

The effects of pregestational prediabetes as a risk factor for preeclampsia in pregnant female Sprague rats and the adverse effects of pregestational prediabetes on the placenta, liver and kidney function

by

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KWAZULU-NATALTM**

**INYUVESI
YAKWAZULU-NATALI**

Preface

Prediabetes and preeclampsia are both pregnancy disorder that are characterized by endothelial dysfunction and end-organ dysfunction. The etiology of preeclampsia is unknown however studies have shown that the pathophysiology of preeclampsia begins with inadequate cytotrophoblast invasion to the maternal uterus. The inadequate cytotrophoblast invasion results in a hypoxic environment which favors the release of antiangiogenic factors, reactive oxygen species, inflammatory cytokines. The production and release of the substances leads to vasoconstriction due to reduced bioavailability of nitric oxide, ultimately resulting in endothelial dysfunction and reduced uteroplacental blood flow. The risk factors of preeclampsia not only arise from trophoblastic but also from non-trophoblastic maternal risk factors which include chronic hypertension, gestational diabetes (GDM) and T2DM. T2DM is often preceded by prediabetes which can be defined by intermediate hyperglycaemia, often associated with moderate hypertension and moderate reactive oxygen species. The development of prediabetes is associated with western modern styled diets that are high calorie diets and sedentary lifestyle. Prediabetes in male Sprague-Dawley rats has been shown to lead to the genesis of macro and microvascular complications such as cardiovascular, renal dysfunctions and non-alcoholic fatty liver disease. However, no studies have reported on prediabetes-associated complications in pregnant female Sprague-Dawley rats. To advance from this knowledge, the current study sought to investigate the effects of pregestational prediabetes as a risk factor for preeclampsia in pregnant female Sprague-Dawley rats and the adverse effects on the placental, hepatic and renal function.

Declaration

I, **Anelisiwe Siboto** (student number: **212518629**) hereby declare that the dissertation entitled: “The effects of pregestational prediabetes as a risk factor for preeclampsia in pregnant female Sprague rats and the adverse effects of maternal prediabetes on the placenta, liver and kidney dysfunction” is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. The research done in this study was carried out under the supervision of Dr P.S. Ngubane, Prof A. Khathi and Dr N Sibiya.

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

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Plagiarism declaration

I, Anelisiwe Siboto declare that:

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Presentations

Symposium presentation

- **Siboto A**, Ludidi A, Khathi A, Ngubane P.S. “PREGESTATIONAL PREDIABETES AS A RISK FACTOR OF PREECLAMPSIA: THE ADVERSE EFFECTS OF MATERNAL PREDIABETES ON PLACENTAL DYSFUNCTION” Annual School Research Day (School of Laboratory Medicine and Medical Science) on **4TH OCTOBER 2023** University of Kwazulu-Natal, South Africa.
- **Siboto A**, Ludidi A, Xulu N, Nkosi A, Sibiya N, Khathi A and Ngubane PS. “PREECLAMPTIC LIKE ADVERSE EFFECTS OF PREGESTATIONAL PD ON RENAL FUNCTION” Annual CHS research symposium on **27-28 August 2024** K-Rith tower building, Nelson R Mandela school of medicine. University of KwaZulu-Natal, South Africa

Dedication

This thesis/ dissertation is dedicated to my late father and brother and my loving family. To the young one in my family: “you can do anything that you put your mind on.”

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Table of Contents

Title page	i
Preface	ii
Declaration	iii
Plagiarism declaration.....	iv
Presentations	v
Symposium presentation	v
Dedication	vi
Acknowledgements	vii
Table of Contents	ix
List of figures	xiv
List of tables	xviii
List of abbreviations	xx
List of Appendices	xxv
Study outline	xxvi
Abstract	xxviii
Chapter 1: Introduction	1
1. Background	1
2. Overarching Aim	3
3. Research Questions	3
4. Aims and Objectives	3
Chapter 4 Aim:	3
Chapter 5 Aim	4
Chapter 6 Aim	5
5. Hypothesis	6
6. Null hypothesis	6
Reference list	7
Chapter 2: Literature review	9
1.1 Normal pregnancy	9
1.1.1 Physiological and hormonal changes during pregnancy	9
1.1.2 Maternal metabolism of carbohydrates	10
1.2 Placentation	11
1.2.1 Vasculogenesis and angiogenesis during normal pregnancies	13
1.2.2 Role of growth factors, angiogenic proteins and hormones in vasculogenesis and angiogenesis	14
1.2.3 Oxidative stress and placental development	15

1.2.4 Placenta glucose transport.....	17
2. Effects of T2DM on the placenta.....	17
2.5 Preeclampsia	23
2.5.1 Pathophysiology of PE.....	23
2.5.2. Treatment of PE	25
2.5.3 Kidney dysfunction in PE	27
2.5.4 Liver dysfunction in PE	28
2.6 Correlation on the incidence of T2DM and PE in humans	31
3 Animal model for PE and PD	31
3.1 The prevalence of PD.....	35
4 . Justification of the study	36
Reference list	37
Chapter 3: Methodology	47
Introduction.....	47
Chemicals/reagents	47
Ethical considerations	47
Study site, population, and design	47
Study site.....	47
Study Design.....	47
Sample size calculation.....	48
Induction of prediabetes mellitus (PD)	48
Criteria for pre-diabetes	49
Mating.....	49
Induction of preeclampsia (PE).....	50
Plasma and Tissue collection	50
Experimental design of all 3 studies	50
Statistical data analysis	52
Chapter 4.....	53
Prologue 1	53
Manuscript 1	55
Abstract.....	56
Keywords.....	56
1. Introduction.....	57
2. Materials and methods	60
2.1 Drugs and chemicals	60
2.2 Animals.....	60
2.2.1 Experimental design.....	60

2.2.2 Induction of PD.....	61
2.2.2.1 Criteria for PD.....	62
2.2.3 Experimental diet	62
2.3 Mating.....	63
2.4 Induction of PE	64
2.5 Plasma and Tissue collection	64
2.6 Biochemical tests	65
2.6.1 Nitric Oxide levels	65
2.6.2 Placental Malondialdehyde (MDA) Measurements.....	65
2.6.3 Antioxidant enzyme activities.....	66
2.6.5 Total RNA extraction and cDNA synthesis for the analysis of placenta VEGF, PlGF and sFlt expression levels by Real time-PCR.....	67
2.7 Statistical analysis.....	69
3. Results.....	70
3.1 Non-fasting blood glucose and triglyceride concentration	70
3.2 Body weight, food intake and water intake.....	72
3.3 Mean arterial pressure.....	74
3.4 Proteinuria.....	76
3.5 Average litter size per group, placenta weight, fetal weights and F;P ratio.....	78
3.6 Placental angiogenic and anti-angiogenic status.....	80
3.7 Plasma NO concentration	82
3.8 Oxidative stress status.....	84
4. Discussion.....	86
5. Conclusion	93
6. Acknowledgments.....	93
7. Declaration of interest statement.....	94
8. Contribution to authorship	94
9. Data availability statement.....	94
Reference List	95
Chapter 5.....	99
Prologue 2	99
Manuscript 2	100
Abstract.....	101
Keywords	101
1. Introduction.....	102
2. Materials and methods	105
2.1 Animals	105
2.2 Experimental protocol.....	105

2.3 Induction of PD.....	106
2.3.1 Criteria for PD.....	106
2.3.1 Experimental diet	107
2.4 Mating.....	108
2.5 Induction of PE	108
2.6 24 hr fluid and urinary output	108
2.7 Plasma and Tissue collection	109
2.8 Biochemical analysis.	109
2.8.1 ELISAs.....	110
2.8.2 Real-time PCR	112
2.9 Statistical analysis.....	113
3. Results.....	114
3.1 24-h urine output.....	114
3.2 Aldosterone concentration	116
3.3 Kidney oxidative stress and antioxidant concentrations	118
3.4 Real-time quantitative PCR of urinary podocytes specific proteins (nephrin and podocin).....	120
3.5 Electrolyte handling, albumin, creatinine and urea levels	122
3.6 Kidney function markers.....	124
3.7 KIM-1 concentration.....	126
4. Discussion.....	128
5. Conclusion	131
Reference list	132
Chapter 6.....	136
Prologue 3	136
Manuscript 3	137
Abstract.....	138
Keywords	138
1. Introduction.....	139
2. Materials and methods	140
2.1 Drugs and chemicals	140
2.2 Animals	140
2.2.1 Experimental design.....	140
2.3 Induction of PD.....	141
2.3.1 Criteria for PD.....	142
2.3.2 Experimental diet	142
2.4 Mating.....	143
2.5 Induction of PE	144

2.6 Plasma and Tissue collection	144
2.7 Biochemical tests	145
2.7.1 Lipid profile	145
2.7.2 Liver Inflammatory markers	146
2.7.3 Liver enzymes	146
2.7.4 Haematological Analysis	146
2.7.5 Lipid Peroxidation and Antioxidant Profile	147
2.8 Statistical Analysis	149
3. Results.....	150
3.1 Liver weight and relative liver weights.....	150
3.2 Liver triglyceride (TGs) concentration	152
3.3 Oxidative stress marker.....	154
3.4 Inflammatory Markers (IL-6 and TNF α)	156
3.5 Plasma Liver Enzymes and bilirubin	158
3.6 Plasma albumin concentration	160
3.7 Haematological parameters.....	162
4. Discussion.....	164
5. Conclusion	167
Reference list	168
Chapter 7.....	171
Synthesis	171
Conclusion	176
Shortfalls and future studies.....	176
Reference List	178
Appendices.....	180
Appendix 1: AREC Ethics Approval Letter	180
Appendix 2 : Published manuscript 1 of this study.....	182
Appendix 3: OGTT results of normal pregnant group, pregnant PD, pregnant PE taken on GND 0, 9 and 18.....	183
Appendix 4: Establishing a Female Animal Model of Prediabetes Using a High-Carbohydrate, High-Fat Diet	184
Appendix 5: GAPDH results	185
Appendix 6: NO standard curve and the graph generated	186
Appendix 7: Abstract of CHS symposium 2023 oral presentation	187

