid dysregulation (17, 29, 28). Increased insulin levels in males can activate pathways like the mammalian target of rapamycin (mTOR) signalling, promoting cardiomyocyte proliferation and hypertrophy, leading to abnormal heart-to-body weight ratios (17, 28, 29). Females, however, may experience less severe cardiac alterations, potentially due to oestrogens protective effects, which enhance endothelial function, promote vasodilation, and mitigate cardiac stress (12, 17). Both genders are affected by the hyperglycaemic environment, which can induce oxidative stress and inflammation (12, 17). Nevertheless, male offspring may be more vulnerable to these adverse conditions, resulting in

heightened levels of cardiac stress markers, such as cardiac troponin I (cTnI) (17, 28, 29). This susceptibility may be exacerbated by differences in hormonal responses between sexes, with oestrogen potentially providing a buffering effect against cardiac stress in females (17, 28, 29).

2.4.2 Haematological development in pregestational type 2 diabetes

In pT2D, foetal erythropoiesis and blood markers are significantly affected by maternal hyperglycaemia and associated oxidative stress. Maternal diabetes can lead to increased levels of erythropoietin (EPO), which is critical for stimulating erythropoiesis, as the foetus adapts to hypoxic conditions induced by placental dysfunction (90-93). Chronic hyperglycaemia results in elevated foetal insulin levels, which can further enhance erythrocyte production, leading to conditions such as polycythaemia (90-93). This overproduction of red blood cells (RBCs) reflects a compensatory mechanism against a background of placental insufficiency and reduced oxygen availability (90-93). Additionally, maternal obesity and gestational diabetes mellitus can influence placental size and function, impacting foetal oxygenation and nutrient transfer (90-93). Alterations in RBC indices, such as increased mean cell volume (MCV) and red blood cell distribution width (RDW), have been documented in foetuses exposed to maternal

diabetes, indicating a dysregulated erythropoietic response (90-93). Moreover, oxidative stress, a hallmark of pT2D, can damage erythrocytes and impair their functionality (90-93). Elevated levels of MDA, superoxide, peroxynitrite and hydrogen peroxide are often observed in these pregnancies, signifying oxidative damage to foetal cells, including those involved in erythropoiesis (90-93). This oxidative burden may lead to compromised integrity of RBC membranes, contributing to alterations in RBC size and function (90-93). The inflammatory state associated with maternal diabetes also plays a role in the dysregulation of haematopoiesis (90-93). Increased levels of granulocyte colony-stimulating factor (G-CSF) can promote excessive granulocyte production, while simultaneously inducing alterations in normal immune responses, which may predispose the infant to postnatal infections (90-93). This environment not only affects haematological parameters but also has long-term implications for the offspring's cardiovascular health and immune function (90-93). The oxidative stress burden is significantly heightened in pregnancies complicated by T2D, leading to increased levels of malondialdehyde (MDA), a marker of lipid peroxidation (90-93). Hyperglycaemia accelerates the production of reactive oxygen species (ROS), which overwhelm the foetal antioxidant defence systems (90-93). Although enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) are upregulated in response to oxidative stress, they are often insufficient to prevent cellular damage to erythroid and myeloid progenitors and other developing tissues (90-93). This oxidative imbalance disrupts normal erythropoiesis, leukopoiesis, and thrombopoiesis, which can lead to anaemia or polycythaemia, leukopaenia or leukocytosis, as well as thrombocytosis or thrombopaenia depending on the severity of placental insufficiency and oxidative damage (90-93). Figure 8 below summaries the events of foetal haematological development in pregestational type 2 diabetes.

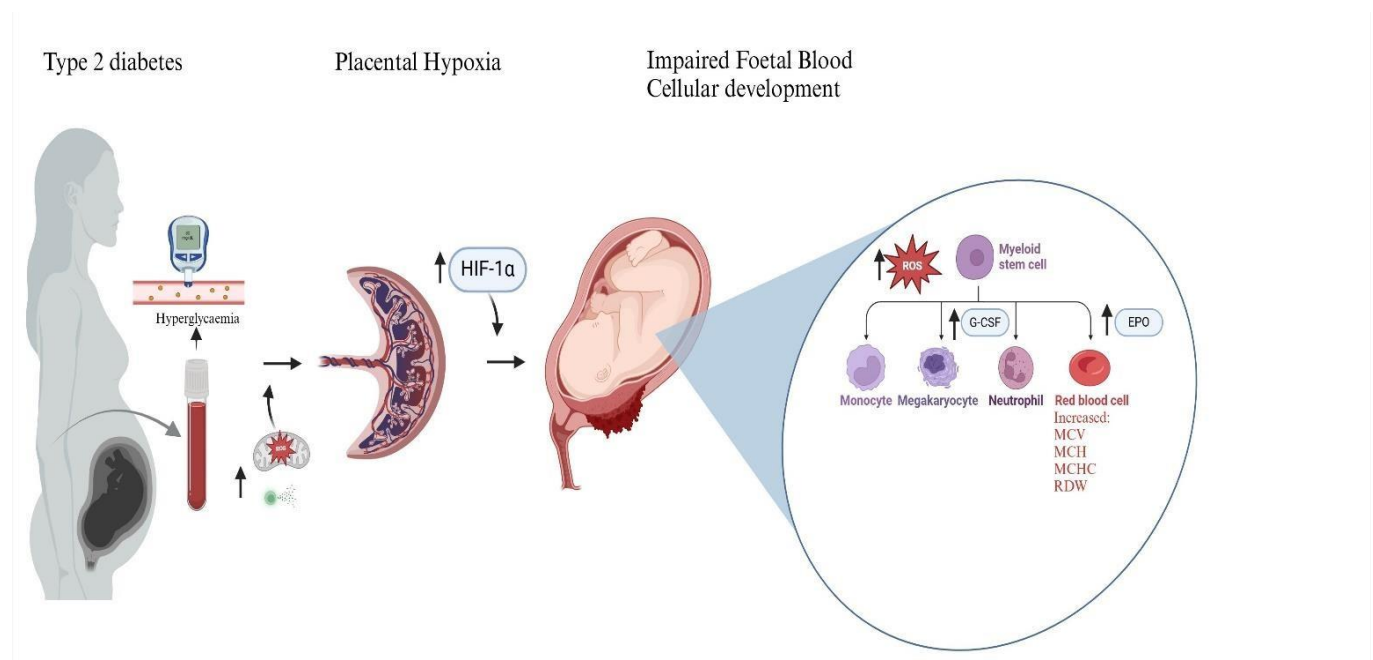


Figure 8: The diagram depicts the effects of pregestational type 2 diabetes (pT2D) on foetal

haematological development. Maternal hyperglycaemia and insulin resistance induce placental dysfunction, characterized by oxidative stress, inflammation, and impaired nutrient transfer. These adverse conditions disrupt foetal erythropoiesis and haematopoietic stem cell function, increasing red blood cell production and altered haemoglobin synthesis.

Adapted from Stewart and Malhotra. (2015) (93), Shashikadze et al. (2021) (91) and Muntean et al. (2023) (92)

In pregestational T2D, gender-specific differences are evident in foetal development, particularly in blood cellular development. Male offspring generally demonstrate more pronounced dysregulation of erythropoiesis compared to females, often exhibiting higher red blood cell counts and greater oxidative damage due to increased vulnerability to maternal hyperglycaemia (9, 14, 15). This vulnerability may lead to elevated insulin levels in males, resulting in increased erythropoietin (EPO) production in response to hypoxia. Consequently, this elevation in EPO stimulates erythropoiesis, resulting in higher red blood cell counts and haematocrit levels. In contrast, female offspring tend to have a more balanced haematopoietic response, potentially due to the protective influence of oestrogen, which helps maintain haematopoietic homeostasis and modulates the immune response (9, 18, 37). Chronic low-grade inflammation associated with maternal diabetes elevates pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF- α), promoting increased neutrophil production and altering white blood cell profiles in both genders (8, 9, 16). However, male offspring may be more susceptible to these inflammatory changes, leading to myeloid lineage expansion and a higher neutrophil count compared to females (9, 15, 43). Genetic and epigenetic factors also play a role, as males may be more vulnerable to hyperglycaemia and inflammation due to variations in gene expression related to metabolic and haematopoietic pathways (9). This vulnerability may contribute to a higher incidence of polycythaemia in male offspring, while females may exhibit subtler shifts in erythropoiesis with less pronounced changes in oxygen transport markers like MCH and MCHC (10, 24, 90).

2.4.3 Neurological development in pregestational type 2 diabetes

Foetal brain development is profoundly affected by maternal hyperglycaemia in pregestational type 2 diabetes (pT2D), particularly due to the combined effects of oxidative stress, inflammation, and disrupted nutrient supply (94-98). The brain-to-body weight ratio and cephalization index are often altered in foetuses exposed to maternal diabetes, indicating suboptimal brain growth relative to overall foetal size (94-98). Placental insufficiency and maternal hyperglycaemia impair the delivery of oxygen and glucose to the developing brain, leading to neurodevelopmental delays and increased risk for long-term cognitive deficits (94-98). Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and fibroblast growth factor 2 (FGF2) are essential for normal neurodevelopment, but their expression is dysregulated in the diabetic intrauterine environment (94-98). Plasma levels and hippocampal gene expression of BDNF are typically reduced, impairing neuronal

differentiation, survival, and synaptic plasticity (94-98). BDNF is highly sensitive to oxidative stress, and the excessive production of reactive oxygen species (ROS) in maternal T2D leads to a reduction in BDNF availability, particularly in brain regions like the hippocampus that are critical for learning and memory (94-98). This reduction in BDNF contributes to long-term cognitive impairments, including deficits in memory, learning, and executive function (94-98). Jaskolski et al. (2022) found that maternal gestational diabetes (GDM) significantly reduced the levels of brain-derived neurotrophic factor (BDNF) in both maternal and neonatal blood, with insulin-dependent GDM showing the lowest levels of BDNF. This reduction in BDNF, a key neurotrophin critical for neuronal survival and development, may have implications for foetal brain development (94-98). Additionally, maternal diabetes has been shown to affect the foetal brain's neurodevelopmental processes, possibly leading to long-term neuropsychiatric disorders (94-98). Their study highlights the negative impact of diabetes on BDNF levels, suggesting potential risks for the foetus under maternal metabolic stress. Furthermore, FGF2, which supports the proliferation and differentiation of neural progenitor cells, is similarly dysregulated in the foetal brain exposed to pregestational diabetes (94-98)). Reduced FGF2 expression disrupts neurogenesis and the development of neural circuits, further impairing brain growth and function (94-98). The abnormal regulation of both BDNF and FGF2 in the foetal brain is a critical factor in the increased risk of neurodevelopmental disorders observed in offspring of mothers with T2D (94- 98). Inflammation plays a central role in the neurodevelopmental complications associated with maternal diabetes (17). Elevated levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) in both plasma and brain tissue are indicative of a chronic inflammatory state

(18). This inflammation disrupts the blood-brain barrier and contributes to neuronal damage and apoptosis (19). IL-6, in particular, has been shown to interfere with neuronal differentiation and synapse formation, leading to altered brain structure and function (20). This inflammatory environment in the foetal brain increases the risk of neurodevelopmental disorders, including autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), and cognitive impairments (94-98). Oxidative stress in the brain is exacerbated by maternal hyperglycaemia. This oxidative stress damages neural progenitor cells, impairs synaptogenesis, and disrupts myelination, all of which are critical for proper brain function (24). The imbalance between oxidative stress and antioxidant defence contributes to long-term neurodevelopmental impairments and increases the risk of cognitive disorders such as intellectual disabilities and motor dysfunction in offspring (25). In summary, pregestational type 2 diabetes (pT2D) exerts profound effects on foetal blood, heart, and brain development through mechanisms involving oxidative stress, inflammation, and metabolic dysregulation (94-98). These disruptions lead to impaired oxygen transport, cardiac dysfunction, and neurodevelopmental deficits, increasing the risk of long-term health complications such as cardiovascular diseases and cognitive impairments in offspring (94-98). Understanding these mechanisms is crucial for developing targeted interventions to mitigate the adverse effects of maternal diabetes on foetal outcomes (94-98). Figure 9 below summaries the events of foetal cognitive development in pregestational type 2 diabetes.

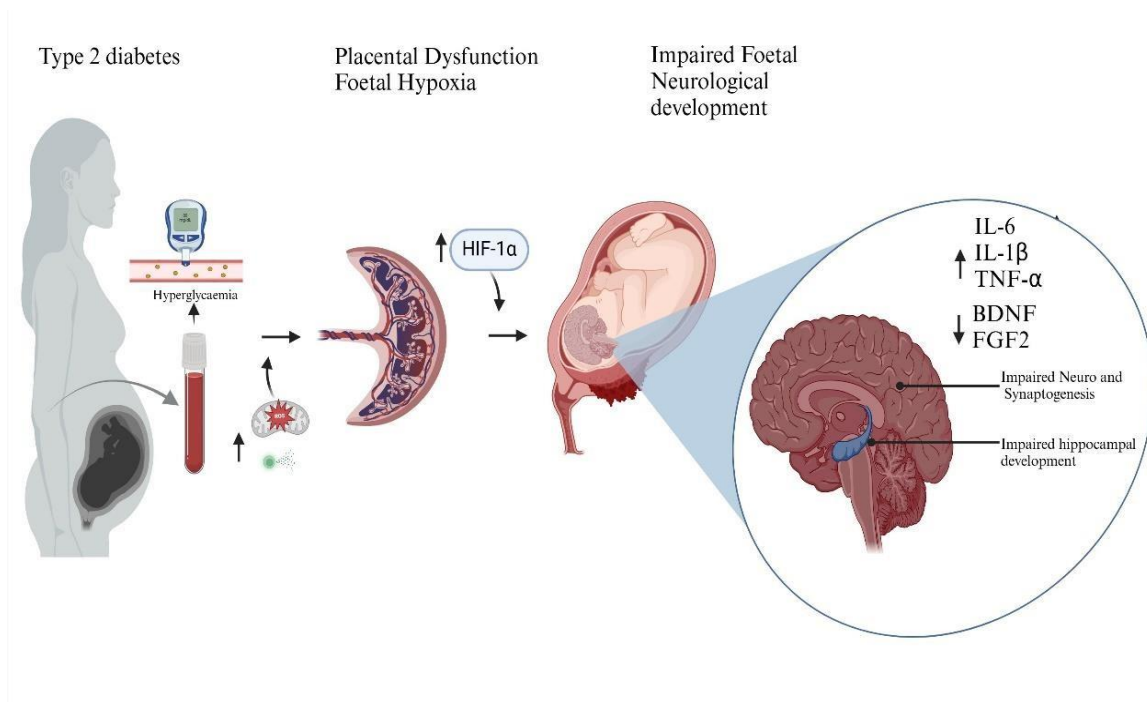


Figure 9: The diagram illustrates the effects of pregestational type 2 diabetes (pT2D) on foetal cognitive development. Maternal hyperglycaemia, insulin resistance, and associated metabolic dysfunction lead to placental oxidative stress, inflammation, and altered nutrient supply to the foetus.

These conditions disrupt key processes in brain development, including neurogenesis, synaptogenesis, and myelination.

Adapted from Rodolaki et al. (2023) (95), Vafaei-Nezhad et al., 2016 (96)

Gender-specific differences in foetal cognitive development are also evident in pregestational type 2 diabetes. Males and females respond differently to the oxidative and inflammatory stressors induced by maternal T2D. Male offspring often experience more significant neurodevelopmental delays, characterized by reductions in critical neurotrophic factors like BDNF, which may increase the risk of cognitive impairments and neurodevelopmental disorders (50, 48). Elevated cytokine levels, such as interleukin-1 β (IL-1 β) and tumour necrosis factor-alpha (TNF- α), can disrupt neurogenesis and neuronal differentiation, potentially leading to cognitive and behavioural deficits later in life (41, 52). In contrast, female offspring may benefit from a more robust antioxidant defence system and a different inflammatory profile, which could confer some neuroprotective effects, although they are not immune to the detrimental effects of maternal diabetes (1). Additionally, hormonal differences, particularly the influence of oestrogen, may help modulate the inflammatory response and support neurodevelopment in females (15). Overall, the interplay between maternal metabolic health, inflammation, and sex-specific hormonal influences ultimately shapes the neurodevelopmental trajectories of both male and

female offspring in the context of pregestational type 2 diabetes (48). However, before the developmental of pT2D, precursor state known as pregestational prediabetes can occur, which could have similar foetal developmental implications to cardiovascular, haematological, and cognitive systems (10).

2.5 Pathophysiology of Prediabetes and Its Distinctions from Overt Diabetes

Prediabetes represents a metabolically intermediate state characterized by impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT), in which blood glucose levels are elevated but not yet reaching diabetic thresholds (8-10). At this stage, pancreatic β -cell function begins to decline, and peripheral insulin sensitivity particularly in muscle and adipose tissues is compromised (104). Unlike overt type 2 diabetes mellitus (T2DM), where hyperglycaemia is sustained and chronic, prediabetes exhibits intermittent or moderate elevations in glucose that may still be amenable to restoration of normoglycaemia with lifestyle or pharmacological interventions (105). This distinction is underscored by varying degrees of insulin resistance and β -cell dysfunction: in prediabetes, insulin resistance often predominates early, whereas in overt T2DM, β -cell failure is more severe and persistent (104-105). Additionally, prediabetic individuals may show subtle dysregulation in hepatic glucose production, dyslipidaemia, and low-grade inflammation, but generally lack the macrovascular and microvascular complications seen in long-standing diabetes (106). Hence, prediabetes is both pathophysiologically distinct yet on a continuum with T2DM, representing an early, potentially reversible metabolic derangement.

2.6 Mechanistic Pathways Linking Insulin Resistance and Vascular/Endothelial Dysfunction to Preeclampsia

Insulin resistance (IR), a hallmark of prediabetes, contributes to endothelial dysfunction through multiple interrelated pathways implicated in the pathogenesis of preeclampsia (10,12). IR exacerbates oxidative stress and systemic inflammation, resulting in reduced nitric oxide (NO) bioavailability and increased endothelin-1 secretion, thereby impairing vasodilation (107). Elevated circulating insulin can enhance sympathetic nervous system activity and augment sodium retention, promoting hypertension, which is a defining feature of preeclampsia (6). Moreover, IR is associated with increased levels of pro-inflammatory cytokines (e.g., TNF- α , IL-6) and adhesion molecules (e.g., VCAM-1, ICAM-1), which further amplify vascular inflammation and endothelial activation, favoring vascular permeability and proteinuria characteristic of preeclampsia (6-8,). Adipokines such as leptin and adiponectin are also dysregulated in IR states—elevated leptin and decreased adiponectin levels further impair endothelial function and contribute to placental insufficiency (8). Collectively, these mechanistic pathways illustrate how insulin resistance-driven endothelial dysfunction fosters the maternal vascular maladaptation that underlies the development of preeclampsia (6).

2.7. Theoretical Rationale for Extrapolating Preeclampsia Risk from Diabetes to Prediabetes

Given the established association between overt diabetes mellitus and increased preeclampsia risk, it is theoretically plausible that prediabetes may similarly confer heightened susceptibility to this hypertensive disorder of pregnancy (9). Women with T2DM exhibit significantly elevated rates of preeclampsia, largely attributed to chronic hyperglycaemia, insulin resistance, endothelial dysfunction, and systemic inflammation all pathophysiological features that overlap with those observed in prediabetes (9). Although prediabetes does not meet the glycaemic severity of diabetes, the presence of subclinical insulin resistance, vascular dysregulation, and inflammatory mediators can be sufficient to perturb the delicate balance of placental perfusion and maternal vascular adaptation required for healthy pregnancy (10). Furthermore, the subthreshold hyperglycaemia of prediabetes may still promote oxidative stress and advance the release of antiangiogenic factors implicated in preeclampsia, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and endoglin (11). Therefore, extrapolation from the well-described preeclampsia risk in diabetic pregnancies to those with prediabetes rests on shared mechanistic underpinnings and a pathophysiological continuum, supporting the hypothesis that even modest metabolic derangements may elevate preeclampsia risk (9-10).

2.8 Pregestational prediabetes

Pregestational prediabetes (PP) refers to the state of dysregulated glucose metabolism present in women prior to conception, characterized by fasting plasma glucose levels between 100 and 125 mg/dL or oral glucose tolerance test results ranging from 140 to 199 mg/dL (9). PP is a precursor to pregestational type 2 diabetes mellitus (pT2D) and is associated with a significant risk of developing preeclampsia (PE) (10). As such, PP has profound implications for maternal and foetal health. The prevalence of pregestational prediabetes is concerning, with an estimated 10% of reproductive-aged women affected, highlighting a critical need for early identification, prevention, and intervention (11). PP can negatively impact pregnancy outcomes through several pathophysiological mechanisms (12). This condition is linked to systemic insulin resistance, which can result from increased adiposity, inflammation, and alterations in the gut microbiome (9). These factors can lead to dysregulated metabolic processes, including impaired glucose uptake and increased hepatic glucose production, ultimately contributing to elevated blood glucose concentrations (10). One of the major concerns regarding PP is its association with increased risks of adverse pregnancy and foetal outcomes (11). The underlying mechanisms include the promotion of endothelial dysfunction, which compromises placental blood flow and nutrient

delivery to the developing foetus (8). Elevated levels of circulating inflammatory markers, such as C-reactive protein and tumour necrosis factor- α , can exacerbate endothelial impairment, further hindering placental function (12). This compromised vascular environment may lead to inadequate placental perfusion, resulting in adverse foetal outcomes, including low body weight, preterm birth, and increased rates of neonatal complications (13). Moreover, PP may disrupt neuroendocrine function, leading to dysregulation of hormonal pathways essential for normal pregnancy progression (14). Alterations in insulin signalling and increased levels of glucagon can further exacerbate maternal hyperglycaemia, creating a cycle of metabolic dysregulation that poses risks to both maternal and foetal health (15).

2.9 Prediabetic animal models

In studies of prediabetes and type 2 diabetes (T2D), several animal models are employed to replicate human disease mechanisms (10, 11). One of the most widely used is the high-fat, high-carbohydrate (HFHC) diet-induced model, which simulates Western dietary patterns (10). This model typically involves feeding rodents a diet rich in saturated fats and sugars, leading to insulin resistance, obesity, and metabolic dysfunction, resembling the human T2D phenotype (10). Key advantages of this model include its ability to induce a broad spectrum of metabolic disturbances, including impaired glucose tolerance, dyslipidaemia, and insulin resistance, which closely mirror the pathophysiology observed in human populations exposed to similar dietary habits (10). HFHC models, especially those utilizing a balanced mix of fats, have been shown to induce metabolic changes that are both tissue and organism-wide, making them highly relevant for investigating prediabetes (10). In contrast, high-fat-only prediabetic models tend to overemphasize lipid metabolism dysregulation, often failing to induce the glucose intolerance seen in human prediabetes (99, 100). Similarly, high-carbohydrate-only models disproportionately target hyperglycaemia without adequately reproducing the complex interplay of insulin resistance and dyslipidaemia (100, 101). These limitations make both models less effective for mimicking the multifactorial nature of human prediabetes and its progression to T2D. Other common models for inducing prediabetes include streptozotocin (STZ) and alloxan injection, which directly damage pancreatic beta-cells to reduce insulin production (102). While these models create hyperglycaemia and insulin resistance, they lack the complexity of dietary-induced prediabetes, especially in terms of obesity and metabolic syndrome (99-103). Additionally, genetic models like the Zucker diabetic fatty (ZDF) rat and db/db mice, which harbour mutations in leptin receptor genes or leptin itself, can develop obesity and insulin resistance (100-103). However, these models are more specific to genetic factors and do not fully replicate the environmental influence of diet on prediabetes (99-103).

When comparing the HFHC model to other models, its major advantage lies in its versatility. It allows for the exploration of both genetic predisposition and environmental factors (dietary habits), which

makes it highly relevant for translational research (10, 99, 100, 103). The metabolic disturbances observed in these models, particularly the induction of insulin resistance and glucose intolerance without requiring genetic manipulation, more closely mimic the human condition (10). Moreover, studies have shown that HFHC-induced prediabetes is closely aligned with the features seen in human T2D, especially regarding tissue-specific insulin resistance and changes in lipid metabolism (10, 103). Therefore, the HFHC model reliably replicates metabolic and hormonal disturbances observed in human prediabetes, including insulin resistance, low-grade inflammation, and altered glucose tolerance (10, 100, 103). Furthermore, this model allows for controlled experimentation, making it possible to study complex physiological interactions that are difficult to isolate in human studies (10). This is why, in this study, we specifically used the HFHC diet-induced prediabetic model in Sprague-Dawley rats, as it closely mimics how prediabetes develops in humans due to saturated fatty diets, sedentary lifestyles, lack of exercise, and unhealthy eating habits (10). Moreover, in alignment with the mandates of the NIH and WHO, this study incorporated sex-specific analyses, reflecting a commitment to understanding gender diversity and its implications in pregnancy and medicine (16).

2.10 Gender-specific studies as mandated by the National Institutes of Health (NIH) and the World Health Organization (WHO)

The National Institutes of Health (NIH) and the World Health Organization (WHO) emphasize the importance of studies investigating gender-specific outcomes due to the significant biological, physiological, and social differences that can influence health and disease processes in men and women (16, 17). Understanding these differences is crucial for developing targeted prevention, treatment, and intervention strategies that effectively address the unique health needs of each gender (16, 17). Gender-specific research can reveal how variations in hormonal influences, genetic predispositions, and metabolic responses contribute to disparate health outcomes, particularly in complex conditions such as preeclampsia and pregestational diabetes, where male and female offspring exhibit distinct physiological responses (14-17). Furthermore, recognizing the impact of gender on health is essential for reducing health disparities and improving overall health outcomes for both sexes (14-17). The NIH has implemented policies to ensure that sex and gender are considered in all research protocols, promoting comprehensive data collection and analysis that can inform clinical practices and public health initiatives (16, 17). Similarly, the WHO advocates for integrating gender considerations into health systems to enhance the effectiveness of health interventions and policies worldwide (16, 17). By prioritizing gender-specific studies, these organizations aim to foster a more nuanced understanding of health that ultimately leads to improved healthcare quality and equity for diverse populations (16, 17). Given the importance of understanding gender-specific differences, it is imperative to conduct studies that account for these distinctions (16, 17). Accordingly, our study investigated both male and female offspring.

2.11 Justification of the study

Pregestational prediabetes (PP) serves as a significant risk factor for adverse pregnancy outcomes through its multifaceted physiological effects on maternal metabolic health, endothelial function, and foetal development (19-10). PP is a precursor to pT2D however whether its effects on foetal development particularly in the heart, blood, and brain follow a similar pathophysiological aetiology remains largely unexplored (11-14). By investigating the impact of PP on maternal and foetal health, we can identify early interventions aimed at preventing the progression of prediabetes into full-blown type 2 diabetes and mitigating the risk of preeclampsia (15, 16). These conditions are highly prevalent and have substantial impacts on maternal and foetal health worldwide (15-17). With type 2 diabetes affecting nearly 10% of the global population, seeing an increasing incidence among women of reproductive age, while preeclampsia complicates approximately 2–8% of pregnancies globally, remaining the leading cause of maternal and perinatal mortality (15-17). These statistics highlight the critical need to mitigate prediabetes to prevent its progression to more severe conditions and their associated complication (15-17). This research can lead to better diagnostic tools and management strategies, offering significant potential for reducing the incidence of type 2 diabetes in pregnancy, as well as the associated risks of preeclampsia, gestational hypertension, and preterm birth (16, 17). Moreover, by addressing prediabetes before pregnancy, we can reduce the likelihood of adverse maternal health outcomes, improve foetal heart, blood and brain development, and ultimately enhance long-term health of both mother and child (17, 18). In this way, a deeper understanding of the mechanisms of prediabetes and its effect on pregnancy will contribute to more effective preventative and therapeutic strategies, ultimately improving maternal and neonatal health outcomes globally (16-18).

Furthermore, assessing gender-specific outcomes in offspring is vital due to the evidence that pregnancy disorders like PE and pT2D affect male and female fetuses differently (14). For instance, male offspring of preeclamptic pregnancies are more likely to experience cardiovascular complications in adulthood, while female offspring show a higher predisposition to metabolic dysfunctions such as insulin resistance and obesity (73-75). Similarly, pT2D has been shown to differentially impact neurodevelopmental outcomes in males and females, likely due to sex-specific responses to in utero metabolic and hormonal environments. These differences not only influence disease susceptibility in adulthood but also necessitate gender-specific therapeutic and preventive strategies. Understanding these variations can guide tailored interventions to optimize health outcomes for both sexes, addressing their unique risks and improving long-term health trajectories (14). In summary, addressing prediabetes in pregnancy could yield significant improvements in maternal health outcomes (10, 11). Effective interventions could reduce the risk of pregnancy-induced hypertension, gestational diabetes, and adverse obstetric outcomes, such as preterm birth and stillbirth (16-8). Moreover, mitigating prediabetes

may have implications for long-term maternal health, including a reduced risk of chronic diseases like T2DM and cardiovascular disorders (16-18). For the foetus, understanding how prediabetes impacts the development of critical systems, such as the brain, heart, and haematological function, could lead to strategies that improve neonatal health and decrease the likelihood of adult-onset chronic diseases (16-18). Moreover, incorporating gender-specific studies into this research ensures a more comprehensive understanding of these impacts, ultimately contributing to reducing the intergenerational cycle of metabolic and cardiovascular disease burdens while tailoring interventions to address sex-specific vulnerabilities (14-17).

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CHAPTER 3: METHODOLOGY

Chapter 3 provides an overview of how the research objectives were accomplished

3.1 Animals

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 (n=18) were bred and housed in the Biomedical Research Unit (BRU) of the University of Kwa-Zulu Natal for 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 below 65 decibels in accordance with institutional guidelines. The animals were allowed access to food and fluids ad libitum. The animals acclimatized to their new environment for 1 week while consuming standard rat chow and tap water before the induction of prediabetes by exposure to a well-established experimental diet (HFHC).

3.2 Induction of prediabetes

Female Sprague Dawley rats were randomly assigned to three groups, groups A, B, and C (n = 6 per group). Experimental prediabetes was induced in female Sprague–Dawley rats using a protocol previously described (19). To summarise, control groups A and B were given a standard rat chow diet with normal water for the 36-week period. Group C was given a high-fat, high-carbohydrate (HFHC) diet with 15% fructose added for the same duration of 36 weeks. The composition of the HFHC diet is 30% high fats, 55% of carbohydrates and 15% fructose. At the end of 36 weeks, the diagnostic criteria of the American Diabetes Association (ADA) were applied to identify prediabetes in the animals. This involved measuring diagnostic indicators for prediabetes, such as fasting blood glucose levels ranging from 5.6 to 7.1 mmol/L, oral glucose tolerance test (OGTT) with 2-hour glucose levels between 7.1 and 11.1 mmol/L at 0, 7, 12, and 19 weeks, these numbers represent time points in weeks after induction of prediabetes in the maternal rats and were guided by preeclamptic maternal time points of murine studies. Animals meeting this criterion were categorised as prediabetic (PD), while those with measurements below the prediabetic thresholds were considered healthy controls (CON) A and B.

3.3 Oral glucose tolerance response

An oral glucose tolerance test was performed on GND 0, 7, 12, and 19 on all animals to assess their glucose tolerance response. Following a well-established laboratory technique, this test was completed after carbohydrate loading (19). After fasting for 16 hours, blood glucose levels were determined at time 0. Then, a monosaccharide syrup was administered orally using an 18-gauge gavage 38 mm long and curved needle with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). The glucose concentrations were determined by collecting blood using the tail-prick method (19) and measuring

glucose concentrations using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). Blood glucose levels were assessed at 15-, 30-, 60- and 120 minutes following carbohydrate loading. The OGTT values reported here refer specifically to the 2-hour post-load glucose concentration, which is the standard interpretive timepoint for impaired glucose tolerance in rodent metabolic work. Because glucose was measured from tail prick capillary blood using a handheld glucometer, cut-points were applied to support consistent within-study grouping and to reflect relative glucose handling and insulin resistance rather than to imply direct equivalence with clinical venous thresholds. Accordingly, the thresholds are interpreted as operational criteria for this experimental model, and conclusions are framed around between-group differences and progression patterns within the study design rather than diagnostic translation to clinical practice.

3.4 Mating

Prior to mating, all 18 female Sprague–Dawley rats underwent assessment for the proestrus stage through vaginal smear analysis under a microscope (Leica Microsystems, Illinois, USA). Briefly, two females were housed in one cage to allow for their oestrus cycles to synchronize. After synchronization, those in the proestrus stage were permitted to mate with healthy male control Sprague–Dawley rats (19). 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 (Leica Microsystems, Illinois, USA) (19, 25). The male rats were removed from the cage and returned to the BRU. Pregnant rats were then observed until the end of gestation (21 days).

3.5 Induction of preeclampsia

After pregnancy was confirmed by the visualization of spermatozoa in a vaginal smear, the pregnant dams from healthy control group B were randomly divided into one rat per cage, which were given 0.3g/L a day of L-NAME in drinking water from day 14.5 to day 20.5 of gestation. Preeclampsia (PE) was confirmed by the measurement of blood pressure which was monitored every third day after administration (19). Blood pressure was monitored at GND 0, 7, 12, and 19 using a non-invasive tail-cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA). Urine samples were collected at similar GND days, overnight using metabolic cages, and protein urea was measured immediately via dipsticks (Multistix® 10 SG, Siemens Healthineers, Pennsylvania, USA). Animals presenting with a blood pressure of $\geq 140/90$ mmHg and proteinuria of ≥ 300 mg (3+) were considered preeclamptic.

3.5.1 L-NAME Rationale

The inclusion of an L-NAME-induced preeclampsia group allowed for the differentiation between the effects of pregestational prediabetes and established preeclampsia on maternal and offspring outcomes. This comparative approach strengthened the mechanistic interpretation of whether observed infantile alterations were attributable to prediabetes alone or to a preeclampsia-like intrauterine environment (10).

3.6 Collection of pups

At gestational day (GND) 21, naturally delivered male and female pups from control (PCON), L-NAME preeclamptic (PPE) and HFHC diet-induced prediabetic (PPD) dams were immediately collected and weighed. The pups were then carefully returned to the dams and were placed in Makrolon polycarbonate cages under standard laboratory conditions (for temperature and humidity) in a 12-hour day and 12-hour night cycle and were given access to food and water.

Table 1 below summarizes the developmental stages in rats and humans, facilitating a comparison of their relative ages (25).

Table 1: Postnatal developmental stages in Rats and Human

Developmental stages in rat/human	Rat	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)	16-18 years
Emerging adulthood	70-150 days (10-21 weeks)	18-25 years

Adapted from Barrow (2011), Ojeda and skinner (2006), Parker and Picut (2016)

3.7 Behavioural tests

All the behavioural tests were conducted at 09h00 to evaluate the cognitive function of the pups from all the groups using the following techniques.

3.7.1 Morris water maze (MWM)

The training in the Morris water maze (MWM) was conducted from day 22 to day 34. This paradigm is employed to assess the learning and recall ability of rats and covers exploratory, navigational, spatial, and contextual memory. The MWM is made of a 1 m diameter pool, comprising 4 quadrants. Each quadrant has a cue to help the rat locate the hidden platform placed in one of the quadrants of the

pool. The method consists of placing the rat in a quadrant other than where the hidden platform is located and recording the time taken (escape latency period) by the rat to reach the hidden platform is considered as the animal's ability to learn. The probe test is a post-test for learning that examines the ability of an animal to recall the quadrant in which the hidden platform will be located. The time spent in the quadrant with the hidden platform is considered as the ability to remember (memory).

3.7.2 Training and Learning

Animals were exposed to 5 consecutive training sessions for a period of 5 days (day 22-34). Animals were taken to the behavioural room 1 hour prior to the training to allow for familiarization with the new environment. In the training procedure, each rat was softly placed in the water, head facing the cue in a quadrant. The rat was allowed 120 s to locate the hidden platform. When a rat is unsuccessful in finding the hidden platform in 120 s, it will be physically directed by the experimenter on the way to the platform and will be allowed 60 s to explore the platform before being returned to the home cage. Training recommenced the following day for the next 4 days, and the time (escape latency period) it took for the rat to locate the platform will be recorded.

3.8 Study Design

Prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cardiovascular function was studied in the following groups. Immediately after birth, the pups and their placenta were weighed for placenta-to-body weight ratios and separated into groups. The placentas were then stored in normal saline for HIF- α analysis, and the pups were returned to the dams for further development. On post-natal day (PND) 21, the pups (n=72) were weaned and divided into PCON, PPE, and PPD male and female groups (n=6/group). Group A: Male and female pups born from normal pregnant control rats (PCON) which received normal standard chow and normal drinking water throughout the study (n=24); Group B: Male and female pups born from PE pregnant control rats (PPE) which received normal standard chow (n=24). Group C: Male and female pups born from PD pregnant rats (PPD) which received the HCHF diet+ 15% fructose throughout the study (n = 24). The dams were then sacrificed, and plasma was collected for biochemical analysis (IL-6, TNF- α , MDA, SOD and GPx). Thereafter, to assess for infantile cardiac, haematological and cognitive development, half the pups (n=36) were euthanized for terminal investigations on day 21. The guillotine was used to decapitate pups for blood collection into EDTA tubes (Eppendorf centrifuge 5403, Germany) that were centrifuged at 4 °C, 503g for 15 minutes. Cardiac tissue, whole blood and brain tissue was collected. The hearts and the brains were collected for heart-to-body weight and brain-to- body weight ratio analysis. The Left ventricle of the heart was used for oxidative stress analysis and analysis of Left ventricular mRNA gene expression of cTnI and COL I. The rest of the hearts (n=18) were stored in formalin for histology. Whole blood was collected for haematological analysis and plasma was collected for cardiac troponin I (cTnI), erythropoietin (EPO), granulocyte colony- stimulating factor analysis, (G-CSF), oxidative

stress, brain derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF2), interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). The remaining pups (n=36) were further divided into male (n=6) and female (n=6) and allowed to acclimate for a week. To assess the effects on learning and memory, the pups underwent Morris Water Maze (MWM) training and testing at week 5. Thereafter, the pups were euthanised. Hippocampal tissue was collected for gene expression of BDNF and FGF2, respectively. Refer to **figure 1** below.

3.8.1 Brain-to-body weight ratio and cephalization index

The brains were rapidly removed and weighed. Considering the difficulty and low accuracy of head circumference measures in rats, the CI (calculated in humans as $CI = \text{head circumference} \times 100 / BW$ (g)) was calculated using the weight of the brain. In these animals, a modified version of the human CI was calculated as $CI = \text{brain weight (mg)} \times 100 / BW$ (mg). This modified version of the CI reflects the brain/BW ratio, expressed as a percentage.

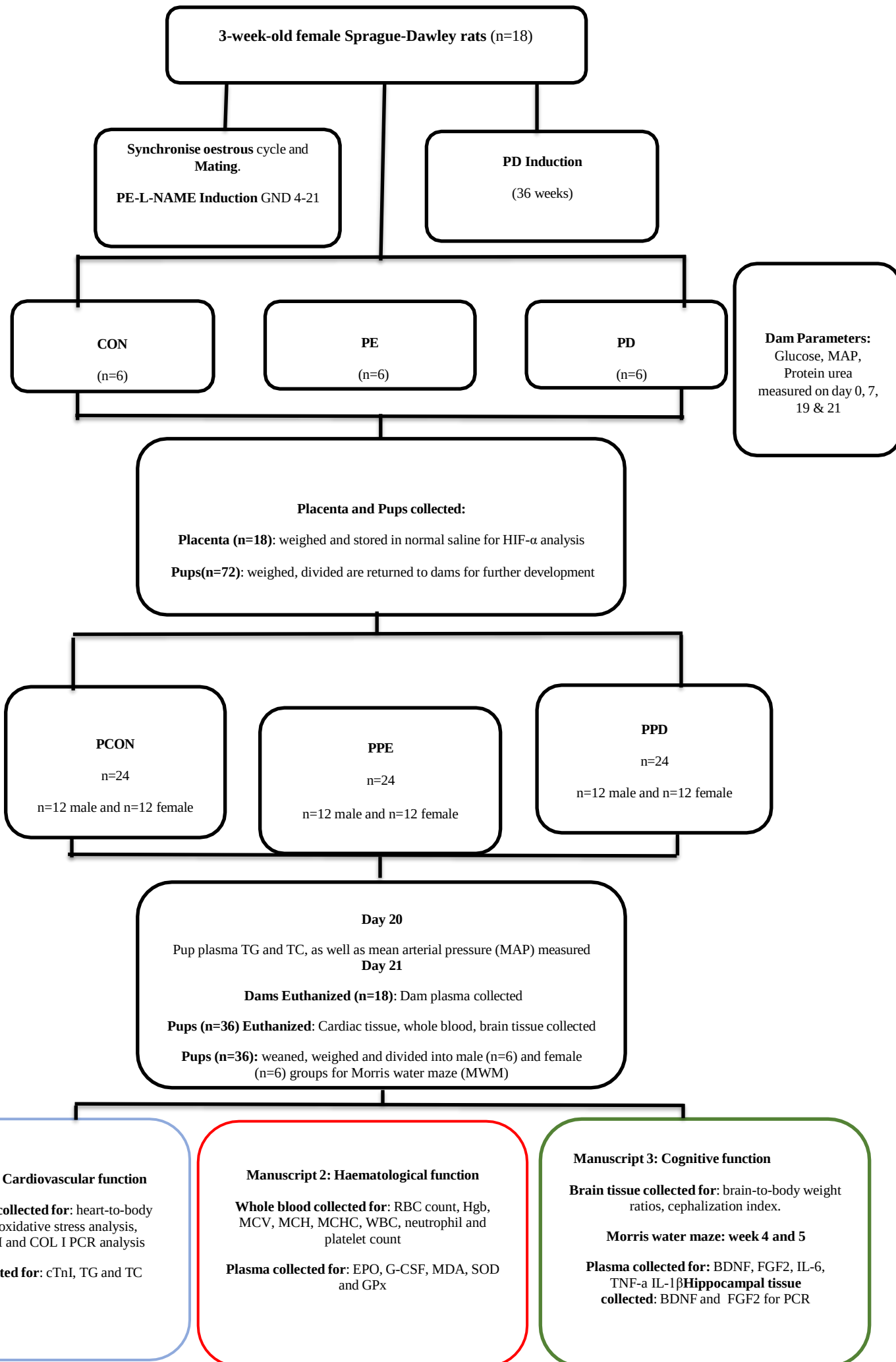


Figure 1: Illustrates the complete study design

3.9 Biochemical Analysis

3.9.1 ELISA analysis of plasma protein concentrations in dams

Hypoxia-inducible factor-1 α (HIF-1 α), interleukin 6 (IL-6), tumour necrosis factor- α (TNF- α), superoxide dismutase (SOD) and glutathione peroxidase (GPx) concentrations in dam plasma were analysed using specific ELISA kits (Elabscience and Biotechnology, Wuhan). Kits included micro-ELISA plates, which were coated with antibodies specific to HIF-1 α , IL-6, TNF- α , SOD, and GPx. Standards and samples (n=36) were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min. The relevant biotinylated detection antibodies (100 μ L) were then added to samples and standards and incubated for 60 min. Avidin-Horseradish Peroxidase (HRP) conjugates (100 μ L) were added to each microplate well-containing samples and standards and incubated for 30 min; Unbound components were washed out using an automated washer (BioTek Instruments, Vermont, USA). Substrate solutions (100 μ L) were added to each microplate well. After incubating for a further 15 minutes, the stop solutions (50 μ L) were added. The optical densities were then measured using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurtemberg, Germany) at the wavelength of 450 nm. The concentration of the samples was extrapolated from the respective standard curves.

3.9.2 Haematological analysis

The erythrocyte markers, namely, red blood cell count (RBC Count), haemoglobin (Hgb) concentration, mean cell volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) and red blood cell distribution width (RDW) were measured together with the white blood cell and neutrophil counts using Coulter Ac•T 5diff CP, USA).

3.9.3 ELISA analysis of plasma protein concentrations in pups

Cardio troponin I (cTnI), erythropoietin (EPO), granulocyte stimulating factor (G-CSF), brain derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF2), interleukin-1 β (IL-1 β), IL-6, TNF- α , SOD, and GPx concentrations in pup plasma were analysed using specific ELISA kits (Elabscience and Biotechnology, WuHan). Kits included micro-ELISA plates which were coated with antibody specific to cTnI, EPO, G-CSF, BDNF, FGF2, IL-1 β , IL-6, TNF- α , SOD and GPx. Standards and samples (n=6) were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min. The relevant biotinylated detection antibodies (100 μ L) were then added to samples and standards and incubated for 60 min. Avidin-Horseradish Peroxidase (HRP) conjugates (100 μ L) were added to each micro-plate well containing samples and standards and incubated for 30 minutes, Unbound components were washed out using an automated washer (BioTek Instruments, Vermont, USA). Substrate solutions (100 μ L) were added to each micro-plate well. After incubating for a further 15 min, the stop solutions (50 μ L) were added. The optical densities were then measured using

a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany) at the wavelength of 450 nm. The concentration of the samples was extrapolated from the respective standard curves.

3.9.4 Malondialdehyde in dam placenta, pup plasma, and pup Left ventricle of the heart

Malondialdehyde was measured using an established protocol (10). Dam tissue homogenate (n=18), pup plasma (n=36) and pup tissue homogenate (n=36) was centrifuged at 400 g for 10 min. Thereafter, 400 mL of the plasma and tissue homogenate was supplemented with 400 mL of 2% phosphoric acid and then separated into two glass tubes, each receiving equal volumes of the solution. Subsequently, 200 mL of 7% phosphoric acid was added into both glass tubes, followed by the addition of 400 mL of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into one glass tube (sample test) and 400 mL of 3 mM hydrochloric acid (HCl) into the second glass tube (blank). To ensure an acidic pH of 1.5, 200 mL of 1M HCl was added to the sample and blank test tubes. Both solutions were heated at 100 °C for 15 min and cooled to room temperature. Butanol (1.5 mL) was added to the cooled solution; the sample was vortexed for 1 min to ensure rigorous mixing and allowed to settle until two phases could be distinguished. The butanol phase (top layer) was transferred to Eppendorf tubes and centrifuged at 13,200 g for 6 min. The samples were aliquoted into a 96-well microtiter plate in triplicate and the absorbance was read at 532 nm (reference 600 nm) on a BioTek mQuant spectrophotometre (Biotek, Johannesburg, South Africa). The absorbance from these wavelengths was used to calculate the concentration of MDA using Beer's Law:

Concentration of MDA = Average Absorbance Absorption/coefficient (156 nm)

3.9.5 Gene expression via real time-PCR in pups

The Left ventricles of the hearts and hippocampuses were collected at day 21 and day 35 respectively. The tissues (n=36) were homogenised, and total RNA was isolated using a reaction kit (Zymo Research Quick-RNA™ Miniprep, Inqaba Biotech) (CA-USA) according to the manufacturer's protocol. Total RNA was reversed transcribed to cDNA using the iScript cDNA synthesis kit [Bio-Rad Laboratory (Pty) Ltd., USA] according to the manufacturer's instructions. PCR amplification was performed using real-time PCR (LightCycler® 2.0, FastStartDNA Master SYBR Green I, Roche Diagnostics, Germany). The mRNA expression levels of cTnI and COL I were expressed in relation to expression levels of the housekeeping gene, glyceraldehyde- 3-phosphate (GAPDH) in the same sample. The PCR reaction mixture per sample comprised of SYBR green; MgCl₂ (25 mM); forward primer (10 mM) and reverse primer (10 mM). Left ventricular cTnI and COL I as well as hippocampal BDNF and FGF2 gene expression was performed using the following oligonucleotide primer sequences (Inqaba Biotechnical Industries, South Africa), The realtime PCR conditions used was as follows: an initial denaturation cycle at 95 °C for 10 min followed by PCR which consisted of 45 cycles at 95 °C

for the 30 s, 65 °C for 30 s and a final elongation at 72 °C for the 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. A standard curve was constructed for each gene using ten-fold serial dilutions of cDNA samples. Lightcycler® analysis software (Version 4.2) was used to produce a standard curve by plotting the log concentration against the crossing point of each standard.

Table 2 complete list of primers used in the study

Gene of interest	Sequence
cTnI (Cardiac troponin I)	Forward: 5'- ATCTGACCCAGCAAATCTATGACCTGCGTG-3' Reverse 5'- CACGCAGGTCATAGATTTGCTGGGTCAGAT-3'
COL I (Collagen I)	Forward: 5'-GCTGAATCCTTCCGTGTT-3' Reverse: 5'-AGGGAGGGGACTTATCTG-3'
BDNF (Brain Derived neurotrophic factor)	Forward: 5'-GCTGCCTTGATGTTTACTTTGA-3' Reverse: 5'-CGTGGACGTTTGCTTCTTTC-3'
FGF2 (Fibroblast growth factor- 2)	Forward: 5'- GAAGGAAGATGGACGGCTGC -3' Reverse: 5'- TGACAGTGTCTAAAGAGAGTC -3'
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	Forward: 5'-AGTGCCAGCCTCGTCTCATA-3' Reverse: 5'-GATGGTGATGGGTTTCCCGT-3'

3.9.6 Cardiac histology in pups

On day 21, the heart tissue from pups (n=36) was removed and immediately stored in formalin for histopathology. The heart tissue was then embedded in paraffin wax, sectioned using a microtome (2µm) and stained through routine protocols with haematoxylin and eosin (H&E) (10). Briefly, the tissue sections were prepared by deparaffinizing in xylene for 5 minutes with two changes, followed by rehydration through a graded ethanol series (100%, 95%, and 70%) for 2 minutes each, and rinsing in distilled water. The sections were stained with haematoxylin for 5 to 10 minutes to visualize nuclei, rinsed in tap water, and differentiated in 1% acid alcohol for 1 to 2 seconds to remove excess staining. To achieve nuclear bluing, the sections were immersed in Scott's tap water substitute for 2 minutes and briefly rinsed in distilled water. Next, the sections were stained with eosin for 1 to 3 minutes to counterstain cytoplasm and extracellular components, followed by a quick rinse in distilled water. The slides were dehydrated through a graded ethanol series, cleared in xylene for 3 minutes, and mounted

with a coverslip using a DPX mounting medium. This method produced nuclei with a distinct blue/purple colour and cytoplasmic regions-stained pink for optimal histological visualization (80).

The prepared slides were then analysed using digital scanning technology (Leica SCN-400). To determine the cardiomyocyte area, we utilized ImageJ (version 1.53, ImageJ Software, LOCI, Wisconsin, USA) (23). First, the image scale was calibrated using a photograph of an object with a known size. We employed the straight-line tool to draw a line along the known object, then selected "Analyse" followed by "Set Scale" to define the scale. Cardiomyocyte measurements were taken at the level of the nucleus. Using the straight-line tool, we drew a line from one side of the cell membrane, through the nucleus, to the opposite side of the membrane, and then clicked "Analyse" and "Measure" to obtain the area. A minimum of 100 cells per section were measured, and the results were exported to Excel by selecting "Results," then "File," and saved as a .csv file. The mean value for each slide was then calculated for area analysis.

3.10 Sample Size Calculation

The sample size (n) for this study was calculated using G*Power (Version 3.1.9.7), a statistical power analysis software (81-83). For the maternal rats, three groups (normal, preeclamptic, and prediabetic) were assigned, each consisting of n = 6, resulting in a total of n = 18 rat mothers. A priori power analysis was performed to ensure the sample size was adequate to detect statistically significant differences between groups while maintaining ethical considerations for animal use.

3.10.1 Rationale for n = 18 (6 mothers per group):

The study design involves three groups: normal, preeclamptic, and prediabetic, with 6 rat mothers in each group. The sample size of 6 per group is often selected based on a priori power analysis using software like G*Power. This analysis considers an effect size calculated from previous studies or pilot data, with common assumptions such as 80% statistical power ($1 - \beta = 0.80$) and a significance level of 0.05. For instance, detecting medium effect sizes ($f = 0.25$) in a one-way ANOVA setup with 3 groups requires approximately 18 total subjects (or 6 per group) for sufficient statistical power.

3.10.2 Rationale for n = 72 (pups):

The total sample size of rat pups (n = 72) is derived from the number of mothers and expected litter sizes, which can vary by strain but are typically around 4-12 pups per mother in Sprague-Dawley rats. This ensures adequate representation of offspring for analysing gender-specific outcomes. Each mother's contribution to the group outcomes is considered, ensuring variability is appropriately accounted for.

3.11 Statistical analysis in dams and pups

All data were expressed as means $\pm$ standard error of the mean (SEM). Normality was assessed prior to statistical testing. Analyses were performed using GraphPad Prism InStat Software (version 9.00, GraphPad Software, San Diego, California, USA). For dam parameters, comparisons were conducted using a one-way analysis of variance (ANOVA), as sex was not a variable of interest. For pup parameters, a two-way analysis of variance (ANOVA) was employed, with Group (Control, Preeclamptic, Prediabetic) and Sex (Female, Male) as fixed factors, thereby allowing simultaneous testing of group differences, sex differences, and their potential interaction. Where significant main effects or interactions were detected, post hoc analyses were conducted using the Tukey–Kramer test for parametric data, or the Bonferroni correction for non-parametric data, to identify pairwise differences between groups. For ANOVAs and other multivariate analyses, assumptions of homogeneity of variance and sphericity were assessed; in cases where sphericity was violated, Greenhouse

BRIDGE 1

The literature review illustrated that pregnancies affected by T2DM and PE impact foetal cardiovascular, haematological and cognitive development, contributing to growth restrictions and obstetric dangers, posing greater risks for cardiovascular, haematological and cognitive disorders later in life. These disruptions, driven by third-trimester hypoxia, proinflammatory cytokines, and oxidative stress, contribute to intrauterine growth restriction and obstetric complications, increasing the risk of long-term cardiovascular, haematological, and cognitive disorders in offspring. Prediabetes, a precursor to T2DM, is also recognized as a risk factor for PE. This suggests that women of childbearing age can unknowingly progress from the prediabetic stage to overt T2DM and increases the likelihood of them developing hypertensive disorders such as PE, leading to high maternal and infant morbidity and mortality rates. Therefore, the occurrence of prediabetes during pregnancy is hypothesized to exacerbate foetal developmental dysfunction, with adverse effects on infantile cardiovascular, haematological, and cognitive systems. This could predispose offspring to cardiovascular diseases, haematological abnormalities, and cognitive impairments in adulthood. Considering this hypothesis, the first manuscript aims to assess diet-induced prediabetes as a risk factor for PE, focusing on gender-specific infantile outcomes related to cardiovascular function.

CHAPTER 4: MANUSCRIPT 1

4. PROLOGUE

Maternal disorders, such as preeclampsia (PE) and pregestational type 2 diabetes (pT2D), profoundly influence foetal cardiovascular, haematological, and cognitive development leading to adverse infantile outcomes. These outcomes are driven by placental insufficiency, endothelial dysfunction, chronic inflammation, and oxidative stress. Recent studies implicate pregestational prediabetes (PP), an intermediate hyperglycaemic state preceding pT2D, as a potential contributor to these disturbances. PP may alter the intrauterine environment through impaired placental function, leading to intrauterine growth restriction (IUGR) and disrupted developmental programming, thereby increasing the risk of long-term metabolic and cardiovascular complications. While PE and pT2D are well-documented contributors to adverse foetal cardiac outcomes, the effects of maternal prediabetes on cardiovascular development in infants, particularly with respect to sex-specific outcomes, remain poorly characterized. The literature review demonstrated that pregnancies complicated by PE and pT2D are associated with significant foetal growth restriction, placental hypertrophy, and structural and functional alterations in the developing cardiovascular system. These disruptions were largely driven by placental hypoxia, elevated oxidative stress, and inflammatory pathways, collectively contributing to a heightened risk of adult-onset cardiovascular diseases. Moreover, given that prediabetes often progresses to pT2D, especially during childbearing years, it represents a critical and modifiable risk factor for adverse maternal and foetal outcomes. Building on these findings, the second manuscript explores the role of pregestational prediabetes induced by a high-fat, high-carbohydrate (HFHC) diet on cardiovascular development in postnatal offspring, focusing on gender-specific differences in key markers of cardiac injury, remodelling, and oxidative stress. By elucidating the impact of prediabetes on foetal programming and cardiovascular health, this work aims to expand our understanding of prediabetes as an early and modifiable risk factor for adverse pregnancy outcomes and long-term offspring health.

The manuscript titled **“High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cardiovascular function”** is authored by N.D. Xulu, A Khathi, N.H. Sibiyi and P.S. Ngubane

The manuscript is under review in *American Journal of Physiology-Heart and Circulatory Physiology*. See Appendix 2. This journal is accredited by the Department of Higher Education and Training South Africa and appears in the Scopus accredited list (2023).

**High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia:
Assessing gender-specific infantile outcomes on selected markers of cardiovascular function**

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4.1 Abstract

Preeclampsia (PE) and pregestational type 2 diabetes (pT2D) are major pregnancy complications associated with impaired foetal growth and increased cardiovascular vulnerability in offspring. Prediabetes, characterised by impaired glucose regulation prior to overt diabetes, has been identified as an important risk factor for the development of PE; however, its direct contribution to placental development and early-life cardiovascular growth indices in offspring remains poorly defined. This study utilised a high-fat, high-carbohydrate (HFHC) diet-induced prediabetes rat model to investigate the impact of maternal prediabetes as a risk factor for PE on placental proportions and postnatal cardiac outcomes in offspring.

Methodology

Male and female pups were born to control (PCON), L-NAME-induced PE (PPE), and HFHC diet-induced prediabetic (PPD) dams. Placentas and pups were collected and weighed immediately at birth, and after 21 days, pups were weaned, weighed, and sacrificed for plasma and heart tissue collection. Biochemical analyses included triglycerides (TG), total cholesterol (TC), oxidative stress markers, cardiac histology, and gene expression of cardiac proteins (cTnI, COL I) and MAP of the pups

Results

Offspring from the PPD and PPE groups showed lower placenta-to-body weight and heart-to-body weight ratios $p < 0.05$ than controls, with the PPD group most affected, suggesting IUGR and placental hypertrophy. Elevated plasma TG, TC, and cardiac markers $p < 0.05$ compared to controls indicated risks for impaired infantile cardiac development in these groups, especially in PPD. Cardiomyocyte enlargement and increased cTnI and collagen I expression in the PPD group pointed to significant cardiac injury according to the developmental stage of the pups.

Conclusion

In this study, our murine/rat model showed that pregestational prediabetes is a potential risk factor for the development of preeclampsia and foetal cardiac abnormalities. Early interventions targeting PP may help reduce the risk of PE and pT2D and associated long-term cardiovascular complications in offspring

4.2 Introduction

Cardiovascular diseases (CVDs) are the most common causes of morbidity and mortality worldwide (1). CVDs can be traced back to impaired foetal cardiovascular development. Therefore, a healthy maternal environment is of paramount importance in ensuring proper foetal development (1,2). The placenta is pivotal to this role as adequate placental function ensures optimal exchange of oxygen and nutrients (3,4). In pregestational type 2 diabetes, placental function is impaired, resulting in abnormal foetal development and intrauterine growth restriction IUGR, which increases the risk of chronic diseases later in life (1,2). Chronic hyperglycaemia, an inherent feature of pT2D, leads to the induction of oxidative stress and endothelial dysfunction, impairing angiogenesis and nutrient transport, which, in turn, culminates into restricted growth of the foetus (3,4). These disturbances in utero predispose individuals to metabolic and cardiovascular disease in later life (8). The risk of developing preeclampsia is further increased by pT2D through shared metabolic, vascular, and inflammatory mechanisms (9). Chronic hyperglycaemia leads to the development of endothelial dysfunction, oxidative stress, and systemic inflammation, all significant contributors to preeclampsia (10). In pregnancies complicated by both pT2D and PE, foetal cardiac development is adversely affected (16). The increased placenta-to-body weight ratio in pT2D and PE is indicative of placental insufficiency and IUGR, which lead to foetal hypoxia and cardiac remodelling mediated by hypoxia-inducible factors (17). This hypoxic state results in increased heart-to-body weight ratios mediated through foetal hyperinsulinemia in pT2D and compensatory hypertrophy due to foetal hypertension in PE, respectively (18). These changes accompany significant alterations in mean arterial blood pressure (MAP), cardiomyocyte size, collagen I (COL I), and cardiac troponin I (cTnI) mRNA expression, serving as indicators of changes in the structural integrity of the developing heart (18). Furthermore, the complications and mechanisms observed in type 2 diabetes are also present in prediabetes but are generally milder due to intermediate hyperglycaemia (19, 20). Prediabetes serves as a precursor to pT2D and is characterized by insulin resistance, leading to impaired glucose metabolism (21). Prediabetes can activate similar pathological pathways seen in pT2D, such as chronic low-grade inflammation and raised oxidative stress (22). These contribute to endothelial dysfunction, which increases the risk of cardiovascular complications, including hypertension and dyslipidaemia (21, 22). Although the severity of these complications generally is not as marked in prediabetes, chronic elevations in glucose levels still pose a significant risk for the development of atherosclerosis and other cardiovascular diseases (23). Furthermore, it is crucial to consider gender-specific outcomes when studying the effects of diabetes on pregnancy (24). Males and females exhibit differential physiological responses to metabolic changes because of distinct differences in genetic, hormonal, and metabolic factors (24). These variations can lead to disturbances in infant cardiac development and growth. In our laboratory, animal studies utilizing the high-fat, high-carbohydrate (HFHC) diet-induced prediabetic model have demonstrated prediabetes (PD) as a risk factor for preeclampsia (19). Moreover, studies using this model have demonstrated adverse alterations to foetal hypothalamic-pituitary-adrenal (HPA) axis function (25). However, the effects of prediabetes

on sex-specific infant cardiovascular function have not yet been established. Therefore, this study sought to assess the effects of HFHC diet-induced prediabetes on gender-specific infantile outcomes on selected markers of cardiovascular function.

4.3 Materials and methods

4.3.1 Animals

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 (n=18) were bred and housed in the Biomedical Research Unit (BRU) of the University of Kwa-Zulu Natal for 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 acclimatized to their new environment for 1 week while consuming standard rat chow and tap water before the induction of prediabetes by exposure to a well-established experimental diet (HFHC).

4.3.2 Induction of prediabetes

Female Sprague–Dawley rats were randomly assigned to three groups, groups A, B, and C (n = 6 per group). Experimental prediabetes was induced in female Sprague–Dawley rats using a protocol previously described (19). To summarize, control group A and B were given a standard rat chow diet with normal water for the 36-week period. Group C was given a high-fat, high-carbohydrate (HFHC) diet with 15% fructose added for same duration of 36 weeks. The composition of the HFHC diet is 30% high fats, 55% of carbohydrates and 15% fructose. At the end of 36 weeks, the diagnostic criteria of the American Diabetes Association (ADA) were applied to identify prediabetes in the animals. This involved measuring diagnostic indicators for prediabetes, such as fasting blood glucose levels ranging from 5.6 to 7.1 mmol/L, oral glucose tolerance test (OGTT) with 2-hour glucose levels between 7.1 and 11.1 mmol/L at 0, 7, 12, and 19 weeks, these numbers represent time points in weeks after induction of prediabetes in the maternal rats and were guided by preeclamptic maternal time points from rodent studies. Animals meeting this criterion were categorized as prediabetic (PD), while those with measurements below the prediabetic thresholds were considered healthy controls (CON) A and B.

4.3.3 Oral glucose tolerance response

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 (19). After fasting for 16 hours, glucose levels were determined at time 0. Then, a monosaccharide syrup was administered orally using an 18-gauge gavage needle that is 38 mm long and curved, with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). The glucose concentrations were determined by collecting blood using the tail-prick method (19) and measuring

glucose concentrations using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). Glucose levels were subsequently assessed at 15-, 30-, 60- and 120-minutes following carbohydrate loading. The Oral Glucose Tolerance Test (OGTT) values reported here refer specifically to the 2-hour post-load glucose concentration, the standard diagnostic timepoint for impaired glucose tolerance in rodent models. The cut-points were interpreted within the context of capillary glucose measurements, ensuring that group allocation reflects relative glucose tolerance and insulin resistance rather than absolute equivalence to venous values. Calibration and quality control were performed according to manufacturer instructions prior to measurement to ensure reliable readings for experimental grouping. These tools were selected due to their rapid assessment capability, minimal blood volume requirements, and established use in small-animal metabolic studies, which is critical for longitudinal monitoring of pups and dams.

4.3.4 Mating

Prior to mating, all 18 female Sprague–Dawley rats underwent assessment for the proestrus stage through vaginal smear analysis under a microscope (Leica Microsystems, Illinois, USA). Briefly, two females were housed in one cage to allow for their oestrus cycles to synchronize. After synchronization those in the proestrus stage were permitted to mate with healthy male control Sprague–Dawley rats (19). Confirmation of pregnancy and assignment of gestational day (GND) zero were determined the following morning by the presence of a vaginal plaque or vaginal smear containing spermatozoa observed under a microscope (Leica Microsystems, Illinois, USA) (19, 25). The male rats were then removed from the cage and were returned to the BRU. Pregnant rats were then observed until the end of gestation (21 days).

4.3.5 Induction of preeclampsia

After pregnancy was confirmed by the visualization of spermatozoa in a vaginal smear, the pregnant dams from healthy control group B were randomly divided into one rat per cage, they were given 0.3g/L a day of L-NAME in drinking water from day 14.5 to day 20.5 of gestation. Blood pressure was measured and the presence or absence of protein in the urine was assessed (19). Preeclampsia (PE) was confirmed by the measure of blood pressure which was monitored every third day after administration. Animals presenting with a blood pressure of $\geq 140/90$ mmHg and proteinuria of ≥ 300 mg (3+) were considered preeclamptic. Blood pressure was monitored at GND 0, 7, 12, and 19. using a non-invasive tail-cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA). Urine samples were collected at similar GND days, overnight using metabolic cages and protein urea was measured immediately via dipsticks (Multistix® 10 SG, Siemens Healthineers, Pennsylvania, USA).

4.3.5.1 L-NAME Rationale

The inclusion of an L-NAME-induced preeclampsia group allowed for the differentiation between the effects of pregestational prediabetes and established preeclampsia on maternal and offspring outcomes. This comparative approach strengthened the mechanistic interpretation of whether observed infantile alterations were attributable to prediabetes alone or to a preeclampsia-like intrauterine environment(10).

4.3.6 Collection of pups

At gestational day (GND) 21, naturally delivered male and female pups from control (PCON), L-NAME preeclamptic (PPE) and HFHC diet-induced prediabetic (PPD) dams were immediately collected and weighed. The pups were then carefully returned to the dams and were placed in Makrolon polycarbonate cages under standard laboratory conditions (for temperature and humidity) in a 12-hour day and 12-hour night cycle and were given access to food and water. On day 20 pup triglycerides (TG) and total cholesterol (TC) were measured using Accutrend Plus Cholesterol meter (Roche Diagnostic, Germany). The mean arterial pressure (MAP) was monitored using the non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA) as previously described (19). The equipment was calibrated daily prior to the measurements. The temperature in the enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodland Hills, California, USA) were maintained at $\pm 30^{\circ}\text{C}$ for at least 30 minutes before MAP measurements. All measurements were performed at 09h00. To assess infantile development, the animals were euthanized for terminal investigations on day 21 (25). **Table 1** below summarises the developmental stages in rats and humans, facilitating a comparison of their relative ages (25).

Table 1: Postnatal developmental stages in Rats and Human

Developmental stages in rat/human	Rat	Human
Neonatal/new born	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)	16-18 years
Emerging adulthood	70-150 days (10-21 weeks)	18-25 years

Adapted from Barrow (2011), Ojeda and skinner (2006), Parker and Picut (2016)

4.3.7 Study Design

Prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cardiovascular function was studied. Immediately after birth, the pups and their placenta

were weighed for placenta-to-body weight ratios and separated into groups. The placentas (n=18) were then stored in normal saline for HIF- α analysis, and the pups were returned to the dams for further development. On post-natal day (PND) 21 the pups (n=36) were weaned, and divided into PCON, PPE, PPD male and female groups (n=6/group). Group A: Male and female pups born from normal pregnant control rats (PCON) which received normal standard chow and normal drinking water throughout the study (n=12); Group B: Male and female pups born from PE pregnant control rats (PPE) which received normal standard chow (n=12). Group C: Male and female pups born from PD pregnant rats (PPD) which received the HCHF diet+ 15% fructose throughout the study (n=12). The dams were then sacrificed, and plasma was collected for biochemical analysis (IL-6, TNF- α , MDA, SOD and GPx). Thereafter, the pups were weighed and sacrificed. Whole hearts were collected, weighed for heart-to-body weight ratio calculations, and subsequently dissected for Left ventricular mRNA gene expression analysis of cTnI and COL I. The other half was stored in formalin for histology. Pup plasma was collected for cardiac troponin I (cTnI) and oxidative stress markers (MDA, SOD and GPx) respectively. The study design is summarised in the figure below (**Figure 1**).