List of figures

Figure	Legend	Page
	Chapter 2: Literature	
Figure 1	A schematic diagram on how T2DM leads to the onset of PE	30
	Chapter 3: Methodology	
Figure 1	Study design	50
	Chapter 4: manuscript 1	
Figure 1	(a) Non-fasting blood glucose and (b) Triglyceride concentration tested over 18 days of gestation measured on GND 0, 9 and 18. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ p<0.05 by comparison with normal pregnant group. α p<0.05 by comparison with the preeclamptic group	73
Figure 2	(a) Body weight, (b) food intake and (c) water intake of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The values are presented as means, and SD (n=6 in each group). ★ p < 0.05 by comparison with normal pregnant group. α p < 0 05 by comparison to pregnant preeclamptic group.	74
Figure 3	Mean arterial blood pressure of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The values are presented as means, and SD (n=6 in each group).★ p < 0.05 by comparison with normal pregnant group. α p < 0 05 by comparison to pregnant preeclamptic group	77
Figure 4	Albuminuria of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats and over the period of 18 gestational	79

	days. The values are presented as means, and SD (n=6 in each group).★ p < 0.05 by comparison with normal pregnant group. α p < 0 05 by comparison to pregnant preeclamptic group.	
Figure 5	Placental mRNA expression of (a) sFlt-1, (b) VEGF and (c) PlGF mRNA expression levels of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats over the period of 18 gestational days. The values are presented as means, and vertical bars represent SEM (n=6 in each group).★p < 0.05 by comparison with normal pregnant group. α p < 0 05 by comparison to pregnant preeclamptic group	83
Figure 6	Plasma nitric oxide concentration of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The values are presented as means, and vertical bars represent SEM (n=6 in each group).★ p < 0.05 by comparison with normal pregnant group. α p < 0 05 by comparison to pregnant preeclamptic group.	85
	Chapter 5: manuscript 2	
Figure 1	Figure 1: 24 h urine output of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats tested over 18 days of gestation measured on GND 0, 9 and 18. Values are presented as means, and (n=6 in each group). ★p<0.05 by comparison with normal pregnant group. α p<0.05 by comparison with the preeclamptic group.	117
Figure 2	Aldosterone measured after 19 days of gestation of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats measured after 19 days of gestation. Values are presented as	119

	means, and SD (n=6 in each group). ★ p<0.05 by comparison with normal pregnant group. α p<0.05 by comparison with the preeclamptic group	
Figure 3	mRNA expression of urinary podocin and urinary nephrin of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats measured after 19 days of gestation. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ p<0.05 by comparison with normal pregnant group.	124
Figure 4	KIM-1 concentration of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats and after 19 days of gestation. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ p<0.05 by comparison with normal pregnant group. α p<0.05 by comparison with the preeclamptic group	130
	Chapter 6: manuscript 3	
Figure 1	(A) Liver weights and (B) relative liver weights of normal pregnant rats, pregnant prediabetic rats after and pregnant preeclamptic rats measured after 19 days of gestation. Values are presented as means, and SD (n=6 in each group). ★ p<0.05 by comparison with normal pregnant group.	153
Figure 2	Liver TGs concentrations tested after 19 days of gestation. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ p<0.05 by comparison with normal pregnant group.	155
Figure 3	Inflammatory markers (A) IL6 and (B) TNFα on the liver of normal pregnant rats, pregnant preeclamptic and pregnant	159

	prediabetic measured after 19 gestational days. The values are presented as means, and vertical bars represent SEM (n=6 in each group).★p < 0.05 by comparison with normal pregnant group. α p < 0.05 by comparison to pregnant preeclamptic group	
Figure 4	Plasma AST (A), ALT (B) and bilirubin (C) concentrations of normal pregnant rats, pregnant prediabetic and pregnant preeclamptic rats measured after 19 gestational days. The values are presented as means, and vertical bars represent SEM (n=6 in each group).★ p < 0.05 by comparison with normal pregnant group	161
Figure 5	Plasma albumin concentrations of normal pregnant rats, pregnant prediabetic and pregnant preeclamptic rats measured after 19 gestational days. The values are presented as means, and vertical bars represent SEM (n=6 in each group).★p < 0.05 by comparison with normal pregnant group.	163
Figure 6	RBC (A), Hb (B) and platelets (C) concentrations of normal pregnant rats, pregnant prediabetic and pregnant preeclamptic rats measured after 19 gestational days. The values are presented as means, and vertical bars represent SEM (n=6 in each group). ★p < 0.05 by comparison with normal pregnant group. α p < 0. 05 by comparison to pregnant preeclamptic group.	165
	Chapter 7: Synthesis	
Figure 1	Summary of findings from the manuscripts	178

List of tables

Table	Legend	Page
	Chapter 4: manuscript 1	
Table 1	Composition of ingredients in the high-fat high-carbohydrate diet	64
Table 2	List of primers used in the study	70
Table 3	Average litter size, placenta, fetal weights and F:P ratio on GND 19 of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats. Values are represented as means, and SD (n=6 in each group).	81
Table 4	The effects of PE and pregestational PD on MDA concentrations and SOD in the placental tissue over the of 19 days of gestation. Values are represented as means and SD (n=6 in each group).	87
	Chapter 5: manuscript 2	
Table 1	List of primers used in the study	115
Table 2	Kidney MDA and TSOD concentrations in the placental tissue of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic. Values are represented as means and SD (n=6 in each group).	122
Table 3	Urine and plasma electrolyte handling, albumin, creatinine and urea levels of normal pregnant rats, preeclamptic pregnant rats and prediabetic pregnant rats. Values are represented as means; the vertical bars represent SEM (n=6 in each group).	126

Table 4	Influences of the PE and pregestational PD on kidney function. Values are presented as means $\pm$ SEM, (n=6) in each group. Values are represented as means; the vertical bars represent SEM (n=6 in each group).	128
	Chapter 6: Manuscript 3	
Table 1	Table 1: The effects of PE and pregestational PD on MDA concentrations and SOD activity in the liver tissue over the of 19 days of gestation. Values are represented as means; the vertical bars represent SEM (n=6 in each group)	157

List of abbreviations

Abbreviation	Name
ACR	Albumin creatinine ratio
AER	Albumin excretion rate
ADA	American Diabetes Association
ALT	Alanine transaminase
ANOVA	One-way analysis of variance
AREC	Animal Research Ethics Committee
AT1-AA	agonistic autoantibodies to the angiotensin-II type-1 receptor
AST	aspartate transaminase
AREC	animal research ethics committee
ATP	Adenosine triphosphate
BMI	Body mass index
BRU	Biomedical Research Unit
BP	Blood pressure
CAT	Catalase
CC	creatinine clearance
CO ₂	Carbon dioxide
CKD	chronic kidney disease
DNA	Deoxyribonucleic acid
DMSO	dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbent assay
eNOS	Nitric Oxide synthase

FFA	Free fatty acids
g	Grams
<i>g</i>	Gravitational force.
GND	gestational day
GP _x	Glutathione peroxidase
GLUT 2	Glucose transporter 2
GPO-PAP	Glycerine phosphate oxidase peroxidase
Hr	Hour
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL-6	Interleukin 6
INF-I	Interferon I
IR	Insulin Resistance
HFHC	High fat high carbohydrates
H ₂ O	Water
IDF	International Diabetes Federation
K ⁺	Potassium
KIM 1	Kidney injury molecule
Kg	Kilogram
KOH	Potassium hydroxide
L	Litre
LNAME	N(ω)-nitro-L-arginine methyl ester
NO	Nitric oxide

Na ⁺	Sodium
Na ₂ SO ₄	Sodium sulphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX 1	NAD(P)H oxidase 1
NOX4	NAD(P)H oxidase 4
ND	normal pregnant group
NF -Kb	Nuclear Factor Kb pathway
No.	Number
NO	Nitric oxide
MAP	Mean arterial pressure
MDA	Malondialdehyde
μ	Micro
μg	Micrograms
μl	Microlitre
mg	Milligram
ml	Millilitre
mmol/L	Millimoles per Litre
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
p.o.	per os (orally)

PBS	Phosphate buffered saline
PKC	protein kinase C
PD	Prediabetes
PE	Preeclampsia
PIGF	Placenta growth factor and
PPD	Pregestational prediabetes
PTP	Protein Tyrosine Phosphatase
RAAS	renin–angiotensin–aldosterone system
RBC	Red blood cell
ROS	Reactive oxygen species
RUPP	Reduced Uterine Perfusion Pressure
TBARS	Thiobarbituric acid reacting substance
TC	Total cholesterol
TG'S	Triglycerides
TNF α	Tumor necrosis factor-alpha
T2DM	Type 2 diabetes Mellitus
TSOD	Total superoxide dismutase
TBIL	Total bilirubin
SEM	standard error of means
sEng	Soluble endoglin
sFlt-1	Soluble fms-Like tyrosine kinase-1
SOD	Superoxide dismutase
STD	Standard
SD	Standard deviation

OD	optical density
UKZN	University of KwaZulu-Natal
USA	United states
VEGF	Vascular endothelial growth factor

List of Appendices

Appendix	Name	Page no
Appendix 1	AREC Ethics Approval Letter	181
Appendix 2	Published manuscript 1 of this study	183
Appendix 3	OGTT results of normal pregnant group, pregnant PD, pregnant PE taken on GND 0, 9 and 18	184
Appendix 4	Establishing a Female Animal Model of Prediabetes Using a High-Carbohydrate, High-Fat Diet	185
Appendix 5	GAPDH results	186
Appendix 6	NO standard curve and the graph generated	188
Appendix 7	Abstract of CHS symposium 2023 oral presentation	189

Study outline

The current dissertation is presented in manuscript format. It consists of 7 sections: Dissertation abstract, chapter 1: background of the study, Chapter 2: introduction/literature review, chapter 3: summary of materials and methods, chapter 4: manuscript 1, chapter 5: manuscript 2, chapter 6: manuscript 3, chapter 7: synthesis and appendices. The dissertation abstract points out the purpose and summarizes the key findings of the study. Chapter 1 is a brief background and chapter 2 is a literature review to highlight the gaps that exist in literature and how the current study aims to fill these gaps. Chapter 3 is a brief on the materials and methods.

Chapter 4 is the first novel research paper that investigated the Pregestational PD as a risk factor of PE: the adverse effects of pregestational PD on placental function. Aneliswe Siboto, Asiphaphola Ludidi, Nombuso Xulu, Ayanda Nkosi, Ntethelelo Sibiya, Andile Khathi, Phikelelani Ngubane are authors. The manuscript is published in the Journal of Obstetrics and Gynaecology.

Chapter 5 is the second research study manuscript which investigated Preeclamptic like adverse effects of pregestational PD on renal dysfunction. Aneliswe Siboto, Asiphaphola Ludidi, Nombuso Xulu, Ayanda Nkosi, Ntethelelo Sibiya, Andile Khathi, Phikelelani are authors. The manuscript is currently under review in the Journal Frontiers in Physiology, section Renal Physiology and Pathophysiology

Chapter 6 is the third research study manuscript which investigated Pregestational PD mellitus exhibits PE associated complications: The adverse effects of pregestational prediabetes on liver dysfunction. Aneliswe Siboto, Asiphaphola Ludidi, Nombuso Xulu, Ayanda Nkosi, Ntethelelo Sibiya, Andile Khathi, Phikelelani are authors. The manuscript is currently under review in the Journal of diabetes and its complications.

Chapter 7 is the synthesis which discusses the link between the three studies and highlights the key findings for the specific aims of the current project. Appendices include the letter of ethical clearance, abstract of presentations to various conferences: and the guidelines to authors applicable to the three journals where the three manuscripts were submitted.

Abstract

Introduction

Preeclampsia (PE) is a pregnancy complication that is diagnosed after 20 weeks of gestation with new onset hypertension and proteinuria. The risk factors of PE arise from trophoblastic and non-trophoblastic risk factors. The risk factors of PE include chronic hypertension, obesity and preexisting type 2 diabetes mellitus (T2DM). The onset of T2DM is preceded by PD and the prevalence of PD is increased exponentially in the developing countries due to urbanization, adapting to westernized diets that have high calories and normalized sedentary lifestyle. This westernized diet is associated with adverse effects on the heart, kidney and the liver. However, there are no reports of PD and its effects on pregnancy. In this study we investigated the effects of pregestational PD as a risk factor for PE in pregnant female Sprague rats and the adverse effects of maternal PD on the placenta, liver and kidney function.

Methods

Eighteen (18) female Sprague Dawley rats (150–180 g) were obtained from Biomedical Research Unit. High fat high carbohydrate (HFHC) with drinking water supplemented with fructose (HFHC+fructose) induced prediabetic female Sprague Dawley rats and LNAME induced preeclamptic female Sprague Dawley rats were used for this study. Parameters including non-fasting blood glucose concentration, body weights, blood pressure, fluid intake and urine output were monitored on gestational day (GND) 0, 9 and 18 during gestation period. Following sacrifice, parameters such as VEGF, PlGF, sFlt-1, NO, liver triglycerides, TNF α and liver function enzymes, lipid peroxidation, antioxidant status, kidney injury molecule (KIM 1), podcin, nephrin, creatinine, urea, albumin and electrolytes, were measured.

Results

The prediabetic group showed impaired non-fasting glucose, dyslipidemia, high blood pressure, impaired placenta function as evidenced by reduction in VEGF and PlGF and an increase in sFlt-1, impaired kidney function showed by increased plasma KIM-1, increased urinary podocin and nephrin and impaired electrolyte handling. The prediabetic group also showed impaired liver function as evidenced by increased plasma liver enzymes, increased liver oxidative stress and increased liver inflammation.

Conclusion

The diet-induced prediabetic rats showed impaired glucose handling and an imbalance in pro-angiogenic and antiangiogenic factors which lead to metabolic disturbances such as endothelial dysfunction, liver and kidney injury.

Chapter 1: Introduction

1. Background

Preeclampsia (PE) plays a serious and potentially life-threatening role, affecting both the mother and baby during pregnancy and after pregnancy. PE is a serious pregnancy complication that is observed after 20 weeks of gestation accompanied by one or more of the following new-onset conditions: proteinuria, other maternal organ dysfunctions, or uteroplacental dysfunction (Abdel-Hamid *et al.*, 2020). Maternal organ dysfunction includes acute kidney injury, liver involvement, neurological complications and hematological complications (Ramos *et al.*, 2017; Abdel-Hamid *et al.*, 2020). The exact cellular and molecular mechanisms underlying PE remain largely unclear (Pankiewicz *et al.*, 2021; Kornacki *et al.*, 2023). However, PE follows a two-stage process: first, impaired uteroplacental perfusion, potentially due to defective trophoblast invasion (Stage 1), and second, widespread endothelial dysfunction and vascular inflammation, ultimately resulting in systemic organ damage (Stage 2) (Redman *et al.*, 2014; Chang *et al.*, 2023). Insufficient trophoblast invasion creates a hypoxic environment which triggers oxidative stress that plays a key role in placental dysfunction and the development of PE (Roberts *et al.*, 2021). This oxidative stress further exacerbates inflammation, worsening the condition in affected pregnancies (Roberts *et al.*, 2021). PE has several well-established risk factors which arise from trophoblastic and also from non-trophoblastic maternal risk such as advanced maternal age, chronic hypertension, Type 2 diabetes mellitus (T2DM) and obesity (Jung *et al.*, 2022; Lee *et al.*, 2022; Johnson *et al.*, 2024). T2DM is often preceded by prediabetes (PD) which is characterized by a moderate increase in blood glucose concentration, impaired fasting glucose, impaired glucose tolerance and an elevation in glycated haemoglobin A1c (HbA1c) levels (Echouffo-Tcheugui & Selvin, 2021). The moderate hyperglycaemia is often associated with moderate hypertension and moderate reactive oxygen species (ElSayed

et al., 2023). The development of PD is associated with western modern styled diets that are high calorie diets and sedentary lifestyle (Tabák *et al.*, 2012; ElSayed *et al.*, 2023). The prevalence of PD has been shown to be escalating in both developed and developing countries. It estimated that 541 million adults have diabetes in year 2021 with the prevalence expected to increase to 730 million by the year 2045 (Magliano *et al.*, 2021). PD and PE share common characteristics such as endothelial dysfunction and end-organ dysfunction (Weissgerber & Mudd, 2015; Lopez-Jaramillo *et al.*, 2018). PE has been shown to have adverse effects on placenta function, kidney function and liver function. PE is characterized by poor placental implantation and insufficient remodeling of uterine arteries which leads to reduced blood supply to the placenta, resulting in fetal hypoxia and intrauterine growth restriction (Chang *et al.*, 2023). Chronic hypoxia in the placenta induced by PE attributes to production of antiangiogenic factors, ROS which contributes to inflammation, endothelial dysfunction, and increased maternal blood pressure (Dickerson *et al.*, 2024). However, the presence and impact of angiogenic factors in placenta during pregestational PD remain unknown. Antiangiogenic factor produced by the stress placenta have been shown to enter maternal circulation and target the fenestrated endothelium in the kidney attributing glomerular endotheliosis which is swelling and damage to the glomeruli (Kattah, 2020). This leads to reduced kidney filtration contributing to proteinuria (Sani *et al.*, 2019). However, the changes in kidney function during pregestational PD are yet to be elucidated. PE can cause elevated levels of liver enzymes which indicates liver inflammation or damage this is observed in severe state of PE (Chandrasekaran & Simon, 2020). severe form of PE known as HELLP syndrome (Alese *et al.*, 2021). However, this is not yet established in pregestational PD. Therefore, the current study sought to investigate the effects of pregestational PD as a risk factor for PE in pregnant female Sprague-Dawley rats and the adverse effects on the placental, hepatic and renal function.

2. Overarching Aim

To investigate the effects of pregestational PD as a risk factor of PE and the adverse effects of the pregestational PD on placental, renal and hepatic function.

3. Research Questions

3.1 What are the effects of pregestational PD on placenta function in pregnant female Sprague Dawley rats?

3.2 How does pregestational PD contribute to renal dysfunction in pregnant female Sprague Dawley rats?

3.3. What are the effects of pregestational PD on liver function including blood parameters in pregnant female Sprague Dawley rats?

4. Aims and Objectives

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

Chapter 4 Aim:

To investigate No, selected angiogenic and antiangiogenic factors gene expression, and oxidative stress status may reveal functional changes demonstrating pregestational PD as a risk factor for pre-eclampsia.

Chapter 4 objectives

Chapter 4 consists of an original research manuscript.

The objectives for the original manuscript include:

- To measure non-fasting blood glucose and TG concentration on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats

- Body weight, food intake and water intake of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats
- To monitor hypertension by assessing MAP throughout gestation on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To study the effects of pregestational PD on antiangiogenic status by measuring sFlt1, VEGF and PlGF on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To study the effects of pregestational PD on endothelial function by measuring NO on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats

Chapter 5 Aim

To investigate whether pregestational PD exhibits PE associated kidney dysfunction in pregnant female Sprague Dawley rats.

Chapter 5 objectives

Chapter 5 consists of an original research manuscript.

The objectives for the original manuscript include:

- To study the effect of pregestational PD on GFR function on prediabetic rats and preeclamptic rats by monitoring urine volume, urine Na⁺, urine K⁺, urine creatinine, urine protein and plasma creatinine during 18 days of gestation on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To study the effects of pregestational PD on concentration of albumin in both urine and plasma of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To study the effects of pregestational PD on kidney injury by looking at KIM 1 on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.

- To study the effects of on blood pressure regulation by measuring aldosterone in plasma on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To study the effects of pregestational PD on podocyte injury by looking at podocyte specific proteins podocin and nephrin on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To measure renal oxidative stress through MDA on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To look at antioxidants activity (TSOD antioxidants) on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To measure TNF- α , and IL-6 as parameters of kidney/renal inflammation on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.

Chapter 6 Aim

To investigate pregestational PD exhibits PE associated complications: The adverse effects of pregestational PD on liver dysfunction.

Chapter 6 objectives

Chapter 6 consists of an original research manuscript.

The objectives for the original manuscript include

- To measure body weight and liver weight to study relative liver weight after 18 days of gestation on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.