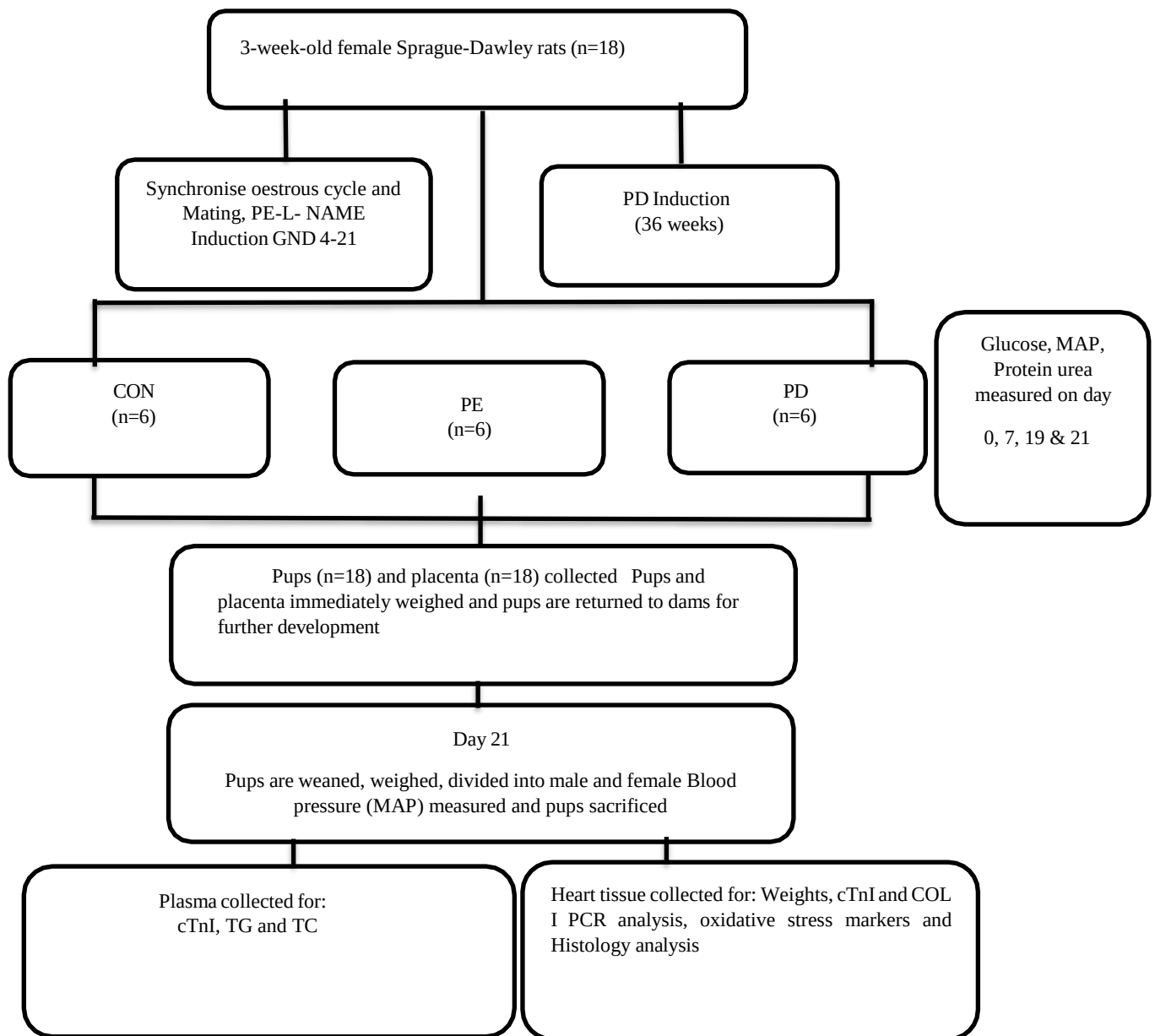


Figure 1: Illustrates a graphical abstract description of the study design.

4.3.8 Biochemical analysis

4.3.8.1 Hypoxia, inflammation, and oxidative stress markers in dam plasma

Hypoxia inducible factor-1 alpha (HIF-1 α) interleukin 6 (IL-6), tumour necrosis factor alpha (TNF- α), superoxide dismutase (SOD), glutathione peroxidase (GPx) Vascular endothelial growth factor (VEGF), Placental growth factor (PIGF) Soluble Endoglin (sEng) and Soluble fsm-like tyrosine kinase-1 (sFlt-1) concentrations in dam plasma were analysed using specific ELISA kits (Elabscience and Biotechnology, WuHan). Kits included micro-ELISA plates which were coated with antibody specific to HIF-1 α , IL-6, TNF- α , SOD, GPx, VEGF, PIGF, sEng and sFlt-1. Standards and samples (n=18) were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min. The relevant biotinylated detection antibodies (100 μ L) were then added to samples and standards and

incubated for 60 min. Avidin-Horseradish Peroxidase (HRP) conjugates (100 μ L) were added to each micro-plate well containing samples and standards and incubated for 30 min, Unbound components were washed out using an automated., washer (BioTek Instruments, Vermont, USA). Substrate solutions (100 μ L) were added to each micro- plate well. After incubating for a further 15 min, the stop solutions (50 μ L) were added. The optical densities were then measured using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden- Wurttemberg, Germany) at the wavelength of 450 nm. The concentration of the samples was extrapolated from the respective standard curves.

4.3.8.2 Plasma cardiac troponin I (cTnI) concentrations in pups

Cardiac troponin I (cTnI) concentrations in pup plasma were analysed using specific ELISA kits (Elabscience and Biotechnology, WuHan). Kits included micro-ELISA plates which were coated with antibody specific to cTnI. Standards and samples (n=36) were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min. The relevant biotinylated detection antibodies (100 μ L) were then added to samples and standards and incubated for 60 min. Avidin- Horseradish Peroxidase (HRP) conjugates (100 μ L) were added to each micro-plate well containing samples and standards and incubated for 30 min, Unbound components were washed out using an automated washer (BioTek Instruments, Vermont, USA). Substrate solutions (100 μ L) were added to each micro-plate well. After incubating for a further 15 min, the stop solutions (50 μ L) were added. The optical densities were then measured using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany) at the wavelength of 450 nm. The concentration of the samples was extrapolated from the respective standard curves.

4.3.8.3 Left ventricular superoxide dismutase (SOD) and glutathione peroxidase (GPx) concentrations in pups

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) concentrations in pup Left ventricle were analysed using specific ELISA kits (Elabscience and Biotechnology, WuHan). Kits included micro-ELISA plates which were coated with antibody specific SOD, and GPx. Standards and samples (n=36) were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min. The relevant biotinylated detection antibodies (100 μ L) were then added to samples and standards and incubated for 60 min. Avidin-Horseradish Peroxidase (HRP) conjugates (100 μ L) were added to each micro-plate well containing samples and standards and incubated for 30 min, Unbound components were washed out using an automated washer (BioTek Instruments, Vermont, USA). Substrate solutions (100 μ L) were added to each micro-plate well. After incubating for a further 15 min, the stop solutions (50 μ L) were added. The optical densities were then measured using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany) at the wavelength of 450 nm. The concentration of the samples was extrapolated from the respective standard curves. The intra-assay coefficient of variation (CV) for the ELISAs ranged from 4.2% to 7.5%, while

the inter-assay CV ranged from 6.8% to 9.1%, consistent with acceptable limits for quantitative immunoassays.

4.3.8.4 Malondialdehyde in dam placenta and pup Left ventricle

Malondialdehyde was measured using an established protocol (10). Tissue homogenate for dams (n=18) and pups (n=36) was centrifuged at 400 g for 10 min. Thereafter, 400 mL of the tissue homogenate was supplemented with 400 mL of 2% phosphoric acid and then separated into two glass tubes, each receiving equal volumes of the solution. Subsequently, 200 mL of 7% phosphoric acid was added into both glass tubes, followed by the addition of 400 mL of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into one glass tube (sample test) and 400 mL of 3 mM hydrochloric acid (HCl) into the second glass tube (blank). To ensure an acidic pH of 1.5, 200 mL of 1M HCl was added to the sample and blank test tubes. Both solutions were heated at 100 °C for 15 min and cooled to room temperature. Butanol (1.5 mL) was added to the cooled solution; the sample was vortexed for 1 min to ensure rigorous mixing and allowed to settle until two phases could be distinguished. The butanol phase (top layer) was transferred to Eppendorf tubes and centrifuged at 13,200 g for 6 min. The samples were aliquoted into a 96-well microtiter plate in triplicate and the absorbance was read at 532 nm (reference 600 nm) on a BioTek mQuant spectrophotometre (Biotek, Johannesburg, South Africa). The absorbance from these wavelengths was used to calculate the concentration of MDA using Beer's Law:

Concentration of MDA = Average Absorbance Absorption/coefficient (156 nm)

4.3.8.4 Left ventricular cardiac troponin I (cTnI) and collagen I (COL I) expression via real time- PCR in pups

The Left ventricles of the hearts were collected at day 21 and the tissues (n=36) were homogenised, and total RNA was isolated using a reaction kit (Zymo Research Quick-RNA™ Miniprep, Inqaba Biotech) (CA-USA) according to the manufacturer's protocol. Total RNA was reversed transcribed to cDNA using the iScript cDNA synthesis kit [Bio-Rad Laboratory (Pty) Ltd., USA] according to the manufacturer's instructions. PCR amplification was performed using real-time PCR (LightCycler® 2.0, FastStartDNA Master SYBR Green I, Roche Diagnostics, Germany). The mRNA expression levels of cTnI and COL I were expressed in relation to expression levels of the housekeeping gene, glyceraldehyde- 3-phosphate (GAPDH) in the same sample. The PCR reaction mixture per sample comprised of SYBR green; MgCl₂ (25 mM); forward primer (10 mM) and reverse primer (10 mM). cTnI and COL I gene expression was performed using the following oligonucleotide primer sequences (Inqaba Biotechnical Industries, South Africa), The realtime PCR conditions used was as follows: an initial denaturation cycle at 95 °C for 10 min followed by PCR which consisted of 45 cycles at 95 °C for the 30 s, 65 °C for 30 s and a final elongation at 72 °C for the 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. A standard curve was constructed for each gene using ten-fold serial dilutions of cDNA

samples. Lightcycler® analysis software (Version 4.2) was used to produce a standard curve by plotting the log concentration against the crossing point of each standard.

Table 2 List of primers used in the study

Gene of interest	Sequence
cTnI (Cardiac troponin I)	Forward: 5'- ATCTGACCCAGCAAATCTATGACCTGCGTG-3' Reverse: 5'- CACGCAGGTCATAGATTTGCTGGGTCAGAT-3'
COL I (Collagen I)	Forward: 5'-GCTGAATCCTTCCGTGTT-3' Reverse: 5'-AGGGAGGGGACTTATCTG-3'
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	Forward: 5'-AGTGCCAGCCTCGTCTCATA-3' Reverse: 5'-GATGGTGATGGGTTTCCCGT-3'

4.3.8.5 Cardiac histology in pups

On day 21, the heart tissue pups (n=36) was removed and immediately stored in formalin for histopathology. The heart tissue was then imbedded in paraffin wax, sectioned using a microtome (2µm) and stained through routine protocols with haematoxylin and eosin (H&E). Briefly, the tissue sections were prepared by deparaffinizing in xylene for 5 minutes with two changes, followed by rehydration through a graded ethanol series (100%, 95%, and 70%) for 2 minutes each, and rinsing in distilled water. The sections were stained with haematoxylin for 5 to 10 minutes to visualize nuclei, rinsed in tap water, and differentiated in 1% acid alcohol for 1 to 2 seconds to remove excess staining. To achieve nuclear bluing, the sections were immersed in Scott's tap water substitute for 2 minutes and briefly rinsed in distilled water. Next, the sections were stained with eosin for 1 to 3 minutes to counterstain cytoplasm and extracellular components, followed by a quick rinse in distilled water. The slides were dehydrated through a graded ethanol series, cleared in xylene for 3 minutes, and mounted with a coverslip using a DPX mounting medium. This method produced nuclei with a distinct blue/purple colour and cytoplasmic regions-stained pink for optimal histological visualization (80).

The prepared slides were then analysed using digital scanning technology (Leica SCN-400). To determine cardiomyocyte area, we utilized ImageJ (version 1.53, ImageJ Software, LOCI, Wisconsin, USA) (23). First, the image scale was calibrated using a photograph of an object with a known size. We employed the straight-line tool to draw a line along the known object, then selected "Analyse" followed by "Set Scale" to define the scale. Cardiomyocyte measurements were taken at the level of the nucleus. Using the straight-line tool, we drew a line from one side of the cell membrane, through the nucleus, to the opposite side of the membrane, and then clicked "Analyse" and "Measure"

to obtain the area. A minimum of 100 cells per section were measured, and the results were exported to Excel by selecting "Results," then "File," and saved as a .csv file. The mean value for each slide was then calculated for area analysis. In Excel, we calculated the arithmetic mean of the “Area” column for that slide:

$$\bar{A}_{\text{slide}} = \frac{\sum_{i=1}^n A_i}{n}$$

Where A_i is the area of the i -th cardiomyocyte and n is the number of cells measured on that slide. When multiple fields of view were captured for a slide, measurements from all fields were pooled for that slide (each cell counted once; no weighting). Obvious artefactual measurements (e.g., truncated/overlapping cells that failed our a priori inclusion criteria) were excluded before averaging. When animals had more than one slide, we computed the animal-level mean as the average of the slide means; these animal-level means were used for group-level analyses and summary statistics (SD/SEM).

4.3.9 Sample size calculation

The sample size (n) for this study was calculated using G*Power (Version 3.1.9.7), a statistical power analysis software (81-83). For the maternal rats, three groups (normal, preeclamptic, and prediabetic) were assigned, each consisting of $n = 6$, resulting in a total of $n = 18$ rat mothers. A priori power analysis was performed to ensure the sample size was adequate to detect statistically significant differences between groups while maintaining ethical considerations for animal use.

4.3.9.1 Rationale for $n = 18$ (6 mothers per group):

The study design involves three groups: normal, preeclamptic, and prediabetic, with 6 rat mothers in each group. The sample size of 6 per group is often selected based on a priori power analysis using software like G*Power. This analysis considers an effect size calculated from previous studies or pilot data, with common assumptions such as 80% statistical power ($1 - \beta = 0.80$) and a significance level of 0.05. For instance, detecting medium effect sizes ($f = 0.25$) in a one-way ANOVA setup with 3 groups requires approximately 18 total subjects (or 6 per group) for sufficient statistical power.

4.3.9.2 Rationale for $n = 72$ (pups):

The total sample size of rat pups ($n = 72$) is derived from the number of mothers and expected litter sizes, which can vary by strain but are typically around 4-8 pups per mother in Sprague-Dawley rats. This ensures adequate representation of offspring for analysing gender-specific outcomes. Each mother's contribution to the group outcomes is considered, ensuring variability is appropriately accounted for

4.3.10 Statistical analysis in dams and pups

All data were expressed as means $\pm$ standard error of the mean (SEM). Normality was assessed prior to

statistical testing. Analyses were performed using GraphPad Prism InStat Software (version 9.00, GraphPad Software, San Diego, California, USA). For dam parameters, comparisons were conducted using a one-way analysis of variance (ANOVA), as sex was not a variable of interest. For pup parameters, a two-way analysis of variance (ANOVA) was employed, with Group (Control, Preeclamptic, Prediabetic) and Sex (Female, Male) as fixed factors, thereby allowing simultaneous testing of group differences, sex differences, and their potential interaction. Where significant main effects or interactions were detected, post hoc analyses were conducted using the Tukey–Kramer test for parametric data, or the Bonferroni correction for non-parametric data, to identify pairwise differences between groups. For ANOVAs and other multivariate analyses, assumptions of homogeneity of variance and sphericity were assessed; in cases where sphericity was violated, Greenhouse–Geisser corrections were applied to adjust the degrees of freedom. Statistical significance was set at $p < 0.05$, and corrected degrees of freedom are reported where applicable.

4.4. Results

4.4.1 Induction parameters for PE and PP in dams

Table 3 shows induction parameter results for preeclampsia (MAP, PU) and prediabetes (FPG, OGTT) in healthy control dams (CON), L-NAME-induced preeclamptic dams (PE), and HFHC diet-induced prediabetes (PP) dams at week 36. The MAP and PU were significantly ($p < 0.05$) higher in PE and PP groups compared to CON. The FPG and OGTT were significantly ($p < 0.05$) lower in PE than PP, whereas the MAP and PU were significantly ($p < 0.05$) higher in PE compared to PP.

Parameters measured	Control (CON)	Preeclampsia (PE)	Pregestational prediabetes (PP)
Mean arterial pressure (MAP) (mmHg)	110 ± 3.12	140 ± 5.57 ★	129 ± 4.35 ★ ^α
Proteinuria (PU) (mg/day)	10-30 (Pale yellow)	300-400 (Orange/red) ★	100-200 ★ ^α (Light orange)
Fasting plasma glucose (mg/dL)	90 ± 2.45	92 ± 2.87 ★	110 ± 4.98 ★ ^α
Oral glucose tolerance test (OGTT) (mg/dL)	120 ± 3.99	121 ± 4.11 ★	160 ± 6.22 ★ ^α

Table 3: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with CON and ^α $p < 0.05$ by comparison with PE.

4.4.2 Angiogenic, antiangiogenic, inflammation, oxidative stress and hypoxia markers in dams

Table 4 shows angiogenic, antiangiogenic, inflammation, oxidative stress and hypoxia markers in healthy control dams (CON) L-NAME-induced preeclamptic dams (PE), and HFHC diet-induced prediabetic (PP) dams at week 36. sEng, sFlt-1, IL-6, TNF- α , MDA and HIF-1 α were significantly ($p < 0.05$) higher in PE and PP groups compared to CON. Whereas VEGF, PlGF, SOD and GPx were significantly ($p < 0.05$) lower in PE and PP groups compared to CON. Moreover, IL-6, TNF- α , SOD, GPx and were significantly ($p < 0.05$) higher in PE than PP, with sEng, sFlt-1, MDA and HIF-1 α showing significantly ($p < 0.05$) lower concentrations in PE compared to PP

Parameters measured	Control (CON)	Preeclampsia (PE)	Pregestational prediabetes (PP)
Vascular endothelial growth factor (VEGF) (pg/mL)	152.4 $\pm$ 5.8	95.6 $\pm$ 4.8 ★	85.2 $\pm$ 4.2 ★ α
Placental growth factor (PlGF) (pg/mL)	135.7 $\pm$ 3.9	80.4 $\pm$ 3.5 ★	75.2 $\pm$ 3.7 ★ α
Soluble Endoglin (sEng) (ng/mL)	48.9 $\pm$ 2.3	74.6 $\pm$ 3.4 ★	86.1 $\pm$ 4.1 ★ α
Soluble fsm-like tyrosine kinase-1 (sFlt-1) (ng/mL)	60.4 $\pm$ 2.8	105.7 $\pm$ 4.9 ★	112.8 $\pm$ 5.2 ★ α
Interleukin-6 (IL-6) (pg/mL)	9.86 $\pm$ 1.12	58.30 $\pm$ 4.20 ★	42.70 $\pm$ 3.15 ★ α
Tumour necrosis factor alpha (TNF- α) (pg/mL)	15.63 $\pm$ 0.67	47.53 $\pm$ 2.35 ★	33.10 $\pm$ 2.04 ★ α
Malondialdehyde (MDA) (nmol/g)	0.28 $\pm$ 0.02	1.15 $\pm$ 0.06 ★	1.45 $\pm$ 0.05 ★ α
Superoxide dismutase (SOD) (ng/mL)	245.80 $\pm$ 10.36	155.45 $\pm$ 9.78 ★	140.60 $\pm$ 8.54 ★ α
Glutathione peroxidase (GPx) (ng/mL)	155.32 $\pm$ 7.90	115.22 $\pm$ 6.50 ★	98.45 $\pm$ 5.60 ★ α
Placental hypoxia inducible factor 1- α (HIF-1 α)	0.21 $\pm$ 0.03	0.76 $\pm$ 0.05 ★	0.82 $\pm$ 0.06 ★ α

Table 4: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with CON and α $p < 0.05$ by comparison with PE.

4.4.3 Pup litter size and pup deaths per litter, and the number of surviving male and female pups per dam

Table 5 Shows the pup litter size and pup deaths per litter, and the number of surviving male and female pups per dam in healthy control (CON), preeclamptic (PE), and prediabetic dams (PP). The PE and PP showed significant ($p < 0.05$) reductions in litter size and increased pup mortality compared to CON. The litter size of the PP was significantly ($p < 0.05$) increased accompanied by a significant decrease in pup deaths per litter compared to PE group. Moreover, the number of surviving males per dam was significantly ($p < 0.05$) decreased compared to surviving female offspring.

PARAMETERS MEASURED	CON	PE	PP
Total Pup litter Size (n=12 each)	100%	100% ★	100% ★ α
Pup Deaths per Litter (%)	0%	25% ★	16.7% ★ α
Number of surviving male pups per dam	50%	33% ★	41.7% ★ α
Number of surviving female pups per dam	50%	42% ★ ϕ	41.7% ★ α

Table 5: Values are presented as percentages. ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. ϕ $p < 0.05$ by comparison with the male offspring.

4.4.4 Placenta weights, pup body weights and placenta-to body weight ratios

Table 6 shows the placenta weights, body weights and placenta-to-body weight ratios of 21-day old pups born from healthy control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and high fat, high carbohydrate (HFHC) diet-induced prediabetes dams (PPD). The PPE- male and female (M and F) and PPD- male and female (M and F) and males heart-to-body weight ratios showed significantly ($p < 0.05$) decreased placenta and body weights as well as increased placenta-to-body weight ratios in comparison to PCON. Interestingly, the placenta and body weights of PPD were significantly ($p < 0.05$) decreased accompanied by a significant increase in placenta-to-body weight ratios compared to PPE group. Furthermore, placenta weights, body weights and placenta-to-body weight ratios were significantly ($p < 0.05$) pronounced in males compared to female offspring

PARAMETERS MEASURED	PCON-M	PPE-M	PPD-M	PCON-F	PPE-F	PPD-F
Placenta weights (g)	0.55 ± 0.08	0.67 ± 0.05 ★	0.72 ± 0.06 ★ α	0.50 ± 0.05 ϕ	0.63 ± 0.07 ★ ϕ	0.69 ± 0.06 ★ $\alpha\phi$
Body weights (g)	5.8 ± 0.30	3.8 ± 0.20 ★	3.3 ± 0.10 ★★ α	5.5 ± 0.30 ϕ	3.5 ± 0.20 ★ ϕ	3.1 ± 0.05 ★ $\alpha\phi$
Ratio	0.095	0.1760 ★	0.218. ★ α	0.091 ϕ	0.180 ★ ϕ	0.223 ★ $\alpha\phi$

Table 6: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. ϕ $p < 0.05$ by comparison with the male offspring.

4.4.5 Pup heart weights, body weights and heart-to-body weight ratios

Table 7 shows the heart weights, body weights and heart-to-body weight ratios of 21-day old pups born from healthy control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and high fat, high carbohydrate (HFHC) diet-induced prediabetes dams (PPD). The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) decreased heart and body weights as well as increased heart-to-body weight ratios in comparison to PCON. Interestingly, the heart and body weights of PPD were significantly ($p < 0.05$) decreased accompanied by a significant increase in heart-to-body weight ratios compared to PPE group. Moreover, heart weights, body weights and placenta-to-body weight ratios were significantly ($p < 0.05$) higher in males compared to female offspring

PARAMETERS MEASURED	PCON-M	PPE-M	PPD-M	PCON-F	PPE-F	PPD-F
Heart weights (g)	1.25 $\pm$ 0.08	1.10 $\pm$ 0.05 ★	1.07 $\pm$ 0.05 ★ ^{α}	1.18 $\pm$ 0.07 ^{ϕ}	1.05 $\pm$ 0.04 ★ ^{ϕ}	1.02 $\pm$ 0.05 ★ ^{$\alpha\phi$}
Body weights (g)	39.2 $\pm$ 4.99	27.8 $\pm$ 4.75 ★	24.5 $\pm$ 5.98 ★	36.8 $\pm$ 5.49 ^{ϕ}	24.8 $\pm$ 4.28 ★ ^{ϕ}	22.5 $\pm$ 5.38 ★ ^{$\alpha\phi$}
Ratios	0.032	0.040 ★	0.044 ★	0.032 ^{ϕ}	0.042 ★ ^{ϕ}	0.043 ★ ^{$\alpha\phi$}

Table 7: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and ^{α} $p < 0.05$ by comparison with PPE. ^{ϕ} $p < 0.05$ by comparison with the male offspring.

4.4.6 Pup plasma triglyceride (TGs) and cholesterol (TCs) concentrations

Figure 2 shows the (A) plasma triglyceride (TG) and (B) cholesterol (TC) in pups born from healthy control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) at the end of the study. The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) increased plasma TG and TC in comparison to the pups born from healthy dams. Furthermore, PPE showed a relative but insignificant decrease in TG and TC when compared to PPD. Also, TG and TC concentrations were significantly ($p < 0.05$) higher in male compared to female offspring.

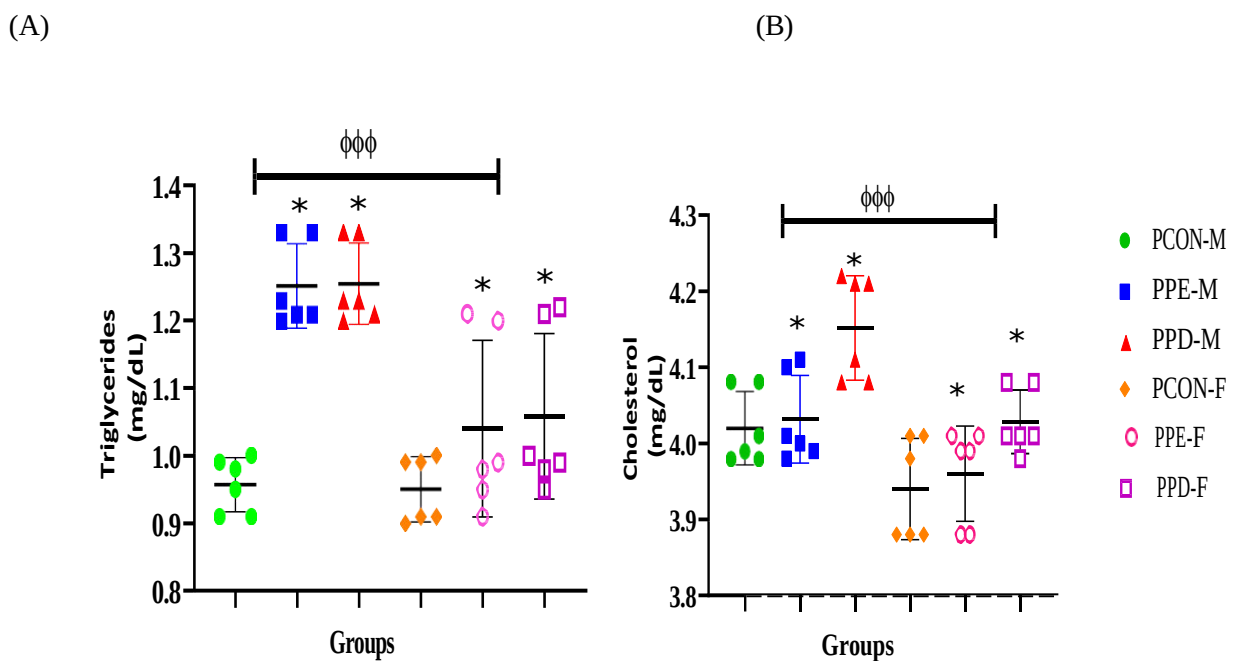


Figure 2: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON. φ $p < 0.05$ by comparison with the male offspring.

4.4.7 Pup mean arterial pressure (MAP)

Figure 3 shows the mean arterial pressure (MAP) in pups born from healthy control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) at the end of the study. The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) increased MAP in comparison to the pups born from healthy dams. Furthermore, PPE showed a significant increase in MAP when compared to PPD. Additionally, the MAP was significantly ($p < 0.05$) higher in male compared to female offspring.

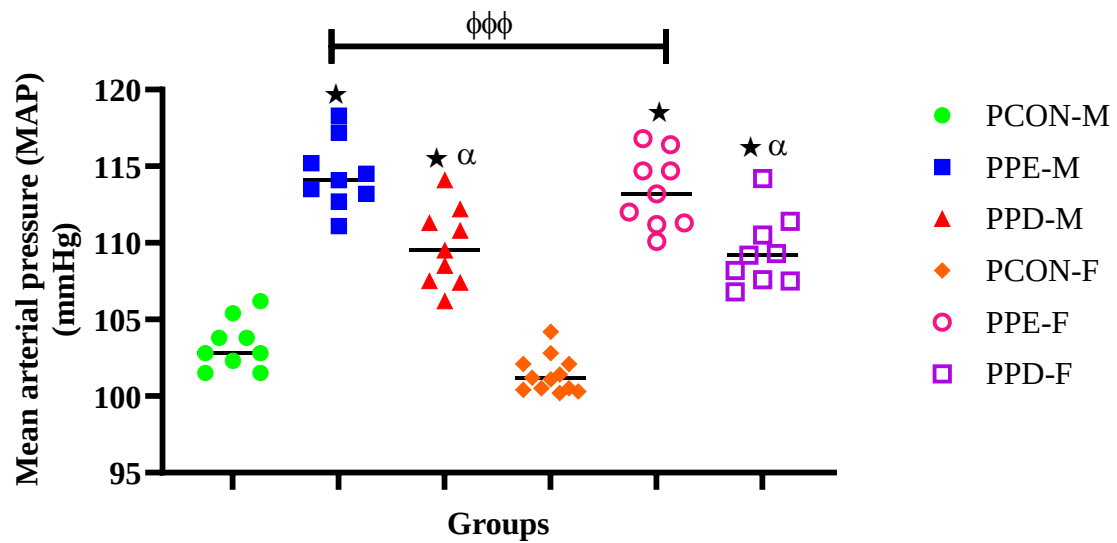


Figure 3: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison with the male offspring.

4.4.8 Pup cardiac troponin I (cTnI) gene expression and plasma cardiac troponin I concentrations

Figure 4 shows the (A) cardiac troponin I (cTnI) gene expression and (B) plasma cardiac troponin I concentrations in pups born from healthy control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) at the end of the study. The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) increased cTnI gene expression and plasma cTnI concentrations in comparison to the pups born from healthy dams. Interestingly, PPE showed a significant increase in cTnI gene expression and plasma cTnI concentrations in when compared to PPD. Moreover, the gene expression and plasma cTnI were significantly ($p < 0.05$) higher in male compared to female offspring.

(A)

(B)

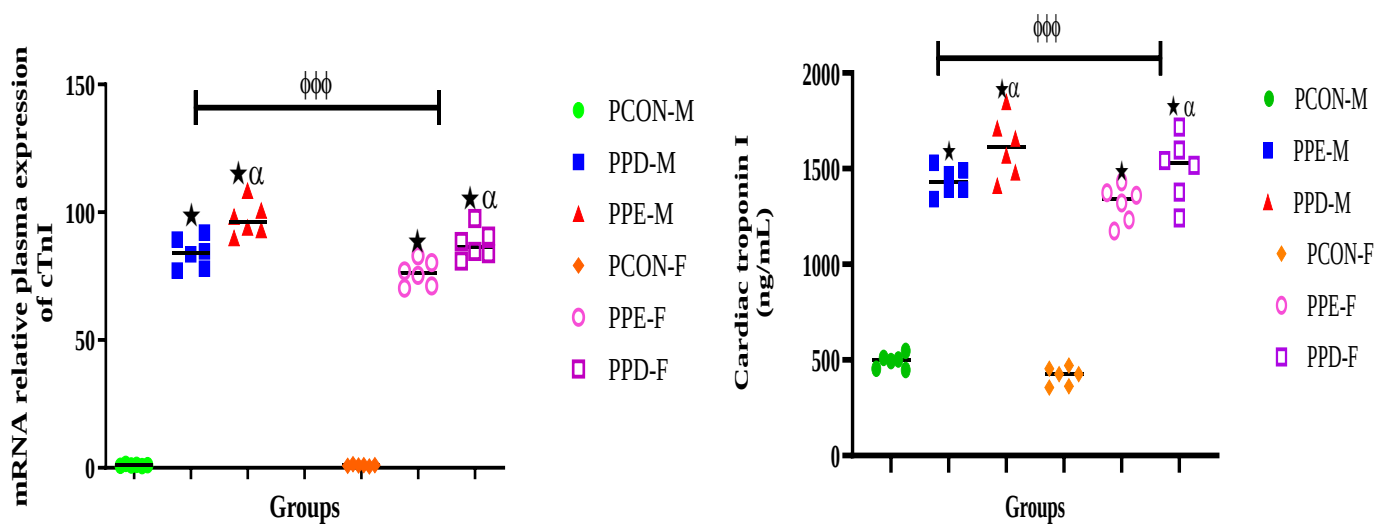


Figure 4: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison with male offspring.

4.4.9 Pup collagen I (COL I) gene expression

Figure 5 shows the collagen I (COL I) gene expression in pups born from healthy control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) at the end of the study. The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) increased COL I gene expression in comparison to the pups born from healthy dams. Interestingly, PPE showed a significant decrease in COL I gene expression when compared to PPD. Furthermore, the gene expression of COL I was significantly ($p < 0.05$) higher in male compared to female offspring.

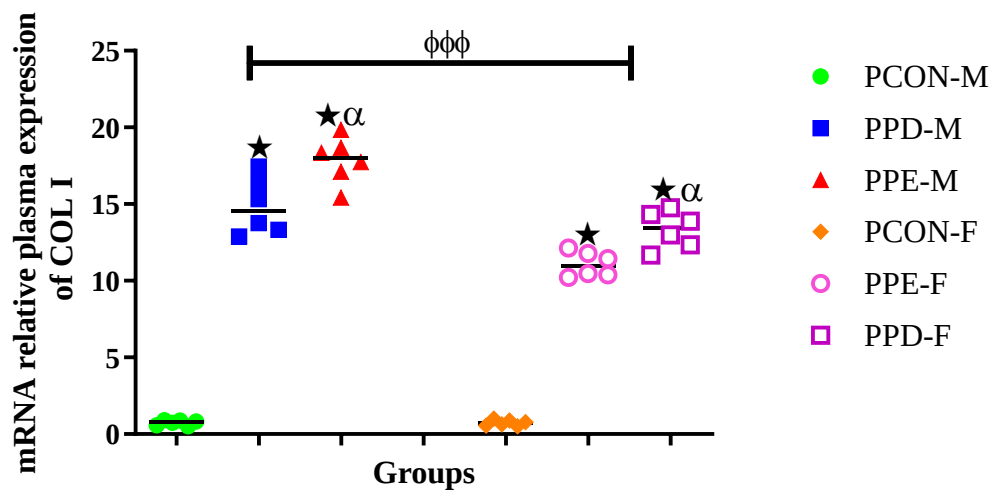


Figure 5: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison with male offspring.

4.4.10 Pup oxidative stress markers

Table 8 shows the Left ventricular malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) of pups born from healthy control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and high fat, high carbohydrate (HFHC) diet-induced prediabetes (PPD). The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) increase in MDA and SOD and significant decrease in GPx in comparison to PCON. Interestingly, MDA and SOD and GPx of PPD were significantly ($p < 0.05$) increased and decreased compared to PPE. Also, the oxidative stress was significantly ($p < 0.05$) pronounced in male compared to female offspring.

PARAMETERS MEASURED	PCON-M	PPE-M	PPD-M	PCON-F	PPE-F	PPD-F
Malondialdehyde (MDA) (nmol/g)	0.25 $\pm$ 0.03	0.43 $\pm$ 0.04 $\star$	0.49 $\pm$ 0.05 $\star^a$	0.22 $\pm$ 0.02 $^\phi$	0.39 $\pm$ 0.03 $\star^\phi$	0.45 $\pm$ 0.04 $\star^{a\phi}$
Superoxide dismutase (SOD) (ng/mL)	198.45 $\pm$ 8.73	140.58 $\pm$ 0.13 $\star$	131.34 $\pm$ 5.92 $\star^a$	207.89 $\pm$ 9.9 $^\phi$	145.72 $\pm$ 6.88 $\star^\phi$	133.85 $\pm$ 6.34 $\star^{a\phi}$
Glutathione peroxidase (GPx) (ng/mL)	145.04 $\pm$ 7.1	85.93 $\pm$ 0.42 $\star$	70.84 $\pm$ 1.13 $\star^a$	150.32 $\pm$ 6.8 $^\phi$	90.15 $\pm$ 0.38 $\star^\phi$	75.91 $\pm$ 1.03 $\star^{a\phi}$

Table 8: Values are presented as means and standard error of means (SEM). $\star$ $p < 0.05$ by comparison with PCON and a $p < 0.05$ by comparison with PPE. ϕ $p < 0.05$ by comparison with male offspring.

4.4.11 Pup cardiac histology

Table 9 and Figure 6 shows longitudinal section and area of Left ventricular cardiomyocytes (H&E stain) in pups born from healthy control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and high fat, high carbohydrate (HFHC) diet-induced prediabetes (PPD). The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) increase in cardiomyocyte size in comparison to PCON. Interestingly, cardiomyocyte size of PPD was significantly ($p < 0.05$) increased compared to PPE. Furthermore, the cardiomyocyte size and area were significantly ($p < 0.05$) higher in male compared to female offspring.

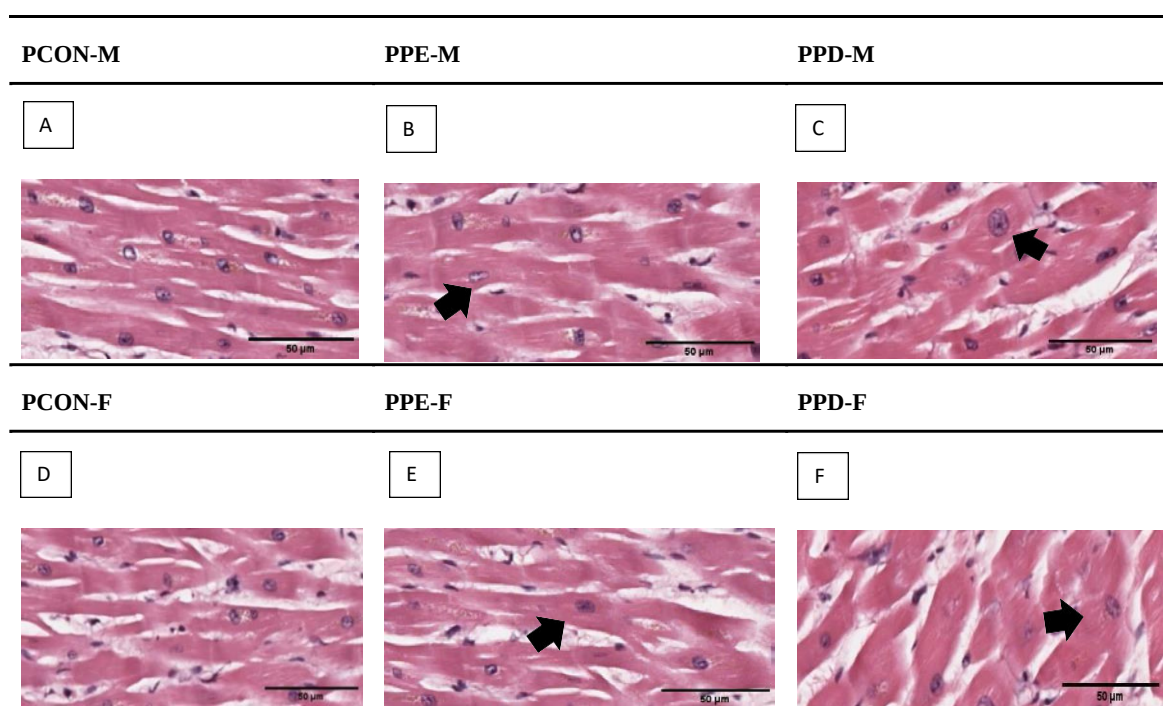


Table 9: Histological sections of longitudinal heart tissue stained for cardiomyocytes in male (A-C) and female (D-F) offspring. (A) PCON-M: Male offspring born from normal control group, showing typical cardiomyocyte structure with no signs of enlargement. (B) PPE-M: Male offspring born from preeclampsia group, with arrow demonstrating notable enlargement of cardiomyocytes compared to the control. (C) PPD-M: Male offspring born from prediabetes group, with arrow displaying pronounced cardiomyocyte enlargement, more severe than in the preeclampsia group. (D) PCON-F: Female offspring born from normal control group, with normal cardiomyocyte structure similar to that of males. (E) PPE-F: Female offspring born from preeclampsia group, with arrow showing cardiomyocyte enlargement similar to males, though slightly less pronounced. (F) PPD-F: Female offspring born from prediabetes group, with arrow showing significant cardiomyocyte enlargement, even more severe than in the preeclampsia group, and consistent with observations in the male group, however less severe.

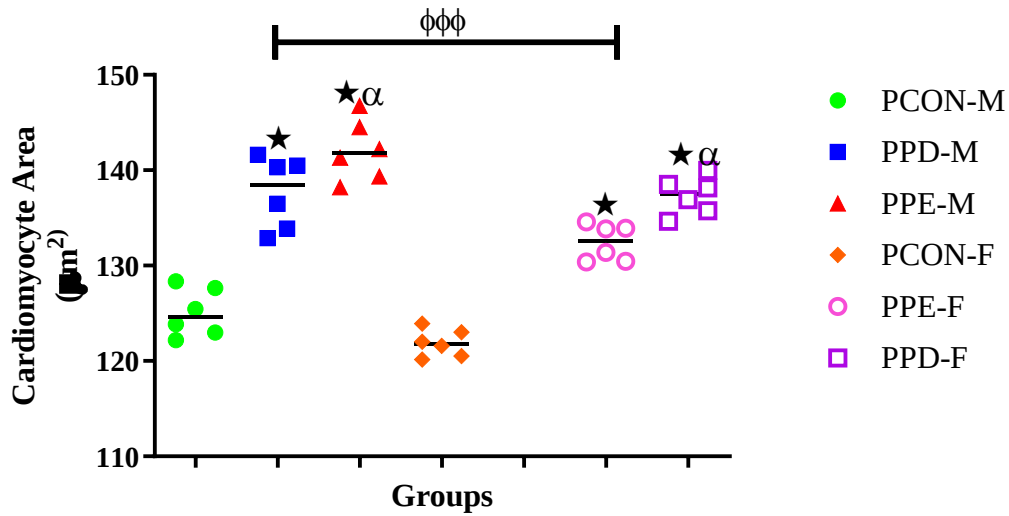


Table 8 and Figure 6: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison with male offspring.

4.5 Discussion

Preeclampsia (PE) and pregestational type 2 diabetes mellitus (pT2D) are conditions that frequently result in intrauterine growth restriction (IUGR), which has significant implications for foetal cardiac development (1, 2). While substantial research focuses on the effects of pT2D on cardiac growth and function, the implications of pregestational prediabetes (PP) on cardiogenesis remain under-explored (1-3). Given that PP is recognized as a precursor to pT2D and a contributing risk factor for PE, it is imperative to investigate its influence on the developing foetal heart (18, 19). This necessitates a thorough exploration of the underlying mechanisms by which PP affects foetal cardiac morphology and function, as well as for the future development of targeted interventions to mitigate these effects, ultimately aiming to prevent the progression of PP to type 2 diabetes mellitus and the onset of preeclampsia. In this study, we assessed the physiological and biochemical markers associated with infant heart development in Sprague Dawley rat pups, including pup and placental weights, heart-to-body weight ratios, mean arterial pressure (MAP), lipid profiles (triglycerides (TG) and cholesterol (TC)), mRNA expression and plasma concentrations of cardiac troponin I and collagen I, as well as oxidative stress markers (malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx)), complemented by histological analyses of the foetal heart.

In this study, induction parameters, and angiogenic factors, anti-angiogenic factors, inflammation, oxidative stress, and hypoxia markers were examined in control (CON), preeclamptic (PE), and prediabetic (PP) dams at week 36 to elucidate the impact of these conditions on maternal and foetal outcomes (**Table 3 and 4**). In **table 4**, the vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) levels were significantly reduced in the preeclamptic (PE) and pregestational prediabetic (PP) groups when compared to the control group (8,16). These pro-angiogenic factors, essential for maintaining placental vascular integrity and supporting foetal growth, were markedly diminished, reflecting compromised placental function (8, 16, 19). This reduction was accompanied by a sharp increase in the anti-angiogenic factors soluble endoglin (sEng) and soluble fms-like tyrosine kinase-1 (sFlt-1) in both the PE and PP groups, indicating a shift toward a highly anti-angiogenic state (19). Interestingly, while both conditions induced substantial changes, the PP group exhibited even lower VEGF and PlGF levels than the PE group, suggesting a more pronounced impairment in angiogenic signalling (8, 16, 19). At the same time, sEng and sFlt-1 levels were significantly higher in the PP group than in the PE group, highlighting the severity of vascular dysfunction associated with PP. This pattern of reduced pro-angiogenic factors and elevated anti-angiogenic factors in the PP group suggests that prediabetes exerts a more extensive disruption of the maternal-foetal interface compared to preeclampsia alone (19). Given that inflammatory cytokines (IL-6, TNF- α), oxidative stress burden (MDA), reduced antioxidant defenses (SOD, GPx), and heightened hypoxia signalling (HIF-1 α) were observed in PE and PP dams (Table 4), the angiogenic shift is best interpreted as occurring within a demonstrably inflammatory and oxidative maternal milieu that is known to impair endothelial and placental vascular function (8, 16, 19, 25). Prediabetes, marked by intermediate hyperglycaemia, hyperinsulinaemia, and chronic low-grade inflammation, likely amplifies the oxidative stress and endothelial dysfunction observed in preeclampsia (19, 25). This combination of metabolic and vascular stress disrupts placental angiogenesis, leading to a maladaptive intrauterine environment (19, 25). Although the findings painted a more or less clear picture of a disturbed angiogenic homeostasis in the presence of inflammation, oxidative stress and hypoxic pathways, no direct significant assessment of the placental structure itself was carried out(19, 25). To be more specific, there was no assessment of placental histology, stereology or transporter studies, so there was no direct quantitation of the villous spatial arrangement, vasculature, or ability to transfer substances. The conclusions of the present paper are based on indirect functional indicators of placenta activity, such as correspondences between the placenta and body size of the foetus, as well as the available profile of maternal angiogenic, inflammatory, oxidative, and hypoxia-inducing indices(19, 25).

Moreover, the significant reduction in VEGF and PlGF levels has profound implications for foetal development (8, 16). These factors are critical for the formation and maintenance of the placental and foetal vasculature, which ensures efficient oxygen and nutrient exchange between the mother and child (8, 16). Lower levels of VEGF and PlGF may result in reduced placental perfusion, limiting the supply

of essential resources to the developing foetus (8, 29). This can lead to intrauterine growth restriction (IUGR) and suboptimal organ development, particularly in high- energy-demand organs such as the brain and heart (8, 17, 29). The elevated levels of sEng and sFlt-1 further exacerbate these effects by actively antagonizing the already reduced VEGF and PlGF activity (8, 17). This heightened anti-angiogenic state not only worsens placental dysfunction but also increases the risk of systemic inflammation and oxidative stress in the foetus, further impairing growth and programming (8, 17, 29)

To elucidate these findings, we assessed the inflammatory and oxidative profiles of the dams. IL-6 and TNF- α , are key inflammatory cytokines which were significantly elevated in both PE and PP compared to CON, this finding was consistent with the pro-inflammatory state seen in these disorders (8, 19, 25). The higher levels of these markers in PE compared to PP suggest a more intense inflammatory response

in preeclampsia, likely driven by severe placental ischaemia and endothelial activation (29). In contrast, the inflammatory state in PP may result from systemic effects of chronic hyperglycaemia and the associated metabolic dysregulation, which, while less pronounced than in PE, is still substantial (19, 25). Moreover, oxidative stress markers also showed distinct patterns (18, 19). Malondialdehyde (MDA), a by-product of lipid peroxidation, was significantly elevated in PE and PP compared to CON, with PP exhibiting the higher levels than PE. This may reflect the sustained oxidative burden induced by chronic hyperglycaemia and insulin resistance in PP, which exacerbates mitochondrial dysfunction and free radical generation (19, 25). The higher MDA levels in PP relative to PE suggest that oxidative stress in prediabetes extends beyond placental insufficiency to encompass systemic metabolic disturbances (19, 25). This finding underscores the heightened risk for mothers with prediabetes, as the pervasive oxidative damage could exacerbate maternal vascular health and foetal growth outcomes (25). Antioxidant defences, as indicated by SOD and GPx levels, were markedly reduced in PE and PP compared to CON, with the greatest reduction observed in PP. The more profound decrease in PP could be attributed to the chronic depletion of antioxidant reserves due to sustained oxidative stress, further impairing cellular defences (19, 25). This diminished antioxidant capacity in PP implies a greater susceptibility to oxidative damage, which may compromise maternal vascular health and foetal cardiac development to a greater extent than in PE alone (19, 25, 29). Hypoxia markers, such as HIF-1 α , were elevated in both PE and PP compared to CON, reflecting placental insufficiency and disrupted oxygen homeostasis (8, 25, 29). Interestingly, HIF-1 α levels were higher in PP than in PE. This unexpected result could be due to a more chronic, multifaceted insult of hyperglycaemia-induced oxidative stress and chronic low-grade inflammation which together with endothelial dysfunction cause this heightened hypoxic state (19, 25). For mothers with prediabetes, this heightened hypoxic state underscores the potential for compounded risks, as foetal oxygen delivery is further compromised resulting in infant mortality, intrauterine growth restriction (IUGR) or small for gestational age (SGA) infants (25).

Due to the adverse effects of metabolic dysfunction, vascular impairment, and compromised placental function observed in PE and pT2D, the number of pup births and pup deaths are significantly affected (26, 29). Given that PP is a precursor to PE and pT2D, we hypothesized that similar changes to pup litter size and pup mortality rate would occur (25). Consequently, this study assessed pup litter size and pup mortality per litter to evaluate the impact of PP on reproductive outcomes. Pup litter size and pup mortality per litter were examined in CON, PE, and PP dams (**Table 5**). Both the PE and PP groups showed significant reductions in litter size and increased pup mortality compared to CON. Moreover, while both conditions induced these adverse effects, the PP group exhibited a larger litter size and lower pup mortality than the PE group, suggesting that PP, a precursor to pT2D and PE, disrupts maternal glucose homeostasis and induces systemic inflammation, oxidative stress, and vascular dysfunction, which impair foetal morbidity and mortality (25). Although the PE group exhibited the most severe outcomes, the PP group displayed an intermediate phenotype, highlighting the progressive nature of

metabolic dysfunction (25). Moreover, a gender disparity was observed in the number of surviving pups, with male pups being more affected than female pups in both PE and PP conditions. Studies suggest that male fetuses are more vulnerable to stressors and environmental factors, such as hypoxia or inflammation, which are often associated with PE and PP (24, 34). These findings align with the literature, in which demonstrates that male offspring tend to have poorer survival rates in adverse conditions, likely due to sex-linked differences in immune response and metabolic programming (24, 34, 35). These findings suggest that early intervention to prevent PP could mitigate these adverse outcomes and improve maternal and infant health (25).

Furthermore, to assess the impacts of the changes observed in the maternal environment on infant growth and development, weights and placenta-to-body weight ratios were measured (25, 26). These parameters are essential indicators to check for IUGR as well as to predict infant growth trajectories and developmental milestones (25, 26). Increased placental weight is often associated with enhanced nutrient transfer to the foetus, which may contribute to improved pup weights (19). However, in cases of placental hypertrophy, as observed in preeclampsia and sometimes in pregestational type 2 diabetes, the increase in size may not correlate with enhanced functionality (19, 25, 26). In PE, and as observed in the findings of this study, maternal hypoxia and oxidative stress causes altered vascularization, trophoblastic hypertrophy due to upregulation of anti-angiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1), which disrupts the balance of pro-angiogenic factors, leading to decreased placental perfusion and consequently, placental hypertrophy leading to low body weights (28, 29). Similarly, in pT2D, high maternal blood glucose and elevated insulin levels in the placenta activate pathways such as protein kinase B (AKT) and mechanistic target of rapamycin (mTOR), which contribute to trophoblastic cell proliferation and growth, resulting in placental hypertrophy and reduced body weights (30-32). However, in pregestational prediabetes (PP), placental weights in relation to body weights have not yet been established. In this study, **Table 6** shows a negative correlation between placental weight and pup body weight in pups born from both preeclamptic (PPE) and pregestational prediabetic (PPD) rats compared to those born from healthy controls (PCON). This finding suggests that placental hypertrophy and inadequate placental function lead to disrupted foetal growth and reduced body weights (29, 32). These results align with previous studies indicating that impaired placental growth can lead to foetal underdevelopment and potential retardation of cardiovascular organ growth (25, 33). Moreover, placenta-to-body weight ratios and growth indices appeared more pronounced in male than in female offspring (34). In the literature, sex differences in foetal growth restriction are often discussed in relation to divergent placental adaptive strategies and sex-linked differences in vulnerability to hypoxia and inflammatory exposure, which may shape nutrient allocation and subsequent organ growth trajectories (24, 34, 35). However, because placental transporter activity, endocrine mediators, and sex hormone concentrations were not measured in this study, these explanations should be treated as interpretive rather than confirmatory. Additional work that combines

placental profiling with endocrine and metabolic measurements is required to determine whether the observed sex pattern reflects differential placental adaptation, postnatal growth dynamics, or a combination of both.

Thus, to assess the link between impaired foetal growth and cardiac development we measured the heart-to-body weight ratio in offspring (36). The heart-to-body weight ratio is a crucial marker that monitors cardiac growth and development; it can also signal congenital heart defects or underdeveloped cardiac structures (36, 37). Placental dysfunction and hypoxia observed in PE have been shown to stimulate signalling pathways like hypoxia-inducible factors (HIFs) that may trigger maladaptive foetal programming and altered cardiovascular development, leading to a decrease in heart-to-body weight ratio (37, 38). Likewise, in pT2D, the excess maternal glucose that crosses the placenta, causing hyperinsulinaemia and placental hypertrophy, disrupts cardiomyocyte proliferation, leading to structural abnormalities and impaired cardiac growth, which decreases the heart-to-body weight ratio (39). However, in PP, a precursor to pT2D and a risk factor for PE, the heart-to-body weight has not been elucidated (31, 32). Our study observed variations in this ratio among different groups, suggesting differential cardiac adaptation in response to various in utero environments (**Table 7**). The lower ratio in PPD suggests reduced cardiac growth, potentially due to altered nutrient availability and maternal metabolic stress caused by intermediate hyperglycaemia and hyperinsulinaemia (12, 40). Diminished cardiac growth causes the heart to struggle to meet circulatory demands, leading to compensatory mechanisms like Left ventricular hypertrophy and elevated mean arterial pressure (MAP) (41). Notably, male offspring exhibited greater heart-to-body weight ratios than females (42). This can be attributed to differential growth trajectories explained above and genetic predisposition, suggesting a higher vulnerability to heart disease in males (42).

MAP is the average pressure in the arteries during one cardiac cycle, reflecting the overall blood flow and the force exerted on arterial walls by the circulating blood (43). Foetal MAP is a critical component of the developing circulatory system, reflecting the balance between cardiac output and vascular resistance (44). It is essential for ensuring proper blood flow and oxygen delivery to foetal organs, particularly the brain, heart and placenta (44). In PE, maternal hypertension affects placental perfusion, leading to altered foetal haemodynamics, thus increasing MAP in offspring (45). Whereas, in pT2D, hyperglycaemia and hyperinsulinaemia alter the vascular tone, stimulating vasoconstriction, which increases MAP in offspring (46). However, infant MAP has not been elucidated in PP (47). This study demonstrated an increase in MAP in PPD offspring which could indicate physiological response to abnormal changes to vascular resistance and contractility, resulting in susceptibility to heart disease in adulthood (45). Furthermore, the higher MAP observed is once again due to specific growth needs and genetic predisposition in males and might signal that issues pertaining to cardiac development affect

male offspring more severely than female offspring (41). This highlights the importance of considering sex-specific factors when evaluating cardiac developmental outcomes (41). Moreover, changes to MAP are often associated with abnormal lipid profiles linked to foetal cardiac developmental function (48). Lipid profiles, including triglycerides (TG) and cholesterol (TC), are fundamental to foetal and neonatal development. TGs provide a major energy source for the growing heart and tissues, while TC supports membrane synthesis, steroidogenesis, and the growth of rapidly dividing cells, including cardiomyocytes (49–51). In this study, both PE and PPD offspring demonstrated elevated lipid markers, suggesting in utero exposure to altered maternal lipid metabolism. Such changes reflect not only maternal metabolic disturbances but also adaptive or maladaptive foetal responses to intrauterine stress (52). In PE, maternal endothelial dysfunction and systemic inflammation are known to induce dyslipidaemia (53). The resulting increase in circulating TGs and TCs can cross the placenta and expose the foetus to abnormal lipid environments during critical periods of organogenesis (54). Similarly, in PPD, persistent maternal hyperglycaemia and compensatory hyperinsulinaemia promote hepatic lipogenesis and excess lipid transfer to the foetus (56–58). In our model, elevated infantile lipid markers in PPD were accompanied by increased oxidative stress (Table 8), indicating that lipid abnormalities not only supply substrates for cardiac energy metabolism but may also drive lipid peroxidation and metabolic stress within the developing myocardium (55–57).

This interdependence was further supported by molecular markers of cardiac injury. Cardiac troponin I (cTnI) and collagen I (COL I) expression were significantly upregulated in both PPE and PPD offspring compared to controls, with PPD showing more pronounced elevations. These markers reflect contractile protein injury and extracellular matrix remodelling, respectively, and were associated with increased circulating cTnI levels in PPD (65–67). Importantly, the link between elevated TGs/TCs and increased cTnI suggests that lipid abnormalities and oxidative stress act synergistically to promote cardiomyocyte injury. Concurrent activation of inflammatory and profibrotic pathways, such as TNF-alpha and IL-6 may further explain the observed COL I upregulation and fibrotic remodelling (69, 70).

Neonatal lipid metabolism must also be considered in context. While TGs and TCs are indispensable for cardiac development, excessive exposure during late gestation or early neonatal life has been associated with increased arterial stiffness, impaired cardiac performance, and early markers of cardiovascular risk in preterm and small-for-gestational-age infants (59). Our findings therefore reinforce the dual role of lipids: essential for cardiac growth but, when dysregulated, signalling metabolic stress and predisposing to cardiac injury.

A notable observation in this study was the sex-specific difference in response. Male offspring demonstrated greater increases in cTnI and COL I expression compared to females. This finding suggests that hormonal and genetic factors may render males more vulnerable to lipid-driven oxidative and fibrotic injury, while females benefit from partial oestrogen-mediated resilience (62, 63). Such sex-linked susceptibility aligns with known differences in neonatal lipid handling and may have

implications for long-term cardiovascular health trajectories(62, 63).

Overall, our data highlights that lipid abnormalities, oxidative stress, and cardiac injury markers are interdependent in shaping neonatal cardiac outcomes in PE and PPD pregnancies. Elevated TGs and TCs, while physiologically essential, can indicate or contribute to cardiomyocyte stress when combined with hyperglycaemia-induced oxidative damage (62, 63). This interplay was more pronounced in PPD, suggesting a stronger impact of persistent maternal hyperglycaemia compared to PE alone. The sex-specific differences further underscore the importance of considering gender in understanding neonatal susceptibility to cardiometabolic programming (62, 63).

These findings consolidate the relevance of our results by demonstrating that intrauterine dyslipidaemia and oxidative stress are not isolated phenomena but act together to drive early cardiac remodelling (65-69). This study therefore provides evidence that maternal metabolic conditions, particularly pregestational diabetes, can predispose offspring to early markers of cardiac injury, highlighting the need for interventions targeting lipid and glucose regulation during pregnancy to reduce offspring cardiovascular risk (68-76).

A further limitation is that several downstream cardiometabolic pathways were not directly profiled in the offspring. Although triglycerides and total cholesterol were measured, more granular lipoprotein characterization (for example, HDL and LDL fractions), glucose and insulin dynamics (fasting insulin and insulin sensitivity indices), and renal or vascular mediators that may connect maternal metabolic status to foetal programming were not assessed (62,63). As a result, some mechanistic interpretations remain bound by the available markers. Future studies should extend offspring phenotyping to include glucose tolerance and insulin sensitivity testing, expanded lipid profiling, and longitudinal cardiovascular endpoints, which would allow stronger causal inference regarding the pathways through which maternal PP and PE-like features shape offspring cardiac-related risk.

4.6 Conclusion

In this murine model, diet-induced prediabetes was associated with alterations in cardiovascular parameters in offspring, with sex-specific patterns observed. These findings highlight potential early cardiovascular risk profiles, though the results should be interpreted cautiously given the limitations of murine-to-human translation. Further studies, incorporating longitudinal analyses and human cohorts, are required to establish whether these associations extend to long-term cardiovascular health in children born to prediabetic or preeclamptic mothers.

4.7 Abbreviations

Preeclampsia	PE
Pregestational type 2 diabetes	pT2D
Pregestational prediabetes	PP
High-fat, high-carbohydrate	HFHC
Pups born from normal control	PCON
Pups born from L-NAME-induced preeclamptic dams	PPE
Pups born from HFHC diet-induced prediabetic dams	PPD
Triglycerides	TGs
Cholesterol	TCs
Cardiac troponin I	cTnI
Collagen I	COL I
Malondialdehyde	MDA
Superoxide dismutase	SOD
Glutathione peroxidase	GPx
Intrauterine growth restriction	IUGR
L-N ^G -Nitro arginine methyl ester	L-NAME
Enzyme-linked immunoassay	ELISA
Polymerase chain reaction	PCR
Complementary deoxyribonucleic acid	cDNA
Messenger ribonucleic acid	mRNA
Glyceraldehyde- 3-phosphate	GAPDH
means $\pm$ standard error of mean	SEM
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Mean arterial pressure	MAP

hypoxia-inducible factors	HIFs
Transforming growth factor- β	TGF-β
Messenger ribonucleic acid	mRNA
soluble fms-like tyrosine kinase 1	sFlt-1
mechanistic target of rapamycin	mTOR
Protein kinase B	AKT
Post-Natal Day	PND
Gestational Day	GND

4.8 Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

4.9 Conflicts of Interest

The authors declare that they have no competing interests.

4.10 Funding Statement

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BRIDGE 2

The cardiac muscle is the first organ to develop during embryogenesis. As pregnancy progresses, the foetus gradually reduces its dependence on maternal oxygen supply and establishes an independent haematological system to meet its metabolic demands. Consequently, cardiac muscle function increasingly relies on the ability of foetal blood cells to ensure adequate oxygenation, vascular integrity, and tissue repair. This dependency persists until birth, underscoring the critical role of foetal haematological development in offspring health. In pregnancies complicated by preeclampsia (PE) and pregestational type 2 diabetes (pT2D), foetal haematological development is impaired, leading to anaemia, polycythaemia, and immune dysfunction in the offspring. Given that PP is a precursor to pT2D, it is hypothesized that the haematological abnormalities observed in PP will be exposed to similar pathological mechanisms as those observed in PT2D. However, this has not been elucidated hence, the second study aims to assess the effects of diet-induced PP on gender-specific infantile outcomes related to haematological function.

CHAPTER 5: MANUSCRIPT 2

5. PROLOGUE

Foetal haematological development is a dynamic process susceptible to modulation by the maternal environment. Preeclampsia (PE) and pregestational diabetes type 2 diabetes (pT2D) interfere with essential haematological pathways through inflammation, oxidative stress and hypoxia. Recent studies suggest that pregestational prediabetes (PP), an intermediary state preceding maternal overt type 2 diabetes, equally predisposes the offspring to a similar pattern of haematological dysregulation via mechanisms implicating oxidative stress, placental dysfunction, and chronic low-grade inflammation. Despite this, it is not well established what, if any, are the effect of PP on offspring's haematological function. As discussed in the previous chapter, PP results in poor cardiovascular outcomes with significant gender-specific disparities. Expanding upon these findings, the current manuscript investigates the role of HFHC diet-induced PP on haematological development in postnatal offspring, focusing on gender-specific differences in key markers of haematological function and regulation, such as red and white blood cell indices, critical cytokines, namely erythropoietin (EPO) and granulocyte stimulating factor (G-CSF) as well as parameters of oxidative stress. These findings aim to establish the effects of PP on functional markers of haematological function

The manuscript titled **“High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of haematological function”** is authored by N.D. Xulu, A Khathi, N.H. Sibiyi and P.S. Ngubane

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High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of haematological function

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5.1 Abstract

Introduction

Maternal diseases like pre-eclampsia (PE) and pre-gestational type 2 diabetes (pT2D) may change the oxygen levels in a foetus so the nutrient supply is interrupted, with possible implications for newborn haematological growth. For a-pT2D, a recognised implication for PE and an early stage preceding pT2D, studies on the attributes of neonatal blood indices are less clear. Therefore, this study has attempted to clarify whether pregestational prediabetes induced by HFHC diets, with or without PE-like characteristics, changes neonatal haematological measurements and erythropoietic regulation, with sex-specific patterns.

Methods and Materials

The number of male and female pups born to normal control dams (PCON; n = 6 dams), L-NAME-induced pre-eclamptic dams (PPE; n = 6 dams), or HFHC diet-induced prediabetic dams (PPD; n = 6 dams). Pups were weighed at birth and followed to postnatal day 21. On day 21 of life and weaning, pups were euthanised, and whole blood was collected into EDTA tubes for complete blood count and red-cell indices, while matched plasma was processed for erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and oxidative stress markers (including MDA, SOD, and GPx).

Results

In comparison with controls, PPE and PPD offspring presented with body weights significantly below the norm, with alterations in haematological parameters and erythropoietic stimulation pathways and haematopoietic signalling (G-CSFC). Changes in the oxidative state included alteration in antioxidative capacity and lipid peroxidation markers. The effect of both conditions was more pronounced in offspring from PPD dams than in offspring from PPE dams, with sex-specific effects on certain outcomes.

Conclusion

Summarily, the results suggest that, in offspring, under prediabetic pregestational, there is early-life disruption of haematological characteristics, and present findings propose a maternal phenotype overlapping with regard to PE-like pathophysiology. The strength of the present work is in advocating better screening and monitoring of prediabetes before and during a woman's pregnant life, hence bringing to the fore the necessity for mechanistic-based research aimed at discerning changes in placental, renal, and hepatic contributions towards erythropoietic regulation chaos.

Keywords: Pregestational diabetes, preeclampsia, intrauterine growth restriction, haematological parameters

5.2 Introduction

Red blood cell (RBC) development, or erythropoiesis, is a fundamental yet species-specific process that requires careful consideration when extrapolating from rodent models to humans (83). In humans, erythropoiesis is characterized by a sequential transition in hematopoietic sites: it begins in the yolk sac around weeks 3–8 of gestation, moves to the foetal liver between 6 weeks and birth, and is established in the bone marrow by mid-gestation, which remains the predominant site throughout life (1,2). Sprague-Dawley rats display a broadly similar developmental pattern, with primitive erythropoiesis originating in the yolk sac and definitive erythropoiesis shifting to the foetal liver, but the transition to bone marrow dominance occurs only perinatally or shortly after birth, and the spleen continues to contribute significantly to erythropoiesis throughout adulthood (3,4). At the cellular level, both species progress through conserved stages of erythroid differentiation beginning with hematopoietic stem cells, advancing through erythroid progenitors (BFU-E and CFU-E), proerythroblasts, basophilic, polychromatic and orthochromatic erythroblasts, to reticulocytes and ultimately enucleated erythrocytes with erythropoietin (EPO) acting as the central survival and maturation factor (5,6). However, rats differ from humans in several critical aspects: they have a shorter erythrocyte lifespan (40–60 days versus approximately 120 days in humans), a greater reliance on splenic reticulocyte maturation, and a more rapid foetal-to-adult hemoglobin switch that occurs close to birth, with fewer hemoglobin isoforms than humans (7,8). These distinctions are physiologically important, as they render rats more sensitive to fluctuations in oxygen demand and metabolic stress, driving higher basal erythropoietic activity regulated by EPO and hypoxia-inducible pathways (9). Sprague-Dawley rats in particular are invaluable for translational research because they are an outbred strain with genetic diversity that better approximates human variability, while also being docile, highly fertile, and physiologically well-characterized (10). Their retention of splenic erythropoiesis provides a unique opportunity to study both steady-state and stress-induced erythropoiesis an aspect directly relevant to maternal metabolic disorders such as prediabetes and preeclampsia, where hypoxia, altered erythropoietin levels, and iron dysregulation may impact infant hematological development. Thus, while differences in lifespan, hemoglobin switching, and hematopoietic niches must be acknowledged, the conserved erythroid developmental cascade and regulatory networks strongly support the use of Sprague-Dawley rats as a robust model system for exploring mechanisms of altered erythropoiesis and testing therapeutic interventions in contexts relevant to human health (3,7,10).

5.3 Materials and Methods

5.3.1 Animals

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 (n=18) were bred and housed in the Biomedical Research Unit (BRU) of the University of Kwa-Zulu Natal for 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 acclimatized to their new environment for 1 week while consuming standard rat chow and tap water before the induction of prediabetes by exposure to a well-established experimental diet (HFHC).

5.3.2 Induction of prediabetes

Female Sprague–Dawley rats were randomly assigned to three groups, groups A, B, and C (n = 6 per group). Experimental prediabetes was induced in female Sprague–Dawley rats using a protocol previously described (19). To summarize, control groups A and B were given a standard rat chow diet with normal water for the 36-week period. Group C was given a high-fat, high-carbohydrate (HFHC) diet with 15% fructose added for the same duration of 36 weeks. The composition of the HFHC diet is 30% high fats, 55% of carbohydrates and 15% fructose. At the end of 36 weeks, the diagnostic criteria of the American Diabetes Association (ADA) were applied to identify prediabetes in the animals. This involved measuring diagnostic indicators for prediabetes, such as fasting blood glucose levels ranging from 5.6 to 7.1 mmol/L, oral glucose tolerance test (OGTT) with 2-hour glucose levels between 7.1 and 11.1 mmol/L at 0, 7, 12, and 19 weeks, these numbers represent time points in weeks after induction of prediabetes in the maternal rats and were guided by preeclamptic maternal time points. Animals meeting this criterion were categorized as prediabetic (PD), while those with measurements below the prediabetic thresholds were considered healthy controls (CON) A and B.

5.3.3 Oral glucose tolerance response

An oral glucose tolerance test was performed on all animals to assess their glucose tolerance response. Following a well-established laboratory technique, this test was completed after carbohydrate loading (19). After fasting for 16 hours, glucose levels were determined at time 0. Then, a monosaccharide syrup was administered orally using an 18-gauge gavage 38 mm long and curved needle with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). The glucose concentrations were determined by collecting blood using the tail-prick method (19) and measuring glucose concentrations using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). Glucose levels were assessed at 15-, 30-, 60- and 120 minutes following carbohydrate loading. The Oral Glucose

Tolerance Test (OGTT) values reported here refer specifically to the 2-hour post-load glucose concentration, the standard diagnostic timepoint for impaired glucose tolerance in rodent models. The cut-points were interpreted within the context of capillary glucose measurements, ensuring that group allocation reflects relative glucose tolerance and insulin resistance rather than absolute equivalence to venous values. Calibration and quality control were performed according to manufacturer instructions prior to measurement to ensure reliable readings for experimental grouping. These tools were selected due to their rapid assessment capability, minimal blood volume requirements, and established use in small-animal metabolic studies, which is critical for longitudinal monitoring of pups and dams

5.3.4 Mating

Prior to mating, all 18 female Sprague–Dawley rats underwent assessment for the proestrus stage through vaginal smear analysis under a microscope (Leica Microsystems, Illinois, USA). Briefly, two females were housed in one cage to allow for their oestrus cycles to synchronize. After synchronization, those in the proestrus stage were permitted to mate with healthy male control Sprague–Dawley rats (19). Confirmation of pregnancy and assignment of gestational day (GND) zero were determined the following morning by the presence of a vaginal plaque or vaginal smear containing spermatozoa observed under a microscope (Leica Microsystems, Illinois, USA) (19, 25). The male rats were removed from the cage and returned to the BRU. Pregnant rats were then observed until the end of gestation (21 days).

5.3.5 Induction of preeclampsia

After pregnancy was confirmed by the visualization of spermatozoa in a vaginal smear, the pregnant dams from healthy control group B were randomly divided into one rat per cage, which were given 0.3g/L a day of L-NAME in drinking water from day 14.5 to day 20.5 of gestation. Blood pressure was measured, and the presence or absence of protein in the urine was assessed (19). Preeclampsia (PE) was confirmed by the measurement of blood pressure which was monitored every third day after administration. Animals presenting with a blood pressure of $\geq 140/90$ mmHg and proteinuria of ≥ 300 mg (3+) were considered preeclamptic. Blood pressure was monitored at GND 0, 7, 12, and 19. using a non-invasive tail-cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA). Urine samples were collected at similar GND days, overnight using metabolic cages, and protein urea was measured immediately via dipsticks (Multistix® 10 SG, Siemens Healthineers, Pennsylvania, USA).

5.3.5.1 L-NAME Rationale

The inclusion of an L-NAME-induced preeclampsia group allowed for the differentiation between the effects of pregestational prediabetes and established preeclampsia on maternal and offspring outcomes. This comparative approach strengthened the mechanistic interpretation of whether

observed infantile alterations were attributable to prediabetes alone or to a preeclampsia-like intrauterine environment (10).

5.3.6 Collection of pups

At gestational day (GND) 21, naturally delivered male and female pups from control (PCON), L-NAME preeclamptic (PPE) and HFHC diet-induced prediabetic (PPD) dams were immediately collected and weighed. The pups were then carefully returned to the dams and were placed in Makrolon polycarbonate cages under standard laboratory conditions (for temperature and humidity) in a 12-hour day and 12-hour night cycle and were given access to food and water. To assess infantile development, the animals were euthanized for terminal investigations on day 21 (25).

5.3.7 Terminal studies

On day 21, the pups (n=36) were euthanised. The guillotine was used to decapitate pups for blood collection into EDTA tubes (Eppendorf centrifuge 5403, Germany) that were centrifuged at 4 °C, 503g for 15 minutes. The plasma was collected for red blood cell parameters, erythropoietin (EPO), Granulocyte Colony-stimulating factor analysis, (G-CSF), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) measurement.

5.3.8 Haematological and Redox Analysis

The erythrocyte markers, namely red blood cell count (RBC), haemoglobin (Hgb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and red cell distribution width (RDW), were analysed together with white blood cell and neutrophil counts and platelet count using an automated haematology analyser (animal mode where applicable). Briefly, whole blood was collected into pre-chilled EDTA microtubes for complete blood count (CBC) analysis. Within 30 minutes of collection, the samples were centrifuged (approximately 1,500 g for 10 minutes at 4 °C) to separate plasma, which was aliquoted and stored at -80 °C until mediator and oxidative stress assays were performed. Because erythropoietin production is developmentally regulated across multiple organs (including renal and hepatic sources), plasma EPO concentrations are interpreted here as a systemic index of erythropoietic signalling rather than as a direct proxy for any single organ's output.

5.3.9 Study Design

Prediabetes as a risk factor for Preeclampsia: Assessing gender-specific infantile outcomes on selected markers of haematological function was studied. The pups were immediately weighed after birth and then returned to the dams for further development. On postnatal day 21, pups (n=36) were weaned and divided into pups born from normal control dams (PCON), pups born from preeclamptic dams (PPE), and pups born from prediabetic dams (PPD) (n=6/group). Group 1: Male and female pups born from

normal pregnant control rats (PCON) which received normal standard chow and normal drinking water throughout the study (n=12); Group 2: Male and female pups born from PD pregnant rats which received the HCHF diet+ 15% fructose throughout the study (n=12); Group 3: Male and female pups born from PE pregnant control rats which received normal standard chow (n=12). On day 21 the pups were euthanised. The guillotine was used to decapitate pups for blood collection into EDTA tubes (Eppendorf centrifuge 5403, Germany) that were centrifuged at 4 ° C, 503g for 15 minutes.

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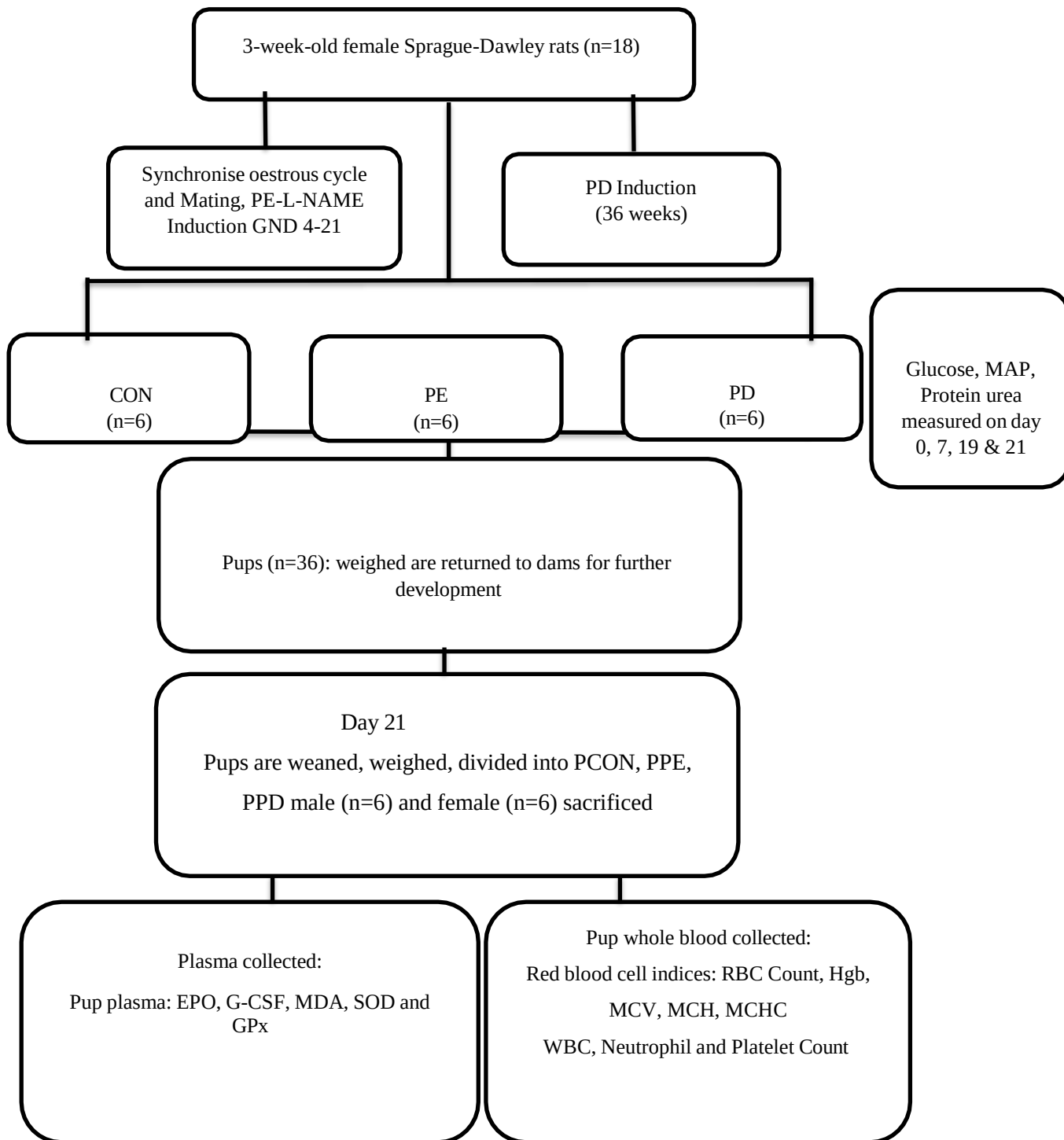


Figure 1: Illustrates a graphical abstract description of the study design.

5.3.10 Biochemical Analysis

5.3.10.1 EPO, G-CSF, SOD and GPx analysis in pup plasma

Rat specific plasma EPO, G-CSF, SOD and GPx ELISA kits (Elabsience and Biotechnology, WuHan) were used following manufacturer's instruction. Briefly, 100µL of standard or sample was added to the wells and incubated for 90 min at 37°C. The liquid was discarded, and 100µL of Biotinylated Detection Ab working solution was added to each well and incubated for 60 min at 37°C. Thereafter the plate was aspirated and washed 3 times. 100µL HRP conjugate working solution was added then the plate was incubated for 30 min at 37°C. The plate was then aspirated and washed 5 times. 90µL of Substrate Reagent was added, and the plate was incubated for 15 min at 37°C. After which 50µL of Stop Solution was added, and the plate was read at 450nm immediately for the calculation of the results.

5.3.10.2 Malondialdehyde in pup plasma

Malondialdehyde was measured using an established protocol (10). Pup plasma (n=36) was centrifuged at 400 g for 10 min. Thereafter, 400 µL of the plasma was supplemented with 400 µL of 2% phosphoric acid and then separated into two glass tubes, each receiving equal volumes of the solution. Subsequently, 200 µL of 7% phosphoric acid was added into both glass tubes, followed by the addition of 400 µL of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into one glass tube (sample test) and 400 µL of 3 mM hydrochloric acid (HCl) into the second glass tube (blank). To ensure an acidic pH of 1.5, 200 µL of 1M HCl was added to the sample and blank test tubes. Both solutions were heated at 100 °C for 15 min and cooled to room temperature. Butanol (1.5 mL) was added to the cooled solution; the sample was vortexed for 1 min to ensure rigorous mixing and allowed to settle until two phases could be distinguished. The butanol phase (top layer) was transferred to Eppendorf tubes and centrifuged at 13,200 g for 6 min. The samples were aliquoted into a 96-well microtiter plate in triplicate and the absorbance was read at 532 nm (reference 600 nm) on a BioTek mQuant spectrophotometre (Biotek, Johannesburg, South Africa). The absorbance from these wavelengths was used to calculate the concentration of MDA using Beer's Law:

Concentration of MDA = Average Absorbance Absorption/coefficient (156 nm)

5.3.11 Statistical Analysis

All data were expressed as means ± standard error of the mean (SEM). Normality was assessed prior to statistical testing. Analyses were performed using GraphPad Prism InStat Software (version 9.00, GraphPad Software, San Diego, California, USA). For dam parameters, comparisons were conducted using a one-way analysis of variance (ANOVA), as sex was not a variable of interest. For pup parameters, a two-way analysis of variance (ANOVA) was employed, with Group (Control, Preeclamptic, Prediabetic) and Sex (Female, Male) as fixed factors, thereby allowing simultaneous testing of group differences, sex differences, and their potential interaction. Where significant main effects or interactions were detected, post hoc analyses were conducted using the Tukey–Kramer test for parametric data, or the Bonferroni

correction for non-parametric data, to identify pairwise differences between groups. For ANOVAs and other multivariate analyses, assumptions of homogeneity of variance and sphericity were assessed; in cases where sphericity was violated, Greenhouse–Geisser corrections were applied to adjust the degrees of freedom. Statistical significance was set at $p < 0.05$, and corrected degrees of freedom are reported where applicable.

5.4 Results

5.4.1 *Pup Body weight*

Figure 2 shows the body weights of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) at the end of the study. The pups born from preeclamptic dams the PPE- male and female (M and F) and prediabetic dams PPD- male & female (M and F) showed significantly ($p < 0.05$) lower body weights in comparison to the pups born from normal dams. However, pups born from preeclamptic dams showed no significant change, but a slight decrease in body weight when compared to pups born from prediabetes-induced animals. Furthermore, the pup body weights were significantly ($p < 0.05$) higher in male compared to female offspring.

5.4.2 *Red blood cell count (RBC) of pups*

Figure 3 shows the red blood cell count (RBC) of pups born from normal control dams (PCON), L-NAME induced preeclamptic dam (PPE), and dams induced with prediabetes (PPD) at the end of the study. The pups born from preeclamptic dams the PPE- male and female (M and F) and prediabetic dams PPD- male and female (M and F) showed significantly ($p < 0.05$) increased RBC in comparison to the pups born from normal dams. As anticipated, pups born from preeclamptic dams showed significantly ($p < 0.05$) decreased RBC when compared to pups born from prediabetes-induced animals. Furthermore, the RBC was significantly ($p < 0.05$) higher in male compared to female offspring.

5.4.3 *Haemoglobin (Hgb) concentration of pups*

Figure 4 shows the haemoglobin (Hgb) of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dam (PPE), and dams induced with prediabetes (PPD) at the end of the study. The pups born from preeclamptic dams The PPE- male and female (M and F) and prediabetic dams PPD- male and female (M and F) showed significantly ($p < 0.05$) increased Hgb in comparison to pups born from normal dams. As expected, pups born from preeclamptic dams showed significantly ($p < 0.05$) decreased Hgb when compared to pups born from prediabetes-induced animals. Furthermore, the Hgb was significantly ($p < 0.05$) higher in male compared to female offspring.

5.4.4 Red blood cell parameters of pups

Table 1 shows the red blood cell indices of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD). The pups born from preeclamptic PPE- male and female (M and F) and prediabetic dams PPD- male and female (M and F) showed a significant ($p < 0.05$) decrease in the mean cell volume (MCV) and mean corpuscular haemoglobin (MCH) and an increase in mean corpuscular haemoglobin concentration (MCHC) and red blood cell distribution width (RDW) levels in comparison to the normal control animals. Interestingly, pups born from preeclamptic dams showed significantly ($p < 0.05$) increased MCV, MCH, MCHC, and RDW levels when compared to pups born from prediabetic animals. Furthermore, the MCV, MCH, MCHC, and RDW were significantly ($p < 0.05$) higher in male compared to female offspring.

5.4.5 White blood cell (WBC), neutrophil (NE), and platelet (PLT) counts of pups.

Table 2 shows the white blood cell (WBC), neutrophil (NE), and platelet counts (PLT) of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dams (PPE) and dams induced with prediabetes (PPD). The pups born from preeclamptic dams PPE- male and female (M and F) and dams induced with prediabetes PPD- male and female (M and F) showed significantly ($p < 0.05$) decreased and increased WBC, respectively, as well as decreased NE levels in comparison to the pups born from normal dams. Furthermore, the PLT of pups born from preeclamptic and prediabetic dams was significantly decreased compared to pups born from normal dams. Interestingly, pups born from preeclamptic dams showed significantly ($p < 0.05$) decreased WBC, and PLT as well as an increase in NE when compared to pups born from prediabetes-induced animals. Furthermore, the WBC, PLT and NE were significantly ($p < 0.05$) higher in male compared to female offspring.

5.4.6 Erythropoietin (EPO) concentrations of pups

Figure 5 shows the erythropoietin (EPO) of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dam (PPE), and dams induced with prediabetes (PPD) at the end of the study. The pups born from preeclamptic dams PPE- male and female (M and F) and prediabetic dams PPD- male & female (M and F) showed significantly ($p < 0.05$) increased EPO in comparison to the pups born from normal dams. As anticipated, pups born from preeclamptic dams showed significantly ($p < 0.05$) decreased EPO when compared to pups born from prediabetes-induced animals. Furthermore, the EPO was significantly ($p < 0.05$) higher in male compared to female offspring

5.4.7 Granulocyte colony-stimulating factor (G-CSF) concentrations of pups

Figure 6 shows the granulocyte colony-stimulating factor (G-CSF) of pups born from normal control

dams (PCON), L-NAME-induced preeclamptic dam (PPE), and dams induced with prediabetes (PPD) at the end of the study. The pups born from preeclamptic dams PPE- male and female (M and F) and prediabetic dams PPD- male and female (M and F) showed significantly ($p < 0.05$) decreased G-CSF in comparison to the pups born from normal dams. As anticipated, pups born from preeclamptic dams showed significantly ($p < 0.05$) increased G-CSF when compared to pups born from prediabetes-induced animals. Furthermore, the G-CSF was significantly ($p < 0.05$) higher in male compared to female offspring

5.4.8 Malondialdehyde (MDA) concentrations of pups

Figure 7 shows the malondialdehyde (MDA) of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dam (PPE), and dams induced with prediabetes (PPD) at the end of the study. The pups born from preeclamptic dams PPE- male and female (M and F) and prediabetic dams PPD- male and female (M and F) showed significantly ($p < 0.05$) increased MDA in comparison to the pups born from normal dams. As anticipated, pups born from preeclamptic dams showed significantly ($p < 0.05$) decreased MDA when compared to pups born from prediabetes-induced animals. Furthermore, the MDA was significantly ($p < 0.05$) higher in male compared to female offspring

5.4.9 Superoxide Dismutase (SOD) concentrations of pups

Figure 8 shows the superoxide dismutase (SOD) of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dam (PPE), and dams induced with prediabetes (PPD) at the end of the study. The pups born from preeclamptic dams PPE- male and female (M and F) and prediabetic dams PPD- male and female (M and F) showed significantly ($p < 0.05$) decreased SOD in comparison to the pups born from normal dams. Pups born from preeclamptic dams showed slightly increased SOD concentrations when compared to pups born from prediabetes-induced animals. Furthermore, the SOD was significantly ($p < 0.05$) lower in male compared to female offspring.

5.4.10 Glutathione Peroxidase (GPx) concentrations of pups

Figure 9 Glutathione Peroxidase (GPx) of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dam (PPE), and dams induced with prediabetes (PPD) at the end of the study. The pups born from preeclamptic dams PPE- male and female (M and F) and prediabetic dams PPD- male and female (M and F) showed significantly ($p < 0.05$) decreased GPx in comparison to pups born from normal dams. Pups born from preeclamptic dams showed slightly increased GPx concentrations when compared to pups born from prediabetes-induced animals. Furthermore, the GPx was significantly ($p < 0.05$) lower in male compared to female offspring

5.5 Discussion

Red blood cell (RBC) development, or erythropoiesis, is an essential physiological process that supports oxygen delivery during early life (83). While differences exist between rodents and humans, important regulatory mechanisms of erythropoiesis, including erythropoietin (EPO) signalling and erythroid

maturation, are conserved across species (5,6, 13). In Sprague-Dawley rats, the continued contribution of the spleen to erythropoiesis and their increased sensitivity to hypoxic and metabolic stress may amplify haematological responses to adverse intrauterine conditions such as maternal prediabetes and preeclampsia (9, 10, 16, 17). These physiological characteristics may help explain the haematological alterations observed in this study. Moreover, the shared erythropoietic pathways support the use of this model to investigate early-life haematological programming (18, 20).

Studies have shown that in pregnancy, infant body weights are strictly monitored as a predictor for infant developmental milestones, as infant body weights give insight to the health and developmental of the infant from neonatal age until adulthood (20, 21). Infants born from pregnancies where preeclampsia and pregestational diabetes, like T2D, are reported have been shown to exhibit low infant body weights (21, 22). Low body weights are a significant risk factor for poor foetal health outcomes in short- and long-term development (23, 24). In the present study, **Figure 2** displays the infant body weights of both male and female preeclamptic pups (PPE) and prediabetic pups (PPD). The body weights of the PPE and PPD groups were significantly lower than the offspring born from the control group. Furthermore, there was a significant increase in the infant body weight of offspring born in the PPE group compared with the PPD group (25, 26). This aligns with the existing literature suggesting that prenatal hypoxia in pregestational and preeclamptic pregnancies leads to IUGR, which causes low body weights.

The present study also divulges how PPD follows the same body weight trend as other pregestational pregnancies. Although the mechanisms are not well elucidated, prenatal hypoxia is assumed to impede foetal growth via placental insufficiency, which impairs foetal oxygenation and nutrition (27, 28). This is because, in healthy pregnancies, maternal spiral artery remodelling occurs via cytotrophoblast invasion (29, 30). This process involves the conversion of the maternal spiral arteries, which are initially narrow and resistant vessels, into wider and more accommodating vessels capable of providing adequate placental perfusion to sustain foetal growth via oxygenation and nutrition (31, 32). We speculate that in PPE, T2D, and the PPD group, the invasion of cytotrophoblast into the maternal spiral artery is unsuccessful (33). This leads to placental insufficiency, resulting in foetal hypoxia, and lowers both oxygenation and nutrition of the foetus, resulting in lower body weight and IUGR (34). Furthermore, the lower infant body weight observed in the PPD group compared to the PE can be attributed to hyperglycaemia added insult, which causes splenomegaly and increases the severity of IUGR in pregestational pregnancies (35, 36). Moreover, the significantly higher body weight observed in the male compared to the female group was attributed to inherent physiological and genetic differences and did not indicate more severe foetal outcomes in females (37, 38). Rather, studies show that females tend to experience better health outcomes, and this is largely due to the influence of sexual hormones such as oestrogen, which confer cellular protection (39, 40).

Moreover, low body weight and IUGR due to prenatal hypoxia have also been implicated in the

disruption of foetal blood cellular development in which erythrocyte production is altered (41-43). For effective maternofoetal oxygen transport to occur, foetal erythroid cells are formed by the embryo in the first week of gestation (44, 45). Once matured, these erythrocytes play an imperative role in ensuring oxygen delivery to the newborn during gestation and postpartum (46, 47). Erythrocyte markers such as RBC, Hgb, MCV, MCH, MCHC, and RDW denote the maturity and health of erythrocytes and are regulated within a narrow range (48, 49). Deviations from this range can have detrimental effects on erythrocyte structure and function (50, 51). In this study, **Figures 3 and 4** show significantly elevated RBC and Hgb concentrations in PPE and PPD compared to normal control offspring. There was also a significant decrease in the RBC and Hgb levels of the PPE group in comparison to the PPD group. We speculate that changes in RBC and Hgb concentrations are a result of the prenatal hypoxia observed in the mothers of PPE and PPD (52). Hypoxia significantly increases erythropoietin (EPO) in PPE and PPD offspring in comparison to normal controls (**Figure 5**) (53, 54). This causes the rate of erythropoiesis in the liver and bone marrow of the foetus to surge, which expands the red cell mass, resulting in elevated infantile nucleated red blood cells/erythroblasts and thus increasing RBC, Hgb, MCHC and RDW levels while resulting in decrease MCV and MCH levels (53, 54). Moreover, the higher RBC and Hgb levels in the PPD group compared to the PPE group are a result of hyperglycaemia which causes foetal polycythaemia by stimulating excess erythropoietin production in the offspring (53, 54). The elevated blood glucose concentration increases foetal blood viscosity and reduces blood flow, which can further contribute to the development of polycythaemia (54). Also, the observed disparities in RBC and Hgb levels between male and female offspring are due to inherent differences between the two sexes (54). The main difference was significantly higher testosterone levels in males, which have been shown to stimulate erythropoiesis increasing erythrocyte production (54).

Oxidative stress is important in pregnancy for foetal blood cell production because it plays a critical role in the development of the foetal circulatory system. During pregnancy, foetal blood cell production is a highly dynamic and complex process that requires a delicate balance of reactive oxygen species (ROS) and antioxidant defences. However, excessive oxidative stress during pregnancy can have detrimental effects on foetal blood cell production. It can lead to the destruction of red blood cells and impair the development of the circulatory system, resulting in conditions such as foetal anaemia or intrauterine growth restriction. Therefore, maintaining a balance of oxidative stress is crucial for ensuring optimal foetal blood cell production and overall development (54). In this study, **Figures 7, 8, and 9** show changes in the antioxidant profile with elevated malondialdehyde (MDA) levels and decreased antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) observed in PPE and PPD groups in comparison to normal control. Furthermore, an increase in MDA is accompanied by further decreased antioxidant enzyme levels in PPD compared to the PPE group. This is due to prenatal hypoxia, which favours reactive oxygen species (ROS) production, overwhelming the antioxidant capacity (54). ROS such as peroxide (O_2^-) and hydrogen peroxide (H_2O_2)

cross the placenta and oxidatively modify antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx), which decrease their availability in foetal circulation (55). ROS also induce DNA damage to erythroid progenitor cells leading to cell cycle arrest and increased immature erythroblast/ nucleated red blood cells (nRBC) in the circulation (56, 57). Moreover, the antioxidant levels in the female offspring were higher than in the male offspring in all groups. This is due to the role of the sexual hormone oestrogen in the form of 17 beta-oestradiol, which influences the production of manganese superoxide dismutase (MnSOD) and extracellular superoxide dismutase (ecSOD) expression and activity, providing vasoprotective effects to the female offspring (58).

Moreover, IUGR interferes with the nutrient supply to the foetus, and as a result, there is limited availability of important nutrients/components such as folate, vitamin B12, and iron (59). These components are crucial for erythrocyte production, growth, and maturation. Their deficiency leads to erythrocyte structural and functional abnormalities (60, 61). Iron deficiency affects haemoglobin production and erythrocyte size, which results in decreased circulating oxygen, whereas folate and vitamin B12 deficits cause irregular erythrocyte shape and size, which impairs their ability to transport oxygen (62). **Table 1** of this study shows the mean corpuscular volume (MCV) and red cell distribution width (RDW) concentrations in PPE and PPD were significantly decreased and increased than those in the normal control group. In PPD this effect could be ascribed to hyperglycaemias-induced prenatal hypoxia and IUGR, which causes iron deficiency anaemia, resulting in haemoconcentration, decreased erythrocyte size (MCV) and size variation in circulation (RDW) (63, 64). Furthermore, the changes between PPE and PPD groups in **Table 1** illustrate that offspring with PPD exhibit significantly lower MCV and RDW values than those from the PPE group. This effect can be a result of hyperglycaemia suppressing hepcidin expression. Low hepcidin levels are an early marker for iron deficiency anaemia (64). Low hepcidin levels have been shown to indicate exhausted iron stores, and the lack of iron affects red blood cell structure and function, leading to iron deficiency anaemia observed in the PPD group

Furthermore, the effects of oestrogen and testosterone may be the cause of the noticeably greater MCV and RDW values seen in males as opposed to females (64, 65). Studies show that testosterone increases haemoglobin production and erythrocyte size, which increases MCV and RDW levels (66,67). Whereas oestrogen decreases erythrocyte size and increases heme production, which decreases the haemoglobin contained in each cell leading to lower MCV and RDW values (67). Moreover, prenatal hypoxia can induce a decrement in the production of granulocyte colony-stimulating factor (G-CSF), which is a cytokine that holds a crucial role in the generation and differentiation of white blood cells, particularly neutrophils (68). Neutrophils are essential in the fight against infections; however, since neonatal immune defences are still inadequate, infants are more susceptible to infection (68,69). **Figure 6 and Table 2** demonstrate that the G-CSF levels, neutrophil in the PPE and PPD groups were lower than those of the pups born to the control group in this study. This could be in part due to prenatal hypoxia and IUGR that impair foetal liver function, decreasing the production of G-CSF (69). Low G-

CSF levels contribute to the failure of downstream myeloid differentiation, resulting in subsequent neutropenia and thrombocytopenia (69). Moreover, there was a significant increase in the G-CSF levels of offspring born in the PPE group in comparison to the PPD group. This could be attributed to severe or prolonged prenatal hypoxia brought about by the hyperglycaemia observed, which causes the dysregulation of several transcription factors and signalling pathways involved in G-CSF synthesis and secretion (70). Hyperglycaemia has been shown to activate the nuclear factor-kappa B (NF- κ B) signalling pathway, which suppresses G-CSF expression (70). ROS have also been shown to further impair G-CSF expression by altering DNA sequences essential for G-CSF gene expression, which lowers G-CSF levels, therefore disrupting neutrophil and platelet differentiation (70, 71). The resulting decrease in neutrophil and platelet counts has detrimental consequences as it leaves the infant vulnerable to infection and at an increased risk of bleeding or haemorrhage (71). Moreover, the G-CSF levels in the female offspring were significantly lower than in the male offspring in all groups (71). This is attributed to the difference in gene expression between sexes (71, 72). Studies have shown that the promoter region of the G-CSF gene has oestrogen response elements (ERE), which are specific genetic sequences activated by oestrogen (72). Oestrogen binds to ERE and interferes with G-CSF transcription, subsequently decreasing the production and secretion of G-CSF, resulting in neutropenia and thrombocytopenia (73). This makes female offspring more susceptible to immune dysfunction and haemorrhage and is further worsened by female offspring born with hyperglycaemic pregnancies (74).

5.6 Conclusion

This study demonstrates that maternal diet-induced prediabetes is associated with changes in neonatal haematological parameters and oxidative stress status, with differences observed between male and female offspring. These results provide an important experimental framework for understanding potential pathways linking maternal metabolic dysfunction to offspring haematological outcomes. However, given the species-specific differences in haematopoiesis and immune regulation, extrapolation to humans should remain cautious. Future studies using longitudinal follow-up and human cohorts are needed to clarify whether these early-life haematological changes persist into later development. While our findings provide valuable insights into the effects of pregestational prediabetes (PPD) and preeclampsia (PE) on neonatal development, it is important to emphasise that these results were obtained in a murine model. Such models are indispensable for dissecting molecular and physiological mechanisms on a tissue-specific level and for establishing causal relationships that are not feasible to investigate in humans (82, 83). However, murine and human systems differ in key aspects of metabolism, placentation, and neurodevelopment, and therefore direct clinical translatability cannot be assumed. The value of our model lies in its ability to reflect features of PE and PPD in a controlled experimental setting, thereby highlighting possible pathways of oxidative stress and haematological adaptation (82, 83).

5.7 References

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FIGURES

5.4.1 Pup body weights

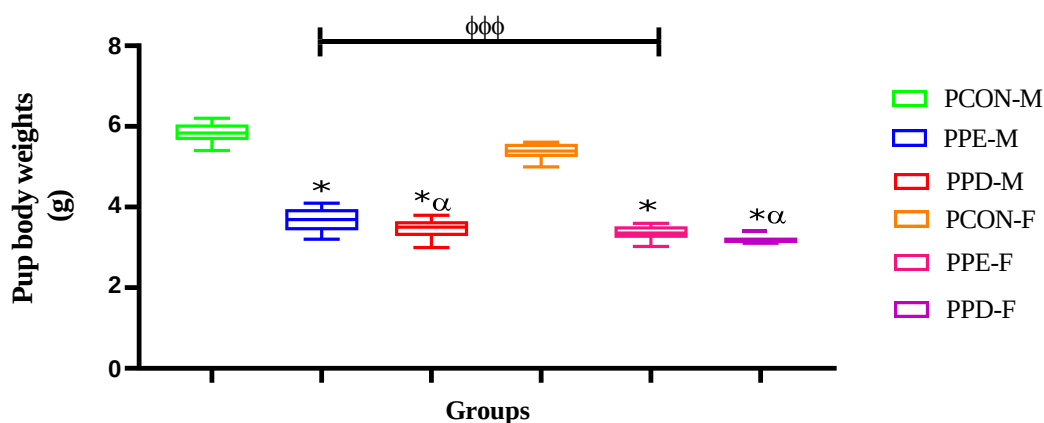


Figure 2: shows the body weights of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. ϕ $p < 0.05$ by comparison with male offspring.

5.4.2 Red blood cell count (RBC) of pups

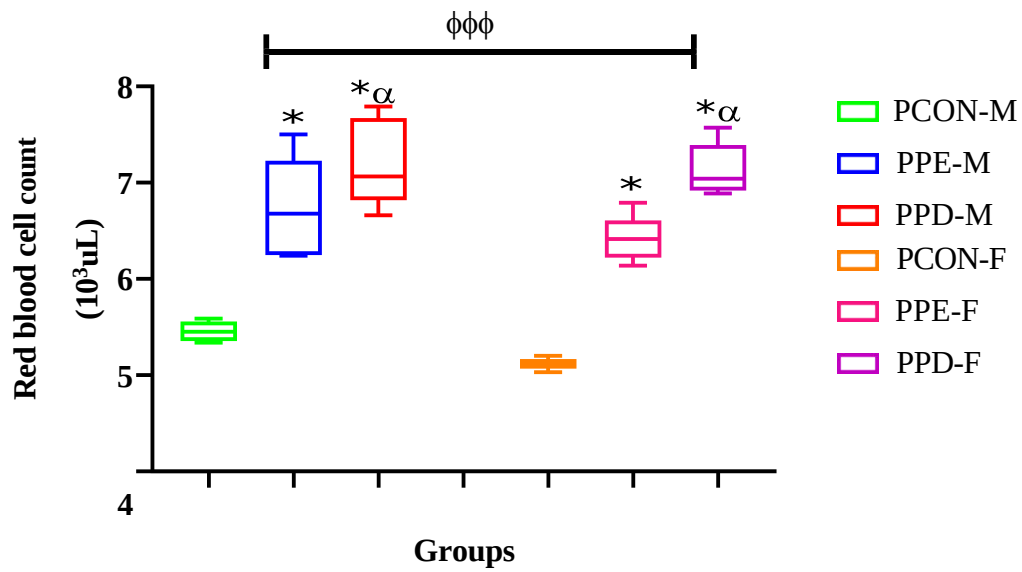


Figure 3: shows the red blood cell count (RBC) of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. ϕ $p < 0.05$ by comparison with male offspring.

5.4.3 Haemoglobin (Hgb) concentration of pups

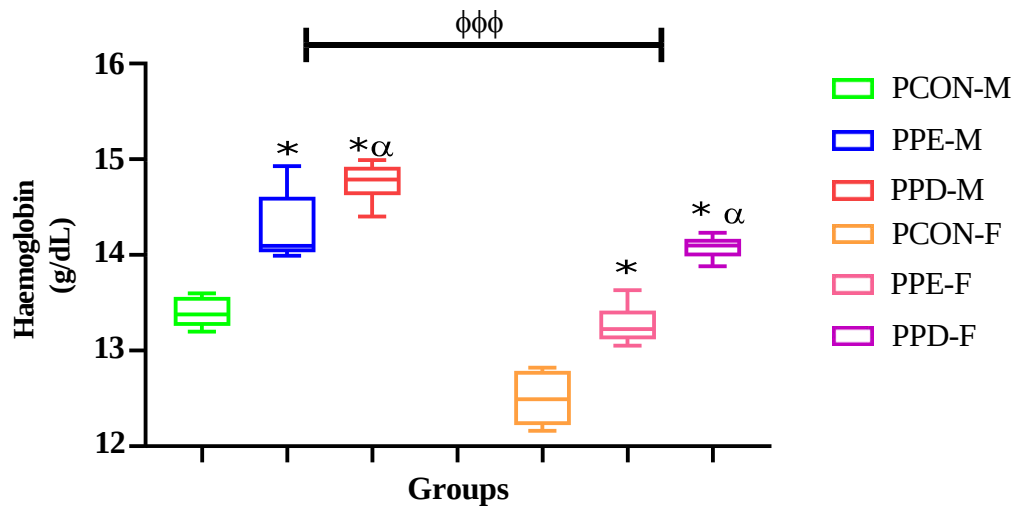


Figure 4: shows the haemoglobin (Hgb) of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison with male offspring.

5.4.6 Erythropoietin (EPO) concentrations of pups

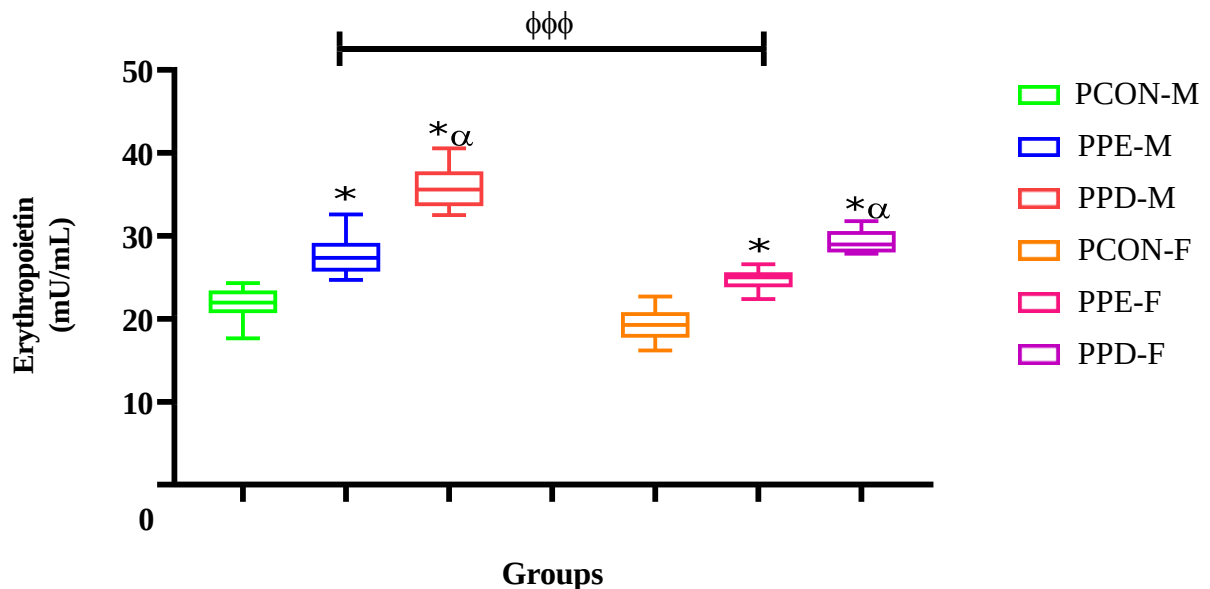


Figure 5: shows the erythropoietin (EPO) of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison

with male offspring.

5.4.7 Granulocyte colony-stimulating factor (G-CSF) concentrations of pups

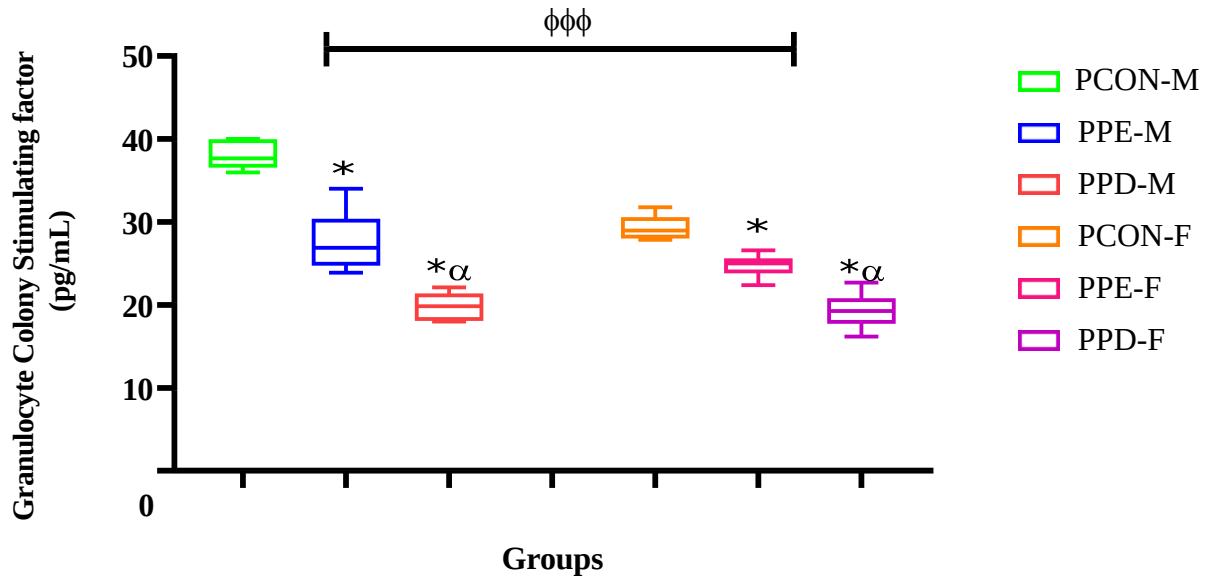


Figure 6: shows the granulocyte colony-stimulating factor (G-CSF) of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison with male offspring.

5.4.8 Malondialdehyde (MDA) concentrations of pups

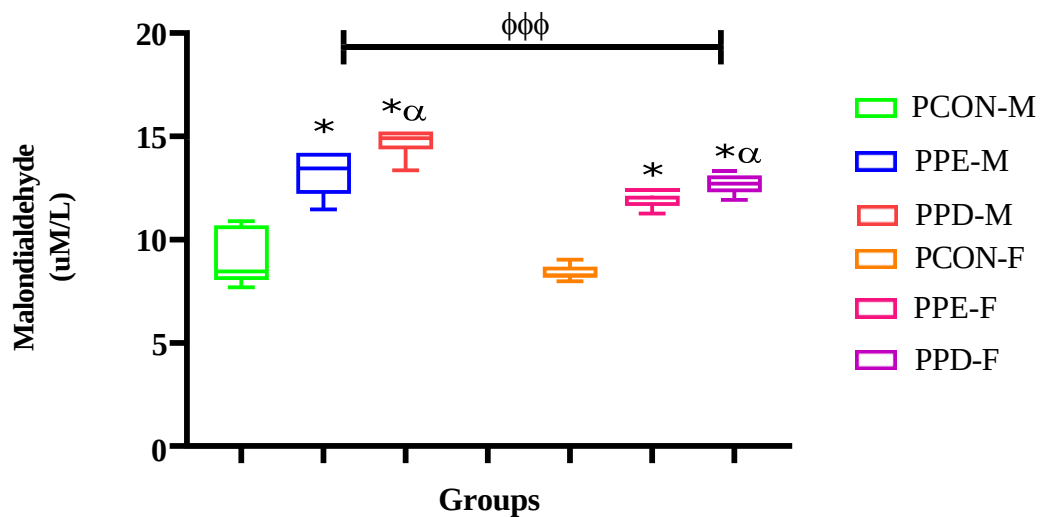


Figure 7: shows the malondialdehyde (MDA) of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. ϕ $p < 0.05$ by comparison with male offspring.

5.4.9 Superoxide Dismutase (SOD) concentrations of pups

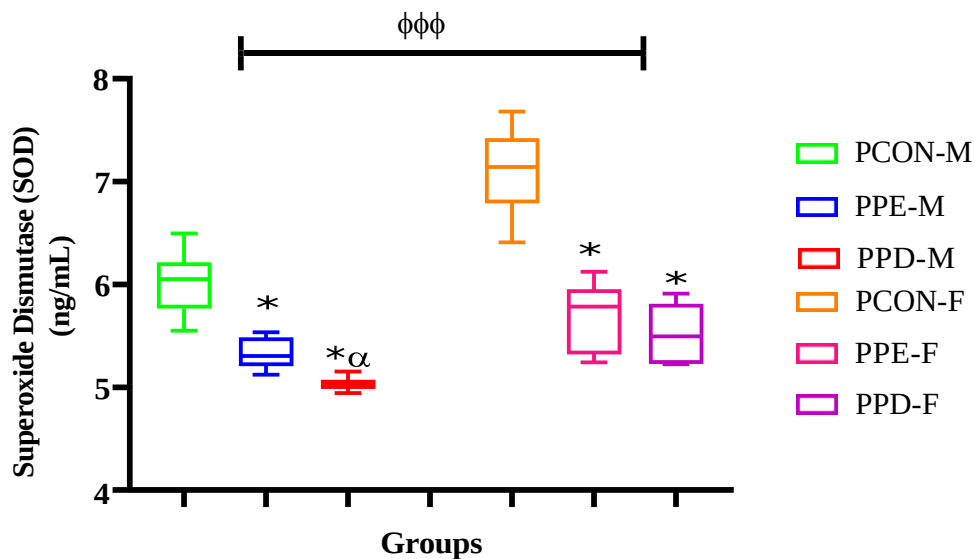


Figure 8: shows the superoxide dismutase (SOD) of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON. ϕ $p < 0.05$ by comparison with male offspring.

5.4.10 Glutathione Peroxidase (GPx) concentrations of pups

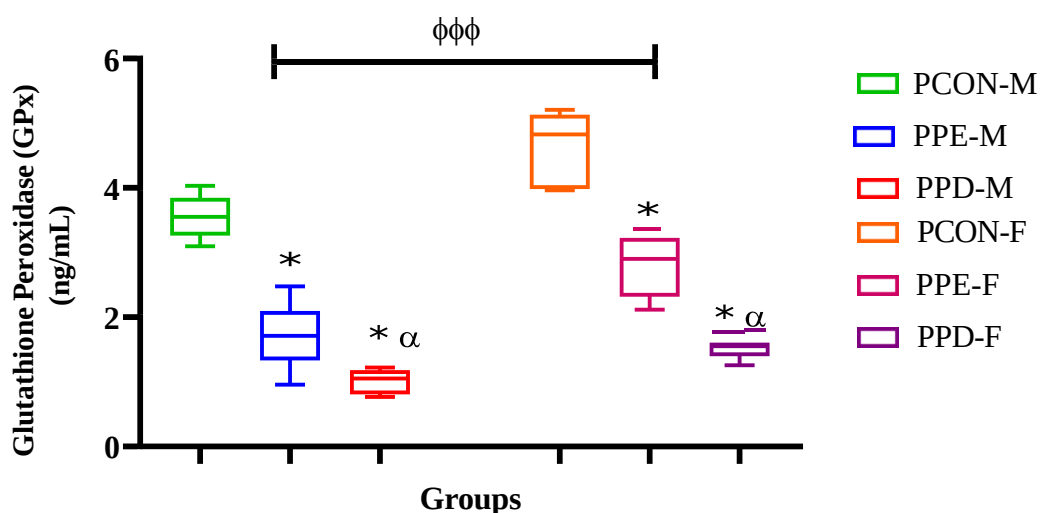


Figure 9: shows the glutathione peroxidase (GPx) of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ p < 0.05 by comparison with PCON. φ p < 0.05 by comparison with male off

TABLES

5.4.4 Red blood cell parameters of pups

PARAMETERS MEASURED	PCON-M	PPE-M	PPD-M	PCON-F	PPE-F	PPD-F
MCV (FL)	75.02 ± 1.51	82.33 ± 0.47 ★	73.78 ± 2.48★ ^α	73.50 ± 1.00 ^φ	79.75 ± 0.50★ ^φ	69.00 ± 2.00★ ^{αφ}
MCH (PG)	26.40 ± 0.42	23.03 ± 1.33★	21.45 ± 0.41 ★ ^α	24.05 ± 1.18 ^φ	21.20 ± 0.23★ ^φ	17.65 ± 0.53 ★ ^{αφ}
MCHC (G/DL)	32.48 ± 0.46	37.33 ± 2.46 ★	33.78 ± 0.22 ★ ^α	29.90 ± 1.63 ^φ	35.98 ± 0.48★ ^φ	31.03 ± 0.29 ★ ^{αφ}
RDW (FL)	11.15 ± 0.94	13.80 ± 0.29 ★	12.13 ± 0.32 ★ ^α	9.65 ± 0.39 ^φ	11.48 ± 0.26★ ^φ	10.68 ± 0.88 ★ ^{αφ}

Table 1: shows the MCV, MCH, MCHC, and RDW of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ p < 0.05 by comparison with PCON and α p < 0.05 by comparison with PPE. φ p < 0.05 by comparison with male offspring.

5.4.5 White blood cell (WBC), neutrophil (NE), and platelet (PLT) counts of pups.

PARAMETERS MEASURED	PCON-M	PPE-M	PPD-M	PCON-F	PPE-F	PPD-F
WBC (10 ⁹ /L)	1,95 ± 0.64	1.40 ± 0.37 ★	5.06 ± 1.29 ★ ^α	1.33 ± 0.54 ^φ	1.15 ± 1.45 ★ ^φ	4.05 ± 0.86 ★ ^{αφ}
NE (%)	22.53 ± 5.07	19.05 ± 5.15 ★	17.43 ± 4.85★ ^α	23.83 ± 7.39 ^φ	17.35 ± 3.10 ★ ^φ	15.33 ± 2.27 ★ ^{αφ}
PLT	122.50 ± 36.04	105.3 ± 36.57 ★	113.5 ± 182.8★ ^α	86.50 ± 24.37 ^φ	73.5 ± 176.60 ★	77.8 ± 130.20 ★ ^φ

Table 2: shows the WBC, NE, and PLT of pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). Values are presented as means and standard error of means (SEM). ★ p < 0.05 by comparison with PCON and α p < 0.05 by comparison with PPE. φ p < 0.05 by comparison with male offspring.

BRIDGE 3

Blood is crucial for neuronal and overall cognitive development, providing essential nutrients and oxygen necessary for the growth and maturation of neural tissues. Any disruption in the blood supply or its composition can have significant consequences on neuronal and cognitive development, affecting processes such as neuronal differentiation, synaptogenesis, and the establishment of neural circuits. Similar to cardiac muscle development, the foetal brain becomes increasingly reliant on a fully functional vascular system to support its metabolic needs as pregnancy progresses. In conditions such as preeclampsia (PE) and pregestational type 2 diabetes (pT2D), impaired blood flow and altered blood composition can lead to deficits in neuronal and cognitive development, resulting in long-term cognitive consequences for the offspring. Since PP is a precursor to pT2D, it is hypothesized that the cognitive development in PP will be exposed to similar pathophysiological mechanisms as those observed in pT2D. However, this has not been established hence the final manuscript, which investigates the impact of high-fat, high-carbohydrate diet-induced PP on gender-specific cognitive outcomes, focusing on markers of cognitive function

CHAPTER 6: MANUSCRIPT 3

6. PROLOGUE

The process of foetal cognitive development is profoundly influenced by maternal health and the intrauterine environment. Conditions such as preeclampsia (PE) and pregestational type 2 diabetes (pT2D) disrupt this development through shared mechanisms, including oxidative stress, chronic inflammation, placental dysfunction, and neuroinflammation. These disturbances compromise critical neurodevelopmental processes, such as neurogenesis, synaptogenesis, and myelination, potentially leading to long-term impairments. Since pregestational prediabetes (PP) is an intermediary state preceding overt pT2D and is a reported risk factor for PE, understanding its potential influence on neurodevelopmental outcomes is essential for addressing research gaps in maternal-foetal health. As discussed in the previous chapter, PP results in adverse haematological outcomes in offspring, with notable gender-specific disparities. Building upon these findings, the current manuscript examines the role of HFHC diet-induced PP on postnatal cognitive outcomes, focusing particularly on gender-specific differences in key markers of cognitive function and regulation, including neuroinflammatory cytokines, oxidative stress parameters, and indicators of neurodevelopmental integrity. These findings aim to provide insight into the gender-specific effects of PP on infant cognitive function, offering a basis for future research and targeted interventions.

The manuscript titled **“High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cognitive function”** is authored by Xulu N.D, Khathi A, Sibiya N.H and Ngubane P.S

The manuscript is under review in *Heliyon*. See Appendix 4. This journal is accredited by the Department of Higher Education and Training South Africa and appears in the Scopus accredited list (2023)

High fat, high carbohydrate (HFHC) diet-induced prediabetes as a risk factor for preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cognitive function.

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6.1 Abstract

Optimal maternofetal nutrient and oxygen supply during gestation is essential for normal foetal neurodevelopment. Preeclampsia (PE) and pregestational type 2 diabetes (pT2D) have been linked to altered neurodevelopment and behavioural outcomes in offspring. Prediabetes, a precursor condition, may represent an early risk factor, yet its impact on offspring cognitive outcomes remains unclear. In this murine model, we assessed neonatal behaviour and cognition, with attention to sex-specific differences. Our findings indicate changes in early behavioural outcomes associated with maternal prediabetes, suggesting potential pathways of vulnerability that require further exploration in human studies.

Methodology

Male and female pups were born from normal control (PCON), L-NAME-induced preeclamptic (PPE), and HFHC diet-induced prediabetic (PPD) dams. The pups were housed with their mothers for 21 days, weaned, and divided into male and female groups. After a week of acclimatization, they underwent Morris Water Maze (MWM) training at week 5. At the end of week 5, the pups were weighed, and sacrificed, and plasma and brain tissue were collected for biochemical analysis of plasma BDNF, FGF2, IL-6, IL-1 β , and hippocampal gene expression of BDNF and FGF2.

Results

Results showed that the PPE and PPD groups had significantly lower hippocampal gene expression rates and plasma concentrations of BDNF and FGF2 compared to PCON, indicating impaired neurodevelopment. Elevated levels of plasma IL-6, IL-1 β , and TNF- α were observed in PPE and PPD compared to PCON, suggesting a pro-inflammatory intrauterine environment. The MWM test revealed that PPE and PPD had increased escape latency, decreased platform crossings, and less time spent in the target quadrant compared to PCON, indicating deficits in spatial learning and memory. Female pups in the PPE and PPD groups showed significantly lower changes in these parameters compared to male pups, highlighting sex-specific differences in neurodevelopmental outcomes.

Conclusion

In this study, our murine/rat model concludes that PE and pregestational prediabetes (PP) negatively impact foetal brain development, with PP posing a potentially greater risk for long-term cognitive impairments. Sex-specific differences were considered, underscoring the importance of tailored interventions. Targeted management of PP and reduction of inflammation during pregnancy could improve neurodevelopmental outcomes in offspring.

6.2 Introduction

During gestation, adequate maternofoetal supply is essential for neurodevelopment because oxygenation, nutrient availability, and tightly regulated immune signalling collectively shape neurogenesis, synaptogenesis, and myelination (1-3). The placenta mediates this environment through vascular development, endocrine signalling, and selective transfer processes that can be disrupted in pathological pregnancies (4-6). In preeclampsia (PE), reduced uteroplacental perfusion and intrauterine growth restriction (IUGR) are frequently reported and have been associated with later neurodevelopment-related differences in offspring, although the strength and specificity of these associations vary across settings and study designs (10-13). In pregestational type 2 diabetes (pT2D) pregnancies, maternal hyperglycaemia and inflammatory-oxidative stress have been linked with altered placental function and with foetal brain vulnerability in both experimental and clinical literature (15-21). Importantly, inflammatory mediators at the materno-foetal interface may influence foetal neurodevelopment through complex pathways that include placental signalling, vascular changes, and downstream foetal immune activation, rather than through a simple assumption of unrestricted transfer (22-26). Against this background, and because pregestational prediabetes (PP) is a precursor to pT2D and a risk factor for PE, the present work investigates whether HFHC diet-induced PP is associated with sex-specific shifts in selected postnatal markers relevant to neurodevelopment, including systemic inflammatory cytokines, oxidative stress parameters, and neurotrophin profiles (10).

6.3 Materials and Methods

6.3.1 Animals

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 (n=18) were bred and housed in the Biomedical Research Unit (BRU) of the University of Kwa-Zulu Natal for 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 acclimatized to their new environment for 1 week while consuming standard rat chow and tap water before the induction of prediabetes by exposure to a well-established experimental diet (HFHC).

6.3.2 Induction of prediabetes

Female Sprague–Dawley rats were randomly assigned to three groups, groups A, B, and C (n = 6 per group). Experimental prediabetes was induced in female Sprague–Dawley rats using a protocol previously described (41). To summarize, control groups A and B were given a standard rat chow diet with normal water for the 36-week period. Group C was given a high-fat, high-carbohydrate (HFHC) diet with 15% fructose added for the same duration of 36 weeks. The composition of the HFHC diet is 30% high fats, 55% of carbohydrates and 15% fructose. At the end of 36 weeks, the diagnostic criteria of the American Diabetes Association (ADA) were applied to identify prediabetes in the animals. This involved measuring diagnostic indicators for prediabetes, such as fasting blood glucose levels ranging from 5.6 to 7.1 mmol/L, oral glucose tolerance test (OGTT) with 2-hour glucose levels between 7.1 and 11.1 mmol/L at 0, 7, 12, and 19 weeks, these numbers represent time points in weeks after induction of prediabetes in the maternal rats and were guided by preeclamptic maternal time points. Animals meeting this criterion were categorized as prediabetic (PD), while those with measurements below the prediabetic thresholds were considered healthy controls (CON) A and B.

6.3.3 Oral glucose tolerance response

An oral glucose tolerance test was performed on all animals to assess their glucose tolerance response. Following a well-established laboratory technique, this test was completed after carbohydrate loading (21). After fasting for 16 hours, glucose levels were determined at time zero. Then, a monosaccharide syrup was administered orally using an 18-gauge gavage 38 mm long and curved needle with a 21/4 mm ball end (Able Scientific, Canning Vale, Australia). The glucose concentrations were determined by collecting blood using the tail-prick method (21) and measuring glucose concentrations using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). Glucose levels were subsequently assessed at 15-, 30-, 60- and 120 minutes following carbohydrate loading. The Oral Glucose Tolerance Test (OGTT) values reported here refer specifically to the 2-hour post-load glucose concentration, the standard diagnostic timepoint for impaired glucose tolerance in rodent models.

The cut-points were interpreted within the context of capillary glucose measurements, ensuring that group allocation reflects relative glucose tolerance and insulin resistance rather than absolute equivalence to venous values. Calibration and quality control were performed according to manufacturer instructions prior to measurement to ensure reliable readings for experimental grouping. These tools were selected due to their rapid assessment capability, minimal blood volume requirements, and established use in small-animal metabolic studies, which is critical for longitudinal monitoring of pups and dams.

6.3.4 Mating

Prior to mating, all 18 female Sprague–Dawley rats underwent assessment for the proestrus stage through vaginal smear analysis under a microscope (Leica Microsystems, Illinois, USA). Briefly, two females were housed in one cage to allow for their oestrus cycles to synchronize. After synchronization those in the proestrus stage were permitted to mate with healthy male control Sprague–Dawley rats (40). Confirmation of pregnancy and assignment of gestational day (GND) zero were determined the following morning by the presence of a vaginal plaque or vaginal smear containing spermatozoa observed under a microscope (Leica Microsystems, Illinois, USA) (40). The male rats were removed from the cage and returned to the BRU. Pregnant rats were then observed until the end of gestation (21 days).

6.3.5 Induction of preeclampsia

After the visualization of spermatozoa confirmed pregnancy in a vaginal smear, the pregnant dams from healthy control group B were randomly divided into one rat per cage, which were given 0.3g/L a day of L-NAME in drinking water from day 14.5 to day 20.5 of gestation. Blood pressure was measured, and the presence or absence of protein in the urine was assessed (40). Preeclampsia (PE) was confirmed by measuring blood pressure monitored every third day after administration. Animals presenting with a blood pressure of $\geq 140/90$ mmHg and proteinuria of ≥ 300 mg (3+) were considered preeclamptic. Blood pressure was monitored at GND 0, 7, 12, and 19. using a non-invasive tail-cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA). Urine samples were collected at similar GND days, overnight using metabolic cages, and protein urea was measured immediately via dipsticks (Multistix® 10 SG, Siemens Healthineers, Pennsylvania, USA).

6.3.5.1 L-NAME Rationale

The inclusion of an L-NAME-induced preeclampsia group allowed for the differentiation between the effects of pregestational prediabetes and established preeclampsia on maternal and offspring outcomes. This comparative approach strengthened the mechanistic interpretation of whether observed infantile alterations were attributable to prediabetes alone or to a preeclampsia-like intrauterine environment (10).

6.3.6 Collection of pups

At gestational day (GND) 21, naturally delivered male and female pups from control (PCON), L-NAME preeclamptic (PPE) and HFHC diet-induced prediabetic (PPD) dams were immediately collected and weighed. Half of the pups (n=36) were sacrificed for brain-to-body weight ratio measurements, and the remainder (n=36) were carefully returned to the dams for further development. The animals were housed in Makrolon polycarbonate cages under standard laboratory conditions (for temperature and humidity) in a 12-hour day and 12-hour night cycle and were given access to food and water.

6.3.7 Behavioural tests

All the behavioural tests were conducted at 09h00 to evaluate the cognitive function of the pups from all the groups using the following techniques.

6.3.7.1 Morris water maze (MWM)

The Morris water maze (MWM) training was conducted from day 22 to day 34. This paradigm is employed to assess rats' learning and recall ability and covers exploratory, navigational, spatial, and contextual memory (42). The MWM comprises a 1 m diameter pool, comprising 4 quadrants. Each quadrant has a cue to help the rat locate the hidden platform placed in one of the quadrants of the pool. The method consists of placing the rat in a quadrant other than where the hidden platform is located and recording the time taken (escape latency period) by the rat to reach the hidden platform is considered as the animal's ability to learn. The probe test is a post-test for learning that examines the ability of an animal to recall the quadrant in which the hidden platform will be located. The time spent in the quadrant with the hidden platform is considered as the ability to remember (memory).

6.3.7.2 Training and Learning

Briefly, pups (n=36) were exposed to 5 consecutive training sessions for a period of 5 days (day 22-34) (42). Animals were taken to the behavioural room 1 hour prior to the training to allow for familiarization with the new environment. In the training procedure, each rat was softly placed in the water, head facing the cue in a quadrant. The rat was allowed 120 s to locate the hidden platform. When a rat is unsuccessful in finding the hidden platform in 120 s, it was physically directed by the experimenter on the way to the platform and allowed 60 s to explore the platform before being returned to the home cage. Training recommenced the following day for the next 4 days, and the time (escape latency period) it took for the rat to locate the platform will be recorded.

6.3.8 Study design

Prediabetes as a risk factor for Preeclampsia: Assessing gender-specific infantile outcomes on selected markers of cognitive function was studied in the following groups. The pups were immediately weighed after birth and then returned to the dams for further development. On the post-natal day (PND), 21, the pups (n=72) were weaned and divided into PCON, PPE, and PPD (n=24/group). Group 1: Male and

female pups born from normal pregnant control rats (PCON) which received normal standard chow and normal drinking water throughout the study (n=24); Group 2: Male and female pups born from PD pregnant rats which received the HCHF diet+ 15% fructose throughout the study (n=24); Group3: Male and female pups born from PE pregnant control rats which received normal standard chow (n=24). Half (n=36) of the pups were sacrificed; whole brain tissue was then collected for brain-to-body weight ratio and cephalization index analysis. Plasma was collected for BDNF, FGF2, IL-6, IL-1 β . The remaining pups (n=36) were further divided into male (n=6) and female (n=6) and allowed to acclimate for a week. To assess the effects on learning and memory, the pups underwent Morris Water Maze (MWM) training and testing at week 5. All behavioural assessments in this manuscript were conducted postnatally, after weaning, and therefore reflect juvenile learning and memory performance rather than direct in utero cognitive function. The Morris water maze was performed at approximately five weeks of age, following acclimatisation, to evaluate spatial learning and memory under standardised testing conditions. Accordingly, references to foetal neurodevelopment in the discussion are framed as developmental programming: an inference that prenatal exposure to a dysregulated intrauterine environment may shape later postnatal behavioural outcomes, rather than as direct measurement of cognitive function in utero. Thereafter, the pups were euthanised. Hippocampal tissue was collected for gene expression of BDNF and FGF2 respectively. The study design is summarised in the figure below (**Figure 1**).

6.3.8.1 Brain-to-body weight ratio and cephalization index

The brains were rapidly removed and weighed. Considering the difficulty and low accuracy of head circumference measures in rats, the CI (calculated in humans as $CI = \text{head circumference} \times 100 / BW$ (g)) was calculated using the weight of the brain (43). In these animals, a modified version of the human CI was calculated as $CI = \text{brain weight (mg)} \times 100 / BW$ (mg). This modified version of the CI reflects the brain/BW ratio, expressed as a percentage.