- To study the effects of pregestational PD on liver damage by measuring liver enzymes (ALT and AST) on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To study effects of pregestational PD on antioxidant activity (SOD and GPx) on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.
- To study the effects pregestational PD on hepatocyte dyslipidemia by measuring selected parameter such as TGS on normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats.

5. Hypothesis

Pregestational PD increases the risk of developing PE leading to adverse impacts the placenta, liver, and kidney function, contributing to systemic dysfunction and poor pregnancy outcomes.

6. Null hypothesis

Pregestational PD does not increase the risk of developing PE and does not lead to impairments in the function of the placenta, liver and kidney.

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Chapter 2: Literature review

1.1 Normal pregnancy

1.1.1 Physiological and hormonal changes during pregnancy

During normal pregnancy, the female body goes through numerous physiological and functional changes to provide an adequate environment for fetal development, meet the increased metabolic demands and prepare for childbirth (Jain *et al.*, 2020; Kazma *et al.*, 2020).

The physiological changes include changes in organ systems such as the cardiovascular, respiratory, hematological, renal, gastrointestinal and endocrine systems (Kazma *et al.*, 2020).

All these changes occur to support and accommodate the developing fetus (Jain *et al.*, 2020).

The most important physiological changes occur in the cardiovascular system as there is increased blood flow to the uterus and maternal organs to support the developing fetus (Saleem *et al.*, 2022).

Hormonal changes are important for the maintenance of normal pregnancy. Human chorionic gonadotropin (hCG) is released from the syncytiotrophoblast after fertilization and supports the development and integrity of the corpus luteum that produces progesterone and estrogen at the beginning of gestation (Kallen, 2004). The progesterone and estrogen concentration increases as pregnancy progresses as these two hormones maintain normal pregnancy. Progesterone has a critical role in implantation and estrogen has a major role in enhancing endothelial functions (Norwitz *et al.*, 2001; Kallen, 2004). Once the level of hCG decreases, the syncytiotrophoblast in the placenta then releases human placental lactogen (hPL) which has similar actions to growth hormone, which promotes the development of protein tissues (Alam *et al.*, 2021). Corticotrophin releasing hormone (CRH) is synthesized by the placenta and the CRH concentration increases as pregnancy progresses. The major functions of CRH include promoting the development of fetal organs such as the lungs, liver and gut, maintaining blood glucose concentration, modulating immune functions and maintaining vascular tone (Duthie &

Reynolds, 2013). CRH stimulates adrenocorticotrophin hormone (ACTH) secretion from the pituitary gland, which has a role in the secretion of fetal cortisol into the bloodstream (Majzoub, 2006). These hormones also influence maternal nutrients metabolism in a manner that supports both the mother and the developing fetus. The following section will focus on maternal metabolism of carbohydrates and lipids during the gestation period.

1.1.2 Maternal metabolism of carbohydrates

Carbohydrate metabolism changes during pregnancy to meet the increased energy needs of both the mother and fetus (Butte, 2000). Studies have shown that peripheral insulin sensitivity is dynamically altered throughout pregnancy, initially increasing following embryonic implantation due to the formation of the placenta and decreasing markedly later in pregnancy due to the elevated of placentally and non-placentally derived hormones such as placentally derived human growth hormone (GH-V), progesterone, cortisol, and prolactin (Kim *et al.*, 2014; Angueira *et al.*, 2015). These hormones contribute to decreasing insulin sensitivity in peripheral tissues such as adipocytes and skeletal muscle by interfering with insulin receptor signalling (Aung *et al.*, 2020; Skajaa *et al.*, 2020). This is important to ensure that glucose is not shuttled back to the maternal cells and that there is enough glucose and fatty acid transported to the fetus (Cohen *et al.*, 2014). The decreased insulin sensitivity helps to maintain normal glucose homeostasis in a manner that is suitable for both mother and fetus (Skajaa *et al.*, 2020). However, in normal pregnancy the body compensates for the observed insulin resistance by producing placental lactogens which stimulate the increase in the size and number of maternal pancreatic β -cells thus enhancing glucose-stimulated insulin secretion at lower than normal blood glucose level (Sorenson & Brelje, 2009; Butler *et al.*, 2010). Maternal hepatic gluconeogenesis also increases as a compensatory mechanism to maintain euglycemia in favor of greater fetal glucose utilization (Angueira *et al.*, 2015).

1.1.3 Maternal metabolism of Lipids

Changes in hepatic and adipose metabolism during pregnancy alter circulating concentrations of triacylglycerols, fatty acids, cholesterol, and phospholipids (Chen *et al.*, 2021). After an initial decrease in the first 8 weeks of pregnancy, there is a steady increase in triacylglycerols, fatty acids, cholesterol, lipoproteins and phospholipids (Butte, 2000). The higher concentration of estrogen and insulin resistance are thought to be responsible for the hypertriglyceridemia during pregnancy (Butte, 2000; Herrera Castellón, 2011). Cholesterol is used by the placenta for steroid synthesis and fatty acids are used for placental oxidation and membrane formation (Kallol & Albrecht, 2020). Changes in lipid metabolism promote the accumulation of maternal fat stores in early and mid-pregnancy and enhance fat mobilization in late pregnancy (Herrera Castellón, 2011). In early pregnancy, increased estrogen, progesterone, and insulin favors lipid deposition and inhibit lipolysis. Lipoprotein lipase (LPL) activity in the adipose tissue from the femoral region, but not from the abdominal region, is elevated at 8–11 week of gestation (Herrera Castellón, 2011). In late pregnancy, human chorionic somatomammotropin (HCS) promotes lipolysis and fat mobilization. The increase in plasma fatty acid and glycerol concentrations is consistent with mobilization of lipid stores (Liu *et al.*, 2017). This shift from an anabolic to a catabolic state promotes the use of lipids as a maternal energy source while preserving glucose and amino acids for the fetus (Herrera Castellón, 2011).

1.2 Placentation

The placenta is a critical organ during pregnancy since the organ plays a central role in mediating fetal growth and development through its metabolic and hormonal functions (Newbern & Freemark, 2011). Metabolic functions include gaseous exchange, nutrient delivery and waste removal (Turco & Moffett, 2019). The placenta produces several hormones such as estrogen, progesterone, thyroid hormone, hPL and CRH (Newbern & Freemark, 2011). Estrogen, and progesterone take over from the corpus luteum and stimulate the endometrial

lining to thicken to provide a nutritive environment by increasing vasculature in the endometrium and play a crucial role in development of the fetus (Turco & Moffett, 2019). Thyroid hormones also play a role in central nervous system (CNS) development (Turco & Moffett, 2019). hPL alters insulin sensitivity to promote glucose transport to the fetus, and CRH stimulates the release of cortisol for lung development (Turco & Moffett, 2019). After fertilization, the fertilized egg grows into a blastocyst and implants/attaches to the endometrium through two connections (Kazma *et al.*, 2020). The first connection forms a loose attachment that involves the outer cells mass of the blastocyst, called trophoblast. The trophoblast has micro villi that allow them to attach to pinopods in the endometrium lining or decidua functionalis (Hemberger *et al.*, 2020). The second connection forms a tight attachment due to the attachment of integrins to selectins which are covered with collagen on the endometrium lining. The trophoblastic cells release chemokines which stabilizes the adherence mechanism (implantation) and allow the blastocyst to start invading into the stroma, invading the endometrium (Rampersad *et al.*, 2011). The trophoblast differentiate into two layers, the cytotrophoblast (internal site (CT)) and the syncytiotrophoblast (external site (SynT)), together these forms part of the placenta (Rampersad *et al.*, 2011). The SynT discharge hydrolytic enzymes that engulf and dissolve adjacent cells of the endometrium and allow penetration into the uterine wall. SynT also releases hCG which stimulates the corpus luteum to secrete progesterone. The increase in progesterone plays a role in vasodilation of blood vessels and allows for adequate amount of blood supply to the endometrial lining and prevents the endometrial lining from becoming ischemic, necrotic and sloughing off from the uterus (Turco & Moffett, 2019). On day 13- 14 of gestation the placental villi development occurs, primary villus are formed when the CT cells proliferates and send out little projections which penetrates through SynT and proliferating around the edge of the intervillous space and forms an outer CT shell (Hemberger *et al.*, 2020). In weeks 3-8 of gestation secondary villus are formed when

the extra embryonic mesoderm invades the primary villus. Tertiary chorionic villus are formed when extra embryonic mesodermal cells differentiate and form capillaries (Kojima *et al.*, 2022).

At about 10 weeks of gestation, when the placental villi is developed, the CT from placental villi invades the space around the spiral artery (Whitley & Cartwright, 2009; Wang, 2017). This process causes the loss of muscle layer and the endothelium is replaced by extravillous CT. This remodeling of the spiral arteries increases the diameter of the vessels about 10-fold which results in increased blood volume to the intervillous space. This system ensures that adequate nutrients and respiratory gases are provided for fetal development and growth (Whitley & Cartwright, 2009; Wang, 2017).

The transfer zone is made up of three distinguished cell layers: CT which lies at the interface of maternal blood, the SynT/spiral arteries, which are located between the CT and the fetal endothelium and the fetal endothelium which lies at the interface of fetal blood, and their development during gestation is precisely regulated to meet fetal demands (Hemberger *et al.*, 2020). In human placentas, the SynT can be characterized as a sort of epithelium, separating fetal and maternal circulation, and is responsible for the transport of nutrients, oxygen and waste products while also producing hormones (Rampersad *et al.*, 2011). Prior to trophoblast invasion two processes that is vasculogenesis and angiogenesis are critical to form a vascular system needed for effective transport of nutrients, oxygen, and waste products. sand

1.2.1 Vasculogenesis and angiogenesis during normal pregnancies

There are two processes involved in formation of blood vessels during embryonic development, namely vasculogenesis and angiogenesis. Vasculogenesis is a process by which blood vessels are formed *de novo* from precursor cell (Boldeanu *et al.*, 2020). During this process, mesenchymal cells are the source hemangiogenic stem cells which differentiate to

hemangioblastic stem cells and giving rise to angioblastic cells (Arroyo & Winn, 2008; Huppertz, 2011). Angioblastic cells then differentiate into endothelial cells forming new vascular networks. The formation of these networks is important for organogenesis during fetal development. Signals that regulate the organization of endothelial cells into tubal networks involve complex processes with a number of factors identified shortly after the endothelial tubes are formed, they become associated with pericytes (vascular smooth muscle cells) derived from the mesenchyme surrounding the endothelium. These pericytes then proliferate and migrate, coating the endothelial cell tubes and forming the vessels (Arroyo & Winn, 2008; Huppertz, 2011). Vasculogenesis eventually evolves into angiogenesis. Angiogenesis involves the creation of new vessels from already existing ones (KV *et al.*, 2021). Angiogenesis can be accomplished by either the migration of endothelial cells from preexisting vessels through the sprouting of endothelial cells (sprouting angiogenesis) to form new vessels or by the elongation or increase in length of the existing vessels (non-sprouting angiogenesis). Through these processes the placenta is able to create a complete vascular tree (Arroyo & Winn, 2008; Rampersad *et al.*, 2011). Vascular growth is necessary to increase placental fetal blood flow over gestation.