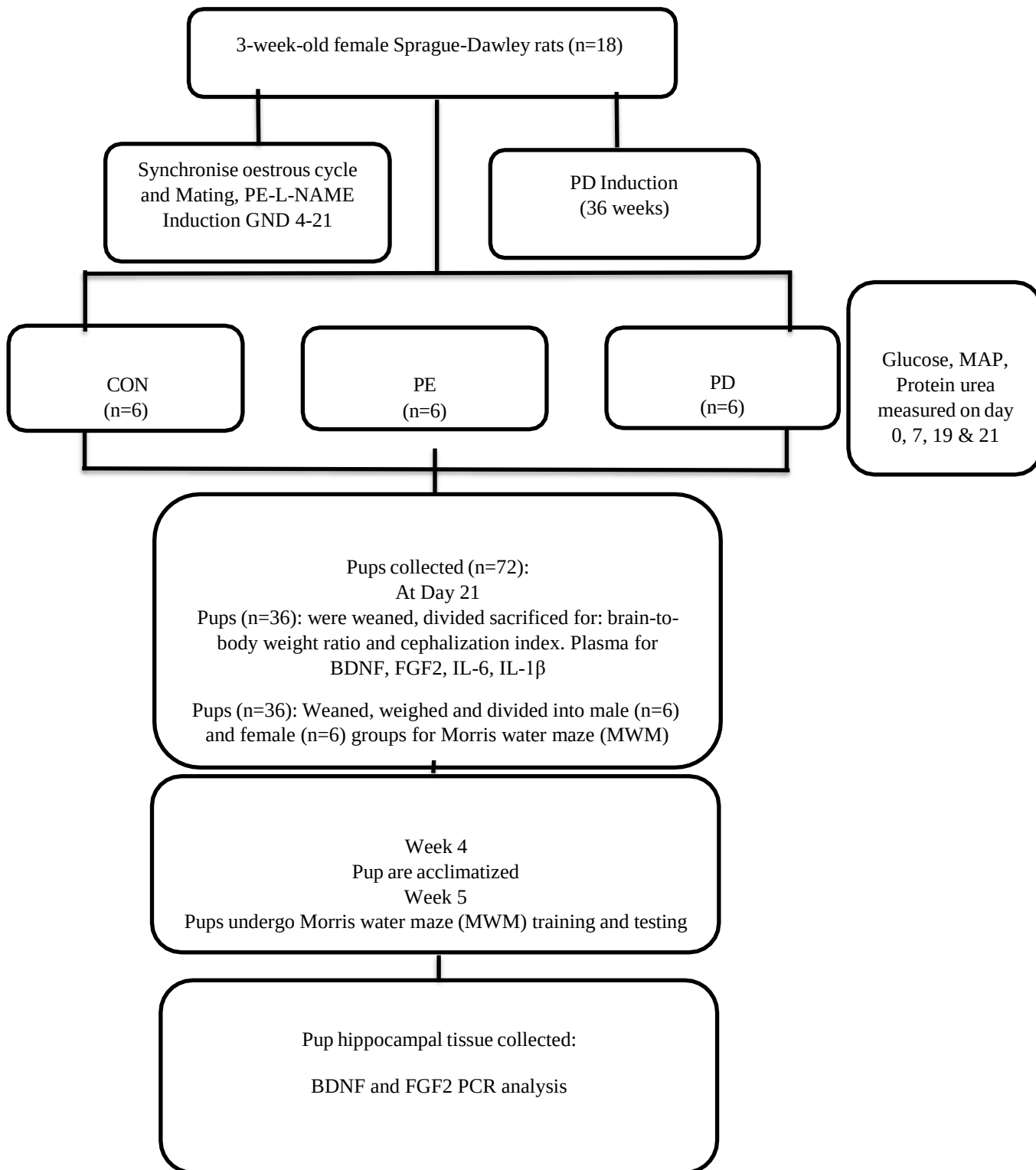


Figure 1: Illustrates a graphical abstract description of the study design.

6.3.9 Biochemical Analysis

6.3.9.1 Neurotrophic factor and inflammatory cytokine analysis in pup plasma.

Plasma Brain-Derived neurotrophic factor (BDNF), Fibroblast growth factor -2 (FGF2), Interleukin -6 (IL-6), Interleukin -1 β (IL-1 β), Tumour Necrosis Factor-alpha (TNF- α) were measured using ELISA assays following the manufacturer's instructions (Elabscience and Biotechnology, WuHan).

6.3.9.2 FGF2 and BDNF expression via Real time-PCR in pup hippocampus

The hippocampus was collected on week 5 and the tissues were homogenised. The total RNA was isolated using a reaction kit (Zymo Research Quick-RNA™ Miniprep, Inqaba Biotech) (CA-USA) according to the manufacturer's protocol. Total RNA was reversed transcribed to cDNA using the iScript cDNA synthesis kit [Bio-Rad Laboratory (Pty) Ltd., USA] according to the manufacturer's instructions. PCR amplification was performed using real-time PCR (LightCycler® 2.0, FastStartDNA Master SYBR Green I, Roche Diagnostics, Germany). Glyceraldehyde- 3-phosphate (GAPDH) was used as an internal control to normalise the data and to determine the relative expression of the gene of interest. The PCR reaction mixture per sample comprised of SYBR green; MgCl₂ (25 mM); forward primer (10 mM) and reverse primer (10 mM). FGF2 and BDNF gene expression was performed using the following oligonucleotide primer sequences (Inqaba Biotechnical Industries, South Africa), The real-time PCR conditions were used as follows: an initial denaturation cycle at 95 °C for 10 min followed by PCR which consisted of 45 cycles at 95 °C for the 30 s, 65 °C for 30 s and final elongation at 72 °C for the 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. Lightcycler® analysis software (Version 4.2) was used to produce a standard curve by plotting the log concentration against the crossing point of each standard.

Table 1 List of primers used in the study.

Gene of interest	Sequence
BDNF (Brain Derived neurotrophic factor)	Forward: 5'-GCTGCCTTGATGTTTACTTTGA-3' Reverse: 5'-CGTGGACGTTTGCTTCTTTC-3'
FGF2 (Fibroblast growth factor- 2)	Forward: 5'- GAAGGAAGATGGACGGCTGC -3' Reverse: 5'- TGACAGTGTCTAAAGAGAGTC -3'
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	Forward: 5'-AGTGCCAGCCTCGTCTCATA-3' Reverse: 5'-GATGGTGATGGGTTTCCCGT-3'

6.3.10 Statistical Analysis

All data were expressed as means $\pm$ standard error of the mean (SEM). Normality was assessed prior to statistical testing. Analyses were performed using GraphPad Prism InStat Software (version 9.00, GraphPad Software, San Diego, California, USA). For dam parameters, comparisons were conducted using a one-way analysis of variance (ANOVA), as sex was not a variable of interest. For pup parameters, a two-way analysis of variance (ANOVA) was employed, with Group (Control, Preeclamptic, Prediabetic) and Sex (Female, Male) as fixed factors, thereby allowing simultaneous testing of group differences, sex differences, and their potential interaction. Where significant main effects or interactions were detected, post hoc analyses were conducted using the Tukey–Kramer test for parametric data, or the Bonferroni correction for non-parametric data, to identify pairwise differences between groups. For ANOVAs and other multivariate analyses, assumptions of homogeneity of variance and sphericity were assessed; in cases where sphericity was violated, Greenhouse–Geisser corrections were applied to adjust the degrees of freedom. Statistical significance was set at $p < 0.05$ and corrected degrees of freedom are reported where applicable.

6.4 Results

6.4.1 Pup body -to-brain ratio and cephalization index

Table 2 shows the body and brain weights, as well as the cephalization index of pups born from normal control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and high fat, high carbohydrate (HFHC) diet-induced prediabetes (PPD). The PPE- male and female (M and F) and PPD- male and female (M and F) significantly ($p < 0.05$) decreased body and brain weights as well as a significantly increased cephalization index in comparison to PCON. Interestingly, the body and brain weights as well as the cephalization index of PPD was significantly ($p < 0.05$) decreased compared to PPE. Furthermore, the brain-to-body weight ratio and cephalization indices were significantly ($p < 0.05$) higher in male compared to female offspring.

PARAMETERS MEASURED	PCON-M	PPE-M	PPD-M	PCON-F	PPE-F	PPD-F
Brain weights (g)	2.50 $\pm$ 0.09	2.01 $\pm$ 0.09 ★	1.94 $\pm$ 0.08 ★ ^{α}	2.22 $\pm$ 0.10 ^{ϕ}	1.65 $\pm$ 0.08 ★ ^{ϕ}	1.55 $\pm$ 0.07 ★ ^{$\alpha\phi$}
Body weights (g)	37.2 $\pm$ 4.99	34.8 $\pm$ 4.75 ★	32.5 $\pm$ 5.98 ★ ^{α}	35.8 $\pm$ 5.49 ^{ϕ}	32.8 $\pm$ 4.28 ★ ^{ϕ}	31.8 $\pm$ 5.38 ★ ^{$\alpha\phi$}
Ratio	0.07	0.06 ★	0.05 ★ ^{α}	0.06 ^{ϕ}	0.05 ★ ^{ϕ}	0.04 ★ ^{$\phi\alpha$}
Cephalization-index (%)	1.04 $\pm$ 0.04	1.20 $\pm$ 0.09 ★	1.23 $\pm$ 0.05 ★ ^{α}	1.00 $\pm$ 0.04 ^{ϕ}	1.16 $\pm$ 0.04 ★ ^{ϕ}	1.20 $\pm$ 0.05 ★ ^{$\alpha\phi$}

Table 2: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and ^{α} $p < 0.05$ by comparison with PPE. ^{ϕ} $p < 0.05$ by comparison with male offspring.

6.4.2 Brain-derived neurotrophic factor (BDNF) concentrations in pups

Figure 2 shows the (A) plasma concentrations of BDNF for day 21(W3) and day 35(W5) and (B) hippocampal brain-derived neurotrophic factor (BDNF) gene expression in pups born from normal control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) at the end of the study. The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) lower plasma and gene expression levels of BDNF compared to the pups born from normal dams. Furthermore, PPE showed a significant increase in plasma and gene expression levels of BDNF compared to PPD. Furthermore, the plasma and gene expression levels of BDNF were significantly ($p < 0.05$) lower in male compared to female offspring.

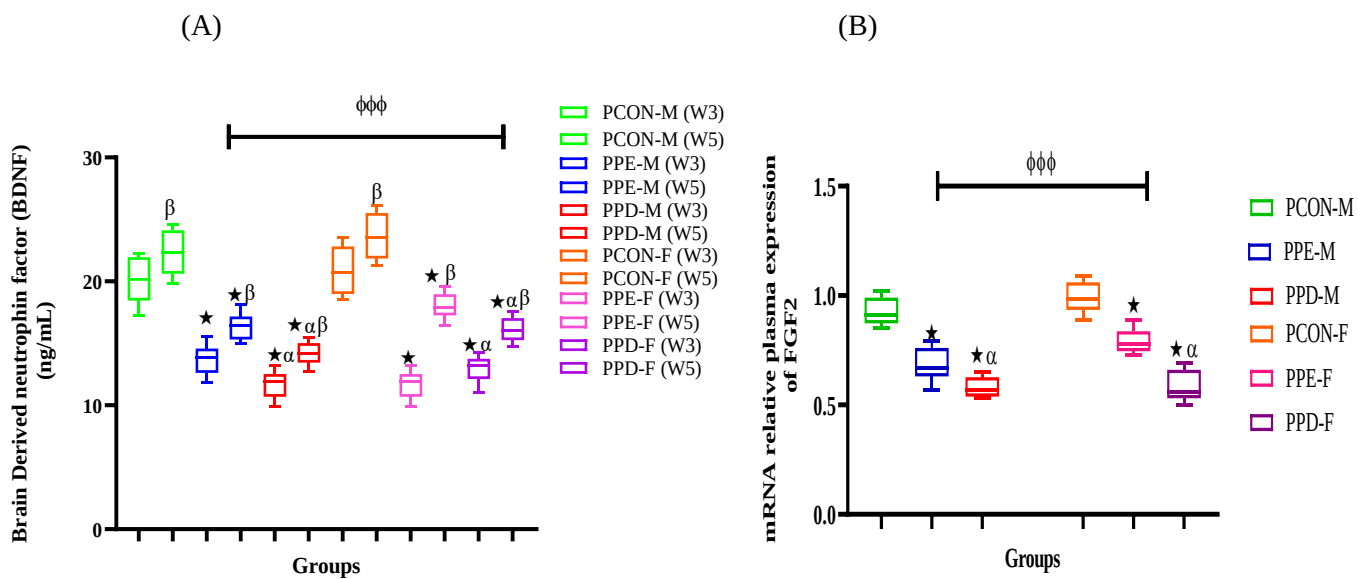


Figure 2: Values are presented as means and standard error of means (SEM). $\star$ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. ϕ $p < 0.05$ by comparison with male offspring. β $p < 0.05$ by comparison with day 21(W3).

6.4.3 Fibroblast growth factor-2 (FGF2) concentrations in pups

Figure 3 shows the (A) plasma concentration of FGF2 for day 21(W3) and day 35(W5) and (B) hippocampal fibroblast growth factor-2 (FGF2) gene expression in pups born from normal control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) at the end of the study. The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) lower plasma and gene expression levels of FGF2 in comparison to the pups born from normal dams. Furthermore, PPE showed a significant increase in plasma and gene expression levels of FGF2 when compared to PPD. Furthermore, the plasma and gene expression levels of FGF2 were significantly ($p < 0.05$) lower in male compared to female offspring.

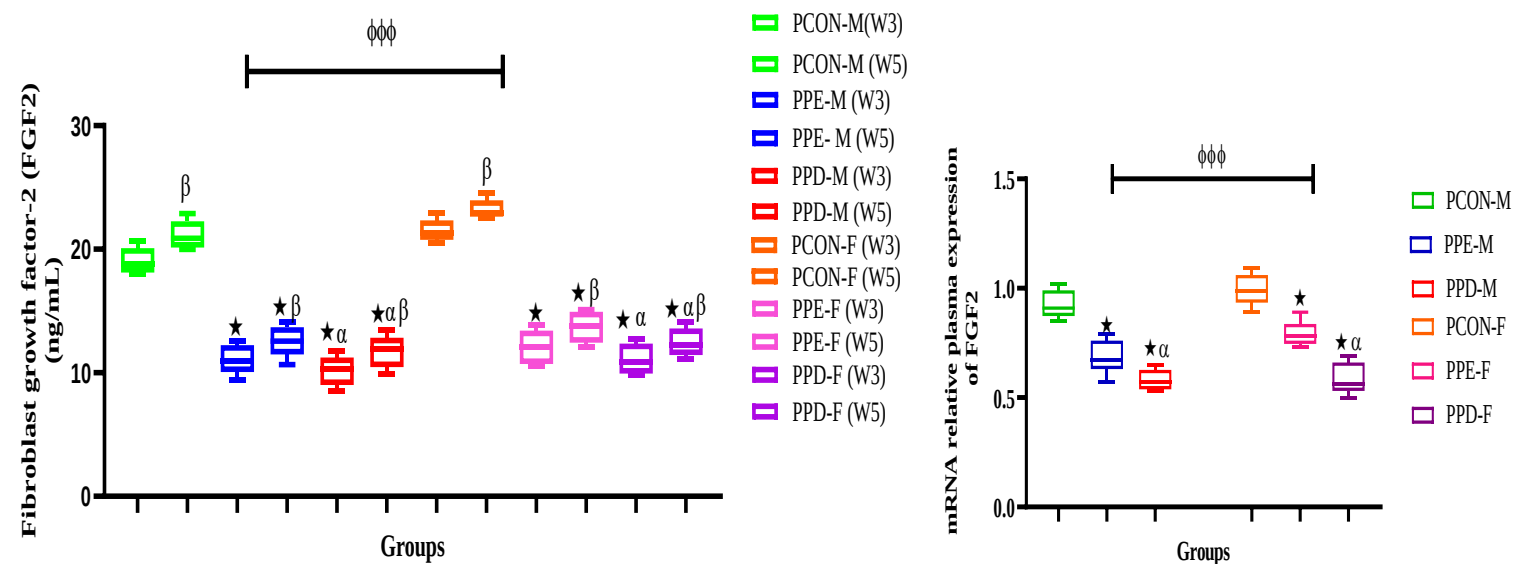


Figure 3: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison with male offspring. β $p < 0.05$ by comparison with day 21(W3).

6.4.4 Interleukin -6 (IL-6), interleukin -1 β (IL-1 β) and tumour Necrosis Factor-alpha (TNF- α) concentrations in pups

Figure 4 shows the plasma concentrations of (A) interleukin -6 (IL-6), (B) interleukin -1 β (IL-1 β), and (C) tumour Necrosis Factor-alpha (TNF- α) in pups born from normal control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) at the end of the study. The PPE- male and female (M and F) and PPD- male and female (M and F) showed significantly ($p < 0.05$) increased IL-6, IL-1 β , and TNF- α in comparison to PCON. Furthermore, PPE showed a significant decrease in IL-6, IL-1 β and TNF- α when compared to PPD. Furthermore, the plasma IL-6, IL-1 β and TNF- α were significantly ($p < 0.05$) higher in male compared to female offspring

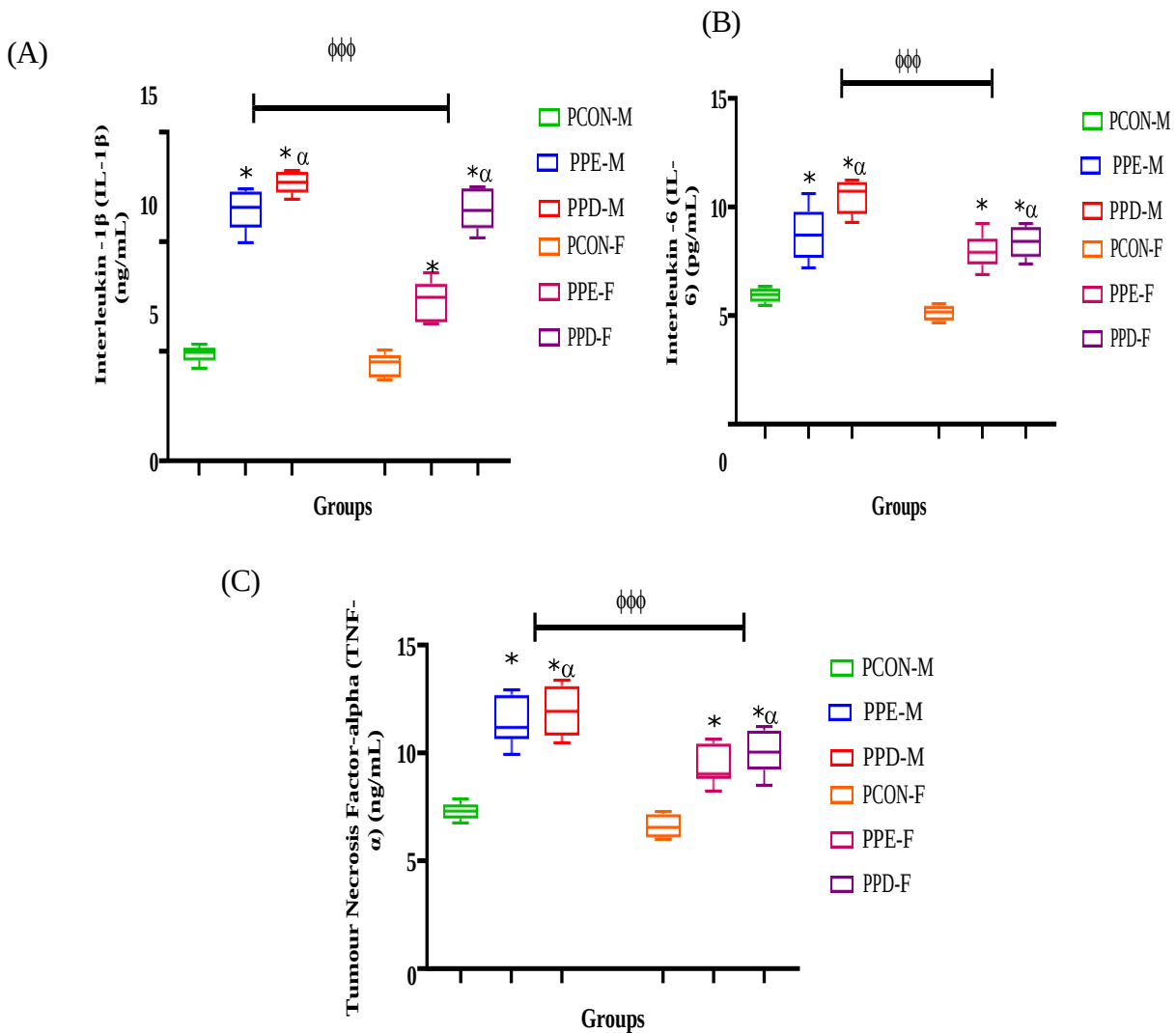


Figure 4: Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. ϕ $p < 0.05$ by comparison with male offspring.

6.4.5 Morris water maze

Figure 5 shows the learning and memory ability in pups born from normal control dams (PCON), L-NAME-induced preeclamptic dams (PPE), and dams induced with prediabetes (PPD) using the MWM experiment. The PPE- male and female (M and F) and PPD- male and female (M and F) showed a significantly ($p < 0.05$) increased escape latency in comparison to PCON. The PPE-F and M and PPD-F and M also showed a significant ($p < 0.05$) decrease in platform crossing and time spent in the target quadrant, when compared to PCON indicating a decline in cognition. Furthermore, PPE showed a significant decrease in escape latency and a significant increase in platform crossing and time spent in the target quadrant when compared to PPD. Furthermore, the male showed a significantly ($p < 0.05$) higher escape latency and significantly ($p < 0.05$) lower platform crossing and time spent in the target quadrant compared to female offspring.

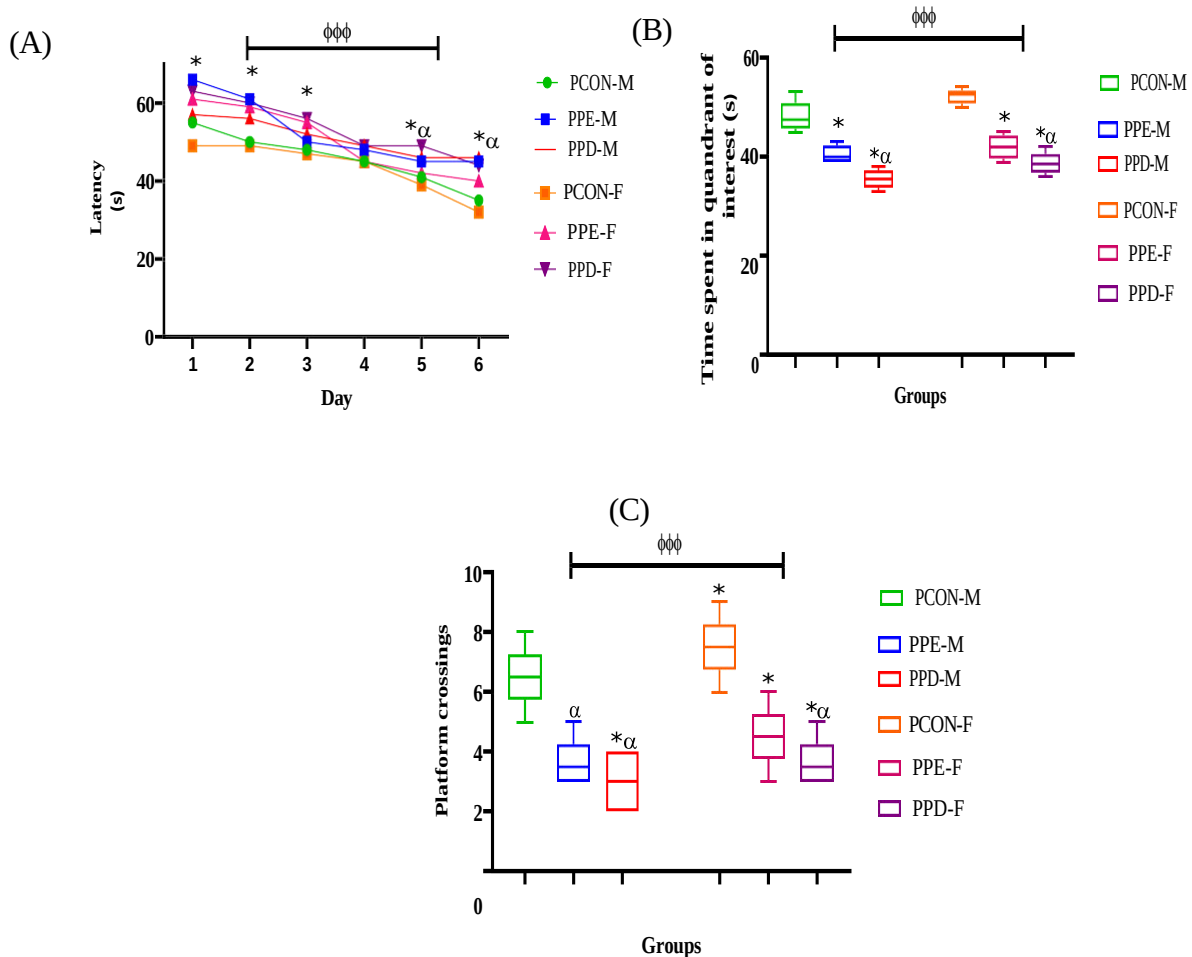


Figure 5: shows the results of the Morris water maze (MWM) test in pups born from normal control (PCON), preeclamptic (PPE), and prediabetic dams (PPD). (A) Shows the escape latency. (B) shows the time spent in the target quadrant after the removal of the platform. (C) shows the number of crossings in the platform area. Values are presented as means and standard error of means (SEM). ★ $p < 0.05$ by comparison with PCON and α $p < 0.05$ by comparison with PPE. φ $p < 0.05$ by comparison with male

offspring.

6.5 Discussion

Adverse maternal health conditions such as preeclampsia (PE) and pregestational type 2 diabetes (pT2D) are frequently complicated by intrauterine growth restriction (IUGR) and are associated with altered offspring developmental trajectories (21). Although substantial work has examined neurodevelopmental correlates of pT2D, the intermediary state of pregestational prediabetes (PP) remains less developed in the literature (12-20). Given its recognised role as a precursor to pT2D and as a risk factor for PE, clarifying how PP relates to offspring outcomes is important, particularly in settings where metabolic risk is increasing (21, 40). In this manuscript, PP and PE were associated with lower growth indices and with differences in selected systemic markers (neurotrophins and cytokines) alongside poorer juvenile performance in a spatial learning task. These outcomes are interpreted as programming-consistent signals rather than as definitive evidence of specific human neurodevelopmental disorders.

The pattern of results is therefore discussed as a convergence across measured developmental indicators rather than as an attempt to assign a discrete disorder label. Growth indices provide an essential developmental context that may co-occur with altered systemic signalling, but they are not, on their own, diagnostic of neurodevelopmental impairment. Similarly, circulating neurotrophin and cytokine profiles are treated as biologically plausible correlates of developmental programming without assuming that peripheral concentrations map directly onto region-specific neurobiology or synaptic function (23, 53). This cautious framing aligns with translational cautions in programming research, where behavioural outcomes and systemic biomarkers can indicate vulnerability but still require direct neural endpoints and longitudinal designs to clarify mechanism and durability (50-52).

The pattern of results is therefore discussed as a convergence across proximate developmental indicators rather than as an attempt to label a discrete disorder outcome. Specifically, growth indices provide a broad developmental context that can co-occur with altered systemic signalling, but they are not, on their own, diagnostic of neurodevelopmental impairment. Likewise, the neurotrophin and cytokine profiles reported here are treated as biologically plausible correlates of developmental programming, without assuming that measured circulating concentrations map neatly onto region-specific neurobiology or synaptic function (23, 53). This framing is consistent with translational cautions in programming research, where behavioural outcomes and systemic biomarkers can indicate vulnerability while still requiring direct neural endpoints and longitudinal designs to clarify mechanism and durability (50-52). The brain-to-body weight ratio and the cephalization index serve as indicators for the allocation of resources and developmental priorities during foetal growth (42). These parameters are used as

significant indicators of relative brain development and overall growth in neonates (42). Studies have outlined how the brain-to-body weight ratio and the cephalization index in disorders of pregnancy such as preeclampsia and pregestational type 2 diabetes (pT2D) are often compromised resulting in neuronal functional impairment (43). Preeclampsia (PE) is characterized by placental insufficiency due to poor trophoblastic invasion and impaired spiral artery remodelling, leading to hypoxia and ischemia (10-13). Similarly, in pT2D chronic hyperglycaemia leads to oxidative stress and inflammation, which can disrupt placental function and nutrient transport to the foetus (14, 21, 40). Both conditions result in reduced delivery of oxygen and essential nutrients to the foetus, impairing brain development (10, 12, 16). However, in pregestational prediabetes (PP), a precursor for pT2D and a risk factor for PE this has not been established (21, 37). As noted in Table 2, pups in both PE and PP rat groups had lower brain-to-body weight ratios compared to pups in the CON group of this experiment. Such a finding indicates that maternal metabolic disorders, such as PP, may impair foetal brain growth and development (36-41). In this study, the lower brain-to-body weight ratios and cephalization indices observed in male offspring suggest possible delays in brain maturation relative to somatic growth. While these findings may point toward altered neurodevelopment, it is important to interpret them within the context of our study design. Complex neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) involve a broad array of cognitive, behavioural, social, and genetic factors, and cannot be inferred from the current dataset. Instead, our findings more cautiously suggest that maternal prediabetes and preeclampsia may influence early structural indices of brain development, which could in turn impact functions such as memory and learning, though further studies are required to confirm this. We also observed sex-specific differences in neurodevelopmental indices. Female offspring appeared to maintain relatively preserved brain-to-body weight ratios compared to males. While prior studies suggest that sex hormones, including oestrogen, play protective roles in female brain development, particularly through modulation of neurotrophic pathways, we did not measure oestrogen or testosterone levels in the present study. Therefore, such explanations remain speculative and should be considered hypotheses for future work. Taken together, these findings highlight the importance of considering sex as a biological variable in developmental programming research. The novelty of this study lies in linking maternal prediabetes and preeclampsia to neonatal hippocampal neurotrophin expression, brain morphology, and early behavioural outcomes. Future studies incorporating broader neurodevelopmental markers, hormonal assessments, and more detailed cognitive testing will be required to fully elucidate these relationships. Brain-derived neurotrophic factor (BDNF) and fibroblast growth factor-2 (FGF2) are critical neurotrophic factors involved in the regulation of major events in brain development, synaptogenesis, as well as neuronal survival (34, 35). BDNF is particularly important as it promotes the survival of existing neurons and the growth and differentiation of new neurons and synapses (46). It exerts these effects by binding to the high-affinity receptor, tropomyosin receptor kinase B (TrkB), which triggers several downstream signalling

pathways, including the phosphatidylinositol 3-kinase/Akt (PI3K)/Akt pathway and the mitogen-activated protein kinase (MAPK) pathway (46, 47). These pathways stimulate neuronal survival, growth, and differentiation by upregulating genes responsible for the synthesis of proteins significant for synaptic plasticity and neurogenesis (46, 47). Similarly, FGF2 plays an important role in neurogenesis and the maintenance of the progenitor cells (48). FGF2 exerts its biological effects through binding to fibroblast growth factor receptors (FGFRs) present on the surface of cells, thereby triggering the activation of the rat sarcoma-mitogen-activated protein kinase (Ras- MAPK) pathway and the PI3K-Akt pathway (47, 48). These pathways are crucial for neural cell proliferation, differentiation, and survival, particularly in neural progenitor cells (48). Due to IUGR observed in PE and pT2D, the concentrations/ levels of the neurotrophic factors are compromised (49). In PE and pT2D, reduced BDNF levels lead to insufficient activation of TrkB receptors, impairing critical neurodevelopmental processes (49). Whereas in PE and pT2D conditions, lower levels of FGF2 result in reduced activation of FGFRs, compromising neurogenesis and the regulation of neural progenitor cells (49). While both ASD and ADHD are characterised by executive function deficits, impaired impulse control, and slower processing speed, the present study cannot make such links. These complex; spectrum-based disorders involve genetic, epigenetic, social, and environmental outcomes. These neurotrophins were selected due to their central roles in regulating neurogenesis, synaptic connectivity, and plasticity, and because of resource limitations that prevented the inclusion of a wider panel of markers (32, 65). We recognise that the evaluation of additional molecular regulators such as PCNA, Ki67, SOX2, and PAX6 (neurogenesis and proliferation), Reelin and Wnt/ β -catenin signalling (structural organisation), and GABAergic synapse-related genes (e.g., GAD67, GABA receptor subunits) would have strengthened the interpretation of our findings. The absence of these measures constitutes a limitation of the present study. Future investigations will aim to incorporate such markers, thereby enabling a more comprehensive understanding of how maternal prediabetes and preeclampsia influence hippocampal development and cognitive outcomes in offspring. In PP, however, the changes to BDNF and FGF2 gene expression and concentrations have not been outlined (21, 37). In this study, Figures 2 and 3 indicated that gene expression and levels of BDNF and FGF2 were significantly lower in PPE and PPD compared to PCON. The decrease in neurotrophic factors and their protein levels is concerning because these factors are essential for the growth and differentiation of neurons during development (44-46). Lower levels of BDNF and FGF2 may result in insufficient support for neural growth and repair, increasing the vulnerability to long-term cognitive issues (44-46). Moreover, PPD exhibited even lower gene expression and levels of BDNF and FGF2 compared to PPE, alluding to the fact that PP may pose higher neurodevelopmental risks than PE (16, 35, 41). This may indicate that endocrine disorders such as PP and pT2D create a more hostile intrauterine environment which impacts the developing foetal brain. This is potentially due to the insult of both hyperglycaemia and inflammation induced-endothelial dysfunction observed in these conditions (16-18). Also, overall BDNF and FGF2 levels were lower in

females compared to males (50-52). This gender-specific difference could be attributed to several factors, including hormonal influences and differential stress responses (50, 51). Oestrogen, which is more prevalent in females, has been shown to modulate BDNF and FGF2 expression, potentially leading to lower baseline levels (50, 52). Moreover, females might experience different compensatory mechanisms in response to intrauterine stress, impacting BDNF and FGF2 levels differently than in males (50, 52). This highlights the importance of considering sex-specific factors when evaluating neurodevelopmental outcomes and suggests that females may have distinct vulnerabilities to prenatal stress and metabolic disturbances, potentially affecting their long-term cognitive health (50, 51).

Inflammatory signalling plays a complex, context-dependent role in neurodevelopment. During pregnancy and early postnatal life, regulated cytokine activity contributes to tissue remodelling and neural patterning, whereas sustained or excessive inflammatory exposure is more consistently associated with disrupted neuronal differentiation and synaptic maturation (23, 53). In this study, higher pro-inflammatory cytokine concentrations in affected offspring may reflect an enduring immune activation phenotype plausibly shaped by the intrauterine environment, but the present data do not localise inflammation to specific brain regions or establish temporal causality. Consequently, cytokine findings are presented as systemic correlates that warrant targeted brain-region analyses in future work. A further interpretive constraint is that cytokines were measured peripherally at the time points sampled and should not be read as demonstrating a continuous inflammatory trajectory from gestation through juvenile testing. Peripheral inflammation can reflect multiple processes, including adaptive immune priming, metabolic-state differences, or postnatal exposures that are not captured by the current design, and these alternative explanations must remain open given the absence of tissue localisation and temporal mapping (23, 53). For this reason, cytokine results are used to contextualise behavioural outcomes and neurotrophin shifts as part of a systems-level profile rather than as standalone proof of neuroinflammation or neuronal injury (50-52).

Cognitive performance was assessed using the Morris water maze (MWM), a well-established procedure for spatial learning and memory in rodents (58). Offspring from PE and PP dams displayed poorer MWM performance than controls, which is consistent with reduced learning efficiency and/or altered memory consolidation reported in prior metabolic and hypertensive pregnancy models (59, 60). Because this test was conducted postnatally, the results provide behavioural evidence compatible with prenatal programming effects, but they do not permit direct inference about foetal cognition. Mechanistically, the co-occurrence of altered neurotrophin concentrations and higher systemic inflammatory markers is compatible with hippocampal vulnerability pathways described in the literature, yet direct hippocampal structural or synaptic measures were not included here and therefore remain interpretive (50-52). Importantly, MWM performance reflects multiple contributors beyond

memory alone, including learning rate, stress reactivity, motivation, and swim strategy, and the study design does not isolate these subcomponents through complementary tasks or strategy scoring (58-60). Accordingly, the behavioural findings are best reported as evidence of altered task performance consistent with programming effects, while recognising that stronger claims about hippocampal microstructure, synaptic integrity, or region-specific plasticity remain inferential without targeted neural endpoints (50-52).

Sex differences were observed for selected measures, aligning with literature indicating that male and female offspring may exhibit different adaptive responses to prenatal stressors and inflammatory environments (56). However, because sex hormones, placental endocrine signalling, and brain-region specific inflammatory responses were not measured, the current findings support a sex-sensitive interpretation without permitting definitive mechanistic attribution (56, 61, 62). The observed sex-sensitive patterns are therefore treated as empirically supported differences at the level of measured outcomes rather than proof of a single sex-specific pathway. A stronger test of sex-specific vulnerability would require designs that explicitly model sex across development, include endocrine and placental measures where relevant, and assess whether behavioural differences are mediated by systemic marker profiles or primarily track growth restriction severity (56, 61, 62).

6.6 Conclusion

Maternal prediabetes was associated with altered growth indices, neurotrophin profiles, systemic inflammatory markers, and juvenile behavioural performance in rat offspring, with evidence of sex-specific patterns (21, 40). These findings support the concept that pregestational metabolic dysregulation may program later-life neurodevelopment-related outcomes, but they should not be interpreted as establishing direct equivalence with clinical neurodevelopmental diagnoses (50-52). Long-term cognitive trajectories were not assessed, and future work should incorporate longitudinal follow-up, broader behavioural batteries, and brain-region specific molecular and histological endpoints to clarify the durability and mechanisms of the observed effects (58-60). In addition, analytic approaches that test mediation and moderation, including sex as a biological variable, would help determine whether behavioural differences are statistically linked to inflammatory and neurotrophin profiles or primarily reflect growth restriction severity (56, 61, 62).

6.7 Abbreviations

Preeclampsia	PE
Pregestational type 2 diabetes	pT2D
High-fat, high-carbohydrate	HFHC
Pups born from normal control	PCON

Pups born from L-NAME-induced preeclamptic dams	PPE
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Pups born from HFHC diet-induced prediabetic dams	PPD
Morris Water Maze	MWM
Brain-derived neurotrophic factor	BDNF
Fibroblast growth factor -2	FGF2
Interleukin-6	IL-6
Interleukin-1 β	IL-1β
Tumour necrosis factor -	TNF-
Pregestational prediabetes	PP
Intrauterine growth restriction	IUGR
Autism spectrum disorder	ASD
Attention deficit disorder	ADHD
L-N ^G -Nitro arginine methyl ester	L-NAME
Cephalization Index	CI
Enzyme-linked immunoassay	ELISA
Polymerase chain reaction	PCR
Complementary deoxyribonucleic acid	cDNA
Messenger ribonucleic acid	mRNA
Glyceraldehyde- 3-phosphate	GAPDH
means $\pm$ standard error of mean	SEM
Analysis of variance	ANOVA
Tropomyosin receptor kinase	TrkB
Mitogen-activated protein kinase	MAPK
phosphatidylinositol 3-kinase	PI3K/Akt
Rat sarcoma-mitogen-activated protein kinase	Ras-MAPK
Fibroblast growth factor receptor	FGFR

Post-Natal Day	PND
Gestational Day	GND

6.8 Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

6.9 Conflicts of Interest

The authors declare that they have no competing interests.

6.10 Funding Statement

National Research Foundation (NRF) and College of Health Science (CHS) Funded- Funding was used to acquire animals (Sprague Dawley rats), equipment, and machinery to perform analytical techniques (Blood analysis and ELISA).

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CHAPTER 7: SYNTHESIS AND CONCLUSION

Maternal health remains a cornerstone of public health priorities in South Africa, aligning with Sustainable Development Goals (SDGs) that aim to reduce maternal and infant mortality while promoting equitable healthcare access (1). Findings from our study emphasize the critical impact of pregestational prediabetes (PP) on foetal development, revealing significant gender-specific outcomes in male and female infants, with males exhibiting greater susceptibility to cardiovascular disease, cognitive deficits, and haematological disorders (2). These vulnerabilities, linked to genetic and hormonal factors, highlight the importance of targeted maternal care and gender-sensitive health interventions (3). Moreover, PP not only predisposes individuals to pregestational type 2 diabetes (pT2D) but also increases the risk of preeclampsia (PE) (4). Addressing and managing PP is vital not only for improving maternal health outcomes but also for reducing neonatal and infant mortality, as early detection and intervention can mitigate long-term risks (5).

PP, defined by intermediate hyperglycaemia and insulin resistance, creates a hostile intrauterine environment with profound consequences for foetal development (6). Hyperglycaemia stimulates the overproduction of reactive oxygen species (ROS), initiating oxidative stress that damages placental and vascular tissues (7). ROS oxidize lipids to form malondialdehyde (MDA), a lipid peroxidation marker that further amplifies oxidative damage by impairing mitochondrial function and endothelial cell integrity (8). Oxidative stress worsens endothelial dysfunction and acts as a major contributing feature commonly shared by the pathological processes leading to PE. Excessive ROS can damage the endothelium and generate an inflammatory state that intensifies the vasoconstrictive component and impairs the vascular reactivity (6, 7). Diminished bioavailability of nitric oxide, increased activation of inflammation, and a deviation towards anti-angiogenesis are often depicted as futures and unequivocally underpinning hypertension in PE and progression to placental malperfusion (8-11). Conversely, placental malperfusion may further deteriorate hypoxia and oxidative stress, resulting in a vicious circle that is evidenced through clinical and experimental assessment of high lipid peroxidation markers and low antioxidant capacity (12-14). The relative sequential order and the impact strength of these pathways may vary depending on the clinical phenotype and patho-anatomical timing; nevertheless, all of them strongly indicate a probable intersource synergism of oxidative stress and inflammation against endothelial and placental vascular performance in PE.

Hyperglycaemia also disrupts the balance between angiogenic and anti-angiogenic factors (10). The high glucose levels decrease the expression of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) via inflammatory cytokines, which interfere with the regulatory mechanisms that promote VEGF and PlGF expression by activating transcription factors like NF- κ B, which downregulate VEGF and PlGF expression, hindering the angiogenic response necessary for proper placental function while upregulating anti-angiogenic factors such as soluble fms- like tyrosine kinase-1 (sFlt-1) and

soluble endoglin (sEng) (11). This imbalance disrupts placental angiogenesis and impairs the remodelling of maternal spiral arteries, which fail to dilate adequately to meet the metabolic demands of the growing foetus (12). The inadequate remodelling of maternal spiral arteries leads to placental hypoxia, triggering a cascade of inflammatory responses (13). Hypoxia-induced factors, such as hypoxia-inducible factor-1 α (HIF-1 α), upregulate pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF- α) (14). These cytokines disrupt placental perfusion and nutrient transport, impairing foetal oxygenation and metabolic homeostasis (15). This results in higher infant deaths, intrauterine growth restriction (IUGR) and abnormal development, particularly in the cardiovascular, haematological, and neurological systems (16). Placental inefficiency further exacerbates these effects, as indicated by our study's elevated placenta-to-body weight ratio marker, reflecting compensatory but inadequate placental growth relative to foetal size (17). VEGF, PlGF, IL-6, sFlt-1, sEng, HIF-1 α measured; findings partially support hypothesis regarding impaired angiogenesis and inflammatory modulation.

The cardiovascular system's development during foetal life depends on balanced haemodynamics and proper nutrient supply from the placenta (18). The heart-to-body weight ratio, a key cardiovascular marker, reflects the growth and proportionality of cardiac tissues relative to overall foetal growth (19). An elevated ratio, as seen predominantly in male offspring in our study, indicates cardiac hypertrophy, a compensatory response to chronic hypoxia and hyperglycaemia (20). Maternal hyperglycaemia increases the transfer of lipids and glucose to the foetus, promoting ectopic lipid accumulation in cardiomyocytes (21). This lipid overload leads to mitochondrial dysfunction, reducing ATP production and impairing cardiac energy metabolism (22). These metabolic disruptions trigger hypertrophic signalling pathways, resulting in enlarged cardiomyocytes, as confirmed through histological analysis in our study (23). The hypertrophy observed suggests reduced cardiac efficiency and diastolic dysfunction, predisposing offspring to heart failure and other cardiovascular diseases later in life (24). These findings are corroborated in studies which link maternal hyperlipidaemia during pregnancy to long-term cardiovascular remodelling in offspring (25). Moreover, the mean arterial pressure (MAP), an indicator of blood flow and vascular resistance, is pivotal in delivering oxygen and nutrients to the developing organs, particularly the brain and heart (26). The MAP reflects the functional state of placental and foetal vasculature (27). Elevated MAP, as observed in male offspring in our study, is indicative of programmed hypertension (28). Hyperglycaemia in PP impairs the vascular environment by increasing oxidative stress through the production of reactive oxygen species (ROS), such as superoxide radicals, which react with nitric oxide (NO) to form peroxynitrite (29). This reduces NO bioavailability, a critical vasodilator, leading to endothelial dysfunction and increased vascular stiffness (30). Studies have shown that impaired NO signalling disrupts vascular tone regulation, resulting in increased MAP during foetal life and persisting as hypertension into adulthood (31). Triglycerides and cholesterol levels are crucial for energy supply and membrane formation in the developing foetal heart

(32). However, elevated levels, as observed in our study, disrupt lipid homeostasis and lead to oxidative damage in cardiac tissues (33). Excess triglycerides and cholesterol contribute to lipotoxicity, further impairing mitochondrial function and increasing ROS production (34). These metabolic imbalances exacerbate the structural and functional disruptions in the foetal heart, laying the foundation for atherosclerosis and myocardial dysfunction in adulthood (35). Elevated cardiac troponin I (cTnI), a marker of myocardial stress and injury, was also observed to be higher in male than female offspring (36). cTnI indicates myocardial cell damage caused by persistent oxidative stress and lipid accumulation (37). Elevated levels, seen at the genetic and protein expression levels, suggest that cardiomyocyte damage is already established in utero, increasing the risk of arrhythmias and cardiomyopathies in adulthood (38). These cardiovascular alterations highlight the profound impact of PP on foetal development (39). The elevated MAP, heart-to-body weight ratio, lipid dysregulation, and cTnI levels in male offspring indicate how the PP in-utero environment programs the cardiovascular system toward a maladaptive trajectory (40). Cardiac morphology, cTnI, COL I; partially supported, male offspring more affected. Female offspring showed milder cardiovascular changes, suggesting a degree of protection potentially mediated by oestrogen, which enhances NO production and mitigates oxidative damage (41). However, the subtle changes observed in females still place them at risk for subclinical cardiovascular conditions later in life (42). Available literature consistently supports these findings, with studies demonstrating that maternal metabolic disorders lead to vascular stiffness, cardiac remodelling, and long-term cardiovascular diseases in offspring (43). These findings underscore the need for early interventions targeting maternal hyperglycaemia to prevent these lifelong cardiovascular consequences (44).

In utero, the foetal heart relies on placental perfusion to meet its metabolic needs, however as pregnancy progresses towards the second and third trimesters, the foetal heart begins to rely on foetal erythropoiesis, leukopoiesis, and other foetal haematological processes to sustain oxygenation and immune protection (45). In addition to oxygen transport by red blood cells (RBCs), the regulation of white blood cells (WBCs) and platelets is crucial for vascular integrity, repair, and immune modulation, further supporting cardiovascular development and function (46). In our study, male and female offspring of PP mothers exhibited elevated RBC counts, increased erythropoietin (EPO) levels, and higher red cell distribution width (RDW), alongside reduced mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) with male offspring exhibiting even higher levels (47). These findings reflect a hypoxia-driven compensatory response mediated by the placenta's inability to adequately oxygenate the foetus due to impaired maternal spiral artery remodelling (48). Hypoxia-induced upregulation of EPO overstimulates erythropoiesis, producing a surplus of RBCs (49). However, these RBCs often display structural abnormalities, as evidenced by the reduced MCV and MCH, which compromise their oxygen-carrying capacity and deformability (50). Functionally impaired RBCs struggle to meet the oxygen demands of developing tissues, including the heart and brain, exacerbating the effects of chronic hypoxia (51). The elevated RDW observed to be higher in male than

female offspring born from PP indicates significant variability in RBC size, a hallmark of ineffective erythropoiesis (52). This variability is likely driven by the hyperglycaemic environment, which disrupts erythropoietic signalling pathways and mitochondrial function within haematopoietic stem cells (53). MDA, contributes to oxidative stress in the bone marrow microenvironment, impairing erythroid progenitor cell differentiation (54). These disruptions lead to a population of dysfunctional RBCs that exacerbate tissue hypoxia, further stimulating EPO production in a maladaptive feedback loop (55). Studies report that maternal hyperglycaemia disrupts foetal erythropoiesis, predisposing offspring to anaemia and/or polycythaemia, and oxygenation deficiencies in adulthood (56). The WBC profile in our study further underscores the compromised immune state induced by PP (57). Though below range, male offspring exhibited higher WBC and neutrophil counts compared to their female counterparts, this could be attributed to slightly higher granulocyte colony-stimulating factor (G-CSF) levels (58). Moreover, the hyperglycaemic environment upregulates pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF- α), creating a systemic state that disrupts normal leukopoiesis (59). Decreased neutrophil counts reflect an impaired innate immune response, which, while adaptive in the short term, may predispose offspring from PP mothers to immune dysregulation later in life (60).

Furthermore, the platelet counts in PP, though below range, were also elevated in male offspring compared to female offspring, highlighting the differences in sex (61). The oxidative stress and inflammation caused by hyperglycaemia, impairs vascular haemostasis resulting in low platelet counts (62). Female offspring showed milder haematological changes, suggesting a partial protective effect mediated by oestrogen, which has anti-inflammatory properties and supports vascular health (63). CBC including RBC indices, WBC, neutrophils, platelets; partially supported However, even these subtle changes could do not disqualify immune irregularities in adulthood (64). Moreover, haematological disruptions have profound implications for the developing cardiovascular system (65). Impaired RBC function limits oxygen delivery to the heart, compounding the metabolic stress caused by lipid overload and mitochondrial dysfunction in cardiomyocytes (66). The inflammatory state exacerbates endothelial damage, increasing vascular resistance and mean arterial pressure (MAP) (67). Dysfunctional leukocyte and platelet activity contribute to vascular remodelling anomalies, reducing oxygen and nutrient transport efficacy to all developing organs (68). Together, these factors create a compounding cycle of hypoxia, inflammation, and oxidative stress that programs offspring for long-term haematological and cardiovascular diseases, including polycythaemia, anaemia, thrombocytosis and cardiomyopathies (69). As the cardiovascular system ensures proper perfusion to organs, the haematological system, including the production and function of erythrocytes and leukocytes, supports the physiological needs of the developing brain (70). Therefore, a compromised haematological system, particularly due to maternal metabolic disorders like PP, can impair brain development, as PP disrupts the oxygen and nutrient transport necessary for proper neurogenesis and cognitive function (71). Thus, disturbances in the

haematological system due to hyperglycaemia directly contribute to adverse cognitive outcomes (72).

Our study measured key markers of cognitive development, namely the brain-to-body weight ratio and cephalization index (73). The brain-to-body weight ratio reflects the proportionality of brain growth relative to overall body size, with a reduced ratio indicating impaired brain development (74). Female offspring born to prediabetic mothers exhibited a higher cephalization index, reflecting a compensatory mechanism in which the brain prioritizes its growth, often at the expense of other organs, and the body's overall growth in response to adverse intrauterine conditions (75). This suggests that while the brain tried to compensate for adverse conditions, the accelerated/faster growth did not result in the proper maturation of neural structures (76). As a result, the offspring are predisposed to cognitive impairments and learning disabilities, as seen in the poor performance of offspring from prediabetic mothers in the Morris Water Maze test (77). The cephalization index and brain-to-body weight ratio were significantly higher in males than females, which is consistent with previous findings indicating that male foetuses may exhibit accelerated brain growth during stress but also show a higher risk for later neurodevelopmental impairments due to suboptimal neurogenesis (78). Further evidence of disrupted neurodevelopment in our study was seen in the expression of key neurotrophic markers. Brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2) are essential for neurogenesis, synaptic plasticity, and cognitive function (79, 80). BDNF, in particular, plays a crucial role in maintaining neuronal health, supporting synaptic strength, and facilitating learning and memory processes (81). FGF2 supports the proliferation of neural progenitors and is involved in neurogenesis, particularly in regions of the brain that are critical for spatial memory and learning, such as the hippocampus (82). As seen in our study, levels of BDNF and FGF2 were significantly reduced in the brains of male offspring, particularly in the hippocampus, which is known to be a key structure for learning and memory (83). This reduction in neurotrophic support impairs synaptic plasticity and reduces the brain's ability to adapt to environmental demands (84). Such deficits in neurogenesis and synaptic connectivity contribute to the cognitive impairments observed in these animals, particularly in males, who showed poorer performance in the Morris Water Maze test, a common test for spatial learning and memory (85). These reductions in neurotrophic factors, combined with elevated pro-inflammatory cytokines such as IL-6, IL-1 β , and TNF- α , further impair neurodevelopment by disrupting neuronal differentiation and synaptic connectivity (86). Chronic inflammation, induced by maternal hyperglycaemia and placental hypoxia, exacerbates the effects of reduced BDNF and FGF2 by impairing the ability of neurons to form new synaptic connections (87). The increased levels of inflammatory markers observed in the hippocampus of male offspring correlate with poor performance in cognitive tests, highlighting the critical role that inflammation plays in disrupting neural development (88). Elevated IL-6 and TNF- α levels are known to affect hippocampal function, impair neurogenesis, and alter the expression of neurotrophic factors, making the brain more vulnerable to stress and injury (89, 90). Hippocampal BDNF/FGF2 and behavioural testing; cautiously supported, noting limitations.

The implications of these inflammatory processes are particularly pronounced in males, as they show a more marked decrease in cognitive function compared to females, further emphasizing the gender-specific effects of maternal prediabetes on offspring cognitive development. The mechanisms by which maternal hyperglycaemia and placental insufficiency impact heart, blood, and brain development underscore the importance of early intervention to mitigate these effects (91). Strategies aimed at preventing prediabetes by managing maternal blood glucose levels could significantly improve cardiovascular, haematological, and neurodevelopmental outcomes for offspring, particularly in males who show greater susceptibility to these disruptions (92).

7.1 Conclusion

Our study highlights the significant impact of PP on foetal development, with male offspring showing heightened susceptibility to cardiovascular disease, cognitive deficits, and haematological disorders. This research addresses critical gaps in understanding gender-specific effects of maternal metabolic disorders such as PP on offspring health (93, 94). A unique aspect of our study is the use of a high-fat, high-carbohydrate diet to induce prediabetes in animal models. This approach closely mirrors the unhealthy dietary patterns common in human populations, making our findings highly relevant to real-world scenarios in South Africa, where dietary habits and lifestyle choices contribute significantly to the rising prevalence of prediabetes and related metabolic disorders (95, 96). By replicating these conditions in a controlled environment, our study bridges a crucial gap in translating animal research to human health outcomes, providing insights that might be applicable to South Africa's public health challenges (97, 98). These findings directly support Sustainable Development Goal 3 (SDG 3), which focuses on ensuring healthy lives and promoting well-being for all at all ages (99). Specifically, our study aligns with Target 3.1, which aims to reduce maternal mortality, and Target 3.2, which seeks to end preventable deaths of newborns and children under five (100, 101). Preventing PP and its progression to PE or pT2D is critical to improving maternal health and mitigating adverse infant outcomes (102). Studies have shown that early intervention through lifestyle modifications, such as dietary improvements and exercise, or pharmacological treatments, such as insulin or metformin, can significantly reduce complications during pregnancy (103). Similarly, antihypertensive therapies, such as labetalol or nifedipine, have proven effective in managing PE and reducing associated risks to both the mother and the foetus (104). Our study also resonates with Sustainable Development Goal 5 (SDG 5), which emphasizes achieving gender equality and empowering women and girls (105). By highlighting the gender-specific vulnerabilities of both male and female infants, our findings underscore the need for gender-sensitive healthcare strategies (106). This is supported by research showing that male foetuses are more prone to adverse outcomes in pregnancies complicated by maternal metabolic disorders due to differences in placental function and foetal adaptation mechanisms (107,108). Addressing these vulnerabilities through targeted interventions such as enhanced maternal monitoring and tailored neonatal care ensures that both male and female infants receive equitable and effective healthcare (109). For infants born to mothers with PP, especially male infants which are at a higher risk,

a range of interventions is available. Cardiovascular risks can be mitigated through pharmacological treatments like beta-blockers or ACE inhibitors, as well as non-pharmacological strategies such as improved nutrition and physical activity (110, 111). Cognitive deficits observed in these infants may benefit from neuroprotective interventions, including omega-3 fatty acid supplementation and early cognitive stimulation programs (112). Moreover, our research also advances public health efforts by providing a foundation for enhanced maternal screening programs, early interventions, and long-term follow-up for at-risk infants (113, 114). By integrating these measures into national healthcare strategies, we can contribute to achieving SDG 3's overarching goal of reducing disease burdens across generations, while simultaneously supporting SDG 5's commitment to gender-sensitive healthcare (115). Collectively, our findings and their alignment with these national and global goals emphasize the critical importance of maternal health in shaping a healthier and more equitable future (116).

7.2 Synthesis Summary

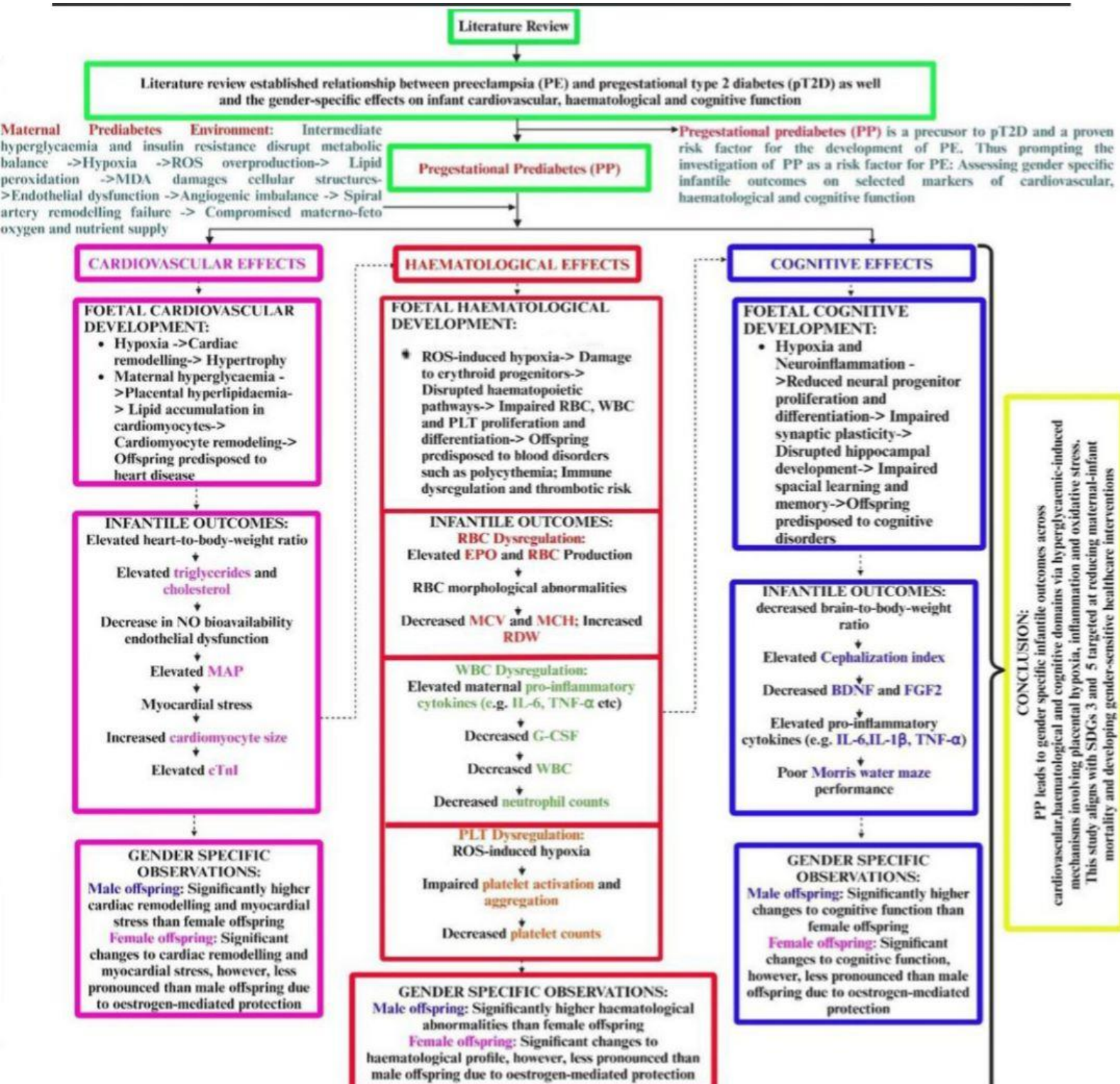


Figure 1: Illustrates the synthesis summary

7.3 Future recommendations and limitations:

This study has several limitations that should be addressed to enhance its depth and translational relevance, many of which arose due to practical constraints such as budgetary limitations, restricted access to advanced laboratory equipment, and logistical challenges associated with long-term studies. While the study explores gender-specific infantile outcomes, the underlying molecular mechanisms driving these differences were not fully elucidated. In addition, long-term offspring health outcomes, including adult-onset metabolic and cardiovascular disease, were not assessed. In addition to echocardiography, other functional assessment approaches that could strengthen future studies include electrocardiography for cardiac conduction analysis, blood pressure telemetry for continuous cardiovascular monitoring, and advanced imaging modalities such as cardiac MRI. Neurobehavioral testing batteries and exercise tolerance assessments may further provide integrated functional insight. These approaches were not incorporated in the present study due to logistical and resource constraints but represent important directions for future research. Although placental analysis was included, further exploration of epigenetic modifications and nutrient transport pathways would strengthen mechanistic understanding. The biomarker panel was relatively focused and may have excluded key pathways such as mitochondrial function, non-coding RNAs, and broader inflammatory signalling networks. Furthermore, the absence of intervention-based studies limited the identification of potential preventative strategies, including antioxidant or dietary interventions. Although plasma EPO provided a systemic index of erythropoietic drive, we acknowledge that hepatic and renal tissues are the primary neonatal sources of EPO; analysis of these tissues was not undertaken in the present study but would strengthen mechanistic interpretation in future work.

Based on the findings of this study, future research should focus on elucidating mechanistic pathways linking maternal prediabetes and preeclampsia to offspring cardiovascular, haematological, and neurodevelopmental outcomes. This includes performing associative and mediation analyses to examine potential sex-specific effects, expanding molecular panels for hippocampal development, and integrating detailed maternal pharmacological and metabolic monitoring. Longitudinal studies in human cohorts, tracking behavioural and physiological outcomes from birth onwards, are essential to validate translational relevance. Also, pathway-specific analyses of other additional inflammatory markers NF- κ B, TGF- β , in maternal and offspring tissues will provide mechanistic insight and inform targeted interventions to improve offspring health outcomes. We also aim to increase collaborative efforts with well-equipped laboratories and clinical research facilities which will facilitate human validation and improve translational applicability. Addressing these gaps would provide more comprehensive insights into the effects of maternal prediabetes on foetal programming and infant development.

Furthermore, specific observations in the manuscripts lead to targeted intervention that would focus on

deriving greater mechanistic implications and translational relevance: it needs investigation to integrate more detailed cardiometabolic phenotyping in offspring, involving glucose and insulin dynamics (fasting insulin values, indexes of insulin sensitivity, and glucose tolerance tests), lipid subfractions, and long-term blood pressure trajectories to see whether early biochemical changes translate into persistent cardiovascular vulnerability.

Secondly, the incorporation of renal markers, for instance, albuminuria quantification beyond that accessed by dipstick grading, creatinine and urea levels, along with information on renal histology, might be beneficial to determine how involvement of the kidneys in terms of certain stressors may, or may as well not, impact either the outcome in offspring or the degree of severity of the disease in the mother. Secondly, renal activity results related to the renin-angiotensin system and associated vasoactive mediators would potentially fill in a critical mechanistic gap cited among examiner feedback. Also, the future research design should include the advancement of placental assessments beyond just weight proportionality indexes to histological/solid-state evaluation of placental architecture, vasculature density, and inflammation infiltration, along with transporter profiling quantification for nutrient transfer capability. This work would address the reliance of current functional-proxying strategies on an angiogenic balance and oxidative and inflammatory markers. It should enhance inferences about placental integrity.

For neurodevelopmental outcomes, mounted designs should consider brain measurements at the region level, such as hippocampal inflammatory signalling, synaptic markers, and myelination indices, as well as more comprehensive behavioural batteries representing multiple cognitive domains across developmental epochs. Longitudinal follow-up will be especially critical for deciding whether learnt behaviour among juveniles disappears, strengthens, or shifts slightly until it can identify the critical interlude for preventative management

7.4 References

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8. APPENDICES

8.1 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.

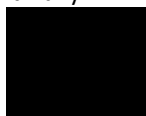
Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

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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Telephone: +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za **Website:**
<http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx>

8.2. APPENDIX 2: CORRELATION AND REGRESSION ANALYSES

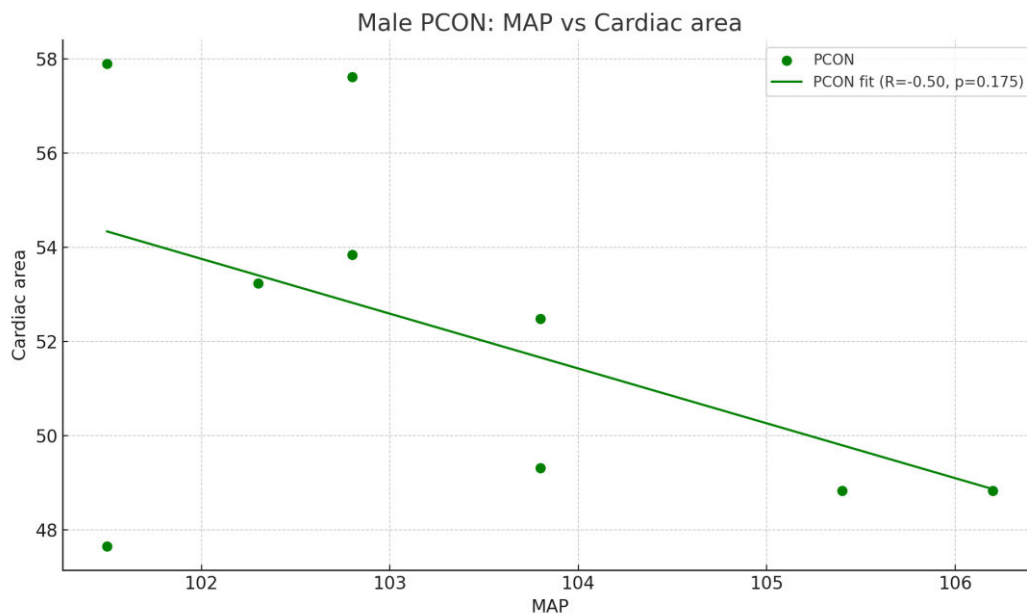
(IL-6, TNF- α , TG, TC, heart-to-body weight ratio, MAP, cardiomyocyte area, cTnI, collagen I expression)

Separated Group Scatter Plots (PCON only, PPE only, PPD only)

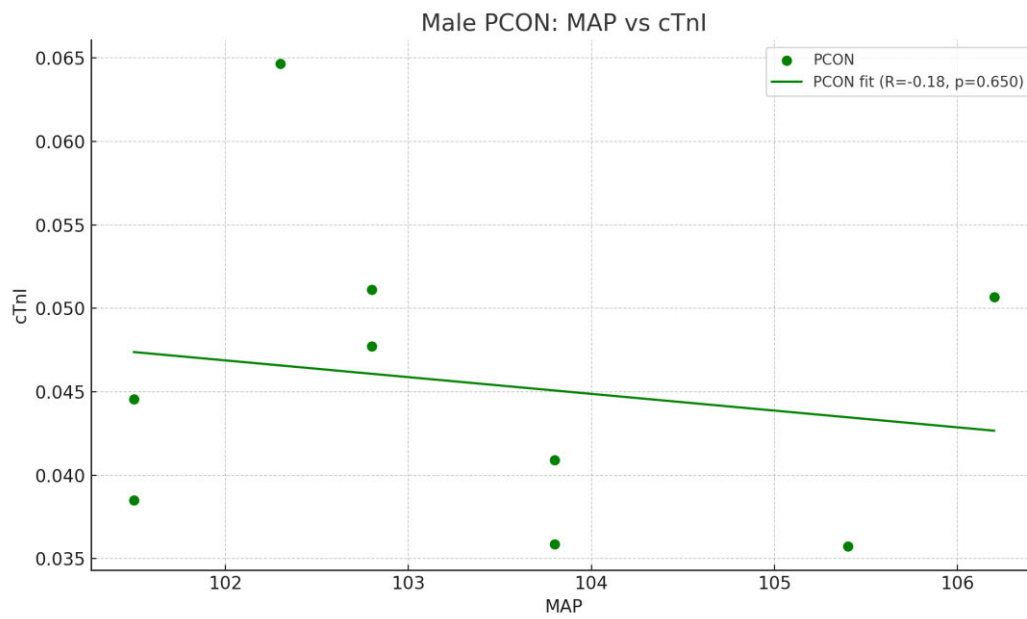
The following figures present each experimental group in isolation (PCON, PPE, PPD) for clarity. Each panel shows a Pearson correlation with a linear regression fit (group-colored line) and reports R and p-value on the figure. Markers are circles.

Male Offspring – Separated by Group

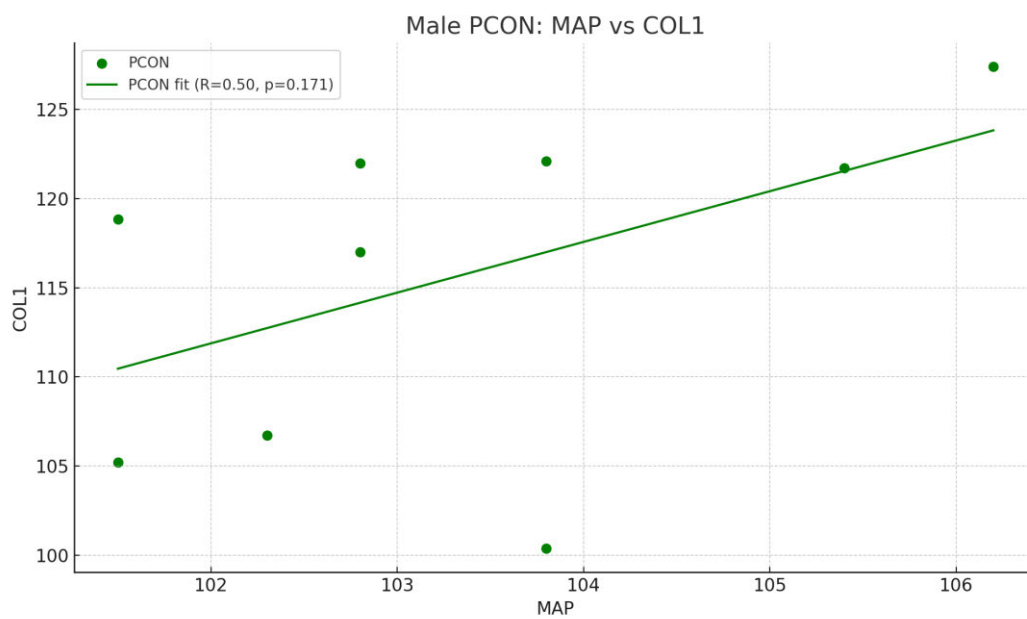
Male PCON MAP vs Cardiac area



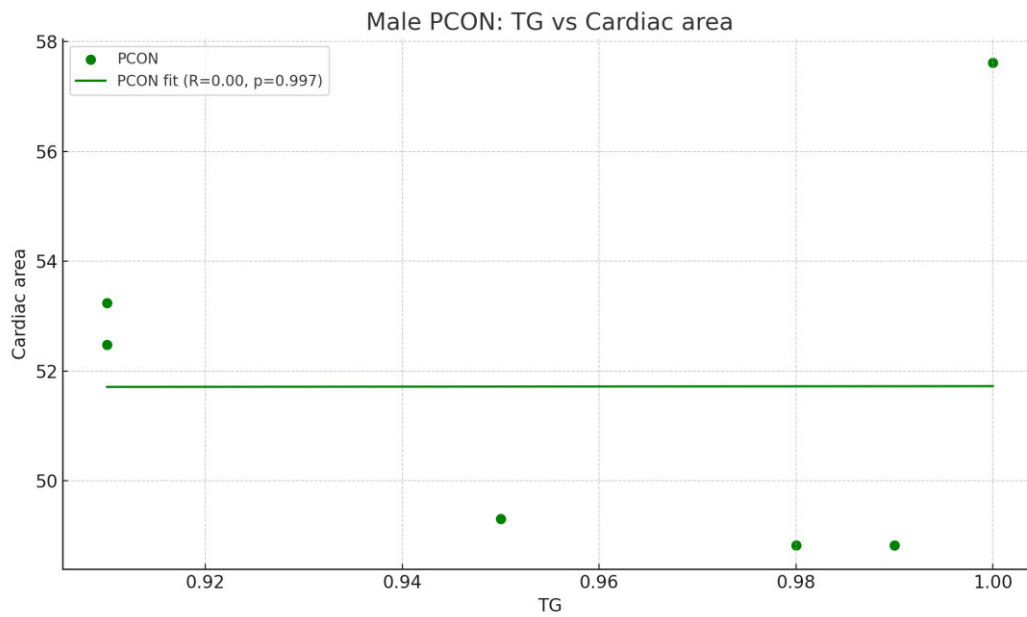
Male PCON MAP vs cTnI



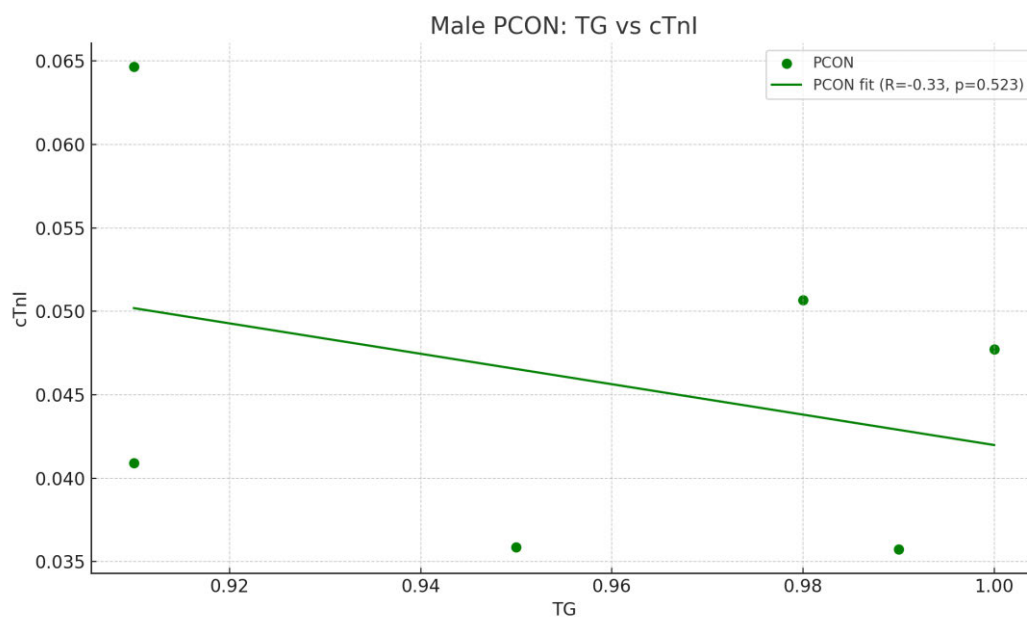
Male PCON MAP vs COL1



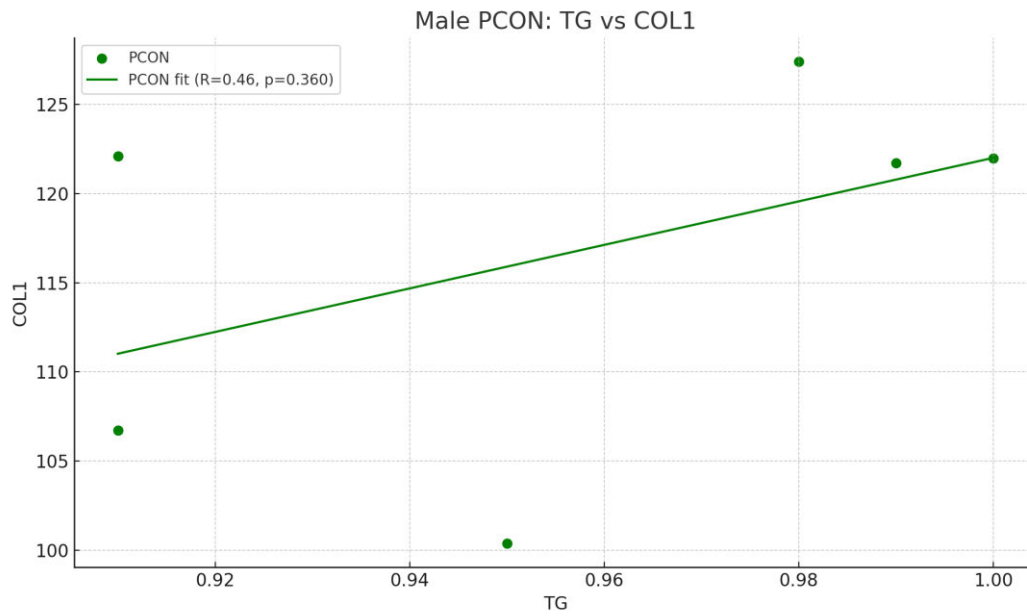
Male PCON TG vs Cardiac area



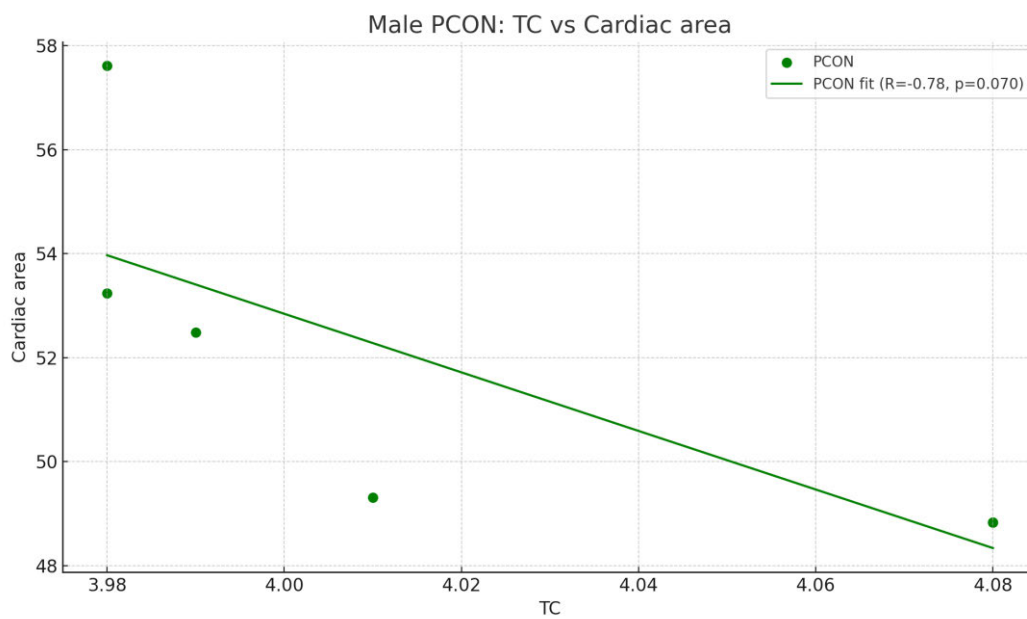
Male PCON TG vs cTnI



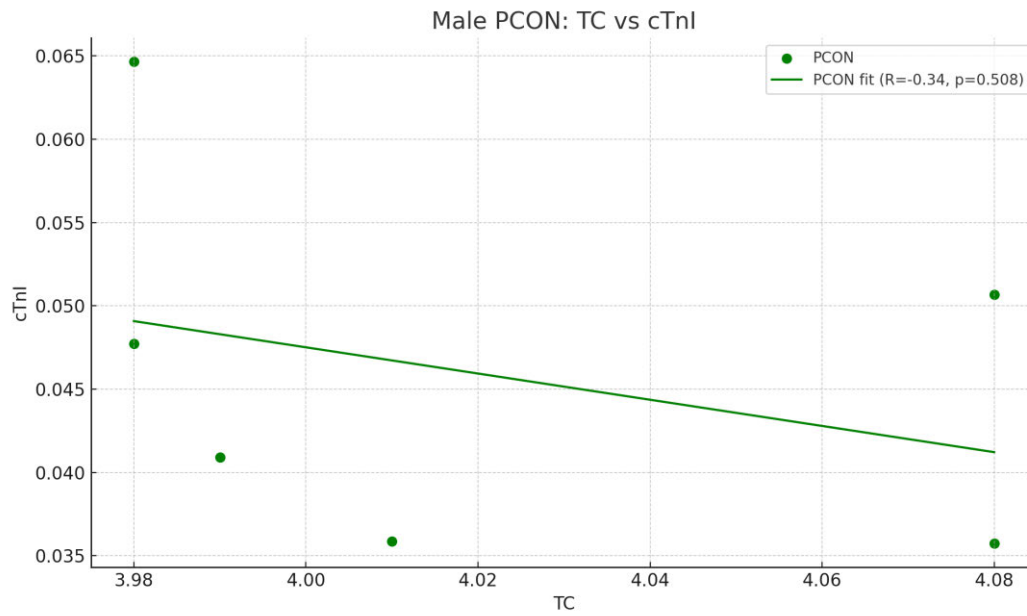
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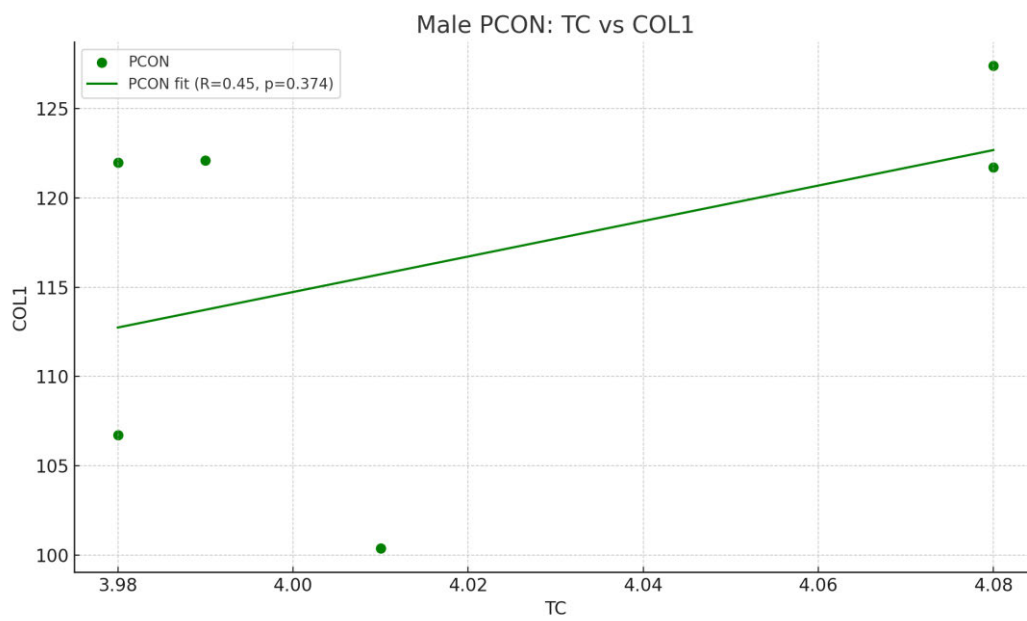
Male PCON TC vs Cardiac area