1.2.2 Role of growth factors, angiogenic proteins and hormones in vasculogenesis and angiogenesis

Vasculogenesis and angiogenesis processes are regulated by several growth factors. These growth factors are secreted by Hofbauer cells which are widely found in villi and secrete primary vascular endothelial growth factor (VEGF) whilst smooth muscle cells secrete growth factors such as VEGF, basic fibroblast growth factor (bFGF), angiopoietins- 1 and -2 (Ang-1 and Ang-2). The secretion of growth factors leads to the formations of villous trees which are the main structure of the placenta (Huppertz, 2011; Andraweera *et al.*, 2012).

Growth factors mainly the VEGF family of proteins are the most studied family of growth factors known to regulate the processes of vasculogenesis and angiogenesis (Wang, 2017; Faraji *et al.*, 2022). The VEGF family consists of a) seven proteins designated VEGF-A (also known as VEGF) to VEGF-F, b) Placenta Growth Factor (PlGF), and c) Receptors for the VEGF family which include VEGFR-1 (also known as fms like tyrosine kinase receptor-1 (flt-1)), VEGFR-2 (also known as kinase insert domain receptor (KDR) and fetal liver kinase 1 (Flk1) in humans) (Andraweera *et al.*, 2012; Ali *et al.*, 2019). These growth factors are known to induce signal transduction by binding to their specific transmembrane receptors. VEGF-A binds to both VEGFR-1 and VEGFR-2, whereas PlGF only binds to the VEGFR-1 receptor (De Falco, 2012).

VEGFR-1 also has a soluble form (sVEGFR-1), which contributes to both physiologic and pathologic conditions by blocking the normal function of the VEGF and PlGF growth factors (Jardim *et al.*, 2015). When these growth factors bind to their transmembrane receptors, a series of downstream proteins are activated, which induce endothelial cell proliferation, migration, and tube formation (Ahmed *et al.*, 2000). VEGF-A, aside from being a potent endothelial survival factor, is also known to induce vasodilation by increasing nitric oxide (NO) production, another function which facilitates blood flow (Jardim *et al.*, 2015). PlGF has similar functions as VEGF, however the expression of PlGF is primarily restricted to the trophoblast cells in the placenta, suggesting that PlGF has a role in placental development (Ali *et al.*, 2019). In addition, PlGF increases proliferation of cells such as fibroblasts and smooth muscle cells (Chau *et al.*, 2017). In the placenta, PlGF is abundantly expressed by the villous syncytiotrophoblast and stem vessels (Lecarpentier *et al.*, 2016).

1.2.3 Oxidative stress and placental development

Pregnancy is a state of oxidative stress due to increased metabolic activity in placental mitochondria (Chiarello *et al.*, 2020). For the first ten weeks of pregnancy in humans, the

process of placentation and embryogenesis takes place in a hypoxic environment (Myatt & Cui, 2004; Hutter & Jaeggi, 2010). Due to general hypoxia in the intervillous space, as many maternal spiral arteries are plugged with extravillous trophoblast cells which results in no blood flow through these vessels and a low degree of oxidative stress (Pereira *et al.*, 2015). The hypoxic environment also stimulates hypoxia inducible factor 1 (HIF 1) to increase the expression of VEGF. After 12–14 weeks of normal gestation, these placental extravillous trophoblasts colonize the maternal spiraled arteries which leads to the arteries to relax and lose contractile properties, which increases blood flow and raises oxygen partial pressure, thus reversing the previously hypoxic environment (Whitley & Cartwright, 2009; Burton *et al.*, 2021). Once maternal intraplacental circulation is fully established the oxygen tension and ROS levels rise promoting oxidative stress (Burton *et al.*, 2021). The increase in oxidative stress is accompanied by an increase in PlGF and a decrease in the expression of VEGF (Jauniaux *et al.*, 2000). Therefore, poor perfusion in the placenta during the first trimester leads to the inhibition of VEGF expression and changes the timing of PlGF growth, resulting in immature vessel development (Marín *et al.*, 2020).

ROS signaling during human pregnancy is important for central processes such as placental vasculature development. As pregnancy progresses and the metabolic demands of the fetus rise, there is an increase in both placental mitochondria mass and mitochondrial electron chain enzyme activity (Hebert & Myatt, 2021). This contributes to elevated ROS production and increased oxidative stress. Therefore, the placenta adapts to the increasing oxygen and ROS levels by increasing cellular antioxidant enzymes such as copper/zinc superoxide dismutase and mitochondrial superoxide dismutase which are stimulated by high estrogen concentration (Bakacak *et al.*, 2015; Schoots *et al.*, 2018).

1.2.4 Placenta glucose transport

The capacity of the placenta to supply nutrients from the mother to the fetus primarily depends on placental nutrient transporters such as glucose transporters and amino acid transporter (Brett *et al.*, 2014). Placental glucose transport is mediated by members of the glucose transporter (GLUT) family distributed across the microvillous and basal membranes of the SynT. In the rat, GLUT1 and GLUT3 are colocalized in the labyrinthine SynT layer of the placenta (Illsley & Baumann, 2020). GLUT1 is the major GLUT in the placenta especially in the early placentation process. GLUT 1 is a primary source of glucose for the fetus. while GLUT3 is required in the metabolically active dividing first trimester trophoblast cells (Stanirowski *et al.*, 2021).. The placental sodium couple neutral amino acid transporter (SNAT) has three transporter isoforms: SNAT1, SNAT2, and SNAT4 (Holm *et al.*, 2017). SNAT1 and SNAT2 facilitate the transport of small neutral amino acids, while SNAT4 can interact with cationic amino acids.

2. Effects of T2DM on the placenta

2.1 The effect of T2DM on angiogenesis and vasculogenesis

Hyperglycaemia leads to the formation of an abnormally large, inefficient placenta that adversely affects fetal growth (Qin *et al.*, 2021). The adverse effects of hyperglycaemia on placentation are due to the direct actions of high glucose on trophoblast cell behavior (Qin *et al.*, 2021). T2DM is associated with micro and macrovascular complications which leads to vascular disorders such as cardiovascular disease. vascular disorders have been shown to change placental function and compromise fetal development (Desoye & Cervar-Zivkovic, 2020).

Studies have shown that under hyperglycaemia conditions, vasculogenesis is affected as early as in yolk sac (Dong & Yang, 2018). In the yolk sac, vasculogenesis is disrupted and cellular structures in the vessels are altered with a concomitant reduction in expression of VEGF and

hypoxic inducible factor 1 α (HIF1 α) (Dong & Yang, 2018). Recent studies have shown that hyperglycaemia reduce the sensitivity of VEGF to hypoxia through the oxidative stress regulated hypoxia inducible factor pathway (Gunton, 2020). VEGF and its receptor expression has been shown to be altered in placenta of hyperglycemic women (Gunton, 2020). Hyperglycaemia is associated with hyperangiogenesis in placenta tissue which leads to hypervascularization of villi due to changes in placental VEGF:VEGF receptor expression ratio. In T2DM, placental morphology is altered due to hypervascularization which enlarges surface area of the placenta. Hyperglycaemia is associated with the deposition of collagen around large arteries in the placenta. The diffusion distance between maternal and fetal circulation is increased because of thickening of trophoblastic membrane with high amounts of collagen deposition. Increased surface area and hypervascularization in the placenta leads to altered oxygen diffusion across the placenta and leads to local hypoxia in the maternal-placental-and fetal axis which eventually upregulate proangiogenic factors such as VEGF, leptin and fibroblast growth factor. High levels of proangiogenic factors lead to enhanced endothelial cell proliferation, vascularization and limited trophoblast invasion. However, the onset of T2DM is often preceded by PD which is characterized by moderate hyperglycaemia and hyperinsulinaemia. Although studies in our laboratory have shown moderate increase in ROS, the effects of intermediate hyperglycaemia seen in PD, as a risk to the development of PE have not been shown (M Luvuno *et al.*, 2018; Gamede *et al.*, 2019). Manuscript 1 of this study sought to provide plausible mechanisms through which intermediate hyperglycaemia can be a risk factor of PE by investigating VEGF and sFlt-1 expression.