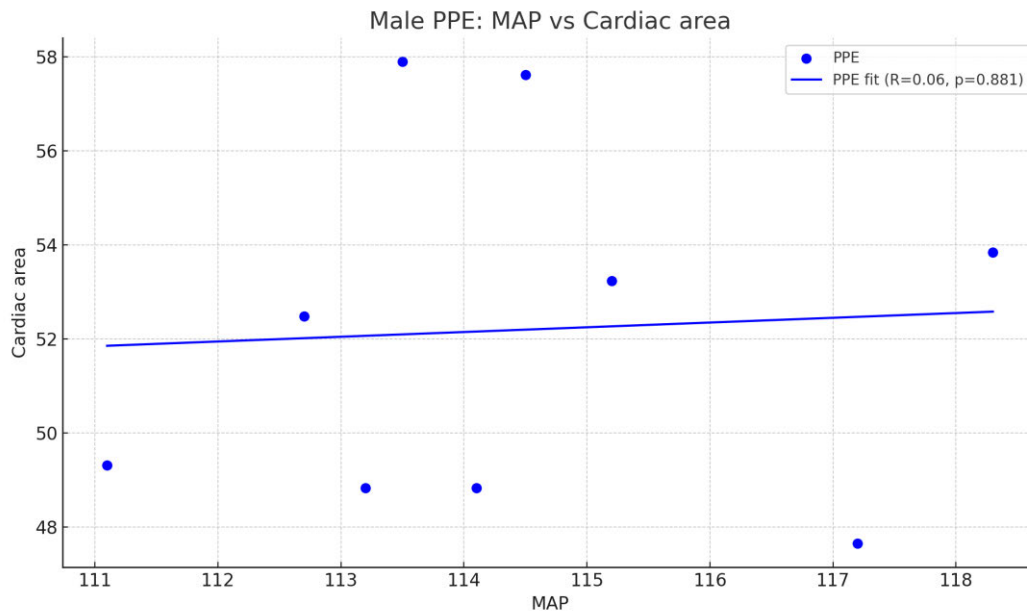
Male PCON TC vs cTnI



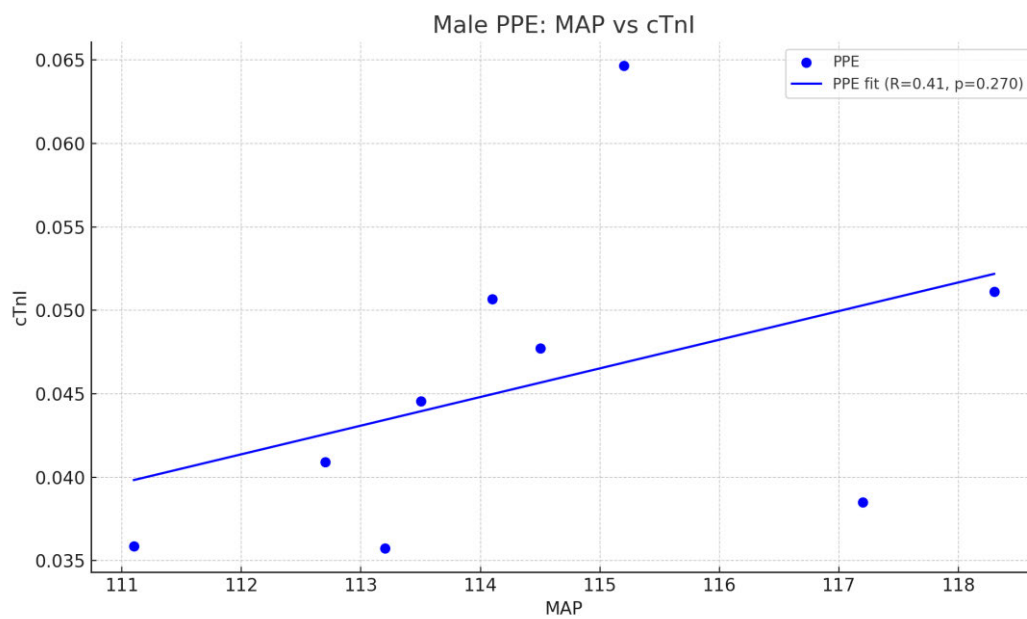
Male PCON TC vs COL1



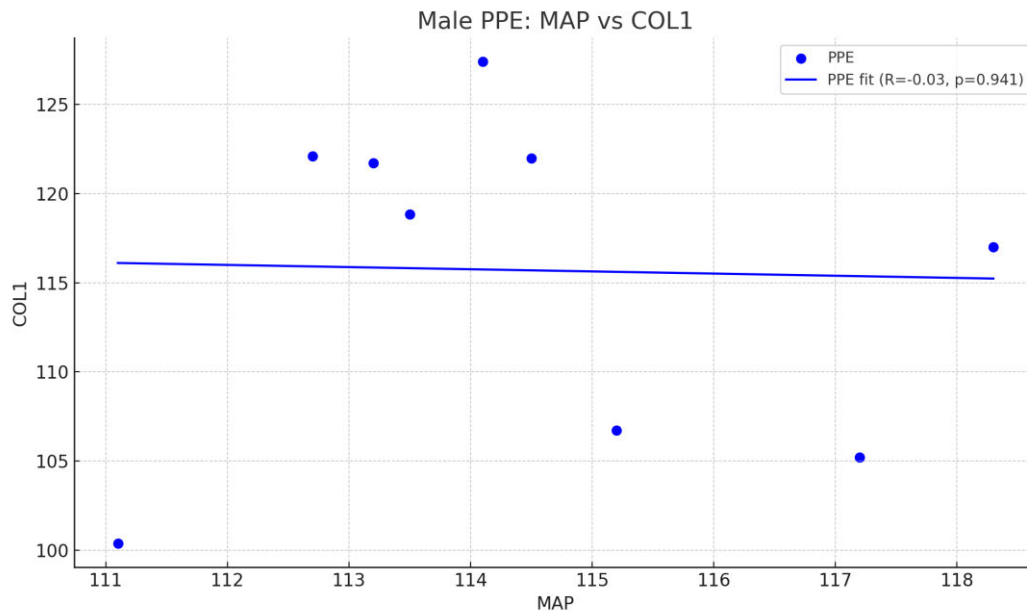
Male PPE MAP vs Cardiac area



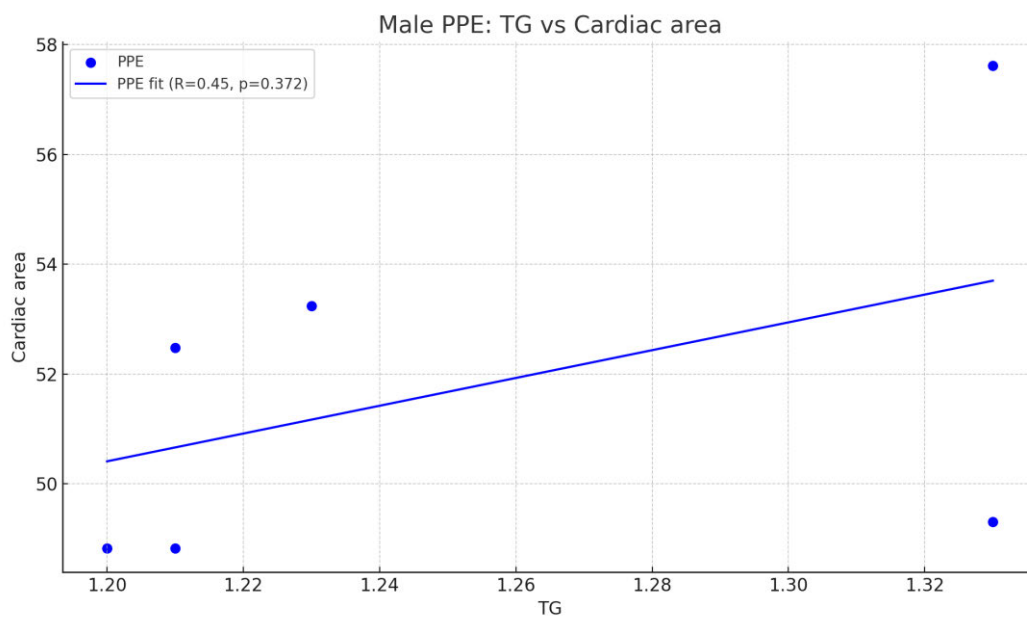
Male PPE MAP vs cTnI



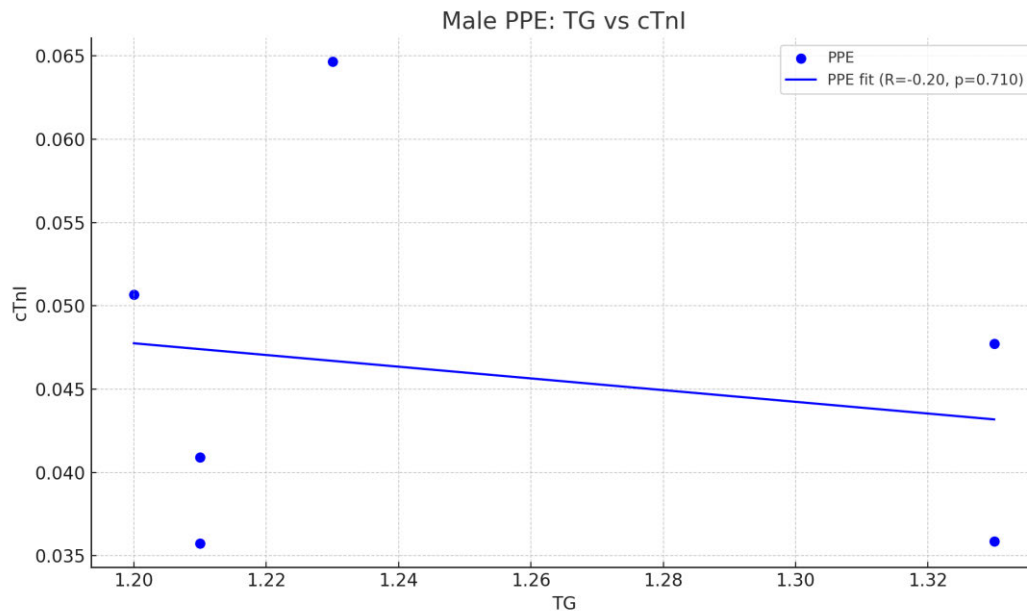
Male PPE MAP vs COL1



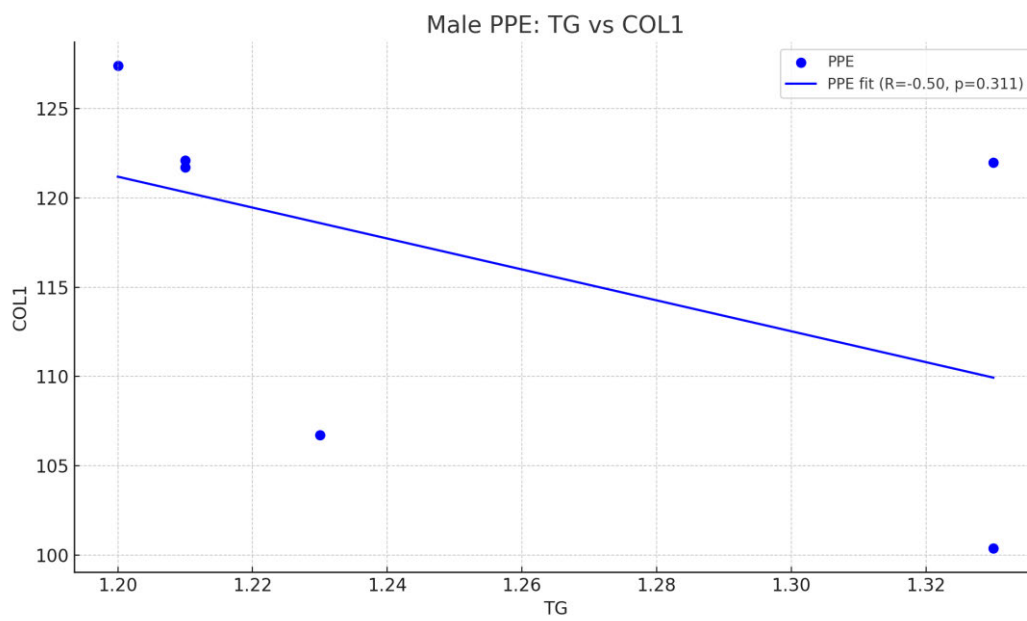
Male PPE TG vs Cardiac area



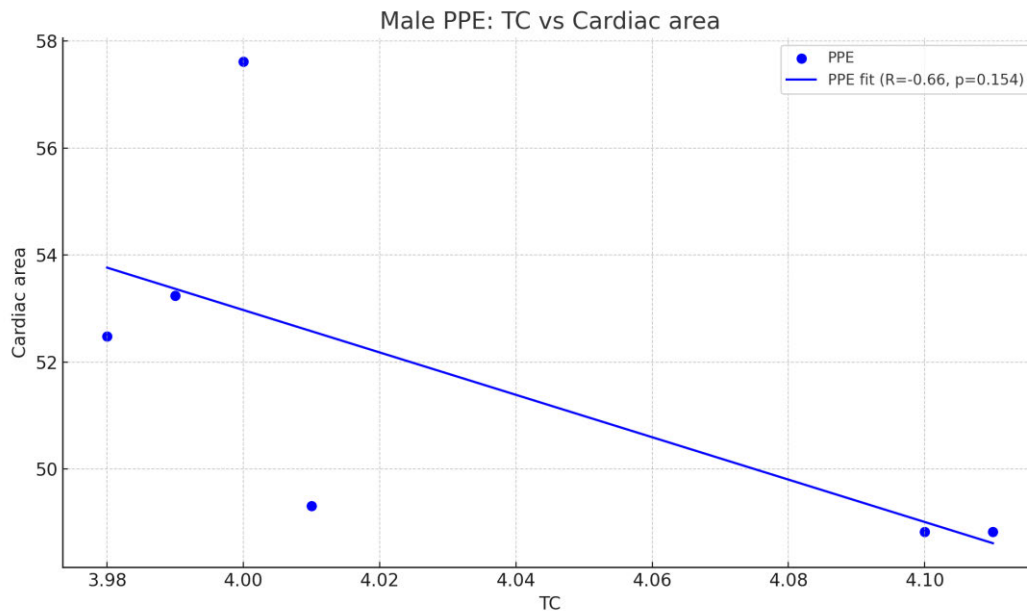
Male PPE TG vs cTnI l



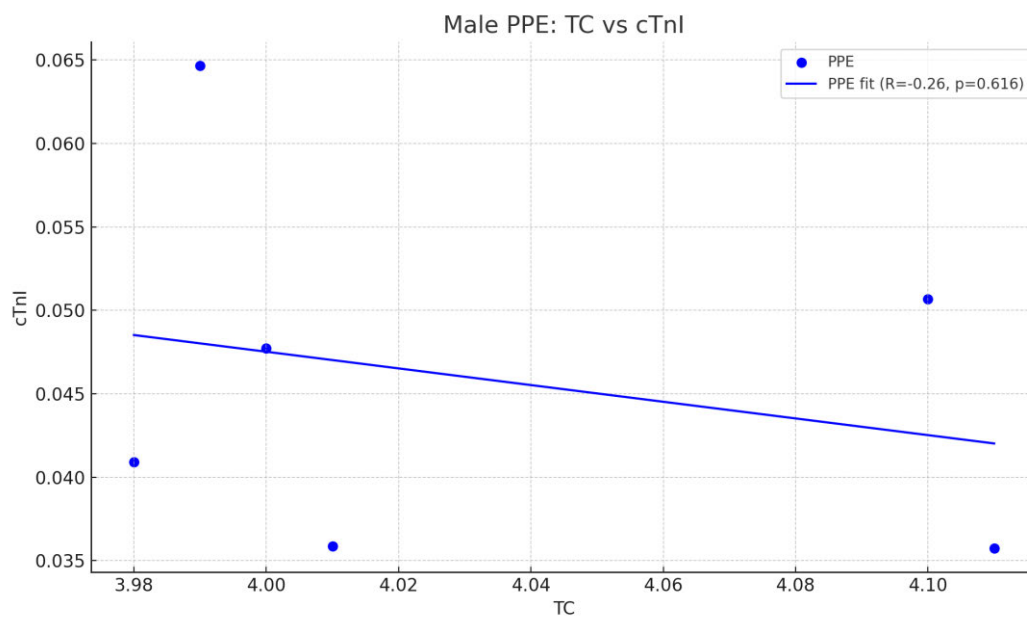
Male PPE TG vs COL1



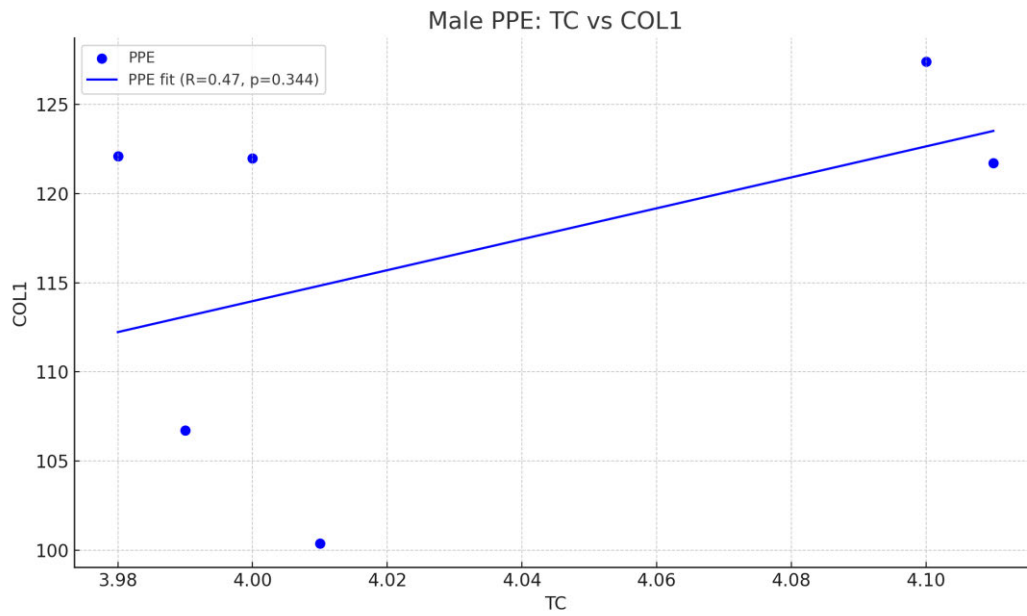
Male PPE TC vs Cardiac area



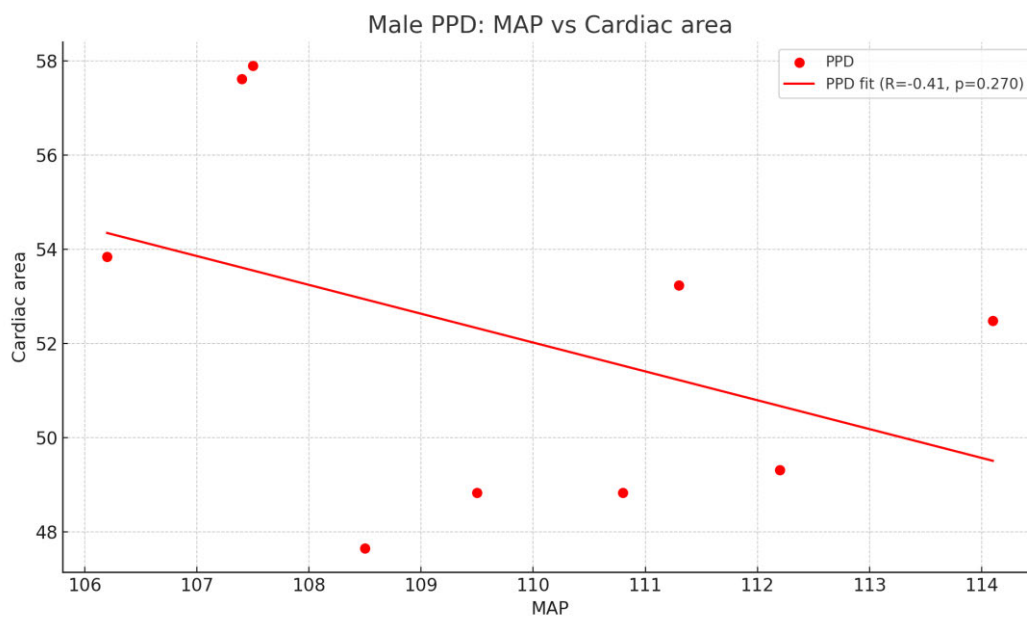
Male PPE TC vs cTnI



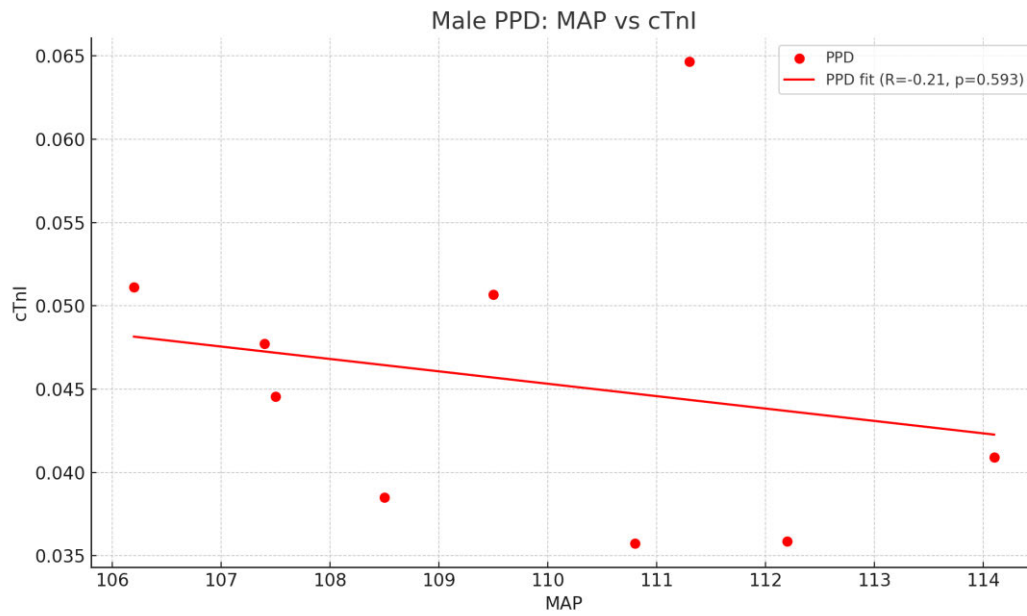
Male PPE TC vs COL1



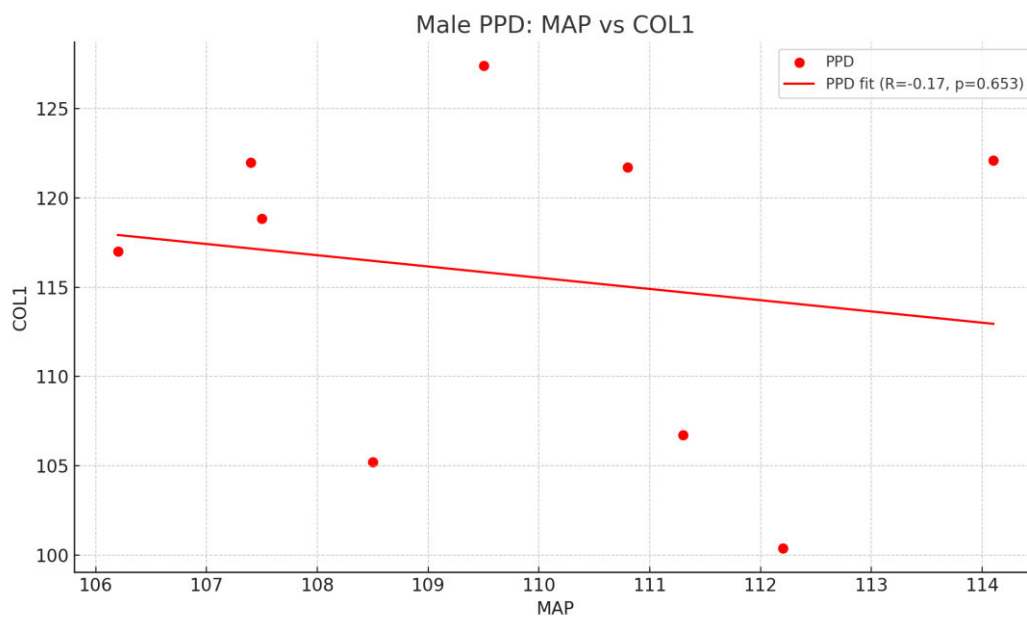
Male PPD MAP vs Cardiac area



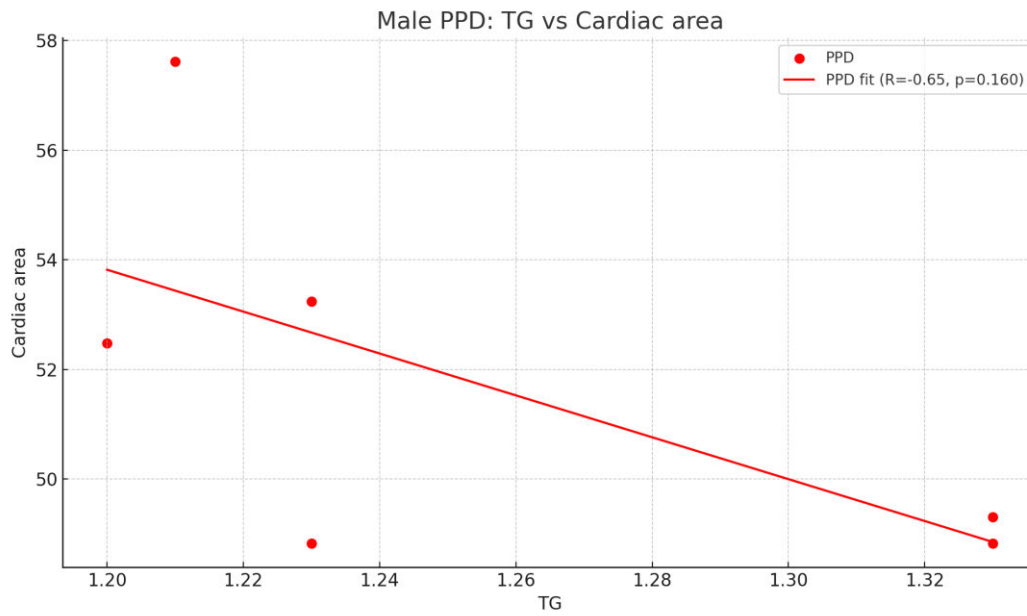
Male PPD MAP vs cTnI



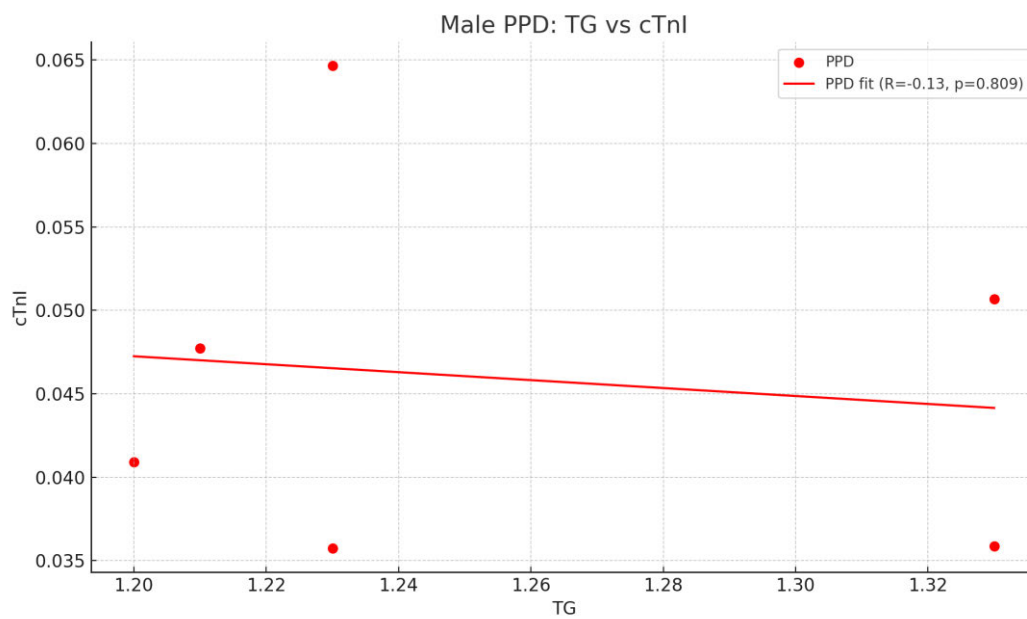
Male PPD MAP vs COL1



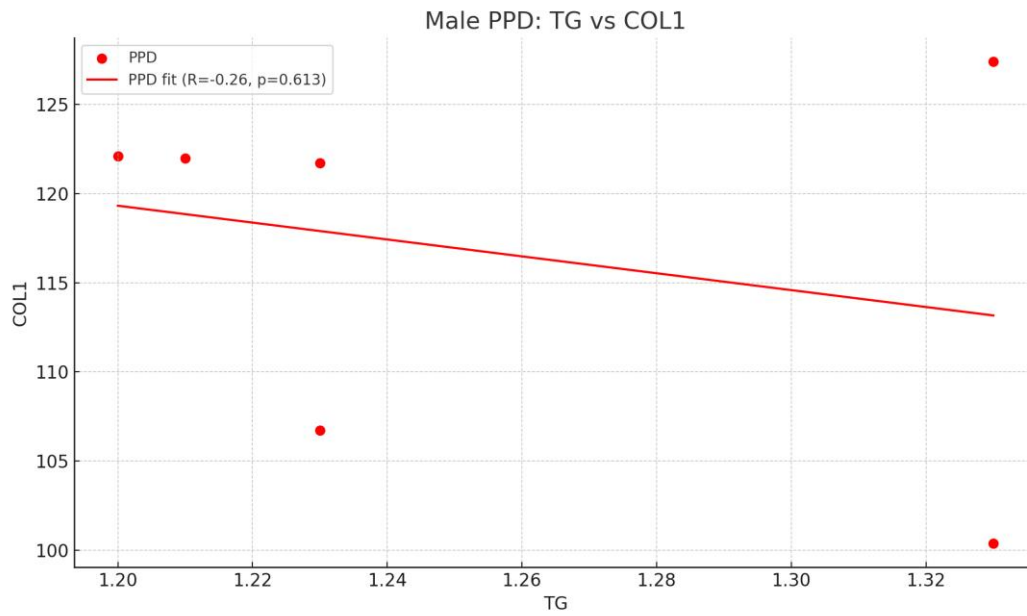
Male PPD TG vs Cardiac area



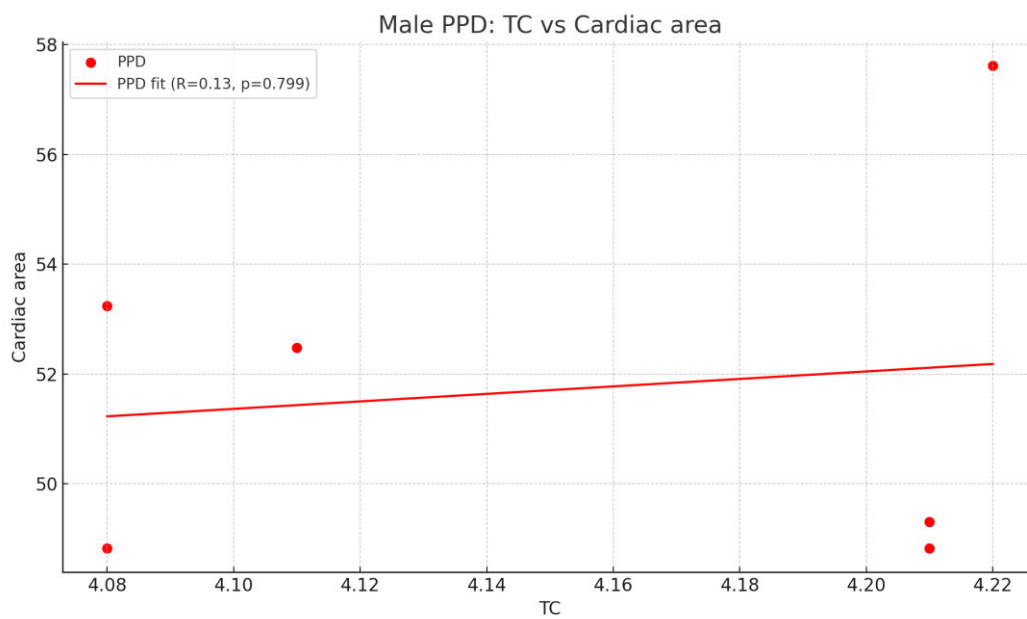
Male PPD TG vs cTnI



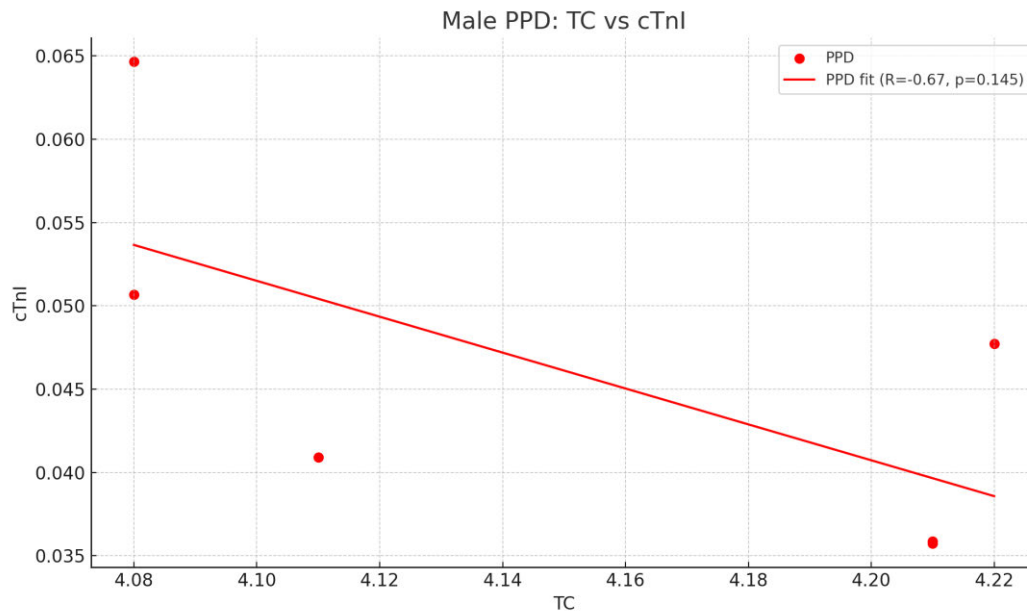
Male PPD TG vs COL1



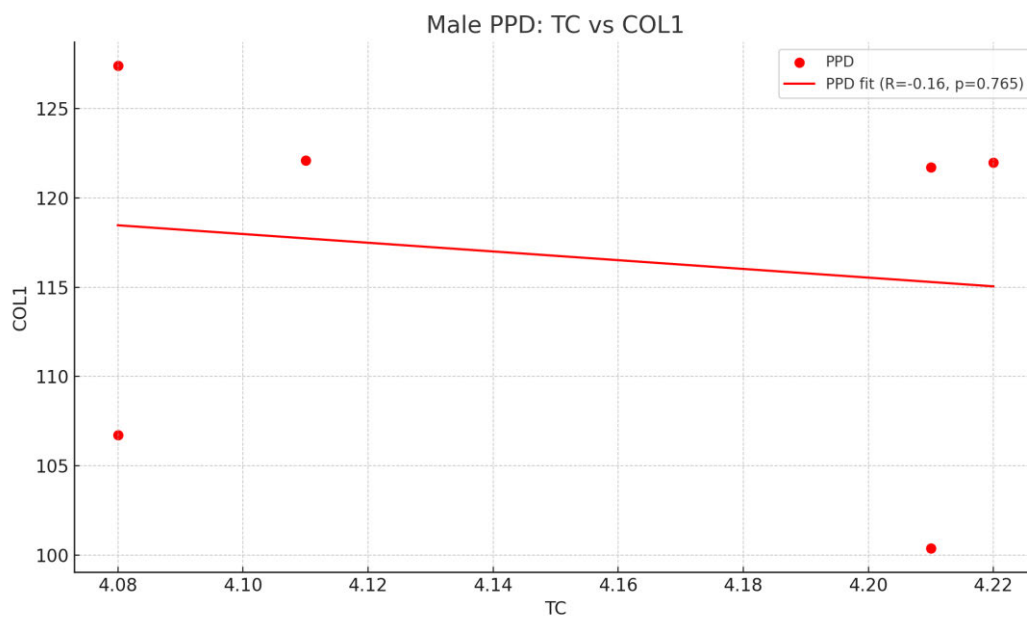
Male PPD TC vs Cardiac area



Male PPD TC vs cTnI

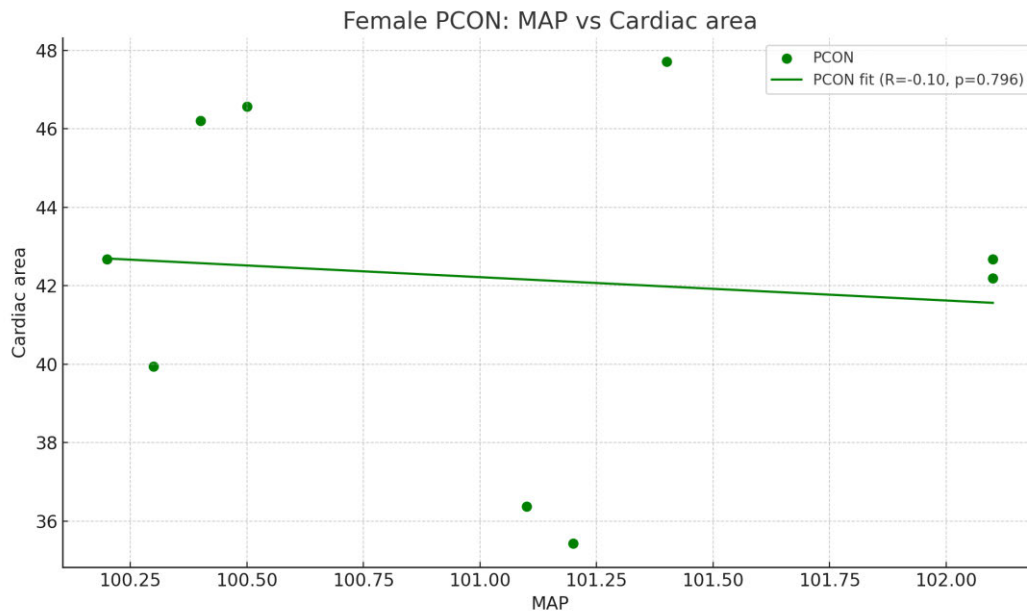


Male PPD TC vs COL1

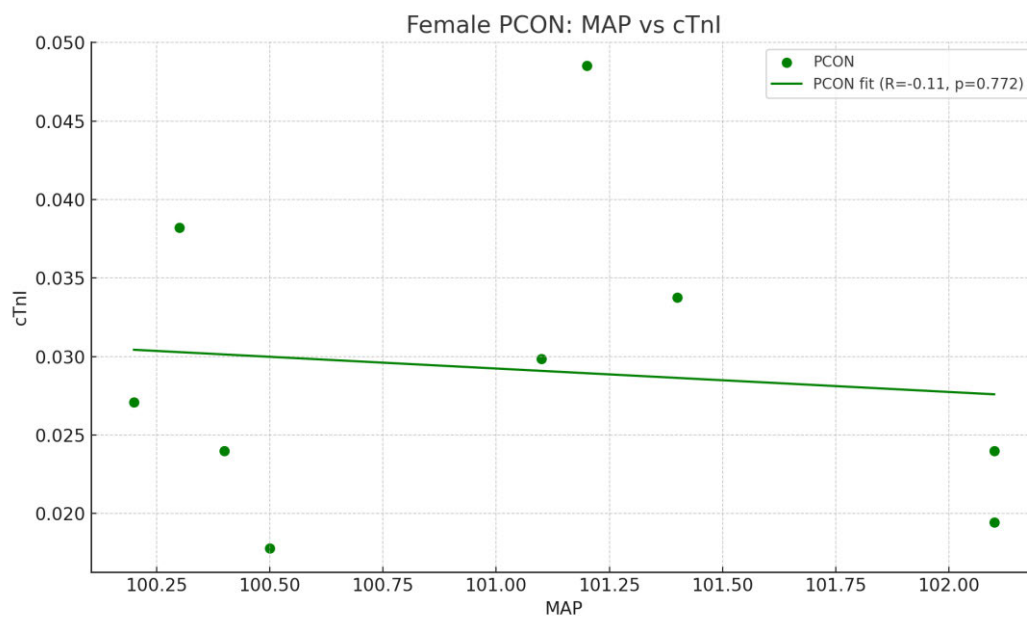


Female Offspring – Separated by Group

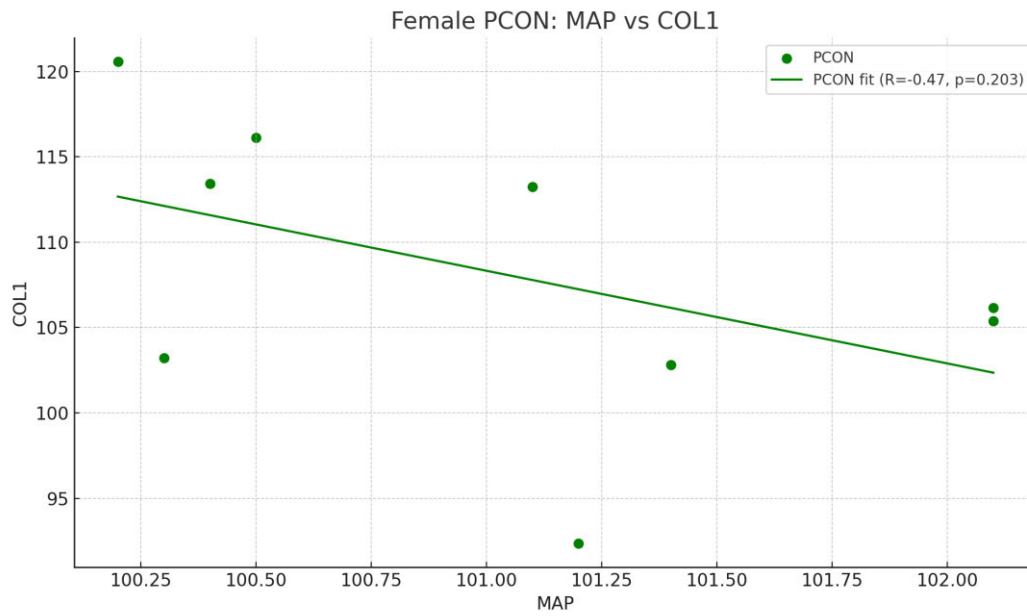
Female PCON MAP vs Cardiac area



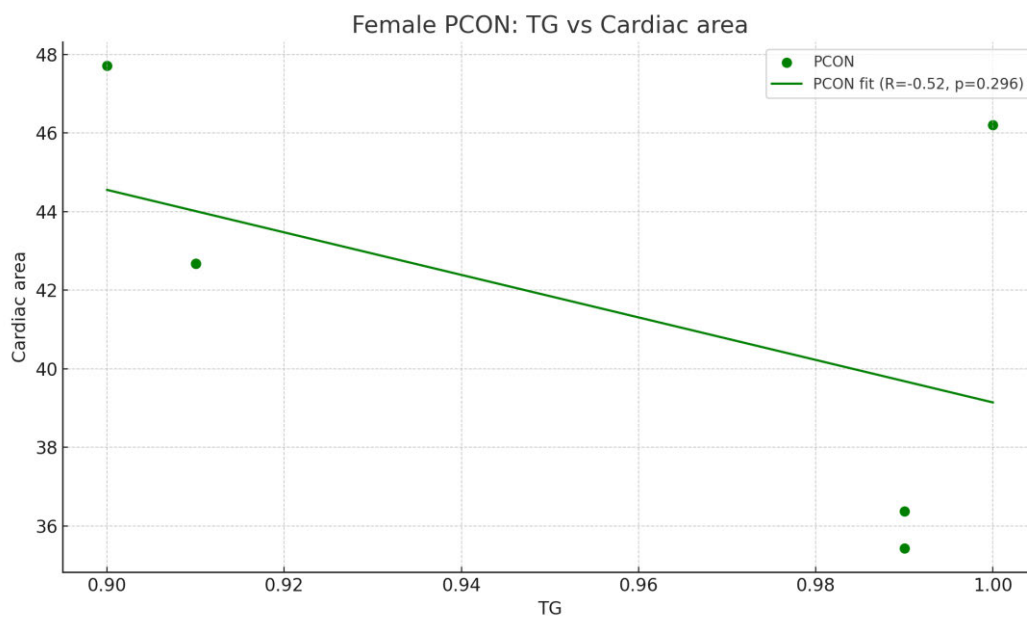
Female PCON MAP vs cTnI



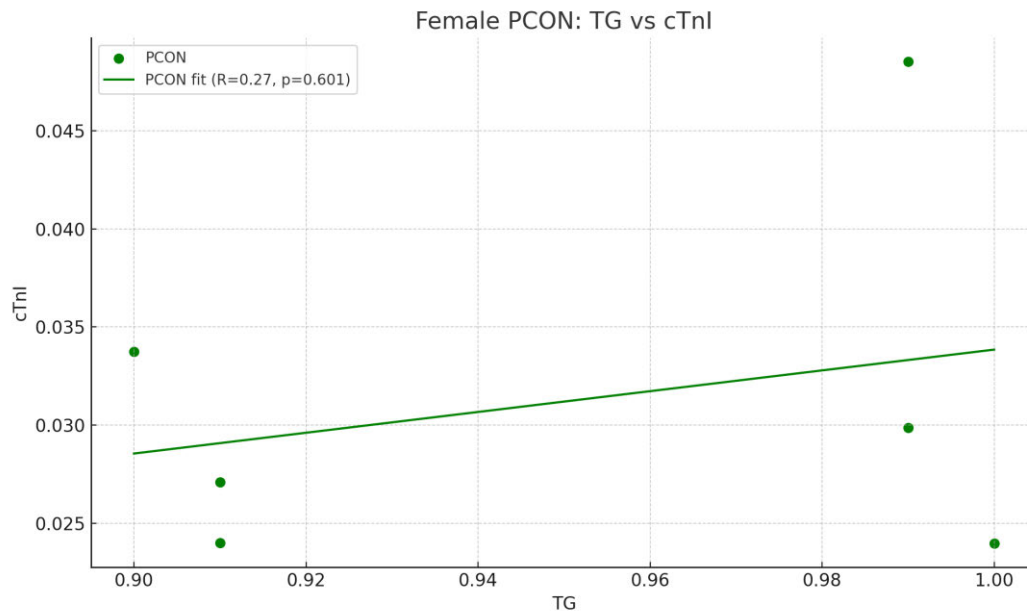
Female PCON MAP vs COL1



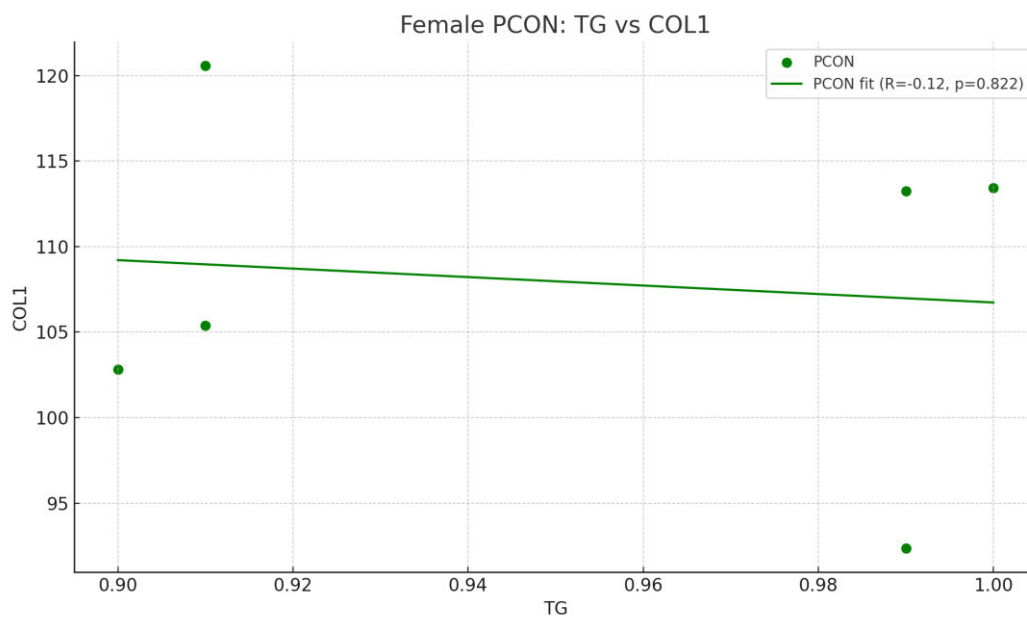
Female PCON TG vs Cardiac area



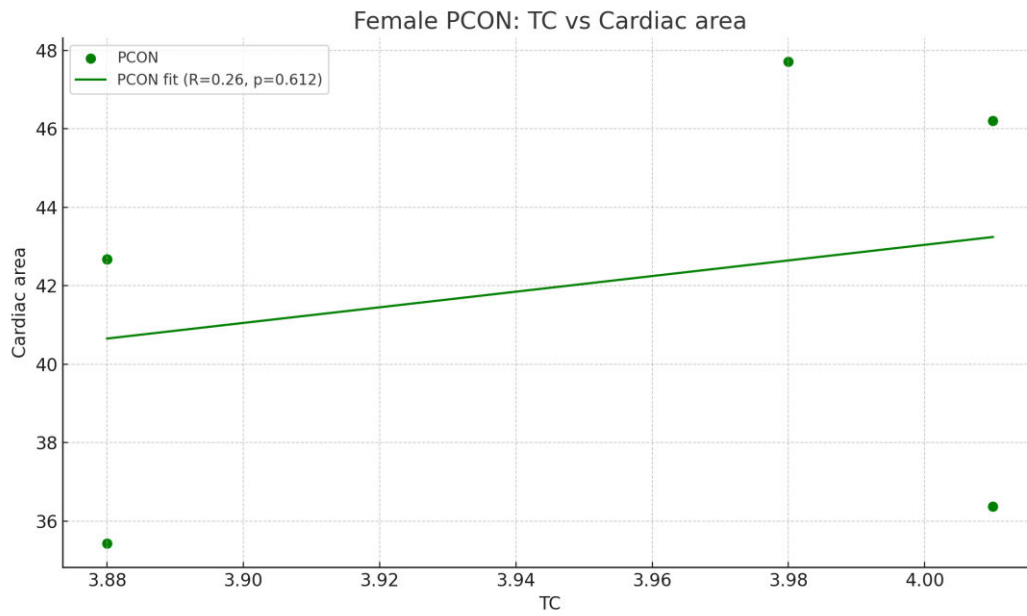
Female PCON TG vs cTnI



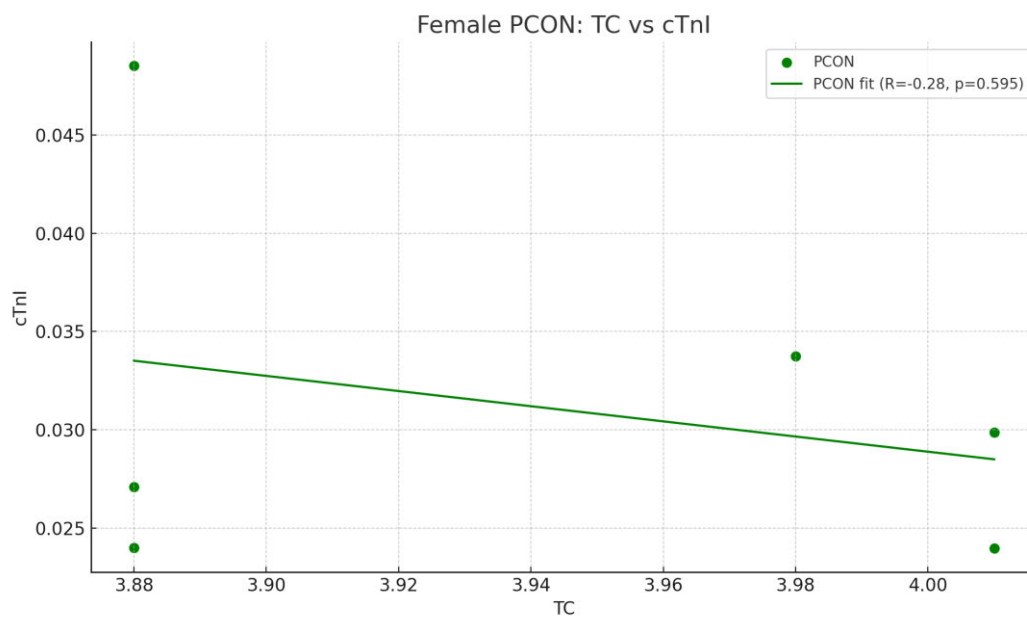
Female PCON TG vs COL1



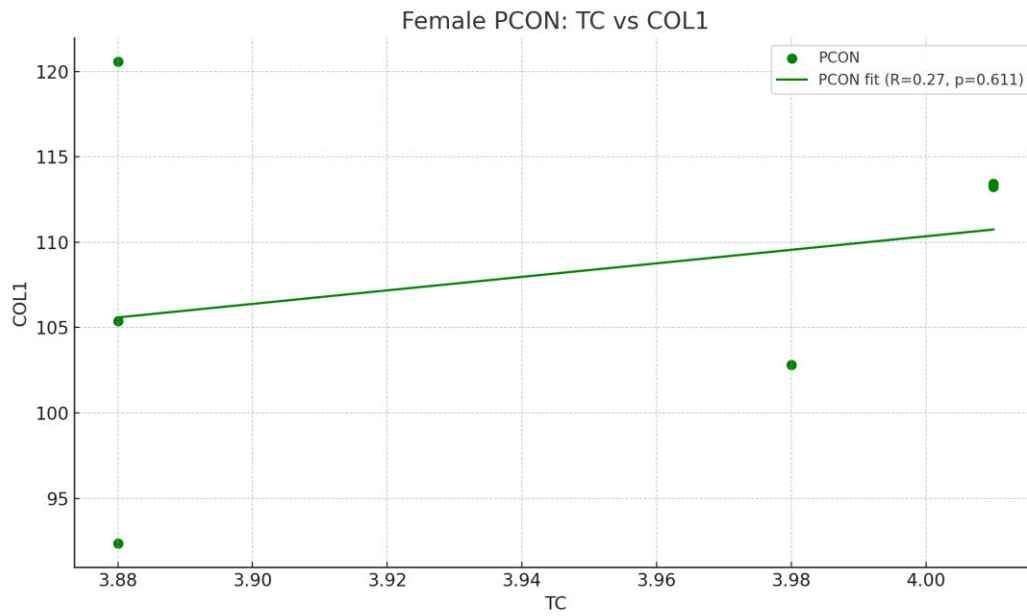
Female PCON TC vs Cardiac area



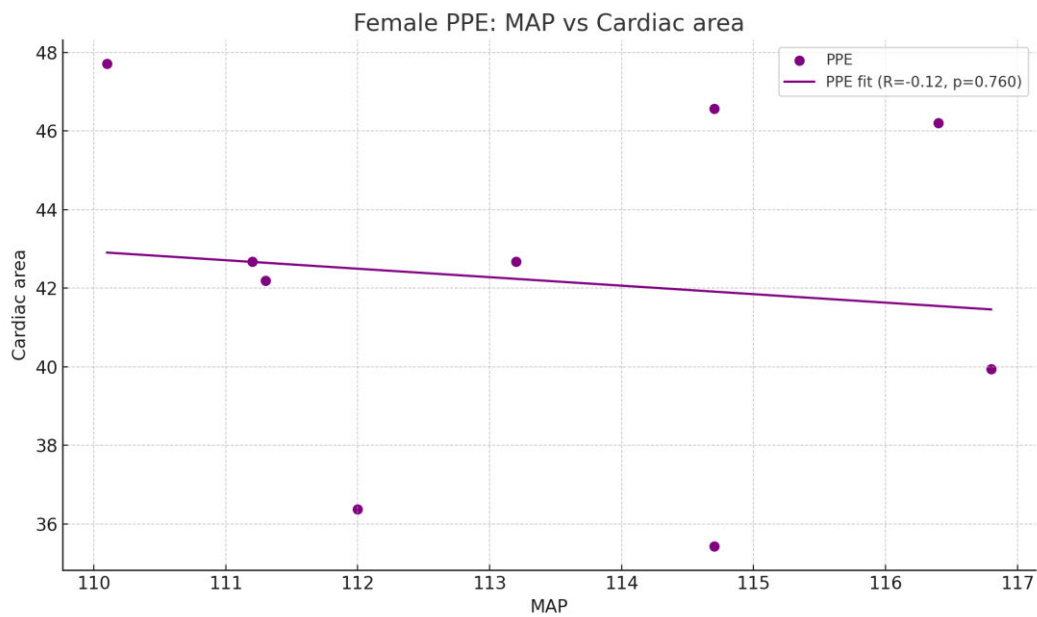
Female PCON TC vs cTnI



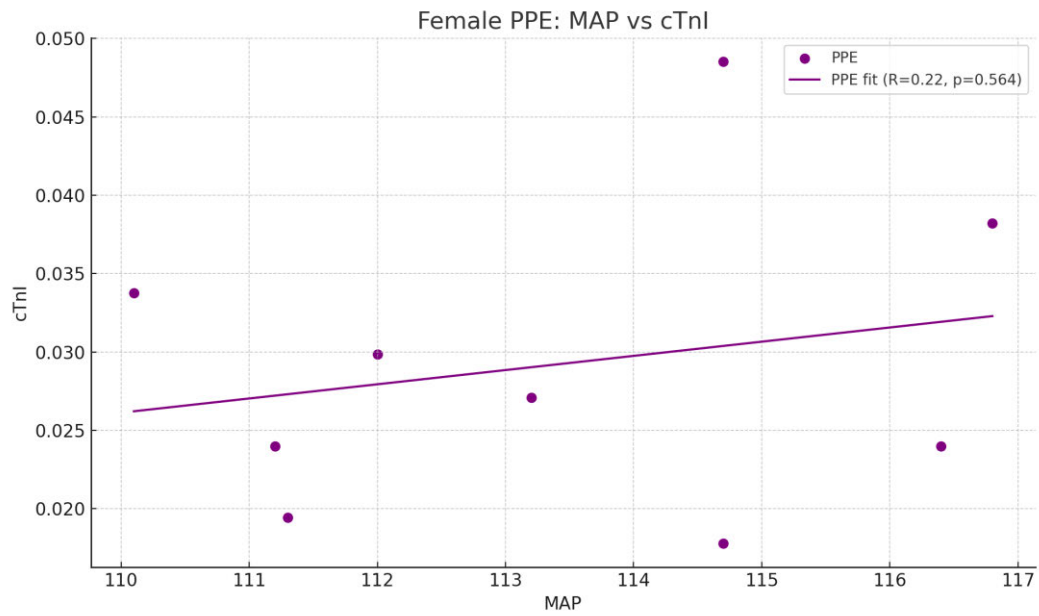
Female PCON TC vs COL1



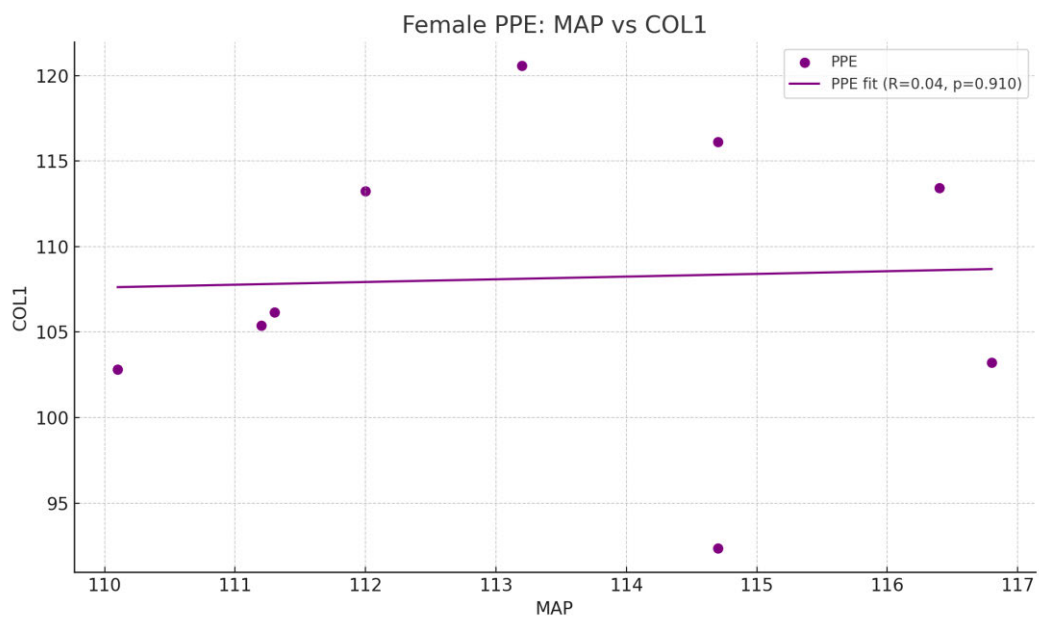
Female PPE MAP vs Cardiac area



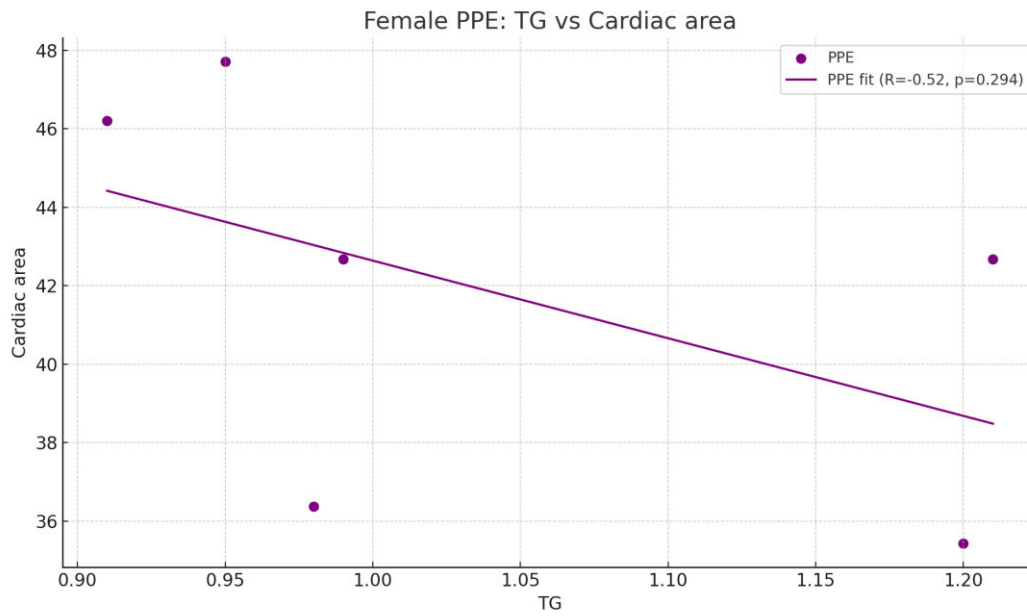
Female PPE MAP vs cTnI



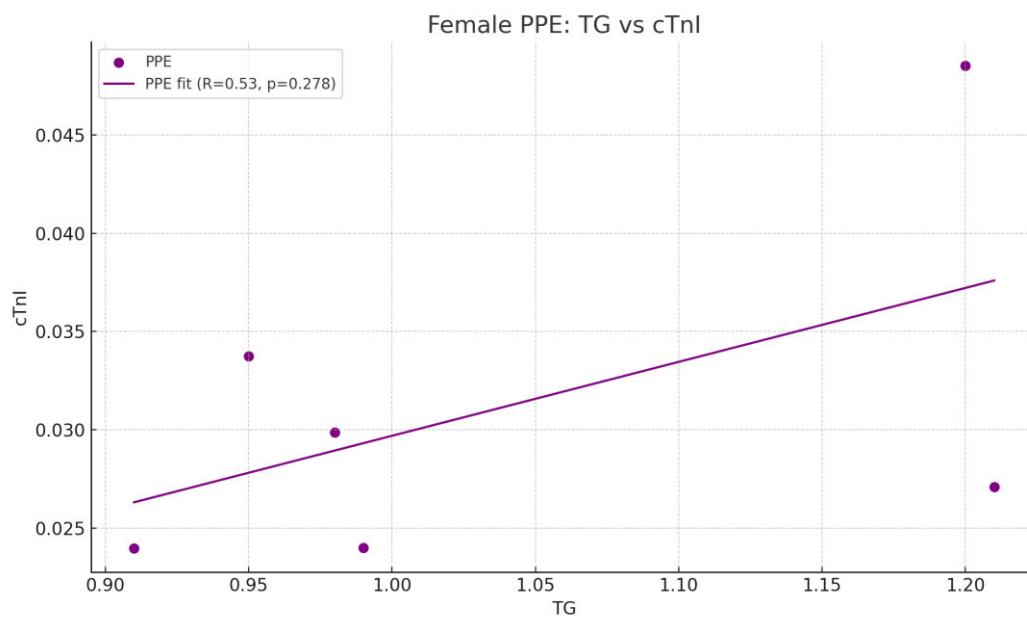
Female PPE MAP vs COL1



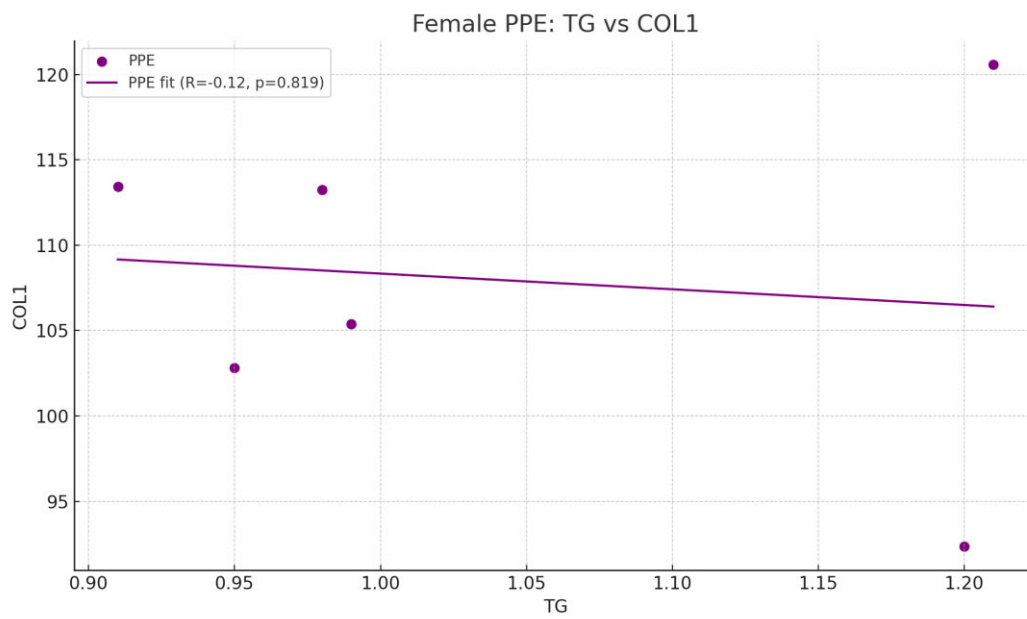
Female PPE TG vs Cardiac area



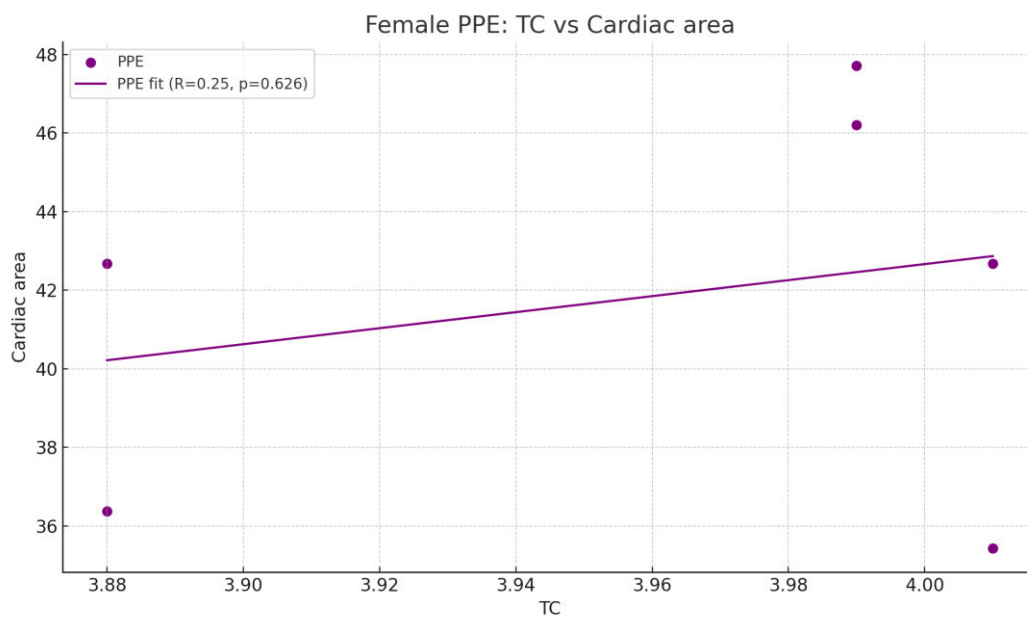
Female PPE TG vs cTnI



Female PPE TG vs COL1



Female PPE TC vs Cardiac area



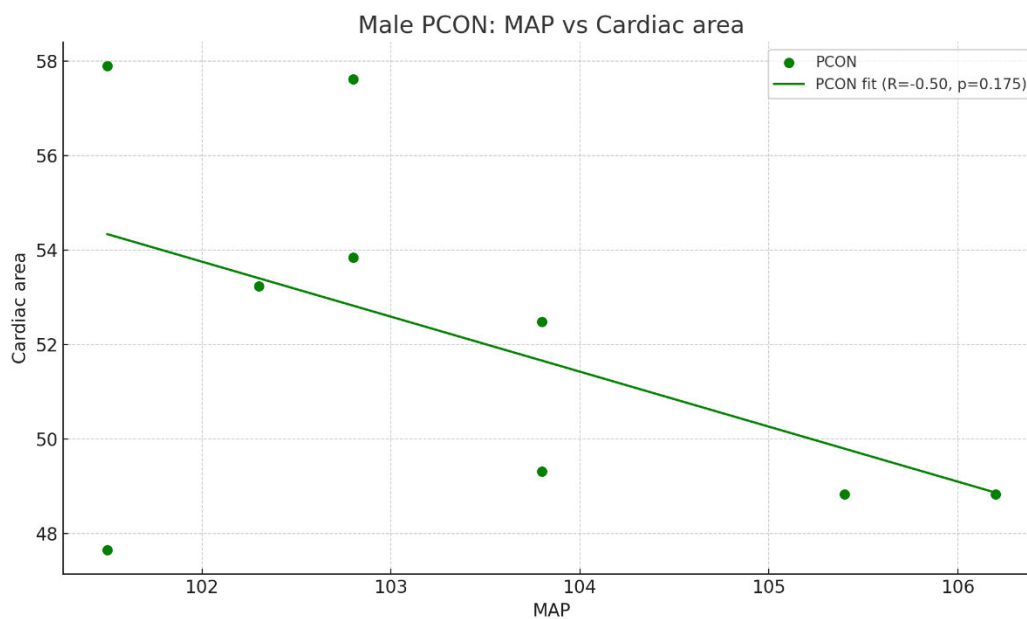
Female PPE TC vs cTnI

Separated Group Scatter Plots (PCON only, PPE only, PPD only)

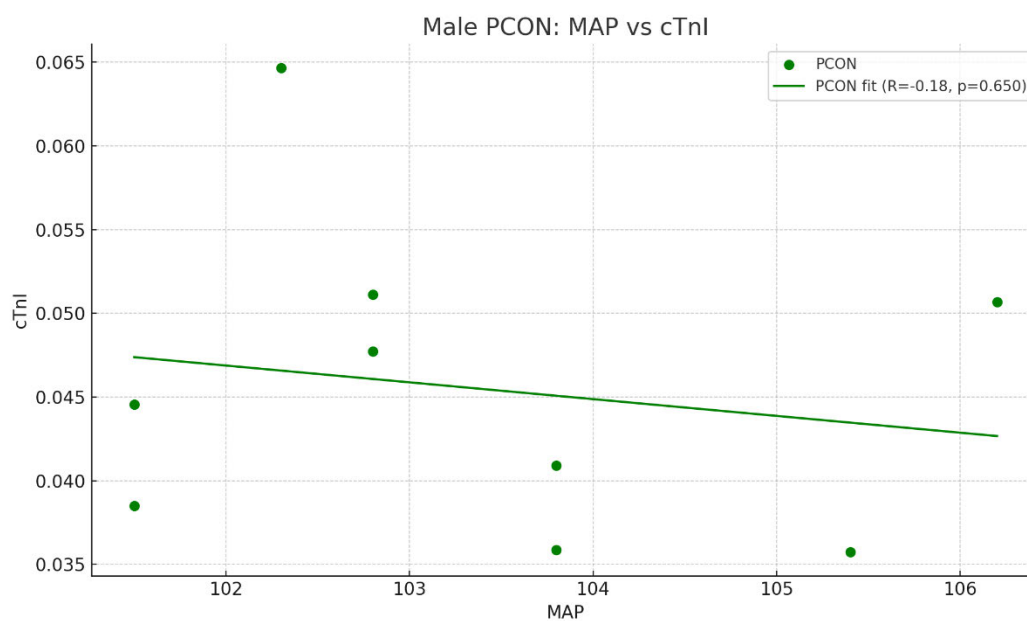
The following figures present each experimental group in isolation (PCON, PPE, PPD) for clarity. Each panel shows a Pearson correlation with a linear regression fit (group-colored line) and reports R and p-value on the figure. Markers are circles.

Male Offspring – Separated by Group

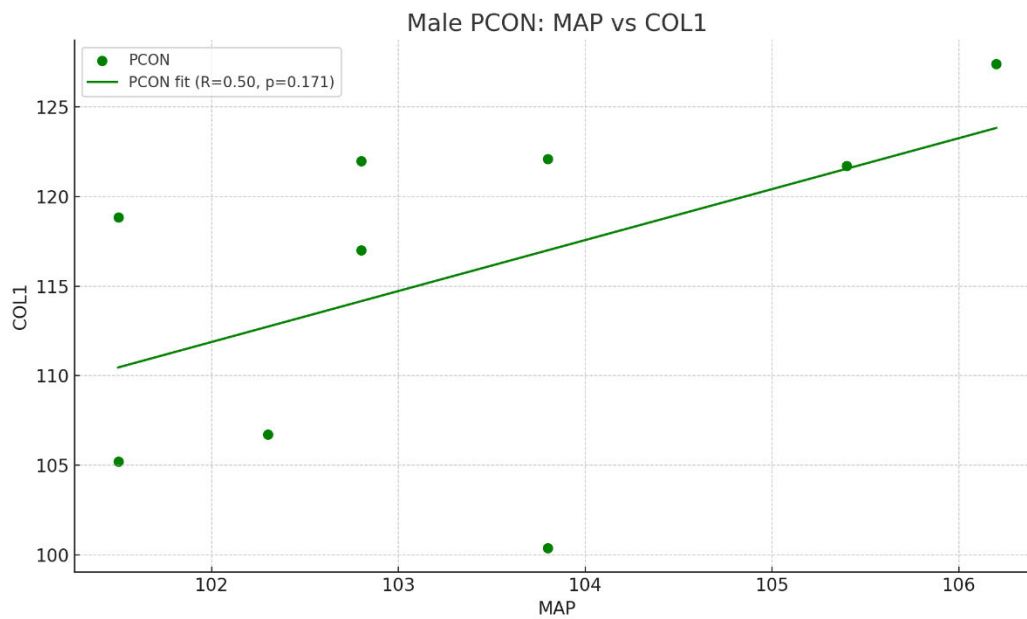
sep Male PCON MAP vs Cardiac area separated



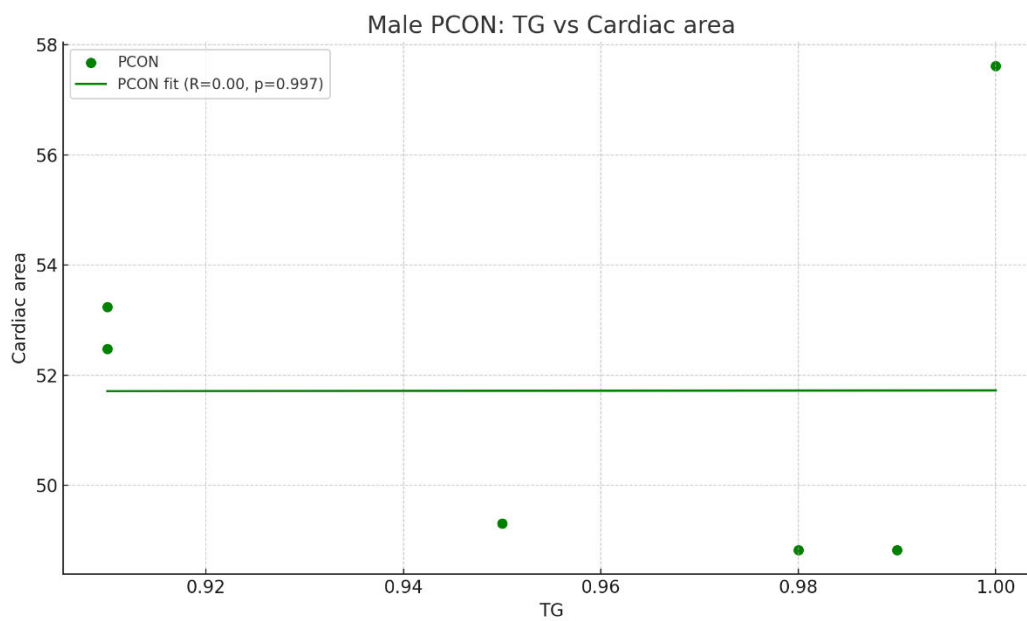
sep Male PCON MAP vs cTnI separated



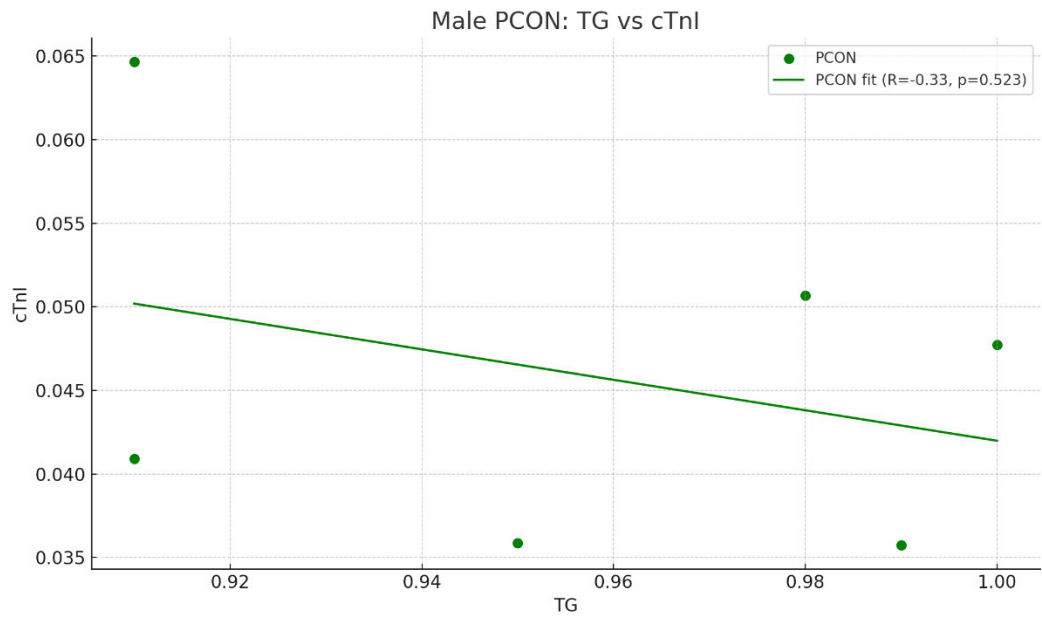
sep Male PCON MAP vs COL1 separated



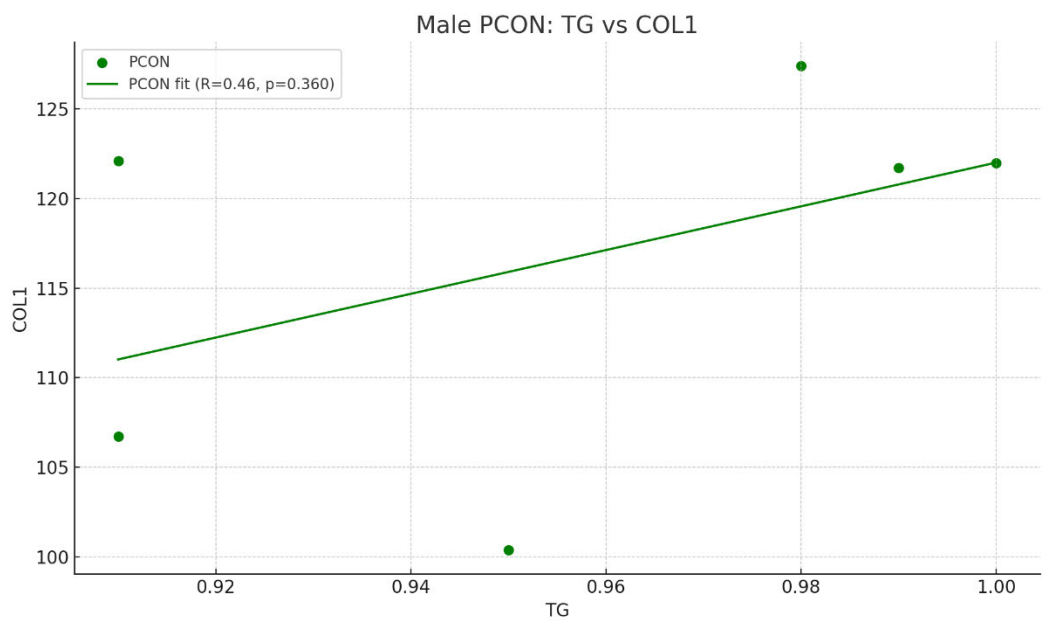
sep Male PCON TG vs Cardiac area separated



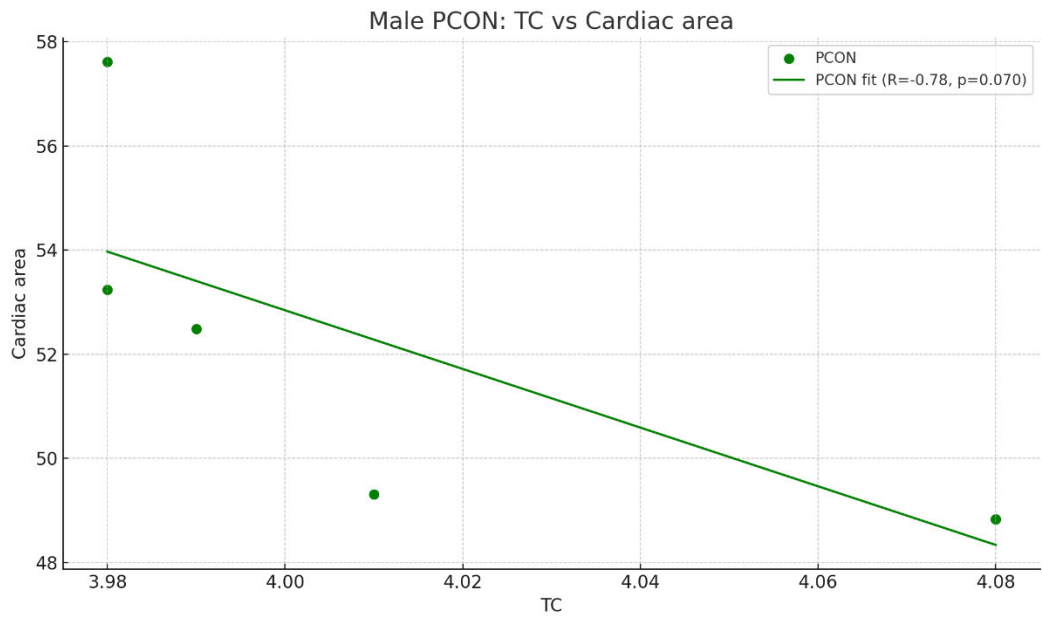
sep Male PCON TG vs cTnI separated



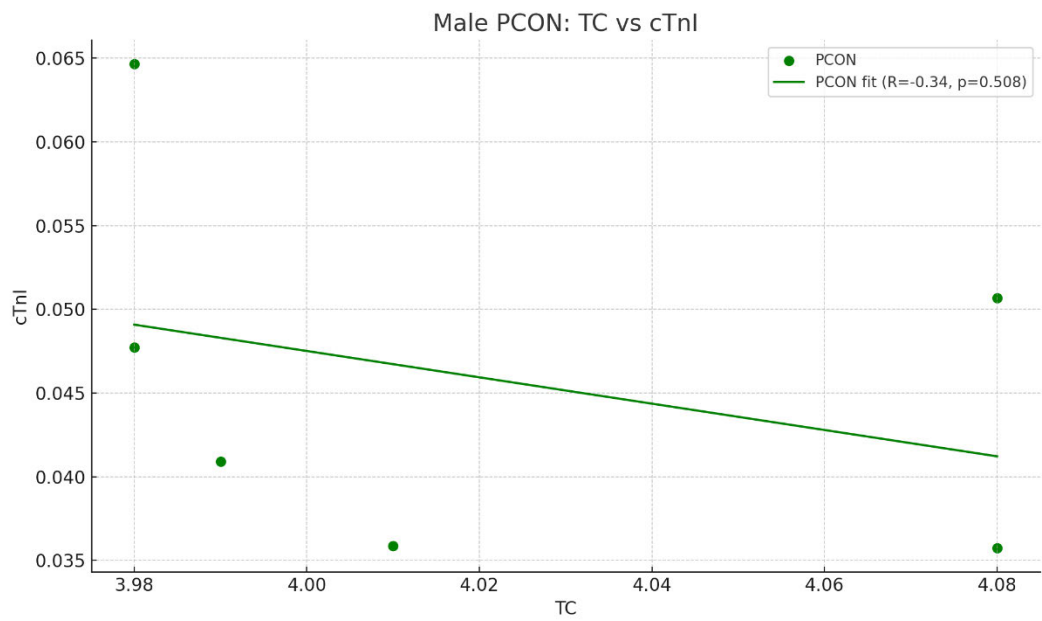
sep Male PCON TG vs COL1 separated



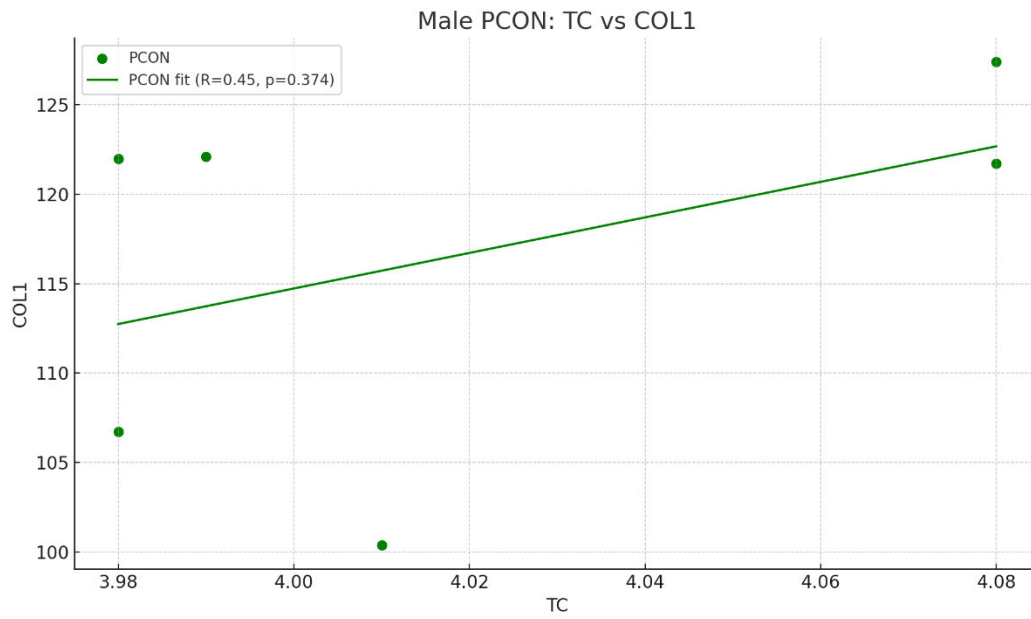
sep Male PCON TC vs Cardiac area separated



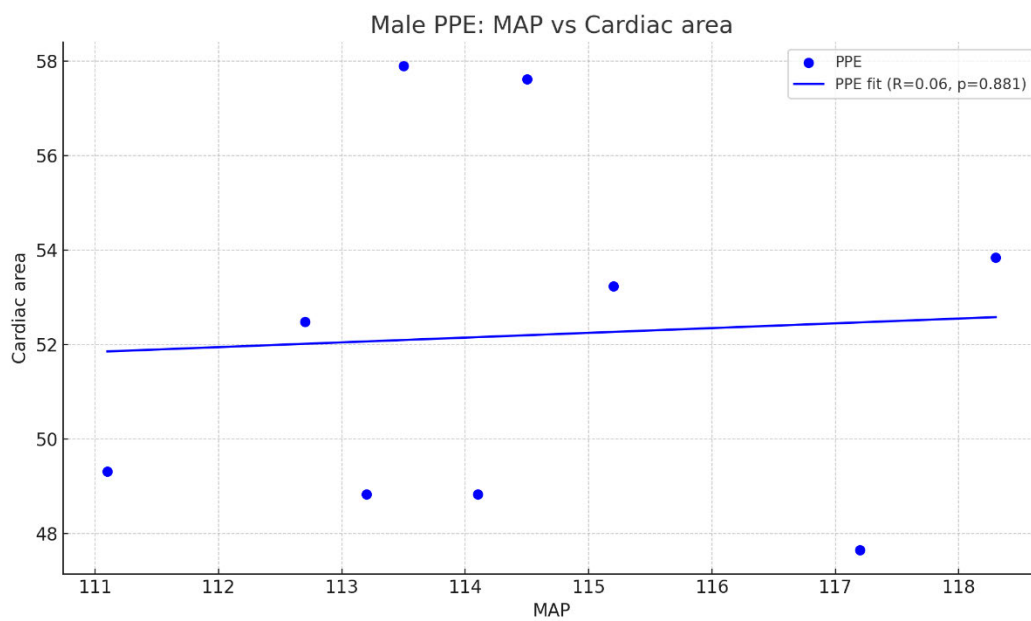
sep Male PCON TC vs cTnl separated



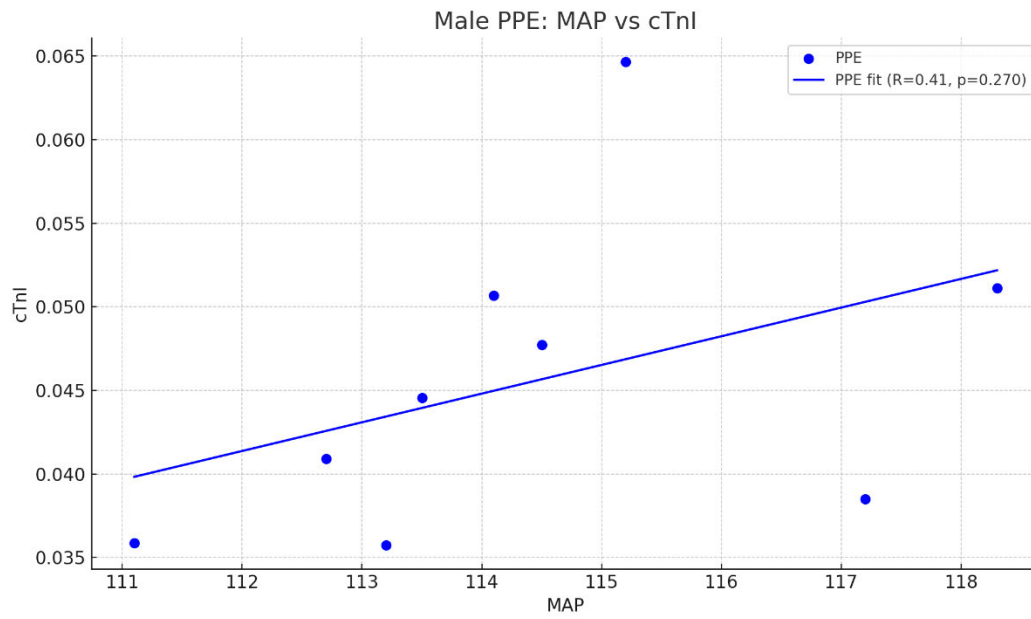
sep Male PCON TC vs COL1 separated



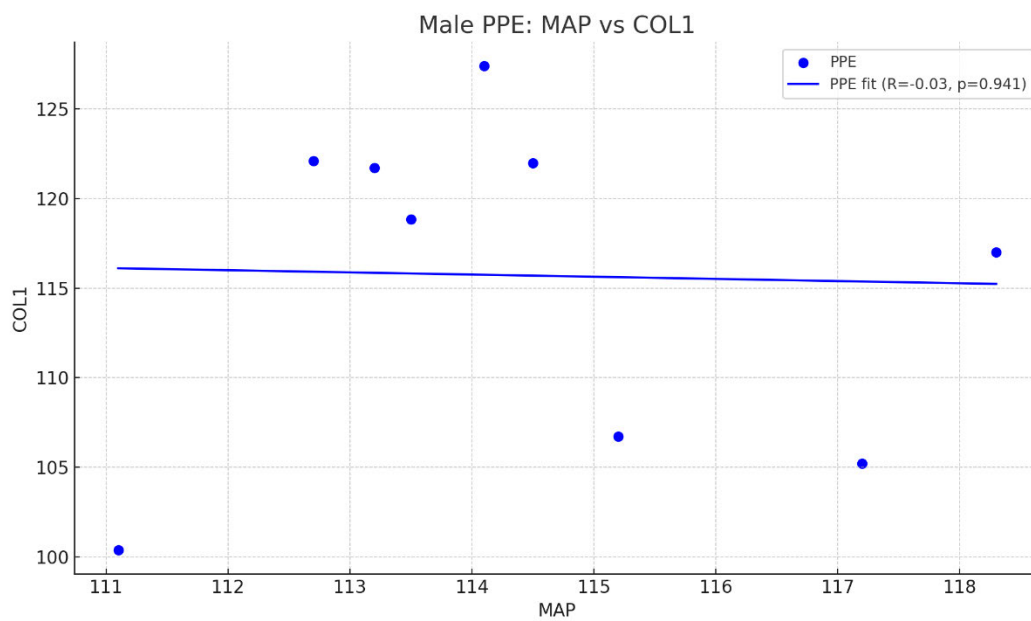
sep Male PPE MAP vs Cardiac area separated



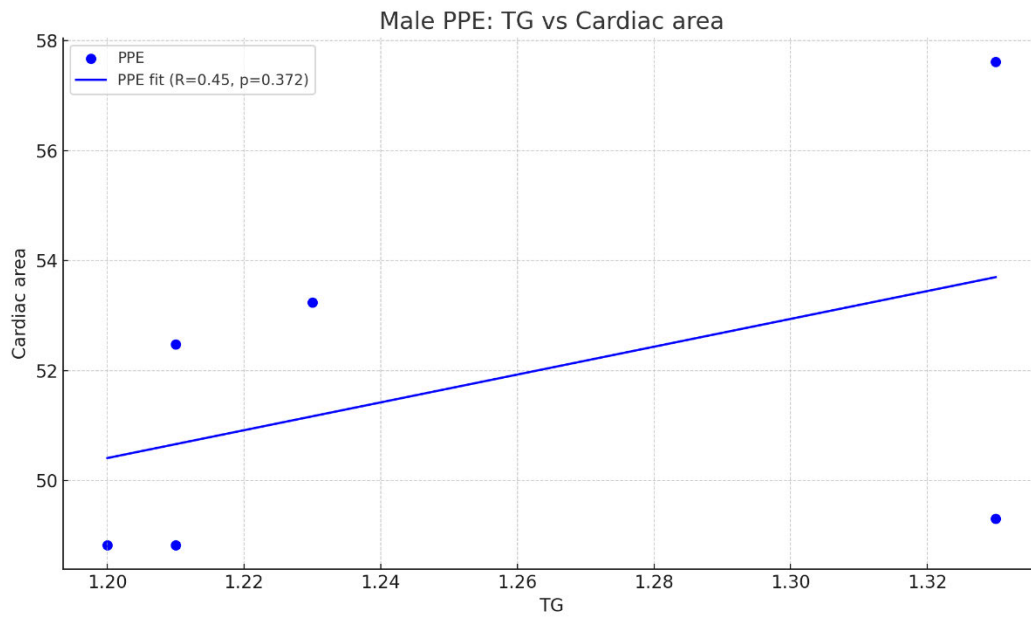
sep Male PPE MAP vs cTnI separated



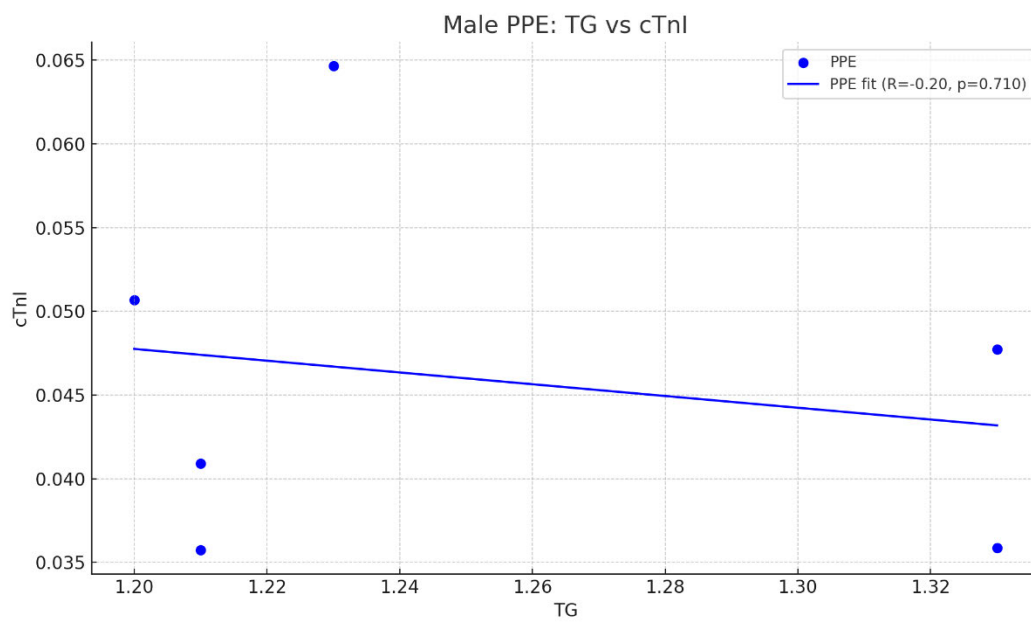
sep Male PPE MAP vs COL1 separated



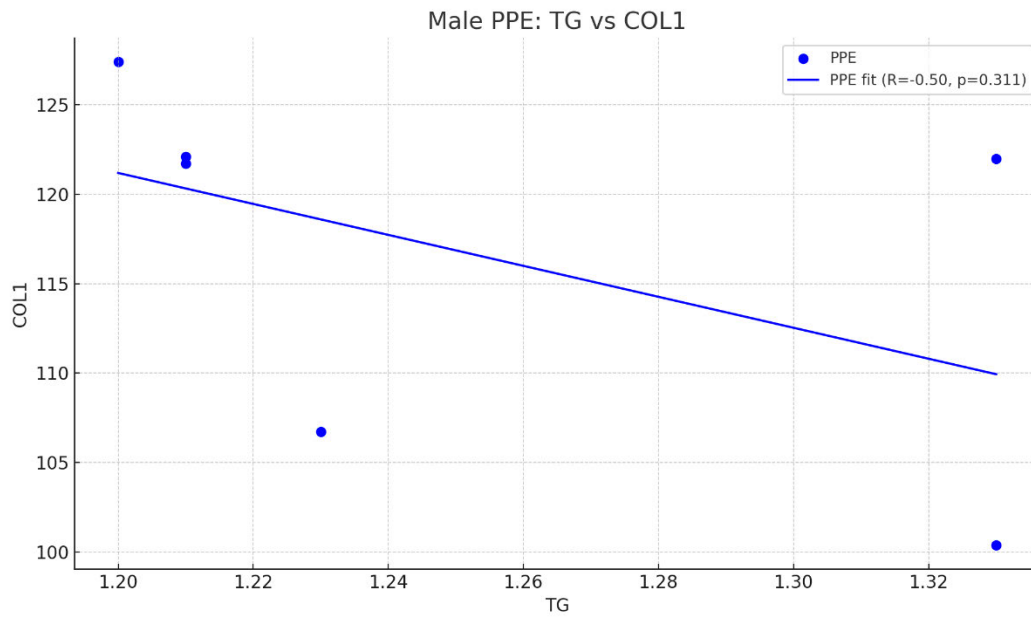
sep Male PPE TG vs Cardiac area separated



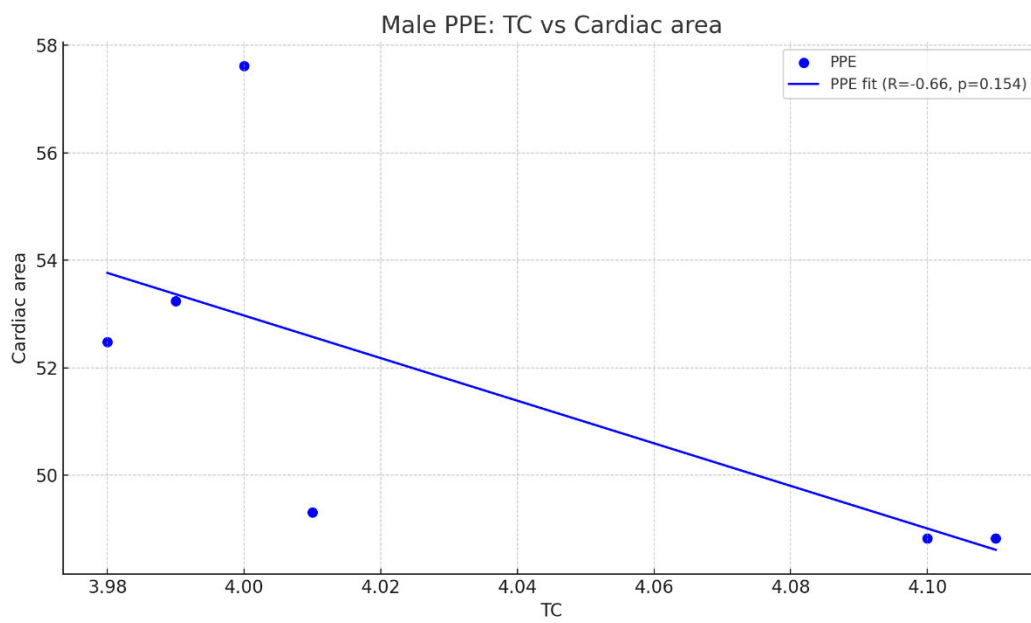
sep Male PPE TG vs cTnI separated



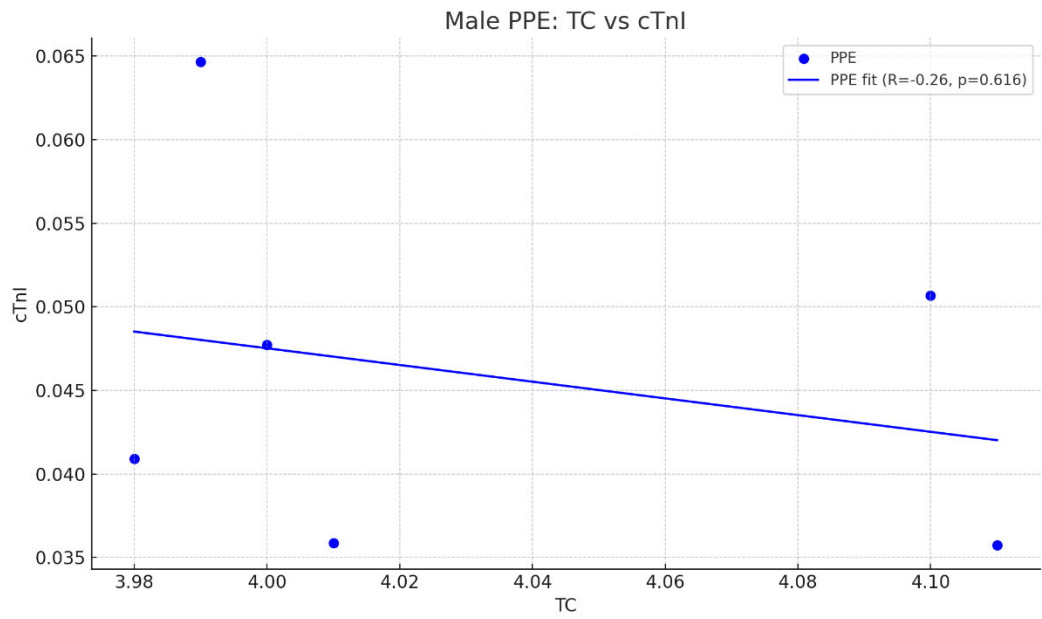
sep Male PPE TG vs COL1 separated



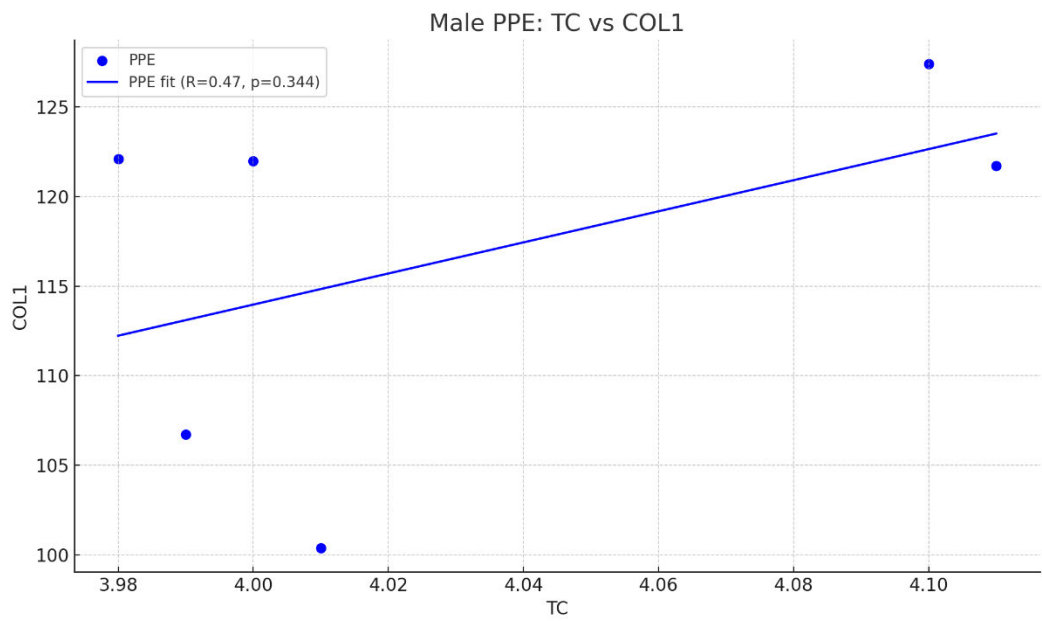
sep Male PPE TC vs Cardiac area separated



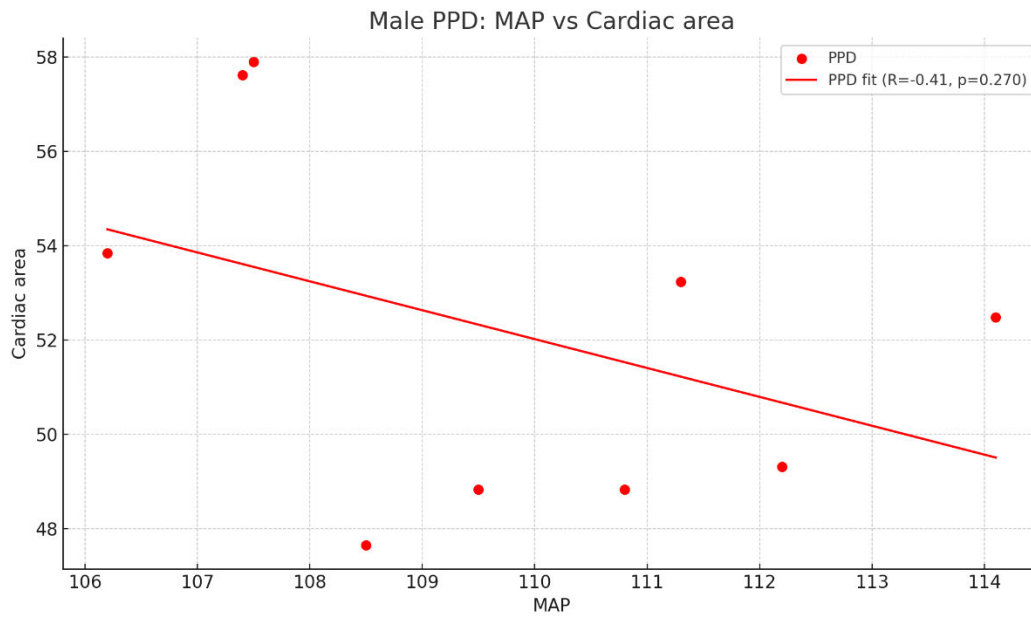
sep Male PPE TC vs cTnI separated



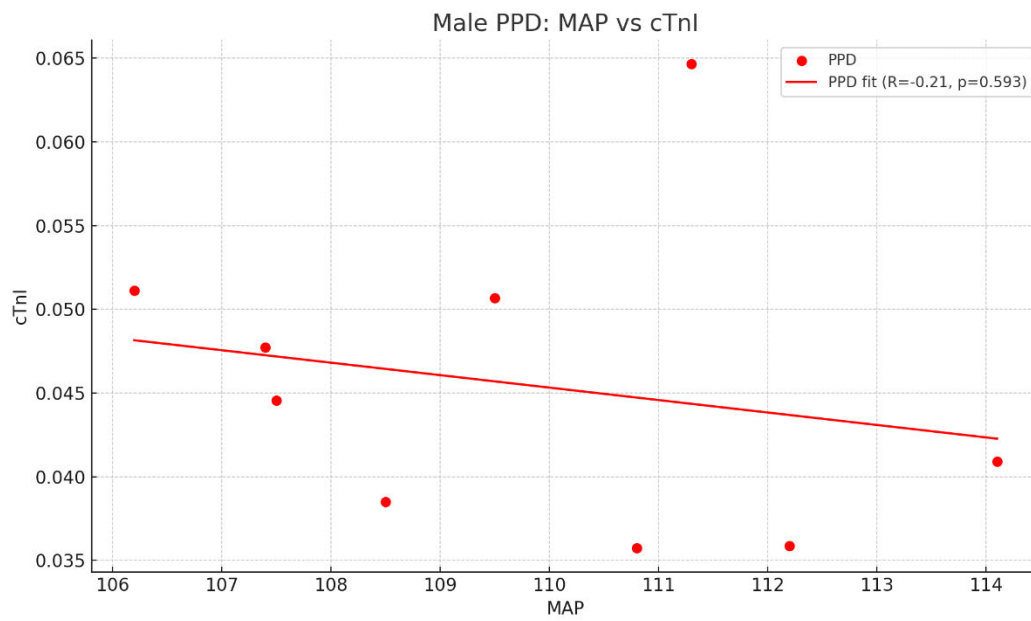
sep Male PPE TC vs COL1 separated



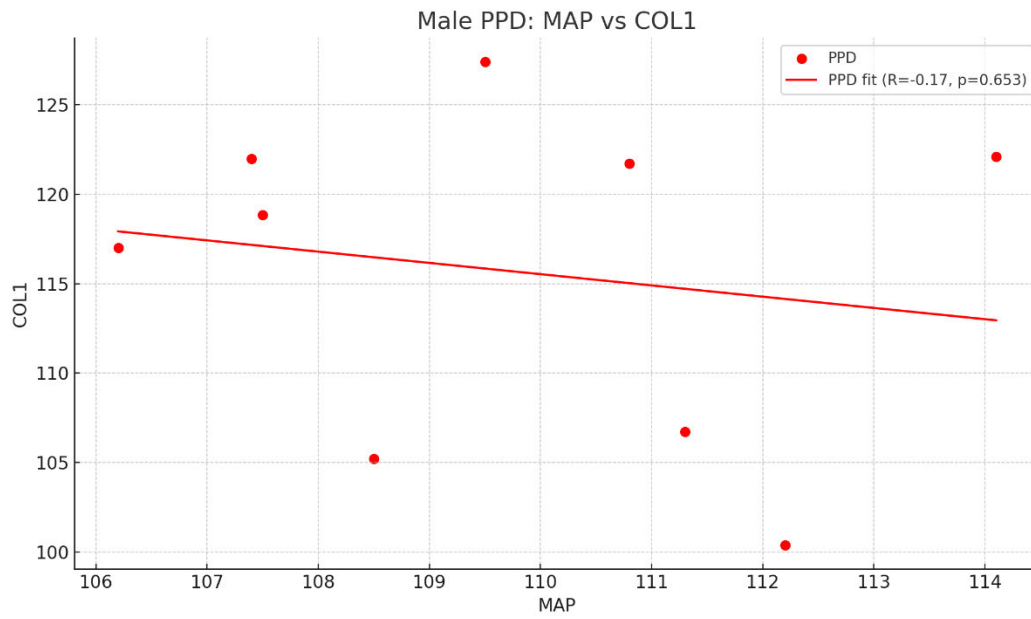
sep Male PPD MAP vs Cardiac area separated



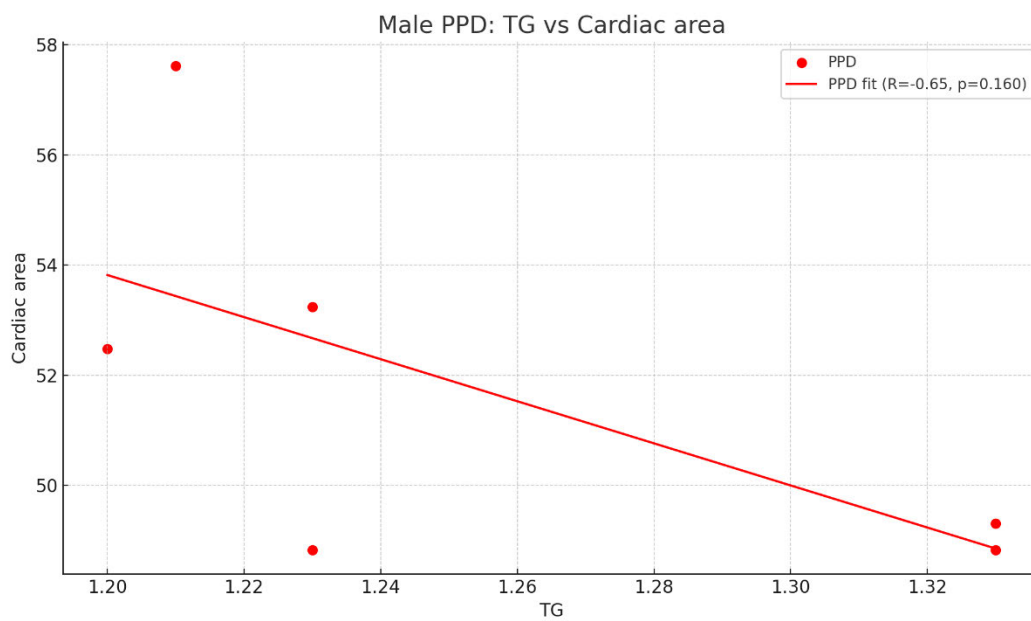
sep Male PPD MAP vs cTnI separated



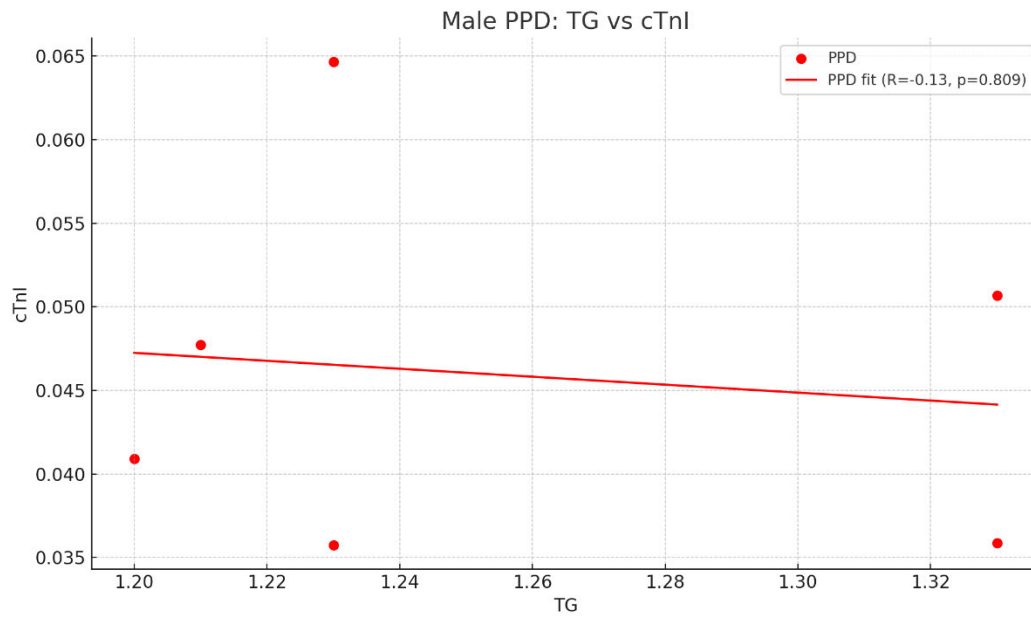
sep Male PPD MAP vs COL1 separated



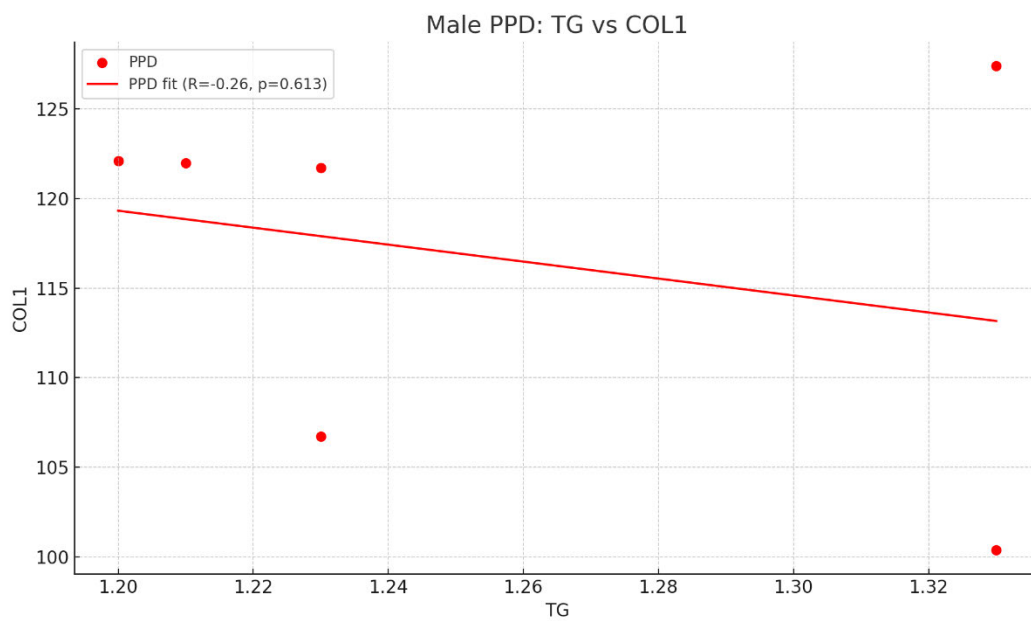
sep Male PPD TG vs Cardiac area separated