It has been shown that T2DM affects vasculogenesis, angiogenesis and vascular remodeling during the initial stages of placental development (Júnior *et al.*, 2017). Histology work on placenta of women with T2DM show placental pathologic features related to uteroplacental malperfusion. Uteroplacental malperfusion is thought to be due to decreased or abnormal

uterine artery blood flow and has been associated with intrauterine fetal growth restriction and fetal death. Pathologic features consistent with uteroplacental malperfusion include placental infarcts, accelerated villous maturation, and an increase in maternal vascular lesions such as decidual vasculopathy. Women with T2DM are more likely to have placental infarcts hence T2DM is considered a risk factor for PE (Beauharnais *et al.*, 2012; Huynh *et al.*, 2015; Vekic J *et al.*, 2019), however thus far, there are very few studies in literature that measure placental antiangiogenic factors in T2DM, studies on the effect of T2DM on the placenta are based on histological confirmation. The effects of PD on antiangiogenic factors in pregnant female are however yet to be established. Therefore, the extent to which PD could be a risk factor to the development of PE is yet to be outlined. Manuscript 1 of this dissertation investigated the effects of PD on antiangiogenic factors in pregnant female Sprague Dawley rats

2.2 Effects of T2DM on endothelial dysfunction in the placenta

Hyperglycaemia and hyperlipidaemia observed in T2DM are associated with endothelial dysfunction. Therefore, individuals with T2DM have vascular endothelium that is particularly sensitive to the placental derived factors under placenta pathologies (Dhananjayan *et al.*, 2016; Li *et al.*, 2021). Furthermore, insulin has a direct effect on endothelial nitric oxide synthase (eNOS). Therefore, hyperinsulinaemia observed in T2DM has been associated with a reduction in NO bioavailability (Kumar *et al.*, 2018; Bahadoran *et al.*, 2020; Vargas *et al.*, 2021). NO plays an important role in vascular relaxation effect contributing to the modulation of vascular tone, thus NO plays an important role in the maintenance of normal pregnancy by influencing cytotrophoblast invasion and mediating spiral artery transformation (Zullino *et al.*, 2018; Cyr *et al.*, 2020). ROS have been shown react with and oxidize NO forming peroxynitrite (ONOO-) which is a potent oxidant that can further react with other molecules leading to damage proteins, lipids and DNA. Peroxynitrite has been shown to reduce the availability and function of NO in the body (Sarmiento-Salinas *et al.*, 2021). Therefore, a reduction in NO during

pregnancy is associated with pregnancy complications such as PE and pregnancy induced hypertension (Beaumann *et al.*, 2020)-

T2DM is characterized by lipid accumulation and insulin resistance which leads to endothelial dysfunction that may diminish the anti-atherogenic function of vascular endothelium. The adverse effects associated with sustained hyperglycaemia produces marked changes in arterial vessels such as a gradual decrease in the elasticity of the vessel wall that leads to hardening and increased susceptibility to atherosclerosis (Cosentino *et al.*, 2020). An increase in glucose promotes atherosclerosis that results in abnormally narrow spiral arterioles which impair uteroplacental blood flow, lead to hypoxia/ischemia and subsequent impaired placental perfusion which results in the onset of PE (Yadav *et al.*, 2018). Placental hypoxia induced by hyperglycaemia and insulin resistance can also be explained by the effect of these markers on haemoglobin structure. Hyperglycaemia is associated with increased glycosylation of haemoglobin protein that can lead to erythrocyte deformation and increased blood viscosity (Alamri *et al.*, 2019). These factors contribute significantly to the reduction of haemoglobin affinity for oxygen, leading to poor oxygen transport and local hypoxia. The hypoxic effect on the microcirculation causes alterations in the basal membrane of arterioles which leads to weakening and dilation of the capillary walls with a tendency to rupture (microangiopathy). The effects of intermediate hyperglycaemia as seen in PD on both maternal and fetal vascular endothelial damage, and fetal IUGR have not been fully outlined. Another factor that plays a role in fetal development is the expression of nutrient transporter on the placenta. The following section will look at effects of T2DM on nutrient transport in the placenta

2.3 The effect of T2DM on nutrient transport in the placenta

T2DM is characterized by chronic hyperglycaemia, hypertension, dyslipidemia, hyperinsulinaemia, insulin resistance and adiposity (Vieira *et al.*, 2018; Košir Pogačnik *et al.*, 2020). During a pregnancy complicated by T2DM, the human placenta undergoes several

functional and structural pathologic changes (Nteebe *et al.*, 2020; Tumminia *et al.*, 2021). Maternal hyperglycaemia induces the thickening of the placental basement membrane and is anticipated to reduce nutrients and oxygen transport which leads to IUGR (Mahmoud *et al.*, 2018; Kapustin *et al.*, 2021), however literature has reported that hyperglycaemia is associated with macrosomia therefore the following section will discuss effects of chronic hyperglycaemia on nutrient transport in the placenta.

An increase in blood glucose concentration and an increase in plasma insulin concentration in T2DM patients alters placental carbohydrate, protein and lipid metabolism which causes macrosomia (Júnior *et al.*, 2017). Fetal macrosomia in maternal T2DM can be explained by different mechanisms which include sustained hyperglycaemia, proinflammatory state, glucose transporters on the placenta and surface area of the placenta under hyperglycaemic conditions (Agudelo-Espitia *et al.*, 2019; Vieira *et al.*, 2020). T2DM is also associated with a proinflammatory state which is proposed to underlie fetal macrosomia (Higgins *et al.*, 2011; Roberts *et al.*, 2011). Studies have shown that inflammatory cytokines such as placental-derived hepatocyte growth factor (HGF) and resistin are associated with obesity (Holm *et al.*). HGF has been shown to increase *de novo* fatty acid synthesis, and accumulation of placental triglycerides. HGF also stimulates placental glucose uptake and utilization, which is facilitated by augmented GLUT1 expression (Visiedo *et al.*, 2015). Studies have shown that Resistin stimulates glucose uptake and GLUT1 expression in trophoblast cells resulting in increased uptake glucose in the placenta (Nicoletta Di Simone, 2009). Birth weight is positively correlated with maternal BMI and is also correlated with GLUT1 expression in basal membranes (Jansson, 2015).

Maternal hyperglycaemia results in increased insulin secretion from the fetus pancreas to achieve normoglycaemia and this condition is compensated by increase glucose uptake in the fetus cells and results in increased macrosomia and adipose mass (Ehrenberg *et al.*, 2004; Kuc

et al., 2011; Ornoy, 2011). In T2DM, the capacity for transplacental glucose transfer across the basal membrane is increased. The expression of GLUT1 remains unaffected in the microvillous membrane but is upregulated in the basal membrane, facilitating enhanced glucose transport across the placental-fetal side (Gallo *et al.*, 2017). Hyperglycaemia has been shown to increase fetal growth hormones such as insulin and insulin-like growth factor-1 (IGF-1) which play a role in development of macrosomia (Borges *et al.*, 2019). In literature, there is contradicting data with regards to the expression of GLUT 1 on the placenta of diabetic mothers.

Other studies have shown that hyperglycaemia downregulate the GLUT 1 glucose transport system of placenta trophoblast by redistribution of the localization of GLUT1 protein (Hahn *et al.*, 2000; Stanirowski *et al.*, 2018). A study by Hahn 2000 showed that the exposure of cultured cells to 25mmol/L glucose significantly reduced the expression of GLUT 1 which shows that hyperglycaemia alters GLUT 1 partitioning between the plasma membrane and intracellular sites and favors internalization of GLUT 1 (Hahn *et al.*, 2000). However, the association between glucose levels and GLUT 1 function in trophoblasts *in vivo* may depend on the compensatory ability of the cells under pregnancies complicated with T2DM and gestational diabetes (GDM) (Hulme *et al.*, 2018). Chronic Hyperglycaemia, as seen in T2DM, also induces oxidative stress by generating ROS which are associated with increased trophoblast apoptosis and deportation, altered placental vascular reactivity and a reduction of glucose transporter 1 (GLUT1) expression in the placenta causing IUGR (Rashid *et al.*, 2018). However, it is yet to be outlined whether intermediate hyperglycaemia, such as seen in PD will also induce IUGR, in this case showing the association of PD and PE. T2DM is often associated with the development of renal, and hepatic-end organ complications and T2DM is often preceded by PD. Therefore in manuscript 2 and manuscript 3 in this study we sought to look at renal and hepatic complications

2.4 Kidney and liver dysfunction in T2DM

Kidney dysfunction in T2DM due to chronic hyperglycaemia which has been shown to cause abnormal morphology of the nephron and hemodynamic dysregulation which are associated with functional changes in nephron such as increased thickness of the glomerular basement membrane, mesangial expansion, extracellular matrix deposition, glomerulosclerosis, overt proteinuria, and decreased glomerular function as well as filtration rate to eventual end-stage renal damage (Jitraknatee *et al.*, 2020; Agarwal *et al.*, 2022). T2DM also linked to the onset and progression of DN through inappropriate activation of the renin–angiotensin system (RAS), elevated levels of proinflammatory cytokines and oxidative stress in the kidney (Roca-Ho *et al.*, 2020). Chronic hyperglycaemia and moderate insulin resistance in male Sprague Dawley rats is associated with non-alcoholic fatty liver disease (NAFLD) (Newton *et al.*, 2016; Targher *et al.*, 2021). NAFLD is associated with alterations in the permeability of the cell membrane and damage in the liver tissue which is shown by abnormal liver enzymes in the circulation (Bhatt & Smith, 2015). Renal dysfunction is a risk factor for PE, as impaired kidney function can lead to retention of sodium and water, increased blood pressure, and fluid retention, all of which are features of PE.

2.5 Preeclampsia

2.5.1 Pathophysiology of PE

PE is a pregnancy complication characterized by hypertension (140/90mmHg), and either proteinuria ≥ 300 mg/L in urine in 24 h or other systemic symptoms after 20 weeks of gestation (Rana *et al.*, 2019; Magee LA *et al.*, 2022). The pathologic process originates in the placenta, with inadequate cytotrophoblast invasion in early pregnancy (Pennington *et al.*, 2012). The pathogenesis of PE has two stages. Stage 1 of PE is thought to begin when trophoblasts are unable to sufficiently invade the decidual side, spiral artery remodeling failure occurs, resulting in ischemia-reperfusion in the intervillous space (Rana *et al.*, 2019). This causes placental

oxidative stress and endoplasmic reticulum stress, leading to Stage 2 of PE. The production of antiangiogenic factors such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) by trophoblasts is promoted, followed by their release into the maternal circulation from the intervillous space (Pennington *et al.*, 2012). This manifests as various clinical symptoms, including both maternal and fetal vascular endothelial damage, maternal hypertension and proteinuria, and fetal IUGR (Hauth *et al.*, 2000).

In PE alternative splicing of sFlt-1 results in production of the secreted protein, sFlt-1, which lacks the cytoplasmic and transmembrane domains but retains the ligand binding domain (Aubuchon *et al.*, 2011). Placental trophoblasts exposed to stress, such as hypoxia and inflammation, release large amounts of sFlt-1, a potent anti-angiogenic molecule that sequesters circulating VEGF and PlGF and prevents their interaction with endogenous receptors on placental and maternal cells leading to abnormal placentation associated with PE and IUGR as well as maternal syndrome (Aubuchon *et al.*, 2011). Along with sEng, another anti-angiogenic protein of placental origin, sFlt-1, has been shown to be elevated in clinical PE suggesting that they contribute to disease pathogenesis (Cim *et al.*, 2017). Studies in experimental pregnancy models suggest that inflammation is a potent trigger of angiogenic dysregulation (Aubuchon *et al.*, 2011). In this study, we will outline the effects of pregestational PD on VEGF and PlGF to establish whether pregestational PD is a risk factor for PE. The elevation of anti-angiogenic factors has adverse effects on the placenta and other maternal organs such as the liver and the kidneys due to the release of antiangiogenic factors into the maternal circulation from the intervillous space and act on the maternal vascular endothelium, inducing oxidative stress and stimulating the production and secretion of pro-inflammatory cytokines (Soobryan *et al.*, 2022).

PE can be classified into early onset and late-onset. Early onset PE is observed at 20- 34 weeks of pregnancy reflecting inadequate placentation, the release of antiangiogenic factors, and ROS

which are responsible for the promotion of endothelial lesions. Early onset PE is referred to as placental PE (Hauge *et al.*, 2024). Late-onset observed at 34 weeks – parturition is characterized by normal or slightly altered placentation with clinical manifestations resulting from a previous endothelial compromise which is exacerbated by pregnancy. Late-onset PE is referred to as maternal PE (Hauge *et al.*, 2024).

2.5.2. Treatment of PE

A major concern of PE is the delay in the appearance of clinical symptoms. The clinical symptoms are detected after 26-28 weeks (severe PE) or more commonly after 34-36 weeks (late onset) (Abdخالimovich & Ro'ziyev, 2024). Studies have shown that the only definitive cure for PE is the delivery of the fetus, and available therapies for PE only have symptom management purposes (Abdخالimovich & Ro'ziyev, 2024). The current clinical management of PE is classified into pharmacological interventions which are aimed at restoring the angiogenic balance, may prevent or modify the course of PE, and antihypertensive therapy which is aimed at lowering blood pressure to less than 160/105 mmHg (mean arterial pressure less than 125 mmHg) (Garovic *et al.*, 2022).

Pharmacological intervention includes using magnesium sulfate, aspirin, and calcium supplementation. Magnesium sulfate is anti-inflammatory and is used to treat maternal seizures and reduce maternal blood pressure (Chiarello *et al.*, 2018; Lin *et al.*, 2023). Magnesium sulfate has been shown to normalize placental interleukin-6 synthesis in a model of PE (Chiarello *et al.*, 2018; Padda *et al.*, 2021). Researchers have explored the role of aspirin in preventing and treating PE, as inflammation appears to play a critical role in the pathophysiology of PE (Atallah *et al.*, 2017; Rolnik *et al.*, 2022). The benefits of aspirin in preventing PE and its associated vascular complications may stem not only from aspirin anti-inflammatory properties but also from its potential ability to restore the balance between

thromboxane and prostacyclin in the vascular system (Henderson *et al.*, 2021; Rolnik *et al.*, 2022). 1 gram per day of calcium supplementation has been linked to a significant reduction in the risk of developing PE, particularly among women with low-calcium diets (Khaing *et al.*, 2017). However, calcium supplementation does not lower the overall incidence of PE, it mitigate adverse outcomes in women who developed PE (Cormick *et al.*, 2020; Dwarkanath *et al.*, 2024).

Antihypertensive therapy is recommended for severe hypertension (systolic BP ≥ 160 mm Hg or diastolic BP ≥ 110 mm Hg) to reduce the risk of maternal complications like stroke (Garovic *et al.*, 2022). The first-line agent is methyldopa which is commonly used due to its safety profile in pregnancy (Sharma *et al.*, 2024). Methyldopa works primarily in the CNS to lower blood pressure, when metabolized into alpha-methylnorepinephrine, which stimulates alpha-2 adrenergic receptors in the brainstem (Afroz *et al.*, 2024; Sharma *et al.*, 2024). This action reduces sympathetic outflow and decreases peripheral vascular resistance, eventually leading to lower blood pressure (Afroz *et al.*, 2024; Sharma *et al.*, 2024). Alternatively, a beta antagonist in particular labetalol, Labetalol is effective in treating and preventing complications of PE by lowering blood pressure through its dual alpha- and beta-blocking effects (Arshad *et al.*, 2024; Sharma *et al.*, 2024). Labetalol provides rapid and sustained blood pressure control, protects maternal organs, and preserves placental blood flow. Dosages range from 200 mg to 1.2 grams per day and taken orally in divided doses (Arshad *et al.*, 2024; Bade *et al.*, 2024). Nifedipine has a vasodilatory effect which helps manage hypertension in PE (Ding *et al.*, 2017; Donel *et al.*, 2023). Nifedipine reduces blood pressure, protects maternal organs, and improves placental blood flow (Overton *et al.*, 2022; Nimbark *et al.*, 2024). PE is associated with reduced secretion of vasodilators and increased secretion of vasoconstrictors, prompting researchers to explore the potential therapeutic benefits of NO donors for managing and preventing PE (Dickerson *et al.*, 2024) .

2.5.3 Kidney dysfunction in PE

During normal pregnancy, the kidneys undergo several changes to support the growing fetus and the mother's changing body. These changes include an increase in kidney size, higher blood flow through the kidneys and alteration in filtration rates to accommodate the increased metabolic demands (Tom Cornelis *et al.*, 2011). These changes aim to help maintain electrolyte balance, manage fluid retention. However, in pathological condition there is significant deviations on these normal changes. The following section will discuss kidney dysfunction in PE.

The kidney dysfunction in PE is caused by glomerular endothelial damage, hypertension and an imbalance between pro-angiogenic and antiangiogenic factors (Karumanchi *et al.*, 2005). The antagonism of VEGF and PlGF by sFlt-1 plays a role in the development of kidney dysfunction in PE. The upregulation of antiangiogenic factors has been shown to occur in hypoxic conditions and prolonged renal hypoperfusion results in acute tubular necrosis (Magee *et al.*, 2022). VEGF is a potent promoter of angiogenesis, VEGF is also known to induce NO and a vasodilatory effect in endothelial cells decreasing vascular tone and blood pressure (Eremina *et al.*, 2008). Studies using animal models have shown that VEGF is important in maintaining glomerular endothelial cell health and healing. Therefore VEGF expression in organs is necessary to induce and maintain the health of the fenestrated endothelium (Ali *et al.*, 2019). The antagonism and absence of VEGF induces proteinuria and glomerular endotheliosis. Studies have shown that tissues with fenestrated endothelium, especially the renal glomerulus, might be particularly susceptible to damage by sFlt-1-mediated VEGF blockade (Holmes *et al.*, 2013).

PE is associated with glomerular endotheliosis a characteristic of renal lesion shown by glomerular endothelial swelling, occlusion of capillary lumens and loss of fenestration due to the injury of podocytes leading to proteinuria (Karumanchi *et al.*, 2005). Both podocyte and

podocyte specific proteins, podocin and nephrin are found in urine of PE women thereby supporting podocyte loss and injury as the mechanism of proteinuria in PE (Henao & Saleem, 2013; Gilani *et al.*, 2017). Women with PE have been shown to have elevated nephrinuria, suggesting that nephrin shedding occurs in PE. Increased nephrinuria was found to be positively correlated with the degree of proteinuria, suggesting that nephrin shedding is associated mechanistically with disruption of the slit diaphragm (Fukuda *et al.*, 2012). Hypertension in PE can also cause kidney damage and dysfunction, high blood pressure can reduce blood flow to the kidneys which impairs filtration in the kidney. In PE glomerular filtration rate (GFR) is reduced due to the damage to the small blood vessels. The reduction in GFR leads to impaired electrolyte handling, accumulation of waste products in the body and fluid retention which eventually leads to proteinuria and oedema (Nityasree Srialluri *et al.*, 2023). However, the adverse effect of PD in pregnancy together with the effects of antiangiogenic factor on kidney function have not yet been shown. In this study therefore, we investigated the effects of PD on kidney function during pregnancy by looking at the following at the effects of pregestational PD podocyte specific proteins (podocin and nephrin), KIM-1, electrolyte handling and kidney oxidative stress. This effort was envisaged to possible shed light on how diet induced PD can be a risk factor for PE.

2.5.4 Liver dysfunction in PE

Maternal body goes through a number of changes during normal pregnancy to support the needs of a growing fetus and the mother's altered physiology. The liver undergoes through the following changes an increase in size due to increased blood flow and hormonal changes, increased glycogen storage to provide energy source for both the mother and the developing fetus and also the altered liver enzyme levels (Di Cianni *et al.*, 2003; Bacq, 2013). However, in pathological condition there is significant deviations on these normal changes. The following section will discuss liver dysfunction in PE and PD.

The clinical findings of severe PE are unified by the presence of systemic endothelial dysfunction and microangiopathy in which the target organ may be the brain (seizures or eclampsia), the liver [the hemolysis, elevated liver function tests, and low platelet count (HELLP) syndrome], or the kidney (glomerular endotheliosis and proteinuria). Of focus for this study is the liver and the kidneys (Arulkumaran & Lightstone, 2013).

Impaired liver function is observed in severe PE due to the an imbalance in pro-angiogenic and anti-angiogenic factors which indicates a hypoxic condition (Frank Wolf *et al.*, 2016). Hepatocytes are arranged in the sinusoids with fenestrated epithelial lining. Therefore, upregulation antiangiogenic factors has been shown exert their adverse effects on the maternal endothelium including all areas of fenestrated endothelium in the liver (Frank Wolf *et al.*, 2016). . Antiangiogenic factors can affect fenestrated endothelium by reducing vessel permeability, altering vessel structure and regulating fluid exchange have the effects on fenestrated endothelium (Andraweera *et al.*, 2012). This causes hepatocytes to leak and release liver enzymes to the maternal circulation indicating liver injury.