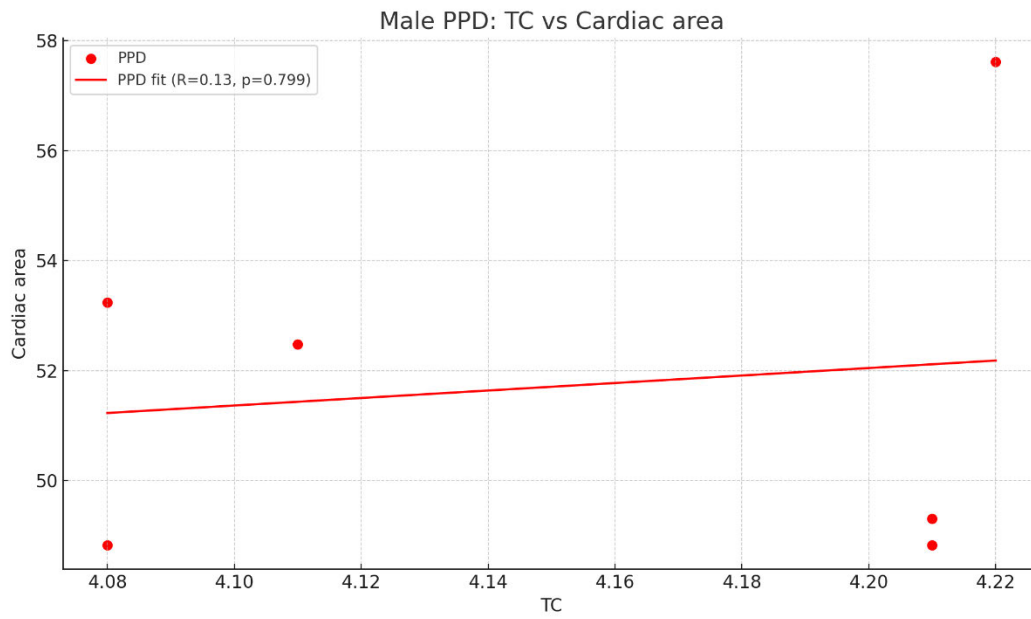
sep Male PPD TG vs cTnI separated



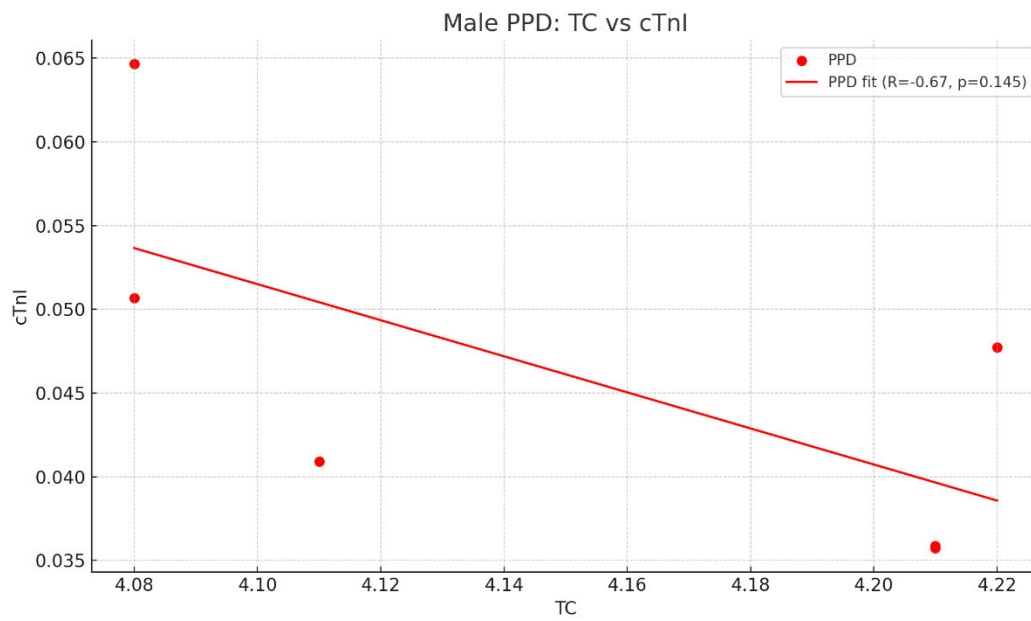
sep Male PPD TG vs COL1 separated



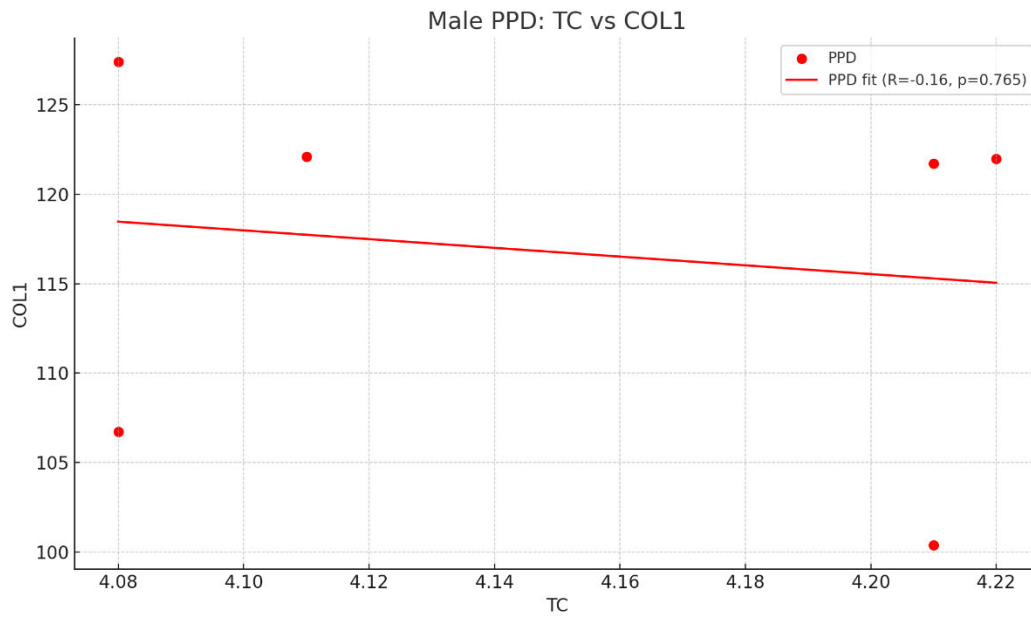
sep Male PPD TC vs Cardiac area separated



sep Male PPD TC vs cTnI separated

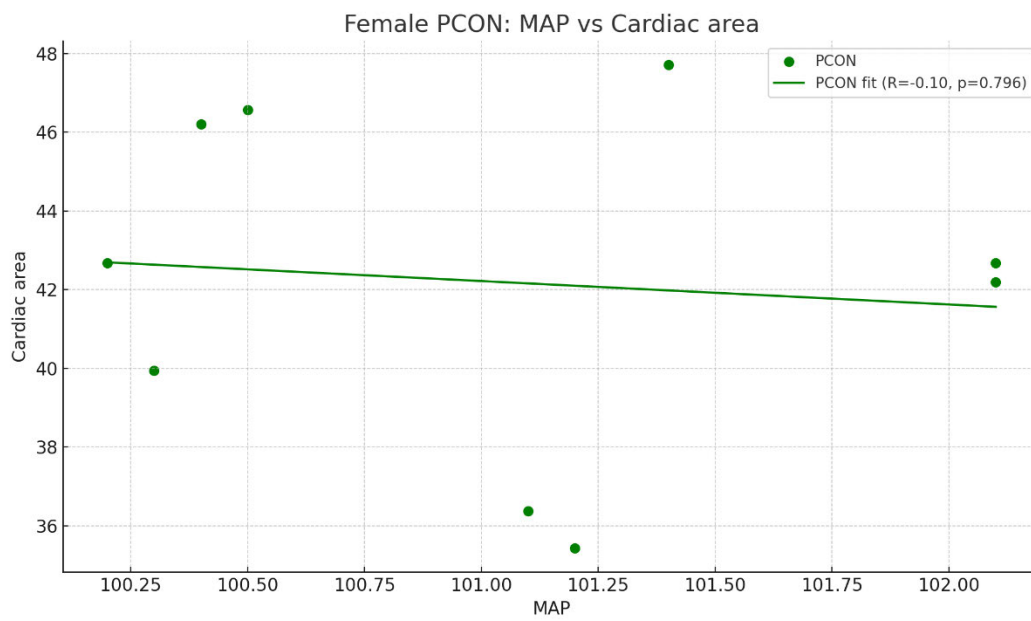


sep Male PPD TC vs COL1 separated

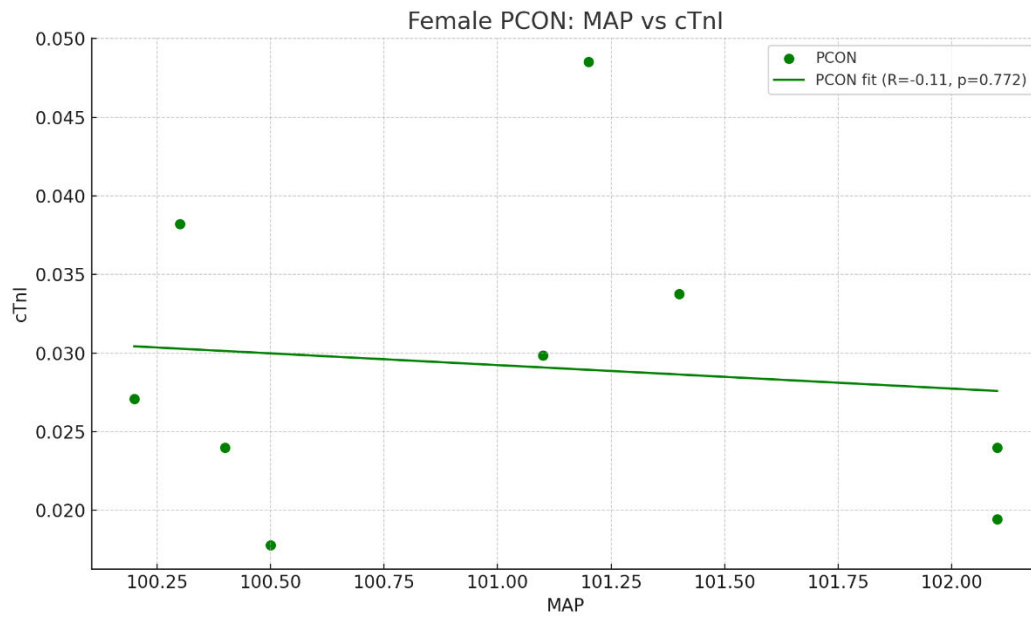


Female Offspring – Separated by Group

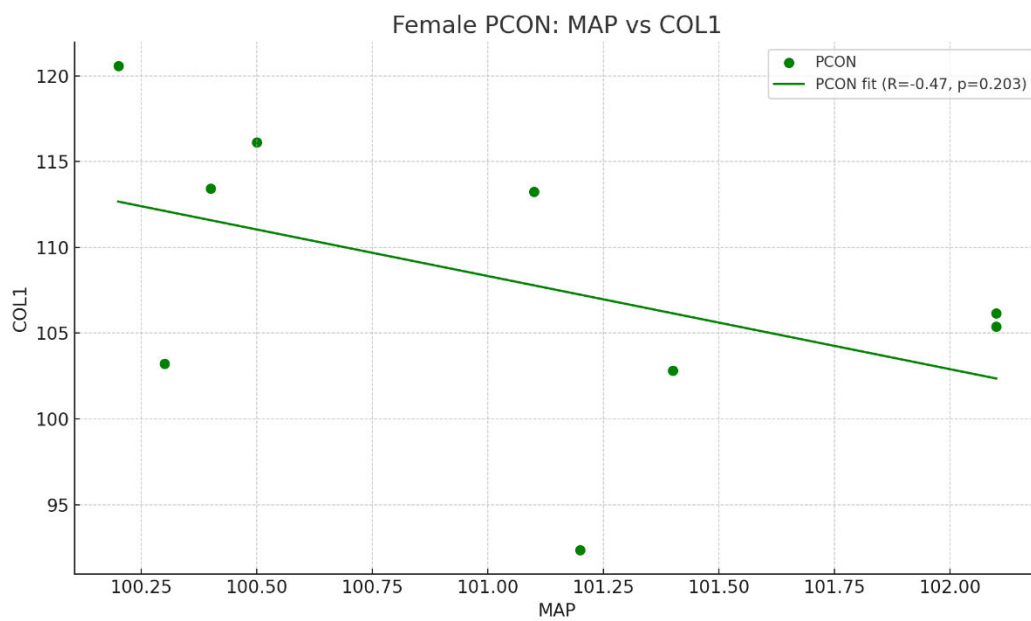
sep Female PCON MAP vs Cardiac area separated



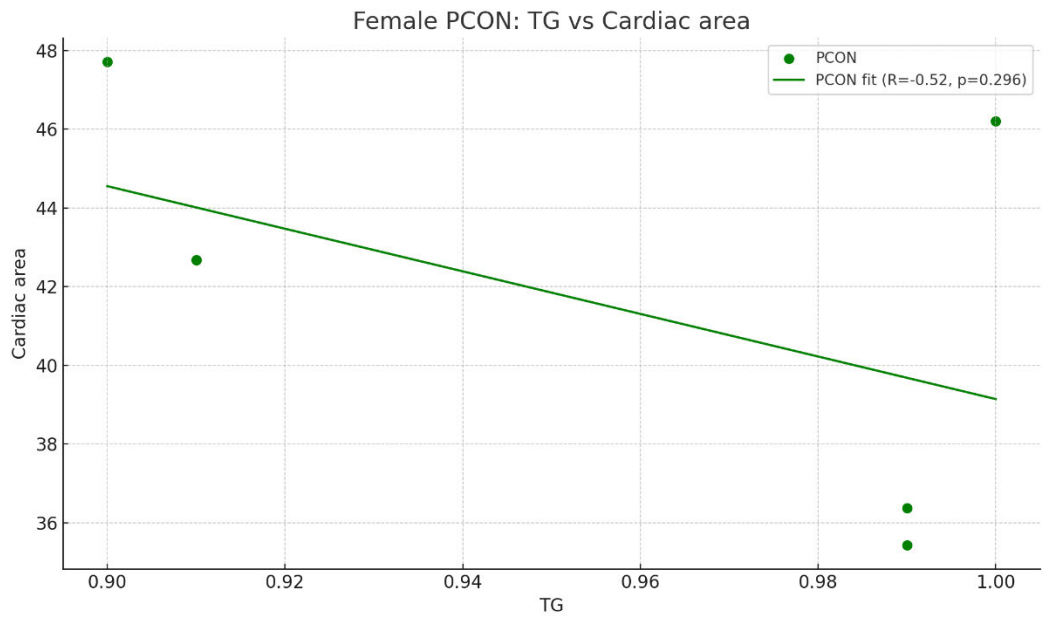
sep Female PCON MAP vs cTnI separated



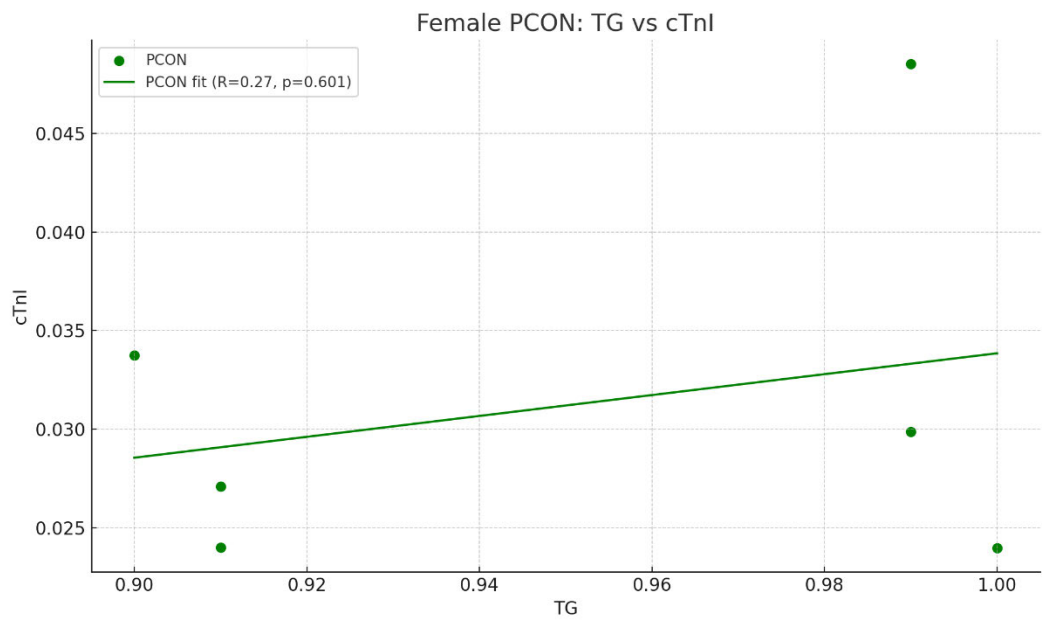
sep Female PCON MAP vs COL1 separated



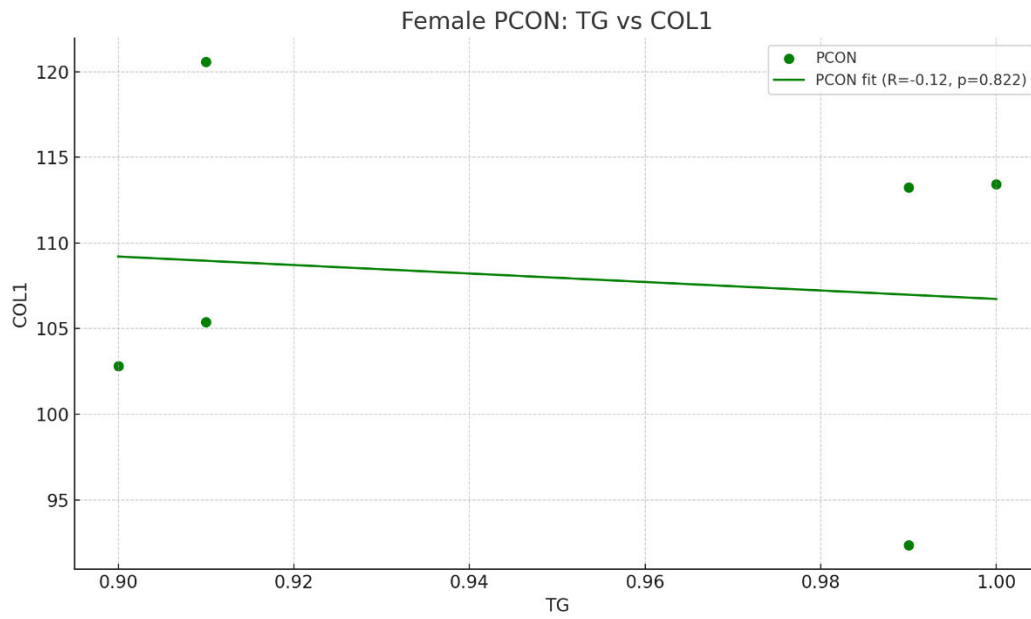
sep Female PCON TG vs Cardiac area separated



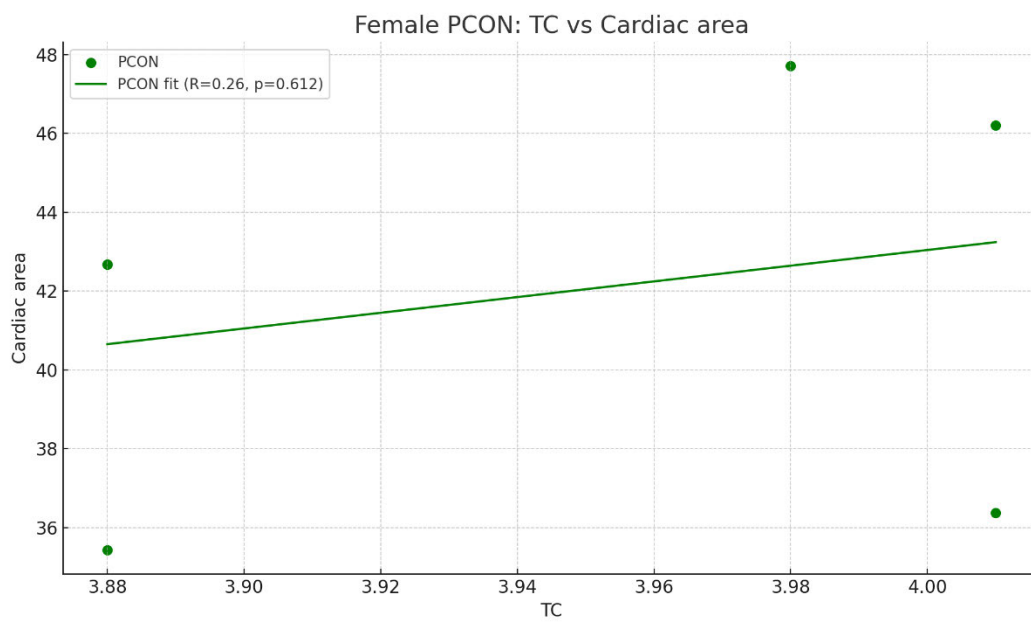
sep Female PCON TG vs cTnI separated



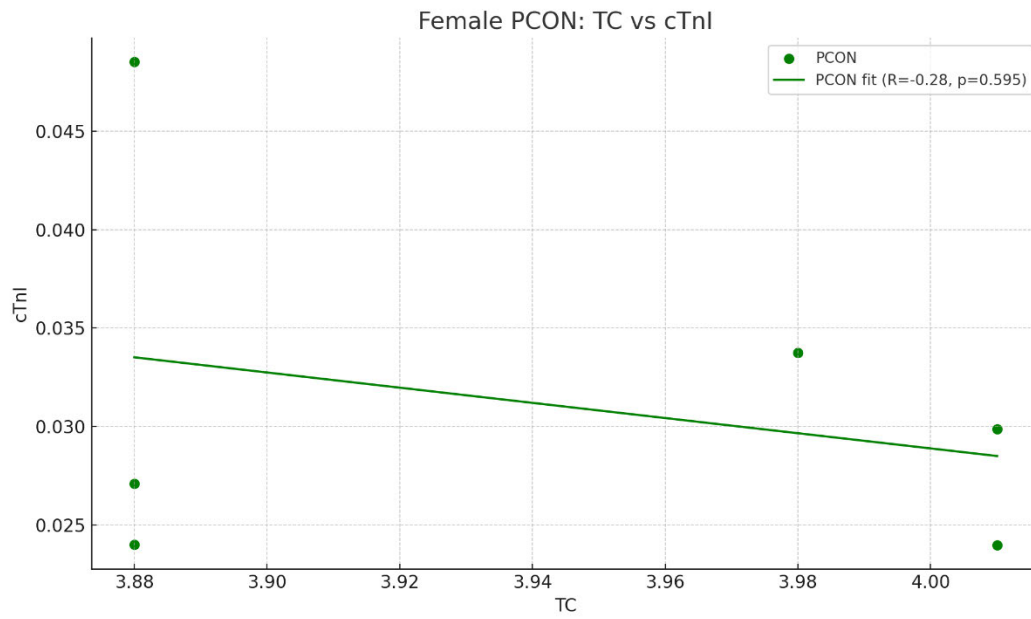
sep Female PCON TG vs COL1 separated



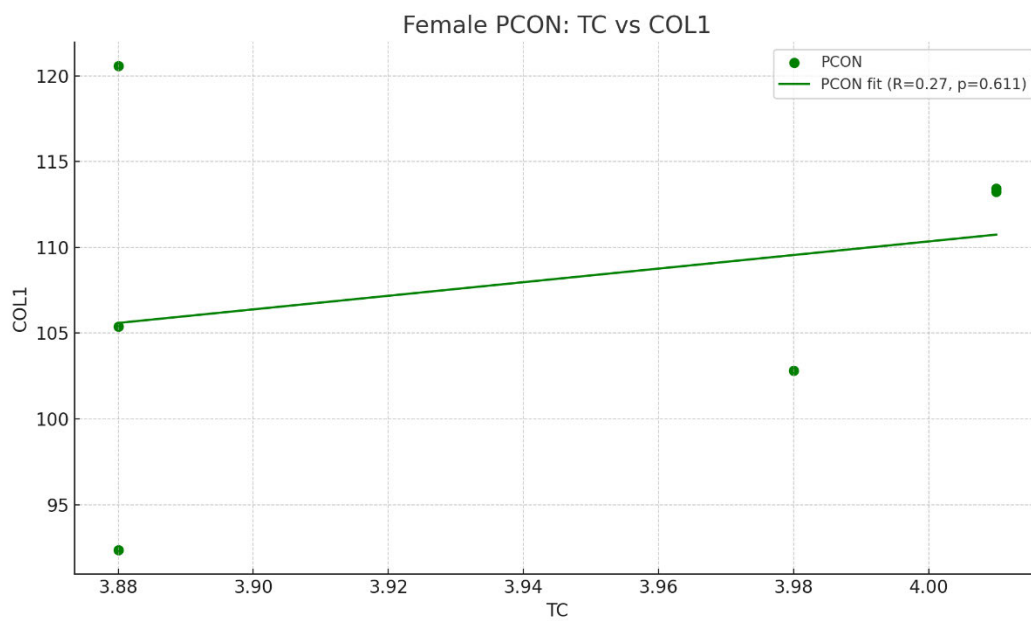
sep Female PCON TC vs Cardiac area separated



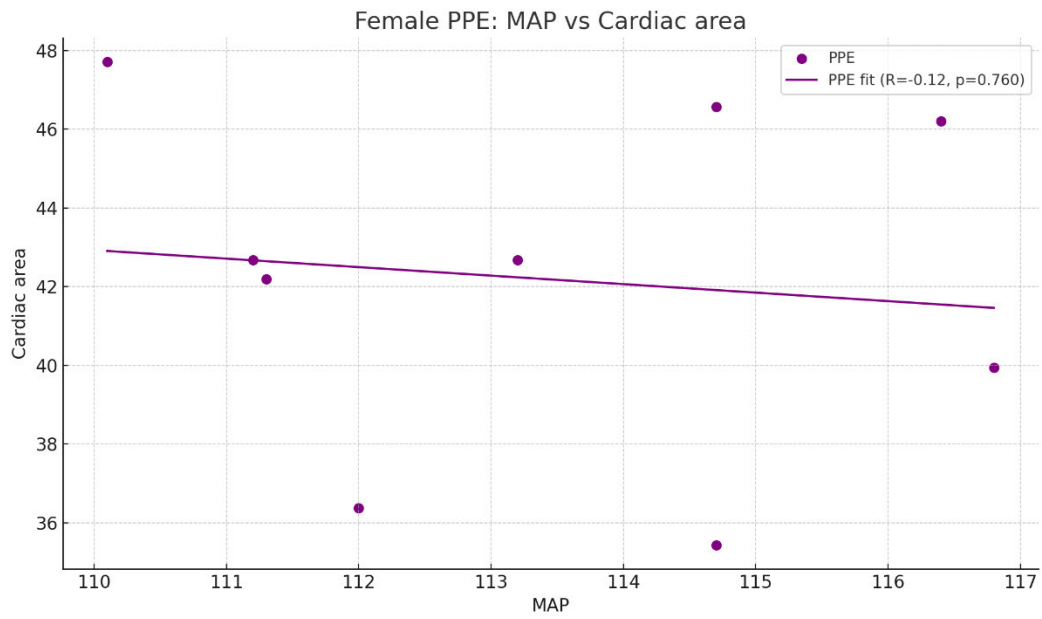
sep Female PCON TC vs cTnI separated



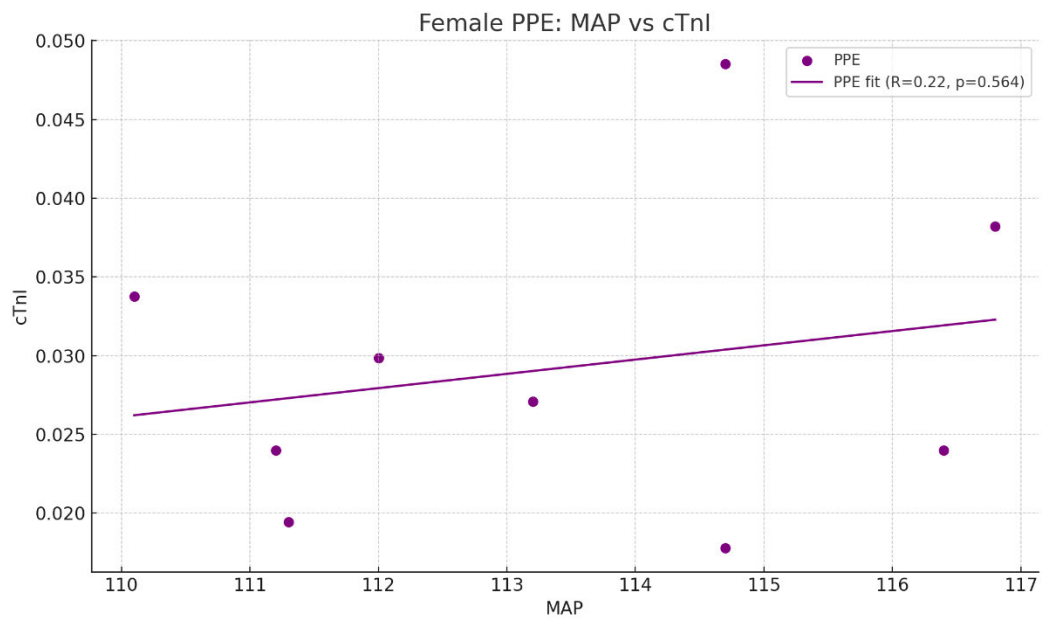
sep Female PCON TC vs COL1 separated



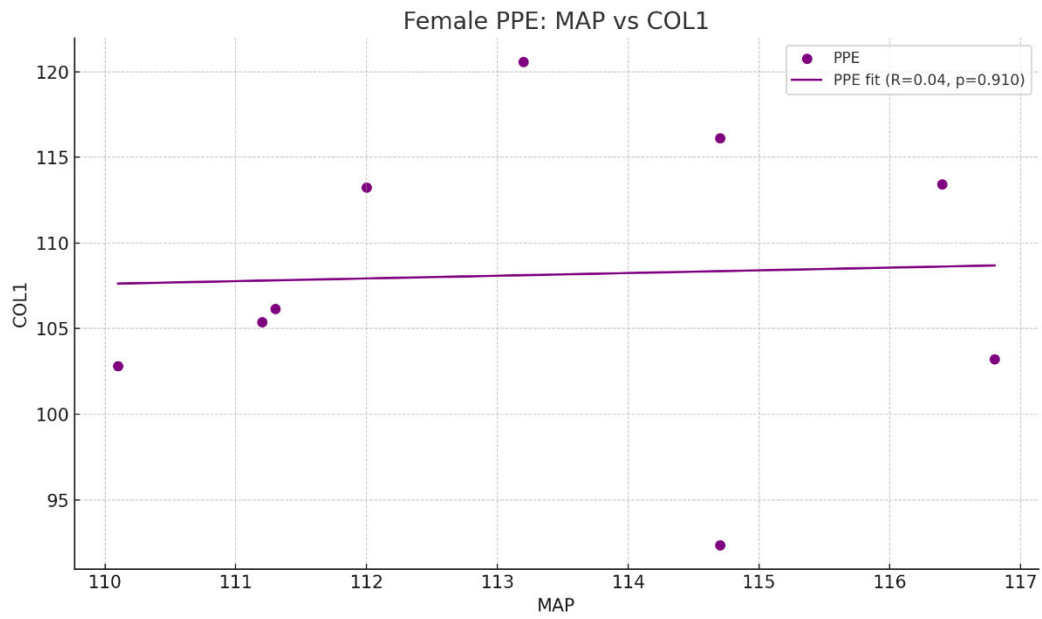
sep Female PPE MAP vs Cardiac area separated



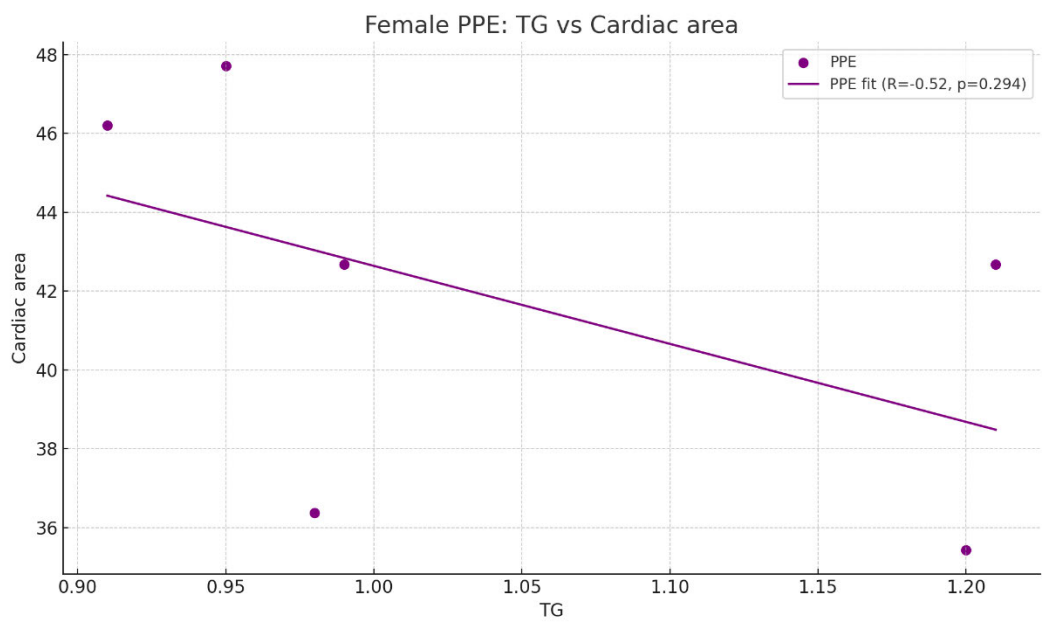
sep Female PPE MAP vs cTnI separated



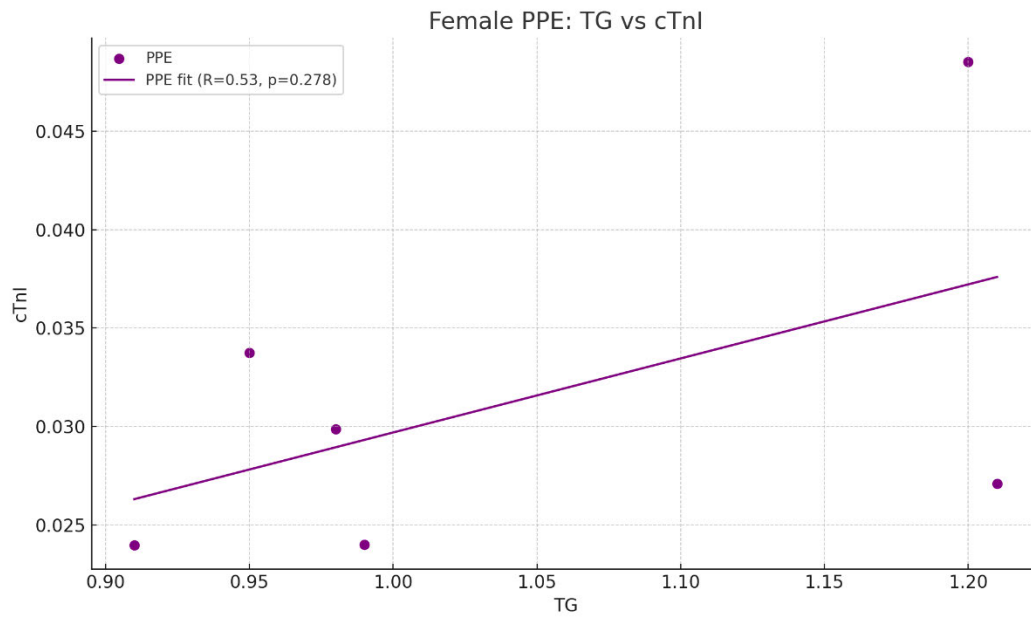
sep Female PPE MAP vs COL1 separated



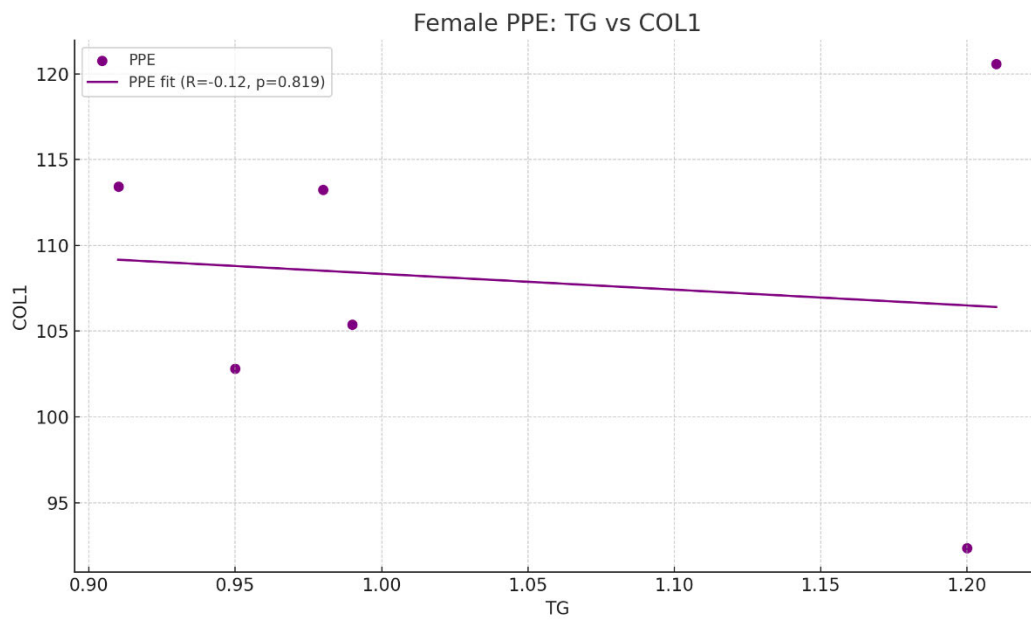
sep Female PPE TG vs Cardiac area separated



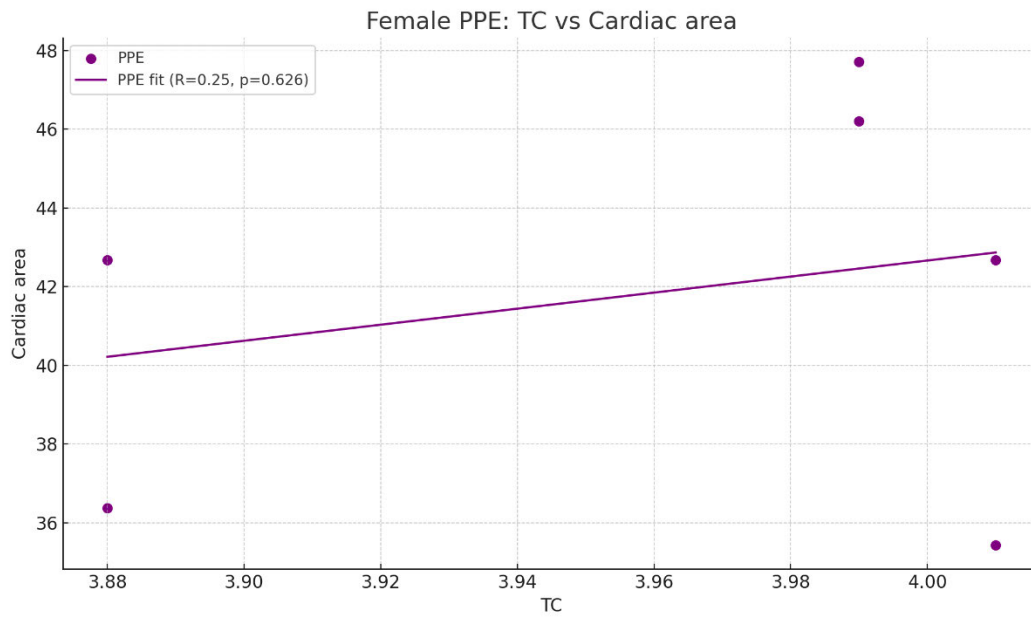
sep Female PPE TG vs cTnI separated



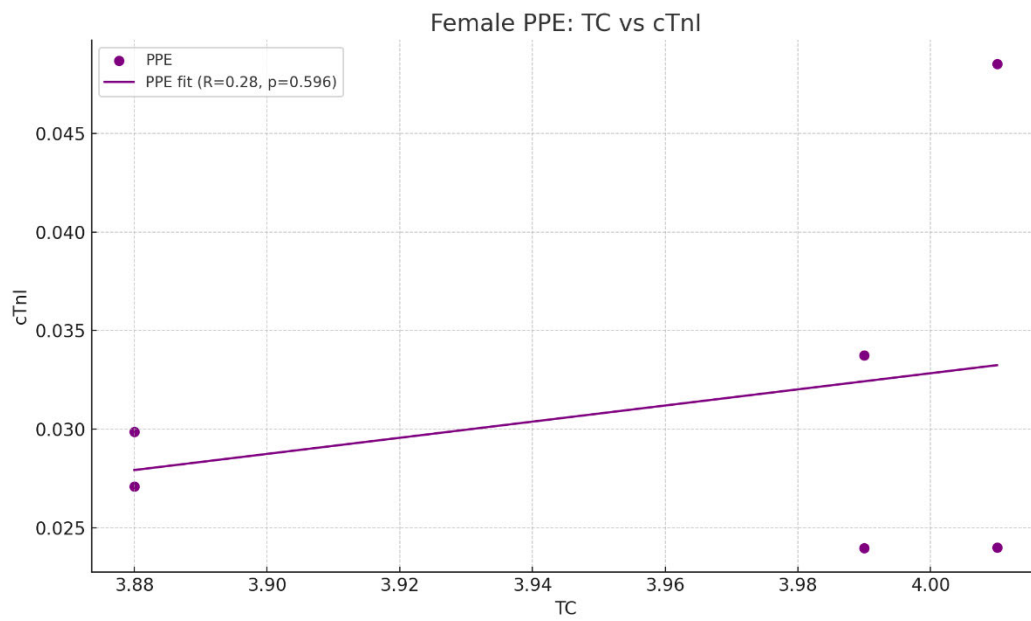
sep Female PPE TG vs COL1 separated



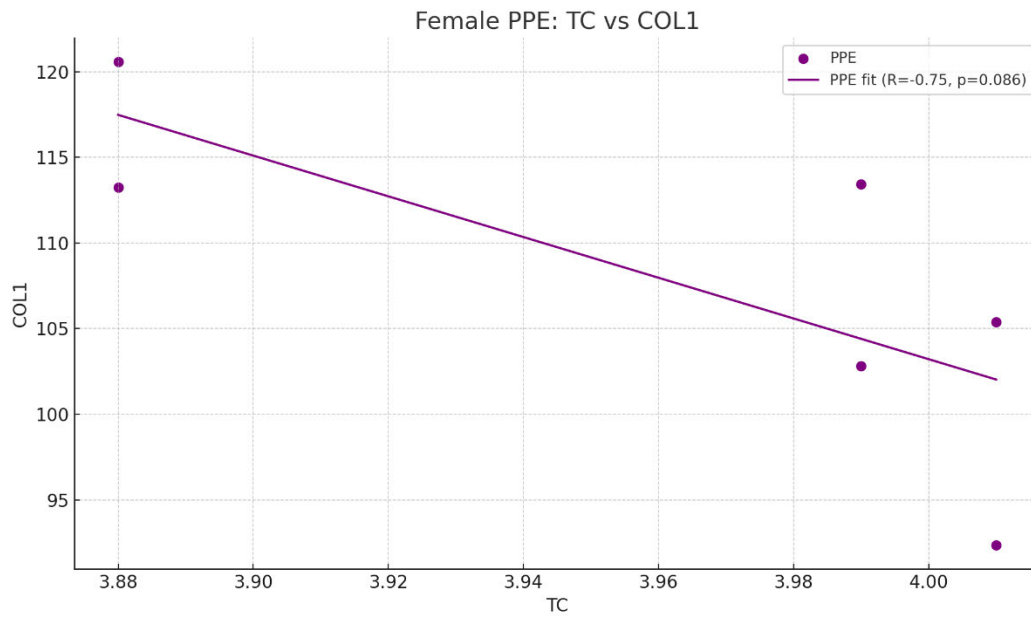
sep Female PPE TC vs Cardiac area separated



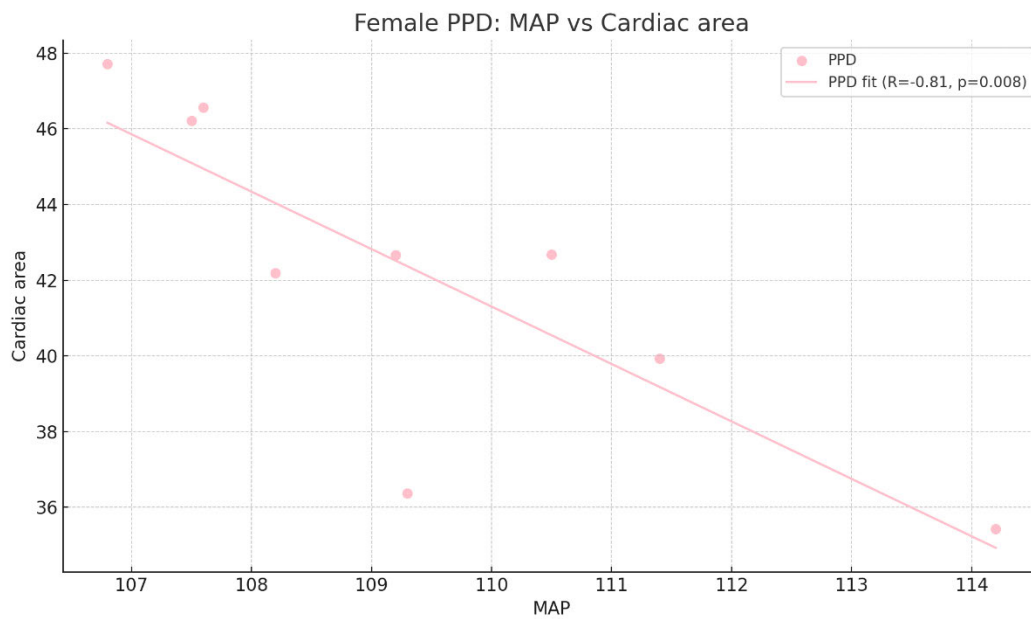
sep Female PPE TC vs cTnI separated



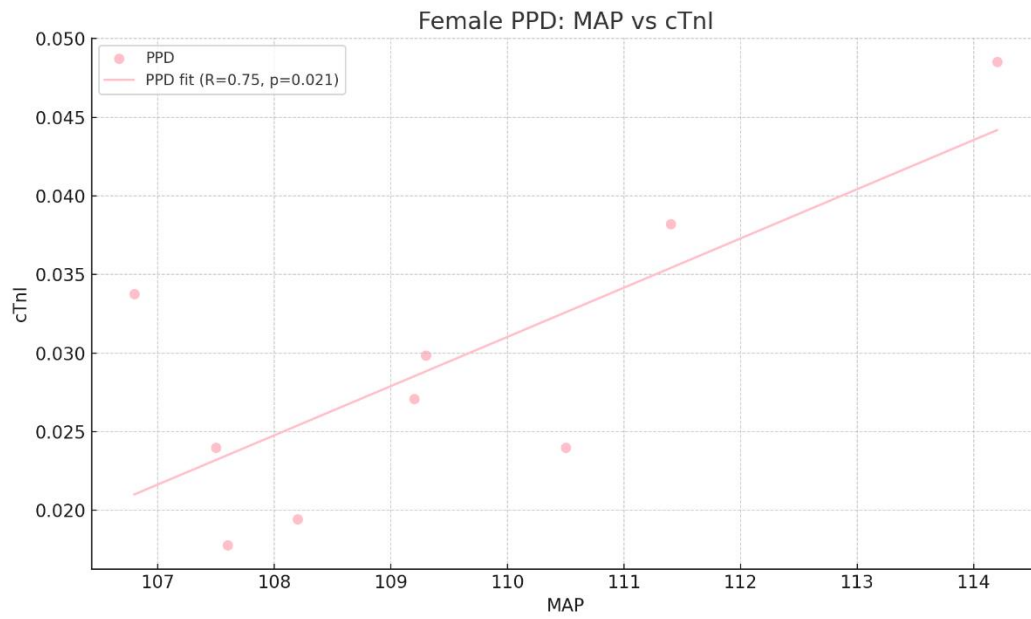
sep Female PPE TC vs COL1 separated



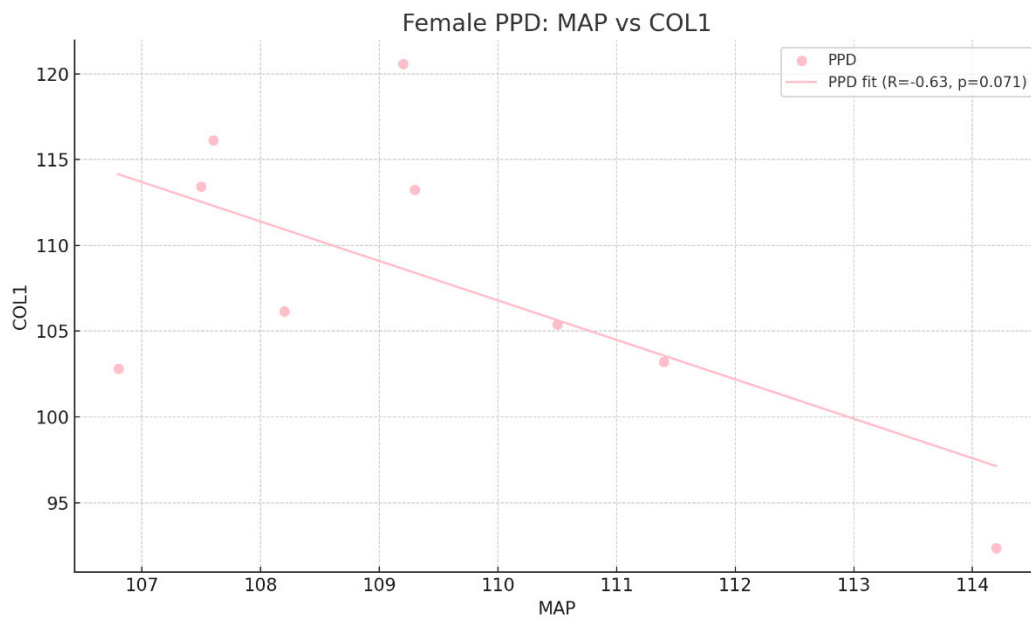
sep Female PPD MAP vs Cardiac area separated



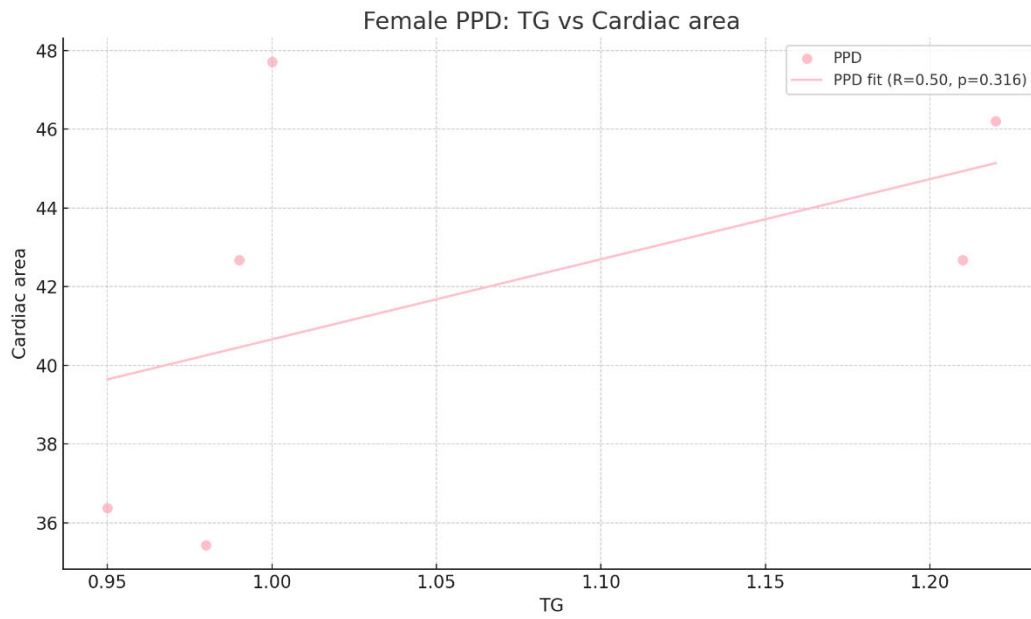
sep Female PPD MAP vs cTnI separated



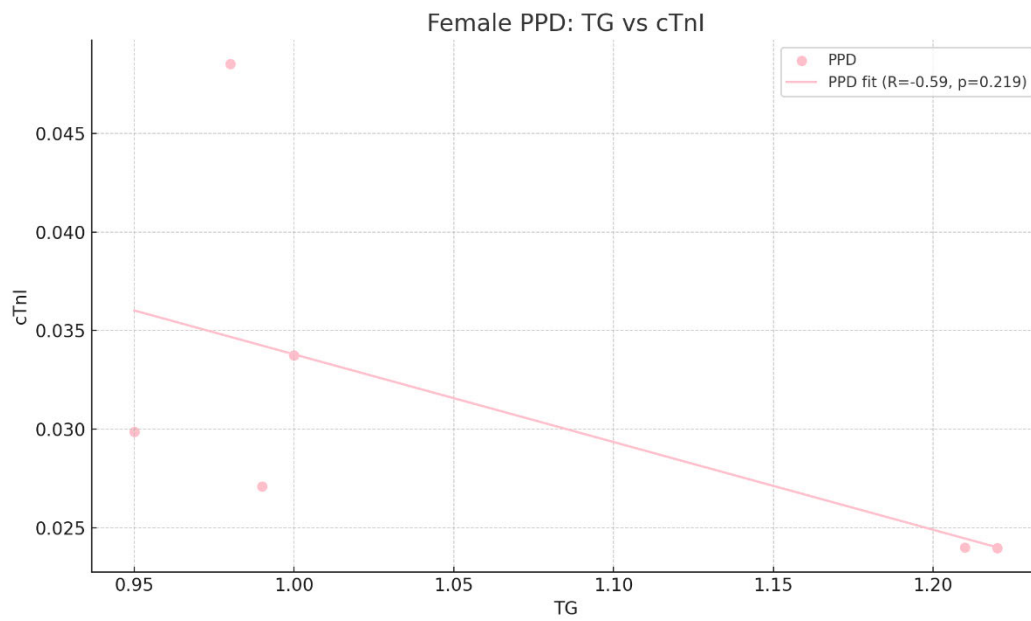
sep Female PPD MAP vs COL1 separated



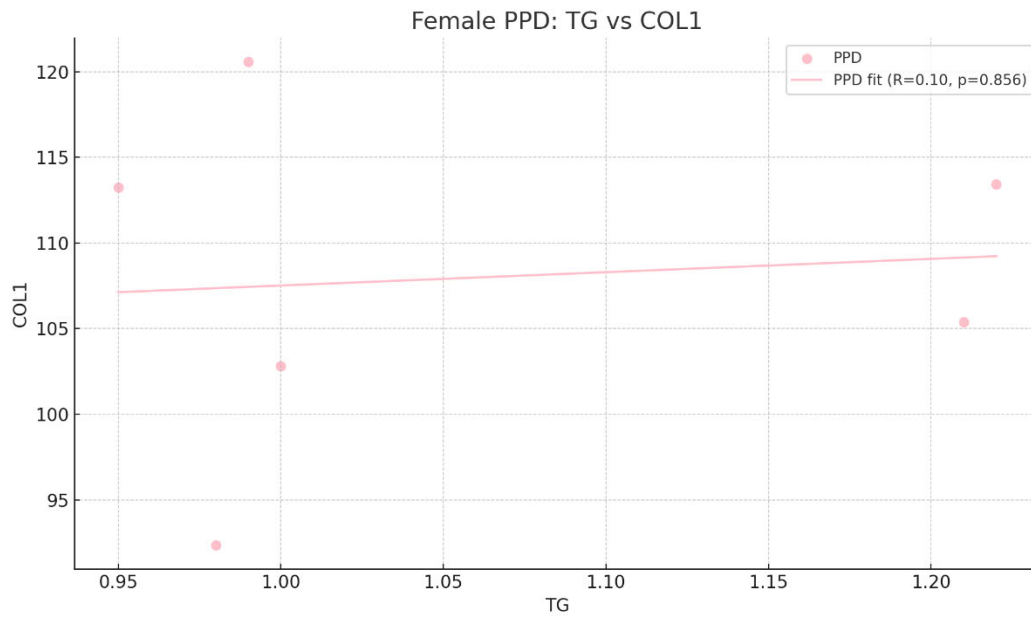
sep Female PPD TG vs Cardiac area separated



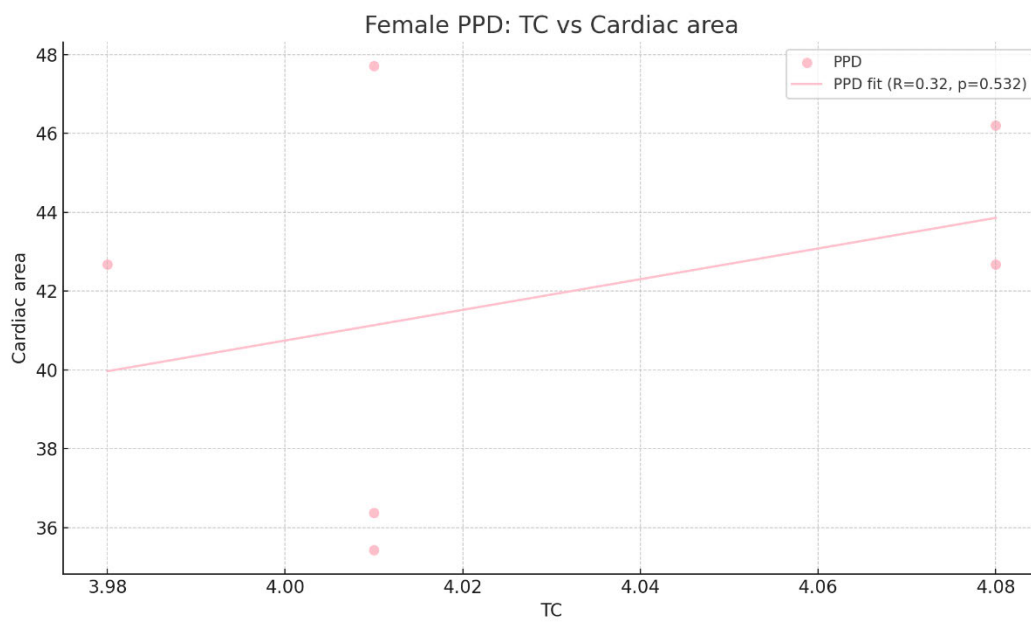
sep Female PPD TG vs cTnI separated



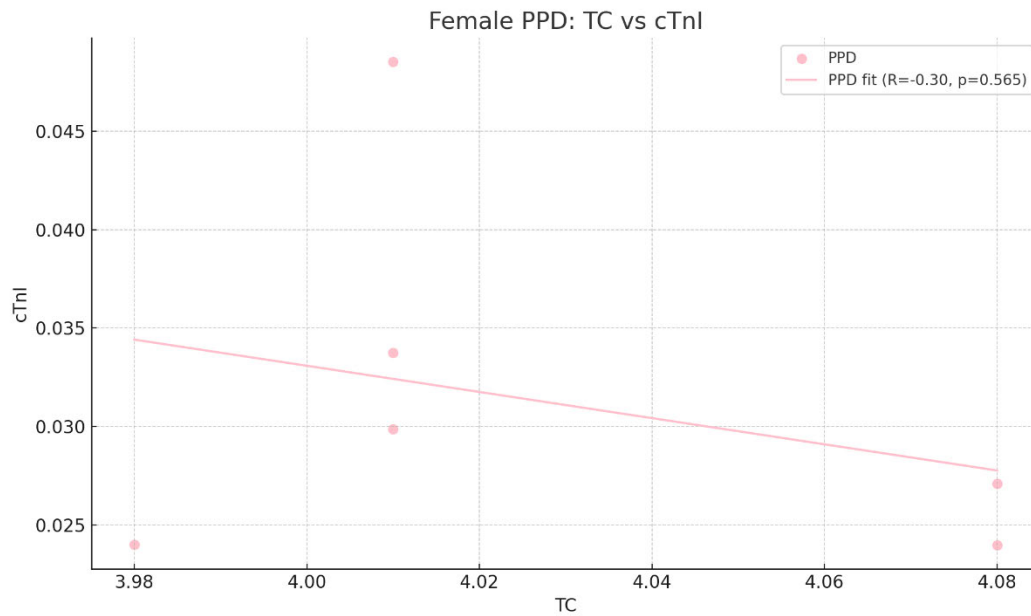
sep Female PPD TG vs COL1 separated



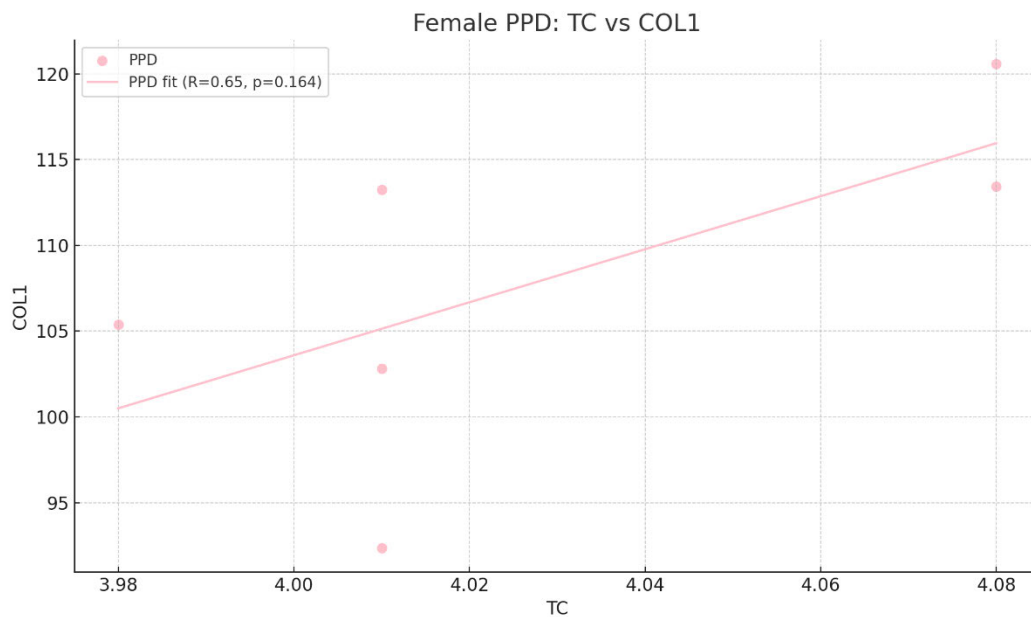
sep Female PPD TC vs Cardiac area separated



sep Female PPD TC vs cTnI separated



sep Female PPD TC vs COL1 separated



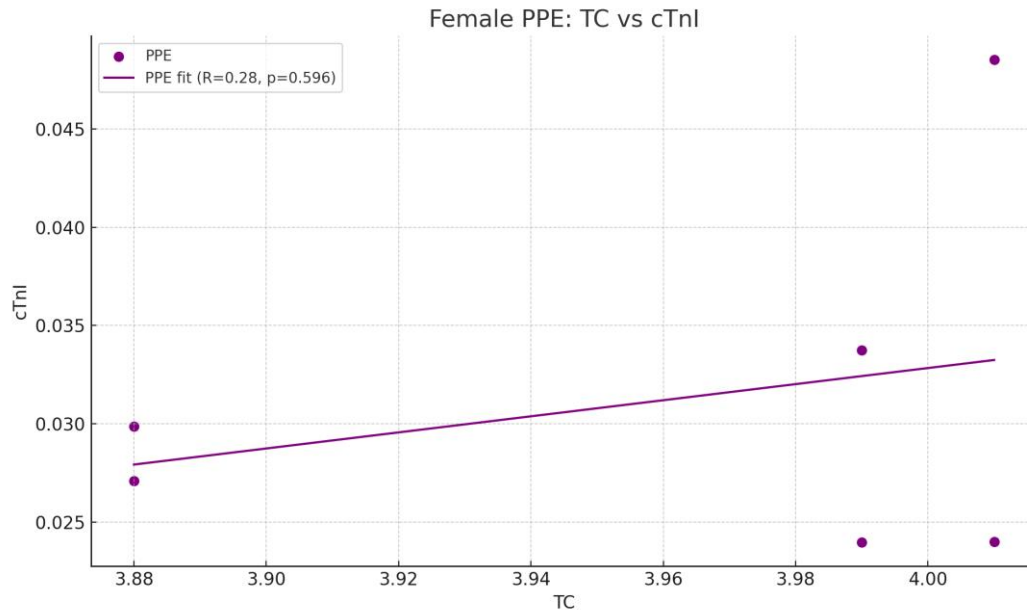
Separated Group Regression Table – How to Read

Each row corresponds to a single sex $\times$ group $\times$ exposure (MAP, TG, TC) $\times$ outcome (cardiac area, cTnI, COL1) comparison. The slope indicates the direction and magnitude of change in the outcome per unit increase in the exposure. R and R^2 capture the strength of the linear association, and the p-value indicates statistical significance ($\alpha = 0.05$). N reflects the number of observations available for that exposure–outcome pair.

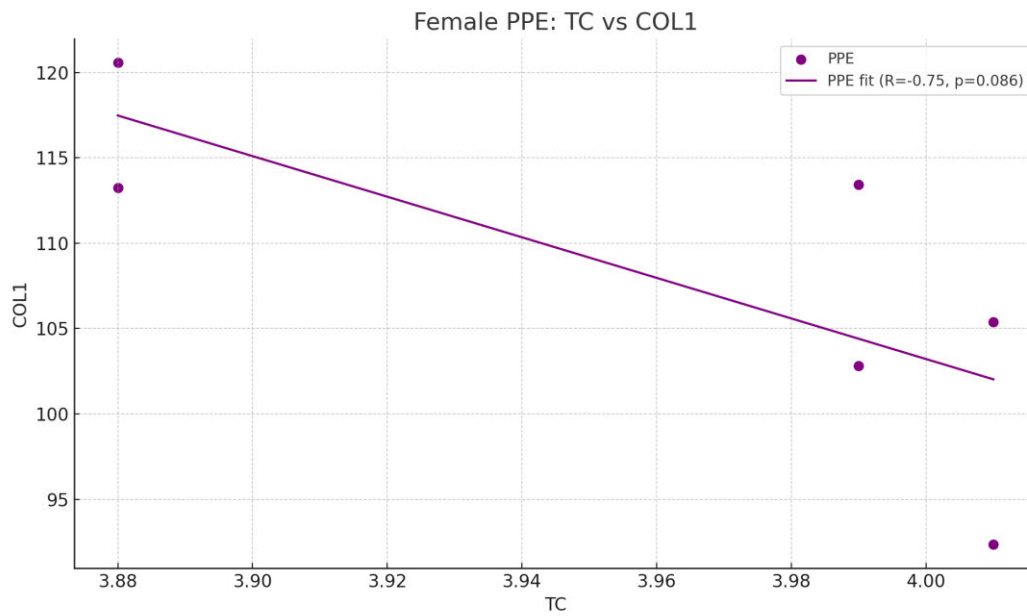
Excerpt of separated group regression results (full tables linked separately):

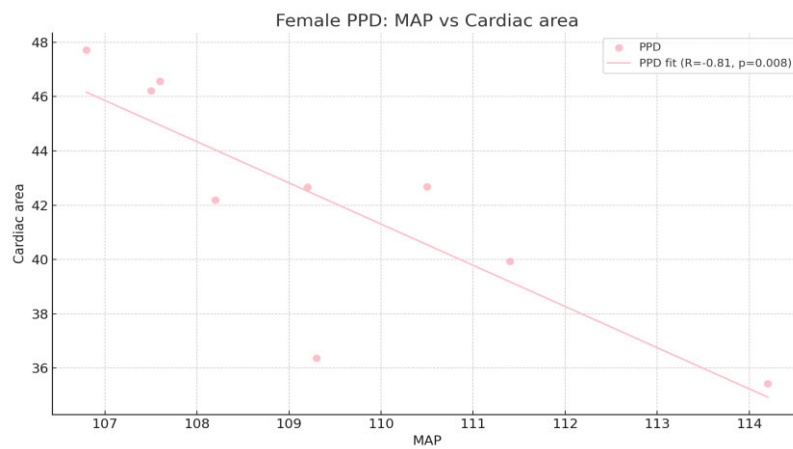
Sex	Group	X	Y	N	Slope	Intercept	R	R^2	p-value
Male	PCON	MAP	Cardiac area	9	-1.165	172.5	-0.4959	0.2459	0.1746
Male	PCON	MAP	cTnI	9	-0.001003	0.1492	-0.1763	0.03107	0.6501
Male	PCON	MAP	COL1	9	2.842	-178	0.4994	0.2494	0.171
Male	PCON	TG	Cardiac area	6	0.1551	51.57	0.001781	3.173e-06	0.9973
Male	PCON	TG	cTnI	6	-0.09114	0.1331	-0.3297	0.1087	0.5234
Male	PCON	TG	COL1	6	122	0.01559	0.4586	0.2103	0.3603
Male	PCON	TC	Cardiac area	6	-56.34	278.2	-0.7754	0.6013	0.06998
Male	PCON	TC	cTnI	6	-0.07865	0.3621	-0.3411	0.1163	0.5082
Male	PCON	TC	COL1	6	99.28	-282.4	0.4474	0.2002	0.3737
Male	PPE	MAP	Cardiac area	9	0.1009	40.65	0.05875	0.003451	0.8807
Male	PPE	MAP	cTnI	9	0.001716	-0.1508	0.4126	0.1702	0.2698
Male	PPE	MAP	COL1	9	-0.1211	129.6	-0.02912	0.0008481	0.9407
Male	PPE	TG	Cardiac area	6	25.31	20.03	0.4484	0.2011	0.3724
Male	PPE	TG	cTnI	6	-0.03514	0.08992	-0.1961	0.03847	0.7096
Male	PPE	TG	COL1	6	-86.48	225	-0.5016	0.2516	0.3107
Male	PPE	TC	Cardiac area	6	-39.63	211.5	-0.6599	0.4354	0.1539
Male	PPE	TC	cTnI	6	-0.04999	0.2475	-0.2622	0.06876	0.6157
Male	PPE	TC	COL1	6	86.71	-232.9	0.4727	0.2235	0.3437
Male	PPD	MAP	Cardiac area	9	-0.6126	119.4	-0.4125	0.1702	0.2698

Male	PPD	MAP	cTnI	9	-	0.1272	-0.2069	0.04282	0.5932
0.0007443									



Female PPE TC vs COL1

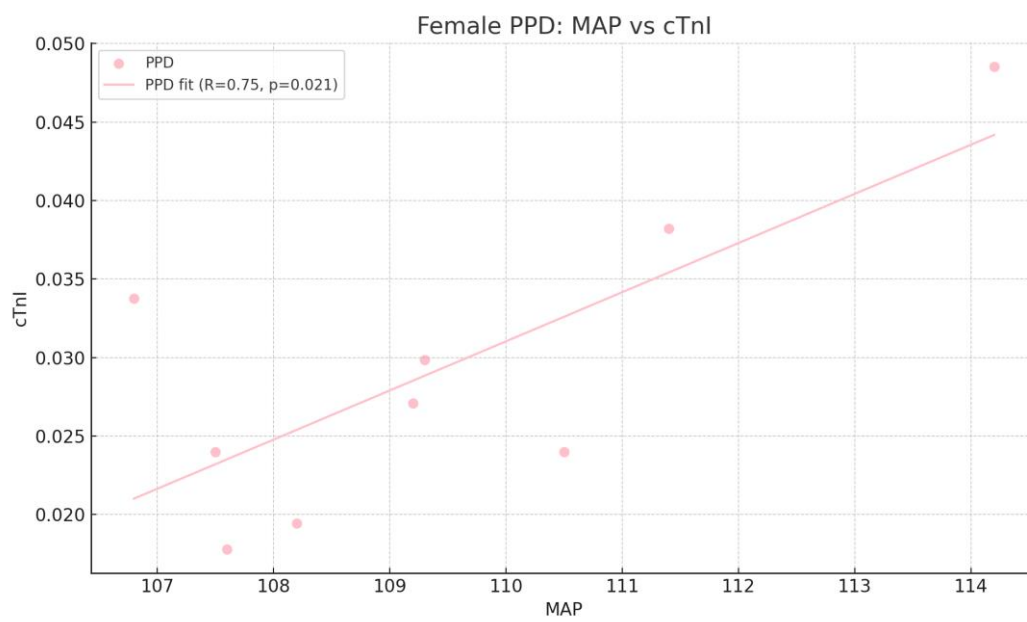




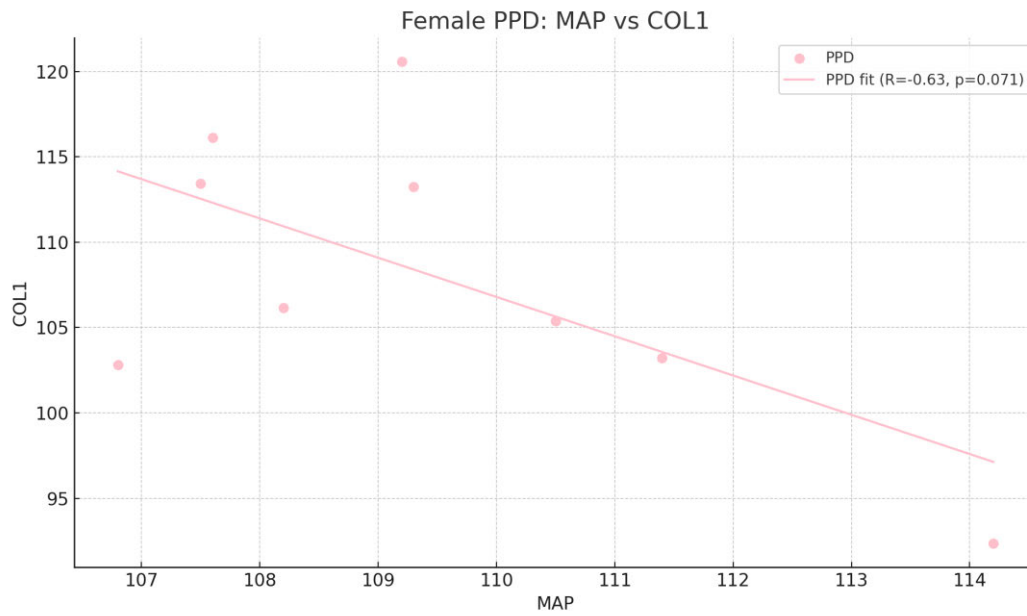
Female PPD MA
area

P vs Cardiac

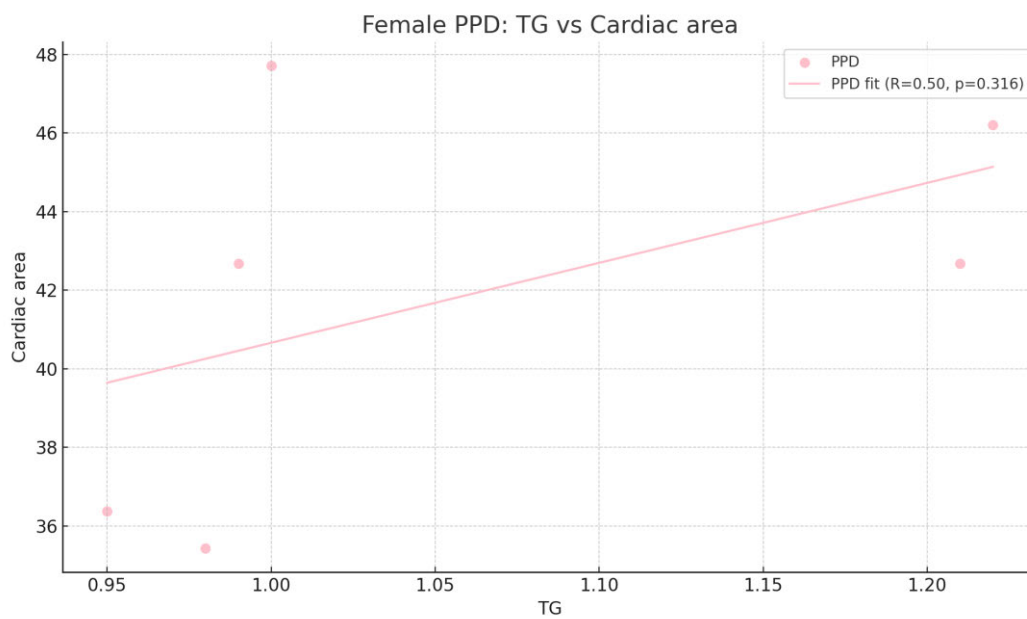
Female PPD MAP vs cTnI



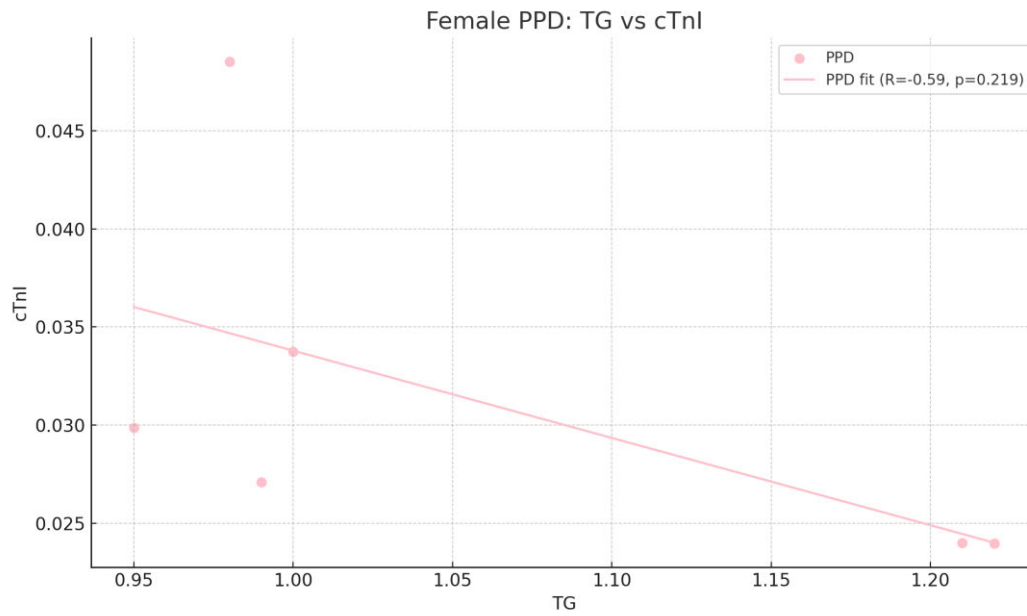
Female PPD MAP vs COL1



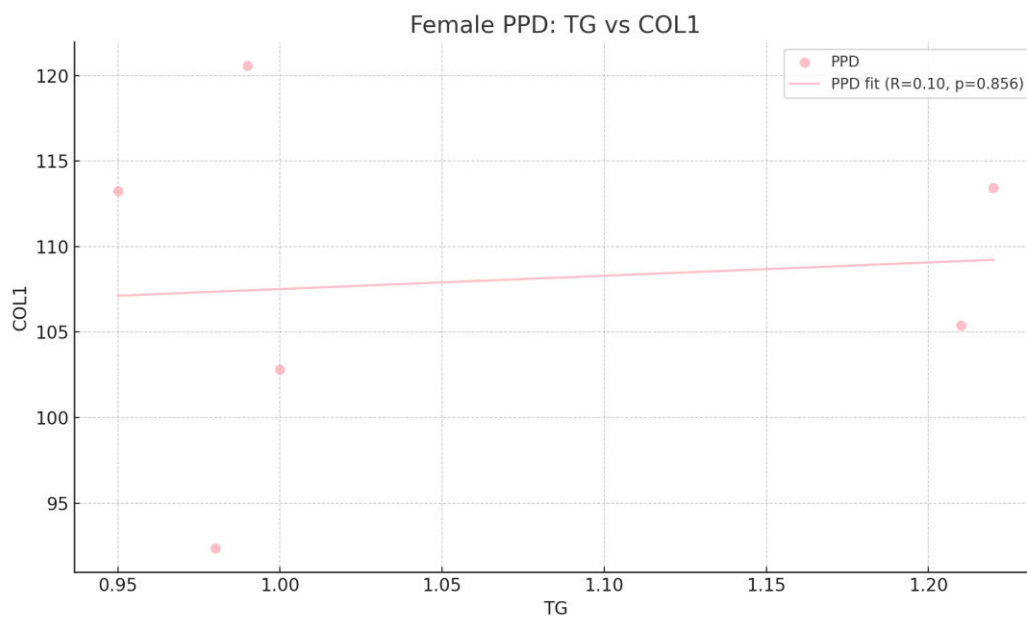
Female PPD TG vs Cardiac area



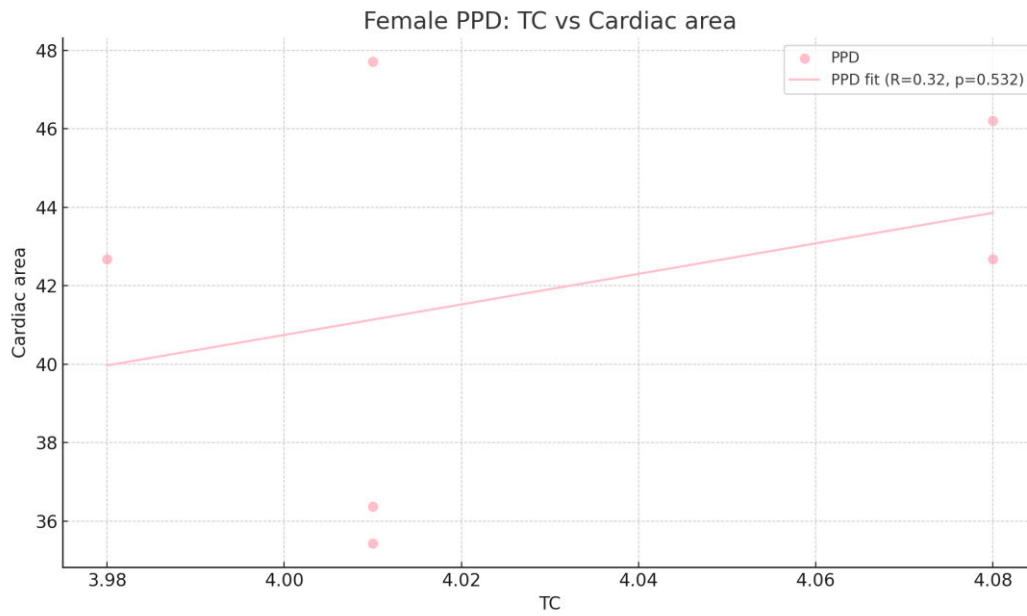
Female PPD TG vs cTnI



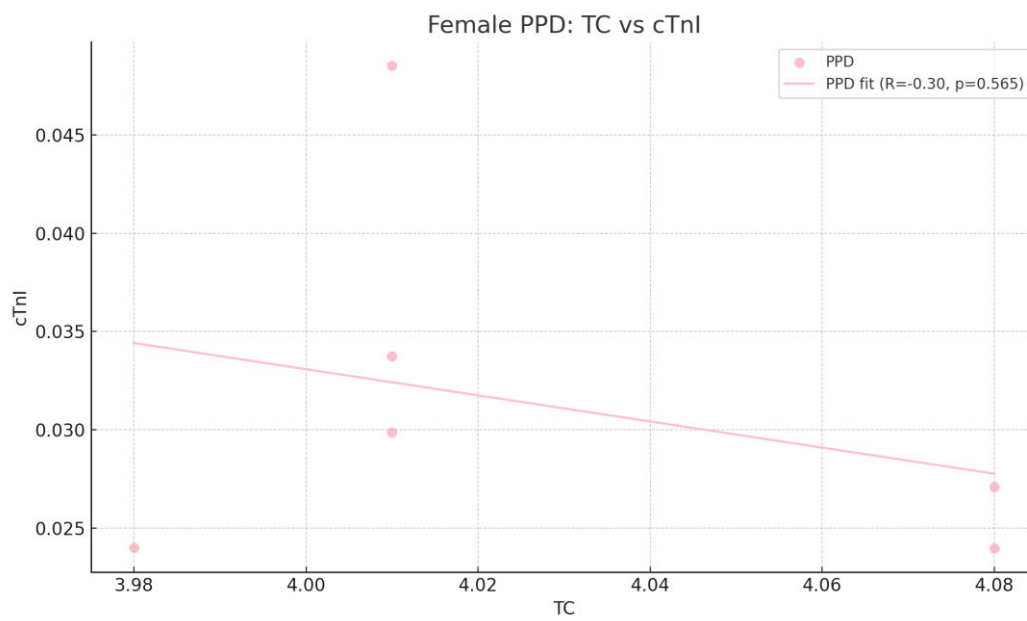
Female PPD TG vs COL1



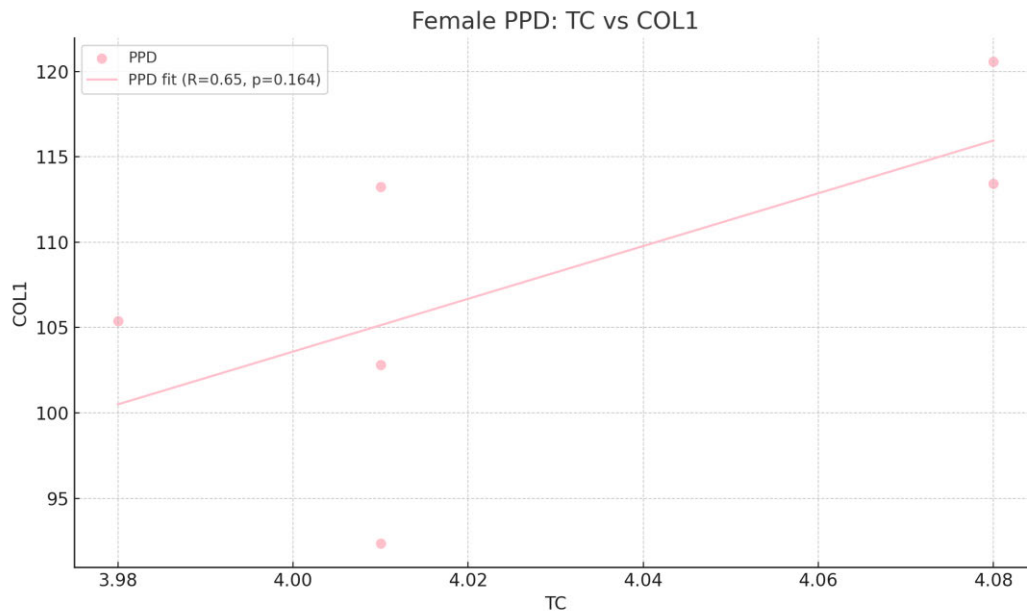
Female PPD TC vs Cardiac area



Female PPD TC vs cTnI



Female PPD TC vs COL1 I



Separated Group Regression Table – How to Read

Each row corresponds to a single sex $\times$ group $\times$ exposure (MAP, TG, TC) $\times$ outcome (cardiac area, cTnI, COL1) comparison. The slope indicates the direction and magnitude of change in the outcome per unit increase in the exposure. R and R² capture the strength of the linear association, and the p-value indicates statistical significance ($\alpha = 0.05$). N reflects the number of observations available for that exposure–outcome pair.

Excerpt of separated group regression results (full tables linked separately):

Sex	Group	X	Y	N	Slope	Intercept	R	R ²	p-value
Male	PCON	MAP	Cardiac area	9	-1.165	172.5	-0.4959	0.2459	0.1746
Male	PCON	MAP	cTnI	9	-0.001003	0.1492	-0.1763	0.03107	0.6501
Male	PCON	MAP	COL1	9	2.842	-178	0.4994	0.2494	0.171
Male	PCON	TG	Cardiac area	6	0.1551	51.57	0.001781	3.173e-06	0.9973
Male	PCON	TG	cTnI	6	-0.09114	0.1331	-0.3297	0.1087	0.5234
Male	PCON	TG	COL1	6	122	0.01559	0.4586	0.2103	0.3603

Male	PCON	TC	Cardiac area	6	-56.34	278.2	-0.7754	0.6013	0.06998
Male	PCON	TC	cTnI	6	-0.07865	0.3621	-0.3411	0.1163	0.5082
Male	PCON	TC	COL1	6	99.28	-282.4	0.4474	0.2002	0.3737
Male	PPE	MAP	Cardiac area	9	0.1009	40.65	0.05875	0.003451	0.8807
Male	PPE	MAP	cTnI	9	0.001716	-0.1508	0.4126	0.1702	0.2698
Male	PPE	MAP	COL1	9	-0.1211	129.6	-0.02912	0.0008481	0.9407
Male	PPE	TG	Cardiac area	6	25.31	20.03	0.4484	0.2011	0.3724
Male	PPE	TG	cTnI	6	-0.03514	0.08992	-0.1961	0.03847	0.7096
Male	PPE	TG	COL1	6	-86.48	225	-0.5016	0.2516	0.3107
Male	PPE	TC	Cardiac area	6	-39.63	211.5	-0.6599	0.4354	0.1539
Male	PPE	TC	cTnI	6	-0.04999	0.2475	-0.2622	0.06876	0.6157
Male	PPE	TC	COL1	6	86.71	-232.9	0.4727	0.2235	0.3437
Male	PPD	MAP	Cardiac area	9	-0.6126	119.4	-0.4125	0.1702	0.2698
Male	PPD	MAP	cTnI	9	-0.0007443	0.1272	-0.2069	0.04282	0.5932

TG raw data

PCON-M	PPE-M	PPD-M		PCON-F	PPE-F	PPD-F	
0,91	1,21	1,2		0,9	0,95	1	
0,95	1,33	1,33		0,91	0,99	1,21	
0,91	1,23	1,23		0,91	1,21	0,99	
1	1,33	1,21		1	0,91	1,22	
0,98	1,2	1,33		0,99	1,2	0,98	
0,99	1,21	1,23		0,99	0,98	0,95	

TC Raw data

PCON-M	PPE-M	PPD-M		PCON-F	PPE-F	PPD-F
3,99	3,98	4,11		3,98	3,99	4,01
4,01	4,01	4,21		3,88	4,01	3,98
3,98	3,99	4,08		3,88	3,88	4,08
3,98	4	4,22		4,01	3,99	4,08
4,08	4,1	4,08		3,88	4,01	4,01
4,08	4,11	4,21		4,01	3,88	4,01

cTnI Raw data

PCON-M	PPE-M	PPD-M		PCON-F	PPE-F	PPD-F
1,16	89,26	93,05		1,05	80,36	83,75
0,69	92,09	108,38		0,62	82,88	97,54
1,51	77,17	89,88		1,39	70,26	80,89
0,83	77,98	98,36		0,75	71,18	88,52
1	84,63	100,72		1,07	75,31	90,65
0,89	83,62	94,12		0,89	77,03	84,71

COL I Raw data

PCON-M	PPE-M	PPD-M		PCON-F	PPE-F	PPD-F
0,9	15,32	18,67		0,78	10,22	12,35
0,57	17,45	19,84		0,52	12,14	14,76
0,83	12,89	15,42		1	10,47	11,67
0,73	13,77	17,11		0,63	10,39	12,99
0,52	13,33	17,74		0,89	11,45	13,88
0,88	16,38	18,34		0,55	11,79	14,33

MAP Raw Data

PCON-M	PPE-M	PPD-M		PCON-F	PPE-F	PPD-F
103,8	112,7	114,1		101,4	110,1	106,8
103,8	111,1	112,2		102,1	111,2	110,5
102,3	115,2	111,3		100,2	113,2	109,2
102,8	114,5	107,4		100,4	116,4	107,5
106,2	114,1	109,5		101,2	114,7	114,2
105,4	113,2	110,8		101,1	112	109,3
101,5	113,5	107,5		102,1	111,3	108,2
102,8	118,3	106,2		100,3	116,8	111,4
101,5	117,2	108,5		100,5	114,7	107,6

cTnI Raw data

PCON-M	PPE-M	PPD-M		PCON-F	PPE-F	PPD-F
510,285	1470,725	1849,975		355,772	1360,674	1717,266
494,809	1491,157	1711,649		470,07	1374,97	1596,82
547,944	1340,71	1657,379		426,548	1233,252	1541,785
454,473	1390,155	1481,105		361,75	1174,847	1377,838
447,944	1530,865	1569,243		426,548	1321,335	1241,785
502,547	1390,155	1411,106		454,835	1431,335	1517,441

Cardiomyocyte Area Raw data

PCON-M	PPE-M	PPD-M		PCON-F	PPE-F	PPD-F
127,65	140,331	138,227		120,526	133,851	135,738
125,446	136,470	139,345		123,922	130,380	139,996
122,197	132,867	144,531		121,572	133,927	136,912
128,343	133,882	142,217		123,033	131,374	138,150
123,836	141,618	141,323		122,002	134,588	138,502
122,974	140,485	146,737		120,163	130,442	134,637

8.3 APPENDIX 3: Journal guideline of american journalof physiology-heart and circulatory physiology

Manuscript Formatting and Composition

FLEXSUBMIT allows initial manuscripts to be submitted in a **format-neutral style**, even as a single merged PDF. If the required information is present (author details, full text, figures and tables, etc.), the manuscript will be evaluated on its scientific merit.

All revised manuscripts should be reformatted according to journal guidelines.

Formats And Styles

Manuscripts may be submitted to the [APS peer review system](#) in the following formats:

8.4.1.1 Microsoft Word (.docx)

8.4.1.2 Rich Text Format (.rtf)

8.4.1.3 LaTeX (.tex)

8.4.1.4 PDF (.pdf)

Manuscript submissions should contain all required elements, such as the title page, abstract, main text, references, and any hyperlinks to data supplements. Please note that PDF (.pdf) cannot be used for production of accepted articles.

LaTeX submissions must contain all necessary components to convert properly: .tex, .bbl / .bib, .cls, .bst. The fewer the number of components submitted, the easier conversion will be.

APS journals follow standard American English style and usage. Authors for whom English is not their native language are encouraged to seek the aid of a professional English language editorial service.

All manuscripts are edited by highly trained professional copy editors, according to the APS house style and guidelines.

Abbreviations, Symbols, and Terminology

Abbreviations should be defined at first usage. However, internationally accepted (or otherwise compellingly conventional) abbreviations do not need to be defined.

For **special characters** not available on the standard keyboard (e.g., Greek characters, mathematical symbols, figure symbols), use the Symbol font or use the “Insert Symbol” function in Microsoft Word; do not use image files (e.g., .gif) within the text.

Proprietary **trade names** should be capitalized, with the spelling carefully checked. The generic name or generic descriptor should accompany the trade name the first time it appears.

For **gene, protein, and species nomenclature**, follow current established conventions for properly presenting symbols and names, in accordance with the appropriate official organization.

8.4.1.5 Human genes: HUGO Gene
Nomenclature Committee
([HGNC](#))

8.4.1.6 Mouse genes: Mouse Genomic
Informatics ([MGI](#))

8.4.1.7 Rat genes: Rat Genome Database
([RGD](#))

Title Page

All submissions must contain a title page: full article title; author name(s); author affiliations where the work was done; a running head; the corresponding author’s information, and abstract elements.

Article Title And Authors

THE **TITLE** should be succinct and informative. It should include key words to aid search discoverability. LIMIT: 160 characters and spaces

List all **AUTHORS** by surname and given names (or initials) *exactly* as they should appear online and in PDF.

“Group authorship” is allowed, with the name of a group (such as a consortium or program) listed as an author, with members of the group listed in the Acknowledgments section.

Authors may present their names in **non-Latin characters** (in their native writing system) alongside the standard English transliteration of their name; for example, “Ta-Ming Wang (王大明).”

For **AFFILIATIONS**, list all departments and institutions where the work was done, with city, state, and country. Affiliation must reflect the organization(s) supporting the

author(s) *while the research was being done*. This may differ from the *current affiliations* of the author(s), which should be listed in the Acknowledgments section as the present address(es) of the author(s).

The **RUNNING HEAD** is an abbreviated version of the title, which will appear at the top of every PDF page after the first page. LIMIT: 60 characters and spaces

Only one author may be designated as the **CORRESPONDING AUTHOR** for manuscript invoicing and proofs; however, additional co-corresponding authors may be included for reader communication purposes.

Abstract

A one-paragraph **ABSTRACT** should accompany all full-length manuscripts (e.g., Research Articles, Short Reports, Reviews). The abstract should state what was done and why, what was found, and what was concluded. LIMIT: 250 words

NEW & NOTEWORTHY is a brief summary of the novel findings in a research article. LIMIT: 75 words

[Physiological Reviews](#) requires a **Clinical Highlights** summary for all Reviews (200 words).

Include up to five **KEY WORDS** most relevant to the article. Avoid abbreviations. This is important for the most optimal discoverability of your article.

Main Text Sections

The **INTRODUCTION** should provide a brief overview of the scope and relevance of the study, especially regarding previous advancements in related fields.

The **MATERIALS AND METHODS** section describes techniques, cell/animal models used (including species, strain, and sex), and lists of reagents, chemicals, and equipment, as well as the names of manufacturers and suppliers, including [Research Resource Identifiers \(RRIDs\)](#) when possible. Also in this section, describe the statistical methods that were used to evaluate the data. See [Rigor and Reproducibility Guidelines \(PDF\)](#) for more information.

All animal or human studies must contain an explicit statement that the protocols were submitted to, and approved by, an institutional review board or committee or that the protocols were performed under a license obtained from such a committee, board, or governing office (see [Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training](#)).

For studies involving humans or animals, the **sex and/or gender** of participants or models must be reported. *AJP-Heart and Circulatory Physiology* expects authors to consider use of more than one sex or gender in experimental design, analysis, and reporting. See [Sex and Gender requirement](#).

Experimental **RESULTS** present the experimental data and their statistical significance. Also see [Rigor and Reproducibility Guidelines \(PDF\)](#).

APS has published an [editorial](#) on the use of statistics, and authors are encouraged to consult it. *Advances in Physiology Education* supports adherence of two of the guidelines:

8.4.1.8 Guideline 5. Report variability using a standard deviation.

8.4.1.9 Guideline 7. Report a precise P value.

Use the **DISCUSSION** to explain your interpretation of the data, especially compared with previously published material cited in the References. Significance and limitations may also be present.

An **APPENDIX** may be included in mathematical modeling or computational papers, e.g., to provide details of a solution strategy.

A **GLOSSARY** may be included in equation-laden articles with many different symbols (such as mathematical modeling or computational papers). The glossary will appear before the Acknowledgments. See [this article](#) for an example.

The **ACKNOWLEDGMENTS** section is where to thank people indirectly involved with the research (e.g., technical assistance, gifts of reagents, loans of equipment, suggestions during writing). Preprints should also be cited here: "Preprint is available at <https://doi.org/...>" Dedications of articles to persons living or deceased are not permitted.

Current addresses of authors (which may differ from those in the affiliation line) should be included here.

List the **GRANTS**, fellowships, and donations that funded (partially or completely) the research. Please include the grant identification number and the recipient name(s).

Authors must list all **DISCLOSURES** at the time of submission to disclose any perceived or potential conflict of interest, financial or otherwise. See [Author Conflict of Interest](#). If the article is accepted for publication, this information, or the lack of potential conflicts, will be published in the Disclosures section.

Any **DISCLAIMERS** by the authors, their institution, or funding body may also be included. Example: “The content is solely the authors’ responsibility and does not necessarily represent the official views of the [institution].”

Identify all **AUTHOR CONTRIBUTIONS** for the study: Conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, approved final version of manuscript. The information must be the same as in the online journal submission site.

Figure Legends And Tables

FIGURE LEGENDS should describe the relevant details of the figure, placing it in the proper context of the manuscript. Legends should sufficiently describe the figure on their own, without reference to the main text. Define all statistical symbols and abbreviations.

TABLES should adhere to the following guidelines:

- 8.4.1.10 Tables must be submitted in Word table format, not as tabbed text or as embedded graphics.
- 8.4.1.11 Table titles should briefly describe the data presented.
- 8.4.1.12 Notes, symbols, and abbreviations should be defined in the table legend.

- 8.4.1.13 Statistical measures of variations, SD, SE, etc., must be identified in the legend.

See [Rigor and Reproducibility Guidelines \(PDF\)](#) for more guidance.

Math and Computer Simulations

EQUATIONS and complex math should be prepared using a math editor such as Microsoft Word Equation Editor or MathType (available from [wiris](#)). In-text math that can be regular text should be prepared using bold, italics, superscript, and subscript together with characters in the Symbol or Greek fonts (e.g., $x_2[n - 2\delta]$).

Mathematical equations should be simplified as much as possible and carefully checked.

- 8.4.1.14 **Symbols** should be defined as they first appear in the text.

- 8.4.1.15 A **glossary** may be included in articles with many different symbols, specifying the units (dimensions) as well as each definition.

Presentation of **MATHEMATICAL MODELS** should be sufficiently clear to allow physiologists with limited experience in modeling to follow the model development, limitations, and physiological relevance. Assumptions concerning the importance of

physiological processes included in the model should be clearly stated. See [Mathematical Models policy](#).

For **COMPUTER SIMULATIONS** using original author-generated code, authors are *encouraged* to deposit software and code via a public repository, such as [GitHub](#), if not already deposited to a public repository. [Computer Simulations policy](#).

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References

Your references should appear in the References section in numerical order, in the order you cited them in your manuscript.

Only published or accepted articles should appear in your reference list. We consider Preprints published work and they should appear in the Reference section.

Reference Manager Templates

We've created reference manager templates for EndNote and Mendeley/Zotero. Here is how you can use them.

8.4.1.16 **EndNote template:**

8.4.1.16.1 [Download the EndNote template](#) (first save the file to your computer (as it will not open directly) and then use EndNote to open the style and save it).

8.4.1.17 **Mendeley/Zotero:**

8.4.1.17.1 [Download the Mendeley/Zotero template.](#)

8.4.1.17.2 To load the CSL file in Mendeley Desktop:

8.4.1.18 Open the "View" menu.

8.4.1.18.1.1 Select "Citation Style," then "More Styles...".

8.4.1.18.2 To load CSL file from Word:

8.4.1.18.2.1 Open the "References" menu.

8.4.1.18.2.2 Select "Mendeley Cite," then "Select another style...".

8.4.1.18.2.3 Choose "Add custom style," enter URL.

Examples

We've provided a list of examples of references to show the APS reference style in context.

JOURNAL ARTICLE

Holowatz LA, Houghton BL, Wong BJ, Wilkins BW, Harding AW, Kenney WL, Minson CT. Nitric oxide and attenuated reflex cutaneous vasodilation in aged skin. *Am J Physiol Heart Circ Physiol* 284: H1662–H1667, 2003. <https://doi.org/10.1152/ajpheart.00871.2002>.

BOOK CHAPTER

Pollock DM. Endothelin receptor subtypes and tissue distribution. In: *Endothelin Molecular Biology, Physiology, and Pathology*, edited by Highsmith RF. Totowa, NJ: Humana, 1998.

ONLINE CONTENT

The general format is Author/editor (if known). (Revision or copyright date, if available). Title of page [Publication medium]. Page publisher. URL (Protocol://Site/Path/File) [Access date].

Dudoit S, Yang YH, Callow MJ, Speed TJ. Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments [Online]. Dept. of Statistics, Univ. of California at Berkeley. <http://www.stat.berkeley.edu/users/terry/zarray/Html/matt.html> [3 Sept. 2000].

CITING UNPUBLISHED OBSERVATIONS AND PERSONAL COMMUNICATIONS

Unpublished materials, observations, or personal communications should be cited only in the text. This includes, but is not limited to the following circumstances:

- 8.4.1.19 to publish information disclosed in a personal communication or unpublished observation;
- 8.4.1.20 to recognize additional individuals who helped in preparation of the manuscript;
- 8.4.1.21 for permission from a copyright holder to discuss information that has been accepted for publication but is “in press” and not yet available, online, or otherwise.
- 8.4.1.22 For both unpublished observations and personal communications, provide the cited person’s last name and initials.

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Preparing Figures

Data Presentation

All accepted materials are evaluated for compliance with [APS policies](#) on image assembly and presentation. See also [Digital Image Ethics Poster](#).

DIGITAL IMAGES (gel, blots, micrographs) should contain a size marker and retain space above and below the band of interest from the original image.

Authors should not move, remove, introduce, obscure or enhance any specific feature within an image. See **R&R Policy link** for additional guidelines.

GRAPHICAL DATA should be formatted to best convey the variability in the results. Authors are strongly encouraged to consider dot-whisker plots instead of bar or line graphs.

Use of **PHOTOGRAPHS** to demonstrate experimental staging, tools, and apparatus in an experimental procedure should be kept to a minimum. Drawings and diagrams representing experimental procedures should be used in place of photographs whenever possible.

PREVIOUSLY PUBLISHED ILLUSTRATIONS may be included in Review articles if scientifically appropriate and permission is obtained from both the original author and publisher. Authors are responsible for:

- 8.4.1.23 obtaining and including permission letters with their submitted manuscript, and
- 8.4.1.24 for providing publication-quality electronic files of the previously published illustrations.

For guidance on securing permission [review our permissions information](#).

For authors using **THIRD-PARTY ART RESOURCES** (e.g., [BioRender](#), [Sevier SMART](#), [Bioicons](#)) to create figures for publication:

8.4.1.25 You must have a paid subscription license (single user, lab, or institutional) at the time of export to have permission to publish in journals.

8.4.1.26 Download proof of publication license and upload it as a “Figure Permission File” with the rest of the manuscript files.

*NOTE: Canva.com does not allow for transfer of licensing, so their images cannot be used in APS journal articles.

Figure Preparation

FIGURE SIZES should match their expected appearance in the journal.

No. of Columns	Inches	Centimeters	Picas
Single	≤ 3.5	≤ 8.9	≤ 21
Double (side legend)	4–5	10–12	25–30
Full Width	6–7.15	15–18	36–43
Maximum Depth	9	22.8	54

Recommended **FONTS** include Arial, Helvetica, Times or Times New Roman, and Symbol, to prevent dropped characters or missing symbols.

Authors are strongly encouraged to create color figures in a manner that will allow the data to be read by **COLORBLIND READERS**. Choose color palettes that can be easily identified by all.

Sites such as [ColorBrewer](#) and [Paul Tol’s Colour Schemes](#) can assist in choosing a colorblind-friendly color palette. Authors are also encouraged to upload completed figures to the [Coblis Color](#)

[Blindness Simulator](#) to ensure readability. Additional resource: [How to make scientific figures accessible to readers with color-blindness](#) (ascb.org)

Graphic File Formats

EPS and **TIFF** are the file formats required for publication. **PDF** is also acceptable.

NOTE: Do not manually increase the resolution of TIFF images once it is set to avoid diminished quality.

Avoid using MS PowerPoint, when possible, to prepare your figures. JPG, GIF, PNG, Excel, and Word files, etc. are also not recommended formats.

IMAGE RESOLUTION requirements only apply to TIFF and JPG. These are most often photographic captures. They do not apply to EPS, AI, PPT, and PDF files UNLESS they also contain raster images.

Image Type	Resolution
Photographs ONLY (color and grayscale)	300 dpi
Photographs with text or line art/graphic elements (color and grayscale)	600 dpi
Line Art (graphs, dot plots)	600–1200

Figure Creation Tips

[Creating PDFs optimized for print \(PC and MAC\)](#) [Microsoft PowerPoint tips](#)

[Adobe Photoshop tips](#) [Adobe Illustrator tips](#) [Go to top](#)

Supplemental Material

Relevant data not published in the main article supplements may be deposited in domain- specific or generalist data repositories to enable sharing with readers.

NOTE: these materials can be embargoed or shared privately until the manuscript is accepted. For specific details, see [Supplemental Data Policy](#).

For **SUPPLEMENTAL MATERIAL** (peer reviewed), authors should provide a complete list of all cited supplemental objects - up to 10 figures, tables, videos, audio, and computer code—with weblinks (preferably DOIs) under its own heading at the back of the manuscript, after the DISCUSSION. Example:

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1–SX: DOI. Supplemental Tables S1–SX: DOI.

NOTE: Methods should not be truncated in the main manuscript to save space since APS research articles do not have page limits.

DATA AVAILABILITY and sharing statements are required by all APS journals, following the SUPPLEMENTAL MATERIAL section. Example:

DATA AVAILABILITY

Source data for this study are openly available at [insert DOI(s) and/or URL(s) with accession numbers].

For other statement options see [Supplemental Data Policy](#).

MATERIALS FOR REVIEWER-USE ONLY (i.e., not published or cited) should be uploaded into the manuscript submission system during submission and labeled as supporting information. These data should not be mentioned in the manuscript text.

General questions regarding data supplements may be directed to the [APS Production Department](#).

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Graphical Abstracts

Graphical abstracts are intended to encourage browsing and help readers identify which papers are most relevant to their research interests. They appear in the final HTML version of full-length articles.

The graphical abstract can be a new image or a key figure from the original paper that clearly summarizes the content.

The graphical abstract should

- 8.4.1.27 be schematic (a drawing, diagram, graph, etc.)
- 8.4.1.28 be simple
- 8.4.1.29 be original-no trademark, copyright, or previously published images
- 8.4.1.30 use color
- 8.4.1.31 use text sparingly, mainly for labels
- 8.4.1.32 not include the heading “Graphical Abstract” within the image file.

Graphical Abstract Features by Journal

	When to Submit	Required Format?	Special Instructions
	Required?		
		Template B	
<u>AJP-Cell Physiology</u>	revision		
<u>AJP-Endocrinology and Metabolism</u>	revision	Template A	Must be used for figures
<u>AJP-Gastrointestinal and Liver Physiology</u>	revision		
<u>AJP-Heart and Circulatory Physiology</u>	optional		
<u>AJP-Lung Cellular and Molecular Physiology</u>	revision	Template B	
<u>AJP-Regulatory, Integrative and Comparative Physiology</u>	optional		

<i>AJP-Renal Physiology</i>	revision	Template A	Must be used for figures
<i>Journal of Applied Physiology</i>	revision	Template A	
<i>Journal of Neurophysiology</i>	revision		
<i>Physiological Genomics</i>	revision		
<i>Advances in Physiology Education</i>	optional		
<i>Physiology</i>	optional		
<i>Physiological Reviews</i>	initial submission		

Image Specifications

- 8.4.1.33 **Size:** Use a 4:3 ratio for your image (optimal: 40 x 30 picas / 6.5 x 5 inches / 17 x 13 cm / 2000 x 1500 px)
- 8.4.1.34 **Font:** Use one sans serif font (such as Arial or Helvetica) consistently, 10–14 points
- 8.4.1.35 **File type:** PDF (preferred), EPS, AI, PPT, TIFF, or PSD
- 8.4.1.36 **Resolution:** The image should have a minimum resolution of 300 dpi.
- 8.4.1.37 **Color:** RGB mode

Image Templates

Some APS journals require the use of templates. If the journal does not require a template, authors may still use one of the templates below.

TEMPLATE A

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Graphical Abstract Title

(not manuscript title)

[Download Template A](#)

TEMPLATE B

Graphical Abstract Title

(do not use manuscript title)

RESULTS

Schematic drawing

CONCLUSION (One sentence)

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Account name: Dove Medical Press Ltd

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Yours sincerely Ms Sandi McIver

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- 2) Your paper should not be under consideration by any other journal or publisher; and
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APPENDIX 5: JOURNAL GUIDELINES

Writing and formatting File format

We ask you to provide editable source files for your entire submission (including figures, tables and text graphics). Some guidelines:

- 8.4.2.1 Save files in an editable format, using the extension .doc/ .docx for Word files and .tex for LaTeX files. A PDF is not an acceptable source file.
- 8.4.2.2 Lay out text in a single-column format.
- 8.4.2.3 Use spell-check and grammar-check functions to avoid errors.

We advise you to read our [“Step-by-step guide to publishing with Elsevier”](#). Double anonymized peer review

While *Heliyon* by default operates a single anonymized peer review process, we offer authors to opt-in for a double anonymized review process which means author identities are concealed from reviewers and vice versa. To facilitate the double anonymized review process, we ask that you provide the following information in your cover letter:

The title page should include:

- 8.4.2.4 Article title
- 8.4.2.5 Author name(s)
- 8.4.2.6 Affiliation(s)

8.4.2.7 Acknowledgements

8.4.2.8 Declaration of Interest statement

8.4.2.9 Corresponding author address (full address is required)

8.4.2.10 Corresponding author email address

The anonymized manuscript should contain the main body of your paper including:

8.4.2.11 References

8.4.2.12 Figures

8.4.2.13 Tables

8.4.2.14 Acknowledgements

It is important that your anonymized manuscript does not contain any identifying information such as author names or affiliations. Some guidelines:

8.4.2.15 Ensure that author names and affiliations do not appear anywhere in the manuscript files (including figures) or supplemental information.

8.4.2.16 Use neutral terms when referring to previous publications by the author group, i.e., use "as shown previously [ref]" instead of "as shown in our earlier work [ref]".

8.4.2.17 Remove the author list, affiliations, and acknowledgements from the main text and provide these in the cover letter instead.

8.4.2.18 Ensure that the experimental procedures section does not contain any identifying information.

8.4.2.19 Ensure that author information is not embedded in any of the manuscript files' metadata. Such information can be found in the "properties" menu in PDF readers and word processor software.

8.4.2.20 If your submission includes datasets hosted on a separate repository, ensure that these are also anonymized for the duration of the review period. This includes the uploading of manuscripts to preprint servers.

Once a manuscript is ready to be accepted, the authors will be asked to include all the required information in the manuscript file.

Read more about [peer review](#). LaTeX

We encourage you to use our [LaTeX template](#) when preparing a LaTeX submission. You will be asked to provide all relevant editable source files upon submission or revision.

Support for your LaTeX submission:

8.4.2.21 [LaTeX submission instructions and templates.](#)

8.4.2.22 Journal Article Publishing Support Center [LaTeX FAQs and support.](#)

8.4.2.23 Researcher Academy's "[Beginners' guide to writing a manuscript in LaTeX](#)". Cover letter

A cover letter is optional for most submissions but required for manuscripts requesting double anonymized peer review.

All authors are welcome to provide a cover letter containing any additional information that you would like us to be aware of, such as information on suggested or excluded editors and referees.

Title page

You are required to include the following details in the title page information:

- 8.4.2.24 Article title. Article titles should be concise and informative. Please avoid abbreviations and formulae, where possible, unless they are established and widely understood, e.g., DNA).
- 8.4.2.25 Author names. Provide the given name(s) and family name(s) of each author. The order of authors should match the order in the submission system. Carefully check that all names are accurately spelled. If needed, you can add your name between parentheses in your own script after the English transliteration.
- 8.4.2.26 Add affiliation addresses, referring to where the work was carried out, below the author names. Indicate affiliations using a lower-case superscript letter immediately after the author's name and in front of the corresponding address. Ensure that you provide the full postal address of each affiliation, including the country name and, if available, the email address of each author.
- 8.4.2.27 Corresponding author. Clearly indicate who will handle correspondence for your article at all stages of the refereeing and publication process and also post-publication. This responsibility includes answering any future queries about your results, data, methodology and materials. It is important that the email address and contact details of your corresponding author are kept up to date during the submission and publication process.
- 8.4.2.28 Present/permanent address. If an author has moved since the work described in your article was carried out, or the author was visiting during that time, a “present address” (or “permanent address”) can be indicated by a footnote to the author's name. The address where the author carried out the work must be retained as their main affiliation address. Use superscript Arabic numerals for such footnotes.

Abstract

You are required to provide a concise and factual abstract which does not exceed 300 words. The abstract should briefly state the purpose of your research, principal results and major conclusions.

- 8.4.2.29 Abstracts must be able to stand alone as abstracts are often presented separately from the article.
- 8.4.2.30 Avoid references. If any are essential to include, ensure that you cite the author(s) and year(s).
- 8.4.2.31 Avoid non-standard or uncommon abbreviations. If any are essential to include, ensure they are defined within your abstract at first mention.

Structured abstract

In certain fields, a structured abstract, by means of appropriate headings, should be used to provide the context or background for your research. Some guidelines:

- 8.4.2.32 State the purpose of your research.
- 8.4.2.33 Outline basic procedures followed such as the selection of study subjects or laboratory animals and observational and analytical methods.
- 8.4.2.34 Include your main findings, providing specific effect sizes and their statistical significance, if possible, and your principal conclusions.

- 8.4.2.35 Emphasize new and important aspects of your study or observations. Keywords

You are required to provide 4 to 8 keywords for indexing purposes. Keywords should be written in English. Please try to avoid keywords consisting of multiple words (using “and” or “of”).

We recommend that you only use abbreviations in keywords if they are firmly established in the field.

Highlights

You are encouraged to provide article highlights at submission.

Highlights are a short collection of bullet points that should capture the novel results of your research as well as any new methods used during your study. Highlights will help increase the discoverability of your article via search engines. Some guidelines:

- 8.4.2.36 Submit highlights as a separate editable file in the online submission system with the word “highlights” included in the file name.
- 8.4.2.37 Highlights should consist of 3 to 5 bullet points, each a maximum of 85 characters, including spaces.

We encourage you to view example [article highlights](#) and read about the benefits of their inclusion.

Graphical abstract

You are encouraged to provide a graphical abstract at submission.

The graphical abstract should summarize the contents of your article in a concise, pictorial form which is designed to capture the attention of a wide readership. A graphical abstract will help draw more attention to your online article and support readers in digesting your research. Some guidelines:

- 8.4.2.38 Submit your graphical abstract as a separate file in the online submission system.
- 8.4.2.39 Ensure the image is a minimum of 531 x 1328 pixels (h x w) or proportionally more and is readable at a size of 5 x 13 cm using a regular screen resolution of 96 dpi.
- 8.4.2.40 Our preferred file types for graphical abstracts are TIFF, EPS, PDF or MS Office files.

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Stereochemistry abstract

For each important chiral compound, you are requested to supply a stereochemistry abstract detailing structure, name, formula and all available stereochemical information for eventual incorporation into a database. An abstract for only one enantiomer per compound is required.

Units

This journal requires you to use the international system of units (SI) which follows internationally accepted rules and conventions. If other units are mentioned within your article, you should provide the equivalent unit in SI.

Nomenclature

- 8.4.2.41 Please consult [IUPAC Nomenclature of Organic Chemistry](#) for further information.
- 8.4.2.42 Please consult [IUPAC Nomenclature of Inorganic Chemistry](#) for further information.
- 8.4.2.43 Please consult [IUBMB: Recommendations on Biochemical & Organic Nomenclature, Symbols & Terminology](#) for further information.
- 8.4.2.44 Please consult [IUGS: Nomenclature for geological time scales and rock names](#) for further information.
- 8.4.2.45 Please consult [IUPAP: Symbols, Units, Nomenclature and Fundamental Constants in Physics](#) for further information.
- 8.4.2.46 Please consult https://www.ncbi.nlm.nih.gov/genome/doc/internatprot_nomenguide/ for further information on protein nomenclature.
- 8.4.2.47 Please consult <https://genenames.org/> for further information on gene nomenclature.
- 8.4.2.48 *Phonetic transcription*

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- 8.4.2.49 Genus and species names must be written in italics through the whole manuscript

Math formulae

- 8.4.2.50 Submit math equations as editable text, not as images.
- 8.4.2.51 Present simple formulae in line with normal text, where possible.
- 8.4.2.52 Use the solidus (/) instead of a horizontal line for small fractional terms such as X/Y.
- 8.4.2.53 Present variables in italics.
- 8.4.2.54 Denote powers of e by exp.
- 8.4.2.55 Display equations separately from your text, numbering them consecutively in the order they are referred to within your text.

Tables

Tables must be submitted as editable text, not as images. Some guidelines:

- 8.4.2.56 Place tables next to the relevant text or on a separate page(s) at the end of your article.
- 8.4.2.57 Cite all tables in the manuscript text.

- 8.4.2.58 Number tables consecutively according to their appearance in the text.
- 8.4.2.59 Please provide captions along with the tables.
- 8.4.2.60 Place any table notes below the table body.
- 8.4.2.61 Avoid vertical rules and shading within table cells.

We recommend that you use tables sparingly, ensuring that any data presented in tables is not duplicating results described elsewhere in the article.

Figures, images and artwork

Figures, images, artwork, diagrams and other graphical media must be supplied as separate files along with the manuscript. We recommend that you read our detailed [artwork and media instructions](#). Some excerpts:

When submitting artwork:

- 8.4.2.62 Cite all images in the manuscript text.
- 8.4.2.63 Number images according to the sequence they appear within your article.
- 8.4.2.64 Submit each image as a separate file using a logical naming convention for your files (for example, Figure_1, Figure_2 etc).
- 8.4.2.65 Please provide captions along with the artwork.
- 8.4.2.66 Text graphics may be embedded in the text at the appropriate position. If you are working with LaTeX, text graphics may also be embedded in the file.
- 8.4.2.67 Scale bar and magnification details must be provided for micrographs. Artwork formats

When your artwork is finalized, “save as” or convert your electronic artwork to the formats listed below taking into account the given resolution requirements for line drawings, halftones, and line/halftone combinations:

- 8.4.2.68 Vector drawings: Save as EPS or PDF files embedding the font or saving the text as “graphics.”
- 8.4.2.69 Color or grayscale photographs (halftones): Save as TIFF, JPG or PNG files using a minimum of 300 dpi (for single column: min. 1063 pixels, full page width: 2244 pixels).
- 8.4.2.70 Bitmapped line drawings: Save as TIFF, JPG or PNG files using a minimum of 1000 dpi (for single column: min. 3543 pixels, full page width: 7480 pixels).
- 8.4.2.71 Combinations bitmapped line/halftones (color or grayscale): Save as TIFF, JPG or PNG files using a minimum of 500 dpi (for single column: min. 1772 pixels, full page width: 3740 pixels).

Please do not submit:

- 8.4.2.72 files that are too low in resolution (for example, files optimized for screen use such as GIF, BMP, PICT or WPG files).

8.4.2.73 disproportionally large images compared to font size, as text may become unreadable. Figure captions

All images must have a caption. A caption should consist of a brief title (not displayed on the figure itself) and a description of the image. We advise you to keep the amount of text in any image to a minimum, though any symbols and abbreviations used should be explained.

Statistical tests and post hoc corrections used in each table, figure, and figure subpanel are mentioned. Number of replicates for each experiment is included, Symbols of p values explained. Same requirements apply for data reported in supplemental figures and tables.

Provide captions in a separate file. Color artwork

If you submit usable color figures with your accepted article, we will ensure that they appear in color online.

Please ensure that color images are accessible to all, including those with impaired color vision. Learn more about [color and web accessibility](#).

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Please read our policy on [the use of generative AI and AI-assisted tools in figures, images and artwork](#), which states:

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Heliyon does not impose a limit on either the length of your article or on the number of figures and tables included. We strongly encourage you to present all information within the main article and eliminate supplementary content whenever possible. *Heliyon* accepts electronic supplementary content when appropriate, such as large dataset files, video and audio files, archives, and any other file containing information that cannot easily be incorporated into the main manuscript.

Some guidelines:

8.4.2.77 Cite all supplementary files in the manuscript text.

8.4.2.78 Submit supplementary materials at the same time as your article. Be aware that all supplementary materials provided will appear online in the exact same file type as received. These files will not be formatted or typeset by the production team.

- 8.4.2.79 Include a concise, descriptive caption for each supplementary file describing its content.
- 8.4.2.80 Provide updated files if at any stage of the publication process you wish to make changes to submitted supplementary materials.
- 8.4.2.81 Do not make annotations or corrections to a previous version of a supplementary file.
- 8.4.2.82 Switch off the option to track changes in Microsoft Office files. If tracked changes are left on, they will appear in your published version.

Video

This journal accepts video material and animation sequences to support and enhance your scientific research. We encourage you to include links to video or animation files within articles. Some guidelines:

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- 8.4.2.84 Clearly label files ensuring the given file name is directly related to the file content.
- 8.4.2.85 Provide files in one of our [recommended file formats](#). Files should be within our preferred maximum file size of 150 MB per file, 1 GB in total.
- 8.4.2.86 Provide “stills” for each of your files. These will be used as standard icons to personalize the link to your video data. You can choose any frame from your video or animation or make a separate image.
- 8.4.2.87 Provide text (for both the electronic and the print version) to be placed in the portions of your article that refer to the video content. This is essential text, as video and animation files cannot be embedded in the print version of the journal.

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It is the responsibility of the authors to anonymize any sensitive data before sharing.

Please read our guidelines on [sharing research data](#) for more information on depositing, sharing and using research data and other relevant research materials.

In general, for this journal, the following instructions from our [research data guidelines](#) apply. However, depending on the nature of your research, uploading your data in a publicly available repository may be a requirement prior to acceptance. In some cases, we may also require that the data be made available for peer-review purposes. Any embargoes on data sharing should be lifted prior to publication of your manuscript. We highly recommend that authors adhere to the highest data sharing standards stated in the [research data guidelines](#) to avoid unnecessary delays in processing their manuscript.

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You are **encouraged** view the Cell Press Author's guide to data sharing [here](#) and to:

- 8.4.2.88 Deposit your research data and code in a relevant repository. Datasets generated from sequencing, genomics, proteomics, peptidomics, metabolomics, structures and similar studies should be deposited in publicly available repositories as recommended in the [Cell Press data deposition guidelines](#). <https://www.cell.com/pb-assets/journals/research/cellpress/data/RecommendRepositories-1723210928843.pdf>
- 8.4.2.89 Provide access to the data for peer-review. If data availability is restricted or data release is embargoed, editors may request anonymous access to the data to conduct the review process. Authors may ask the repository to generate a token for such access which will be shared with the reviewers for anonymous evaluation.
- 8.4.2.90 Cite repository IDs in the Data and Code availability statement within the manuscript. Link the dataset(s) to your article.
- 8.4.2.91 All other raw data and codes should be included as supplemental files, and/ or deposited in a free-to-use open access platform such as Mendeley or GitHub.
- 8.4.2.92 Ensure that the deposited data are released and publicly available at the time of publication.
- 8.4.2.93 If data sharing is not possible, please provide an explanation in the Data and Code availability statement.
- 8.4.2.94 Describe in the Data and Code availability statement how you intend to share the resources produced from your research with the scientific community. Any resources generated in the study that may be subject to restricted sharing / cannot be shared / subject to patent rights should be clearly indicated in this section.

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You may be asked to provide the raw data connected to your paper for editorial review, so you should be prepared to provide access to these data. You should retain all raw data underlying your research manuscript for a reasonable time after publication. Please consult with your research institution on its data retention policy.

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To foster transparency, you are required to state the availability of any data at submission in line with the research data guidance above.

Ensuring data is available may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you can state the reason why (e.g., your research data includes sensitive or confidential information such as patient data) during the submission process. This statement will appear with your published article on ScienceDirect.

Read more about the importance and benefits of providing a [data statement](#). Data linking

Linking to the data underlying your work increases your exposure and may lead to new collaborations. It also provides readers with a better understanding of the described research.

If your research data has been made available in a data repository there are a number of ways your article can be linked directly to the dataset:

8.4.2.95 Provide a link to your dataset when prompted during the online submission process.

8.4.2.96 For some data repositories, a repository banner will automatically appear next to your published article on ScienceDirect.

8.4.2.97 You can also link relevant data or entities within the text of your article through the use of identifiers. Use the following format: Database: 12345 (e.g. TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

Learn more about [linking research data and research articles in ScienceDirect](#). Mendeley data

This journal supports [Mendeley Data](#), enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols and methods) associated with your manuscript in a free-to-use, open access repository.

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Research Elements

Data in Brief and MethodsX: Co-submission

You are encouraged to publish research Data, Methods or Protocols related to your manuscript as an additional Co-submission article in [Data in Brief](#) or [MethodsX](#).

By publishing a Co-submission you further advance research reproducibility and interoperability, as well as to further advance open science. In case both your original research article and your Co-submission article(s) get accepted for publication, they will be linked together on ScienceDirect.

When submitting your original research article to Heliyon please follow the **co-submission process** active for this journal:

8.4.2.98 Describe the research data, methods or protocols in a separate paper to be considered for publication in [Data in Brief](#) or in [MethodsX](#).

8.4.2.99 Adhere to one of the following **mandatory submission templates**:

8.4.2.99.1 [Data article template](#) (*Data in Brief*)

8.4.2.99.2 [Methods article template](#) (*MethodsX*)

8.4.2.99.3 [Protocol article template](#) (*MethodsX*)

- Note that submissions can only be processed when submitted as a **Word file**.

Editorial Manager:

8.4.2.100 In the '**Attach Files**' section of the EM submission process, please select **Item Type** '**Data in Brief**' or '**MethodsX**' from the 'Select Item Type' Dropdown menu to upload your Co- submission.

Article structure Manuscript organisation

Except for protocols, we do not have strict formatting requirements, but all manuscripts must contain the essential elements needed to convey the content in clearly defined sections: Title, Abstract, Keywords, Introduction, Materials and methods, Results, Discussion and/or Conclusions, Figures and Tables with captions, and any Supplementary material required.

Please see our [article type page](#) for manuscript templates.

If your article includes any video, audio or other metadata files, you should include these in your initial submission for peer review purposes. The text should be in single-column format and the layout of the text should be as simple as possible. Please note that the size of each individual submission file must not exceed 150MB; and the collective size of all submitted files must not exceed 700 MB.

Title

Please provide a title that is concise and informative; ideally it should contain no more than 20 words. Titles are often used in information-retrieval systems so please keep in mind that your choice of title may impact how easily readers can discover your article.

Introduction

The introduction should clearly state the objectives of your work in light of previous findings. We recommend that you provide an adequate background to your work but avoid writing a detailed literature overview or summary of your results.

Material and methods

Please provide careful and clear reasoning for the selection of methodology as well as clear and precise description of procedure and materials. The level of detail should be sufficient to allow for the work to be reproduced. Some guidelines:

8.4.2.101 If the method you used has already been published, provide a summary and reference the originally published method.

8.4.2.102 If you are quoting directly from a previously published method, use quotation marks and cite the source.

8.4.2.103 Describe any modifications that you have made to existing methods.

For manuscripts involving animal experiments, authors should clearly detail the species and strain of animals used, their housing conditions, the experimental design, all procedures performed, the sample size, and statistical methods used for analysis in the methods section. Additionally, an ethics statement with information about the ethical approval for the experiment and any steps taken to minimise animal discomfort should be provided under “ethics declarations” (see below).

For studies involving human subjects, the methods section should incorporate information about the study design, participant demographics including sex/gender and age, procedures, outcome measures and the time period during which the study was conducted. An ethics statement detailing approval from an ethics committee or institutional review board and informed consent procedures should also be included under “Ethics Declarations”. Details about how the sample size was determined, and how data, while respecting participant privacy, can be accessed, should also be included under the “Data availability statement”.

Theory and calculation

The theory section should lay the foundation for further work by extending the background you provided in the introduction to your article. The calculation section should represent a practical development from a theoretical basis.

Results

Results should be clear and concise. We advise you to read the sections in this guide on supplying tables, artwork, supplementary material and sharing research data.

Discussion

The discussion section should explore the significance and implications of the findings and their limitations, with reference to all other relevant studies and possibilities these suggest for future research. A discussion should not be a repetition of the results section and often a combined results and discussion section is appropriate.

Conclusion (optional)

The conclusion section should present the main conclusions of your study. You may have a stand-alone conclusions section or include your conclusions in a subsection of your discussion or results and discussion section.

Glossary

Please provide definitions of field-specific terms used in your article, in a separate list. Abbreviations
Our guidelines for abbreviations are:

8.4.2.104 Abbreviations should not be used in the abstract unless they are firmly established in the field.

8.4.2.105 Minimize the usage of abbreviations.

8.4.2.106 Do not use uncommon abbreviations in figure panels, support all abbreviations used in figures and tables with footnotes or in the respective captions.

8.4.2.107 Provide an abbreviation table in the manuscript.

8.4.2.108 All abbreviations which are not standard in the field must be spelled out in their first occurrence, followed by use of the abbreviated form only.

8.4.2.109 Before submission we recommend that you review your use of abbreviations throughout your article to ensure that it is

Footnotes

We advise you to use footnotes sparingly. If you include footnotes in your article, ensure that they are numbered consecutively.

You may use system features that automatically build footnotes into text. Alternatively, you can indicate the position of footnotes within the text and present them in a separate section at the end of your article.

Ethics Declarations

Ethics and Consent Statement: All submissions which include human or animal participation must have an ethics and consent section in the manuscript. More information can be found in the section describing “Human and behavioural research” (above).

Please note, if these details are not included or are incomplete at submission, the manuscript may be rejected.

Acknowledgements

Include any individuals who provided you with help during your research such as help with language, writing or proof reading in the acknowledgements section.

8.4.2.110 For manuscripts submitted using single anonymized peer review, acknowledgements should be placed in a separate section which appears directly before the reference list. Do not include acknowledgements on your title page, as a footnote to your title, or anywhere else in your article other than in the separate acknowledgements section.

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8.4.2.113 Data curation

8.4.2.114 Formal analysis

8.4.2.115 Funding acquisition

8.4.2.116 Investigation

8.4.2.117 Methodology

8.4.2.118 Project administration

8.4.2.119	Resources
8.4.2.120	Software
8.4.2.121	Supervision
8.4.2.122	Validation
8.4.2.123	Visualization
8.4.2.124	Writing – original draft
8.4.2.125	Writing – review and editing

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Research, <https://doi.org/10.1029/2001JB000884>. Reference format

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[1] J. van der Geer, T. Handgraaf, R.A. Lupton, The art of writing a scientific article, *J. Sci. Commun.* 163 (2020) 51 – 59. <https://doi.org/10.1016/j.sc.2020.00372>.

Reference to a journal publication with an article number:

[2] J. van der Geer, T. Handgraaf, R.A. Lupton, 2022. The art of writing a scientific article. *Heliyon*. 19, e00205. <https://doi.org/10.1016/j.heliyon.2022.e00205>.

Reference to a book:

[3] W. Strunk Jr., E.B. White, *The Elements of Style*, fourth ed., Longman, New York, 2000.

Reference to a chapter in a book:

[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones,

R.Z. Smith (Eds.), *Introduction to the Electronic Age*, E-Publishing Inc., New York, 2020, pp. 281 – 304.

Reference to a website:

[5] Cancer Research UK, Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/>, 2023 (accessed 13 March 2023).

Reference to a dataset:

[6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions [dataset], Mendeley Data, v1, 2015. <https://doi.org/10.1234/abc12nb39r.1>.

Reference to software:

[7] E. Coon, M. Berndt, A. Jan, D. Svyatsky, A. Atchley, E. Kikinzon, D. Harp, G. Manzini, E. Shelef, K. Lipnikov, R. Garimella, C. Xu, D. Moulton, S. Karra, S. Painter, E. Jafarov, S. Molins, Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88) [software], Zenodo, March 25, 2020. <https://doi.org/10.1234/zenodo.3727209>. Web references

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8.4.3 APPENDIX 6: HFHC DIET COMPOSITION

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