PE is associated with an increase in fibrosis due to an alteration in lipid metabolism and steatosis (Frank Wolf *et al.*, 2016). Hepatic microvesicular steatosis causes mitochondrial dysfunction and oxidative stress in hepatic subcellular organelles. Oxidative stress in the liver results in endothelial cell injury which exposes subendothelial collagen which is a risk factor for liver cirrhosis and portal hypertension (Alese *et al.*, 2021).

Oxidative stress and increased hypoxic condition in the liver causes a disruption of endothelium resulting in a reduction of prostacyclin level and an increase of thromboxane level (Munazza *et al.*, 2011). There is an increase in the ratio of prostacyclin to thromboxane in favor of thromboxane, resulting in vasoconstriction of blood vessels of the liver which further contribute hypoxia in the liver (Dacaj *et al.*, 2016)

The subsequent effects of hypoxia in the liver will be responsible for necrosis and degeneration of hepatocytes and increased liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) in the circulation (Alese *et al.*, 2021). Pathophysiological abnormalities such as generalized vasospasm, vascular endothelial dysfunction, abnormal lipid metabolism, and insulin resistance could affect the liver and result in partially elevated liver enzymes prior to the development of PE (Dacaj *et al.*, 2016). There are currently limited studies investigating the effects of PD during pregnancy on liver function. Therefore, this study sought to investigate the effects of PD on liver function during pregnancy. This will be in an effort to show how pregestational PD may present with PE like complications to further show PD as a risk factor to PE by looking at the following at the effects of pregestational PD on inflammation, liver enzymes and liver TGs.

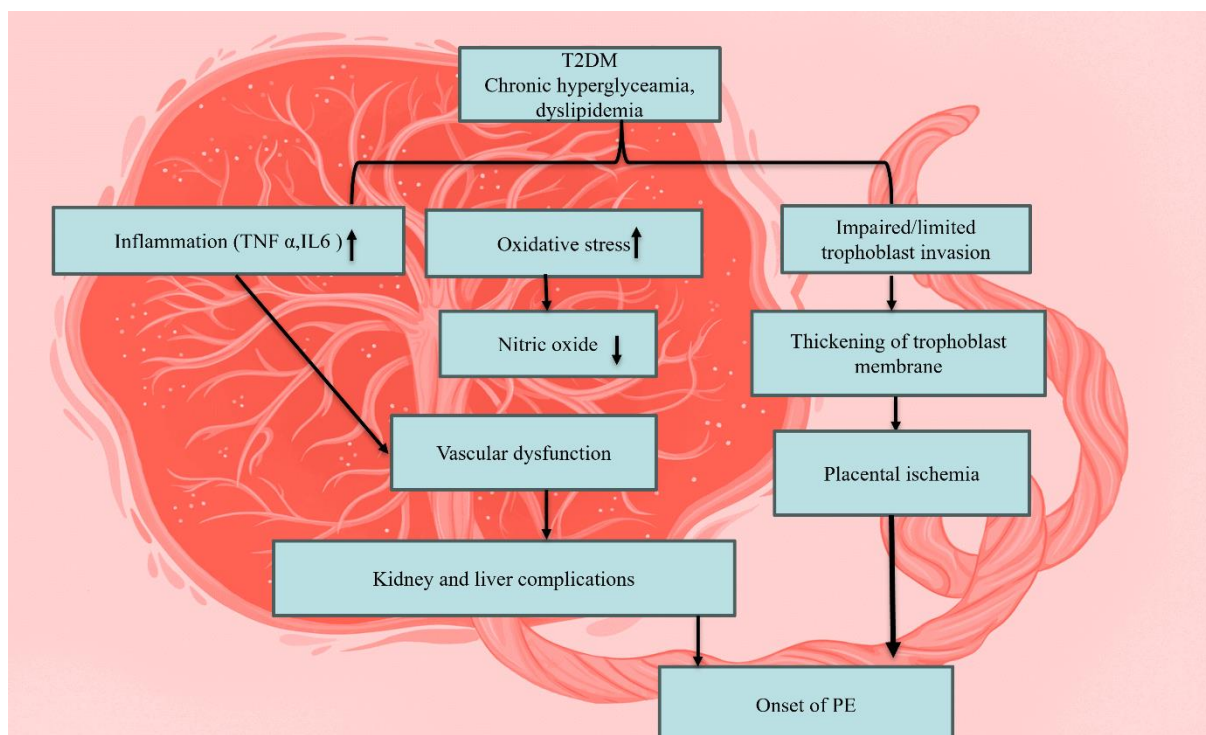


Figure 1: A schematic diagram on how T2DM leads to the onset of PE

2.6 Correlation on the incidence of T2DM and PE in humans

T2DM and PE have common risk factors such as obesity, advanced age, and multiple pregnancy (Weissgerber & Mudd, 2015). Both T2DM and PE share pathophysiological mechanisms characterized by oxidative stress, the release of pro-inflammatory factors, and dysfunction of vascular endothelial cells (Weissgerber & Mudd, 2015). Therefore, there is a correlation between T2DM and PE. Hyperglycaemia can induce trophoblast inflammation and autophagy, inhibit trophoblast migration and invasion resulting in placental ischemia which is related to the occurrence of PE (Han *et al.*, 2015; Heim *et al.*, 2018). Hyperglycaemia induces oxidative stress through various pathways, one of the pathways is the formation of advanced glycation end products (AGEs) (Nowotny *et al.*, 2015).

This process leads to an increase in reactive oxygen species, which reduces the levels and availability of circulating NO, ultimately impairing vasodilation (Molehin *et al.*, 2020). In women with T2DM, there is an elevation of AGEs, which can contribute to the development of PE by promoting oxidative stress and inflammation (Molehin *et al.*, 2020). Additionally, inflammatory markers such as serum tumor necrosis factor-alpha (TNF- α) and Interleukin-6 (IL-6) are elevated in women with both T2DM and PE, indicating endothelial dysfunction. Some studies suggest that TNF- α , IL-6, and C-reactive protein (CRP) act as independent risk factors for PE in women with T2DM (Galicia-Garcia *et al.*, 2020). Hyperinsulinemia and insulin resistance caused by obesity before pregnancy are related to the migration of cytotrophoblast and the reduction of uterine spiral artery remodelling, which is more likely to lead to placental ischemia (Al Beloushi *et al.*, 2020).

3 Animal model for PE and PD

Animal models of PE can be classified as (i) spontaneous, (ii) surgically induced, (iii) pharmacologically induced or substance-related, and (iv) transgenic (Aubuchon *et al.*, 2011). An ideal animal model of PE would exhibit all the symptoms seen in women with PE,

including hypertension, proteinuria, endothelial dysfunction, and an imbalance of angiogenic factors, all of which arise secondary to poor trophoblast invasion and resolve following delivery of the placenta (Gatford *et al.*, 2020). This study focuses on the pharmacologically induced or substance-related PE model. The main focus is on NO-related models of PE. NO is a potent vasodilator that is synthesized from the amino acid L-arginine, by eNOS (Zullino *et al.*, 2018). Acute blockade of NO synthesis in studies using rabbits, guinea pigs and rats has demonstrated a marked rise in systemic blood pressure in these animals (Forstermann & Munzel, 2006; Taylor & George, 2022). Furthermore, chronic inhibition of NOS using nitro-L-arg-methyl ester (L-NAME) in pregnant rats led to the development of a model characterized by increased antiangiogenic factors, hypertension, proteinuria, glomerular sclerotic injury, thrombocytopenia and IUGR which are all symptoms of PE (Lowe, 2000 ; Bakrania *et al.*, 2021). Other models that can be used for PE include the BPH/5 mouse model. BPH/5 mouse when exposed to pro-inflammatory factors such as lipopolysaccharides (LPS) or angiotensin II receptor agonistic autoantibodies to induce hypertension and proteinuria therefore mimicking the features of human PE, including hypertension, proteinuria, and placental inflammation (Davisson *et al.*, 2002). The BPH/5 Mouse model allows investigation of immune dysregulation and the role of inflammation in PE (Davisson *et al.*, 2002). STOX-1 mouse model (storkhead-box 1) uses a transcription factor involved in placental development (Dunk *et al.*, 2021; Bakrania *et al.*, 2022). Overexpression of STOX-1 in transgenic mice leads to PE-like symptoms such as placental insufficiency, hypertension, proteinuria, and fetal growth restriction (Dunk *et al.*, 2021). The placenta from STOX-1 mouse model exhibits features such as oxidative stress and abnormal trophoblast invasion which are similar to human PE (Dunk *et al.*, 2021). STOX-1 mouse model is used in exploring the genetic and molecular mechanisms underlying placental dysfunction (Dunk *et al.*, 2021). Reduced Uterine Perfusion Pressure (RUPP) Rat model which is created by mechanically reducing blood flow to the uterus in

pregnant rats, typically by partial occlusion of the abdominal aorta and uterine arteries (Bakrania *et al.*, 2022; McClements *et al.*, 2022). This induces placental ischemia, oxidative stress, and activation of inflammatory and angiogenic pathways with the following key features hypertension, proteinuria, and fetal growth restriction, closely mimicking the maternal and fetal outcomes of human PE (van Kammen *et al.*, 2024). RUPP is used in studying the impact of placental ischemia on maternal cardiovascular and renal systems (van Kammen *et al.*, 2024).

PD is characterized by moderate hyperglycaemia, hyperinsulinaemia and moderate oxidative stress which may reflect endothelial dysfunction (M Luvuno *et al.*, 2018). C57Bl/6J female mice are commonly used for research related to PD, T2DM and PE. A standard animal model for development of insulin resistance is C57Bl/6J that are exposed to a high fat diet. In C57Bl/6J key characteristics of PE were observed which include elevated blood pressure, proteinuria and impaired fetal and placental growth (de Alwis *et al.*, 2022). Animals used to study obesity, T2DM and PD are diet-induced and genetically modified. These animal models include (i) genetically modified C57BL6 female mice on a high-fat diet, (ii) genetically modified C57BL6 female mice on high fructose diet, and (iii) Sprague Dawley rats on high carbohydrate, high fat, and high fructose diet (HCHF+ fructose). C57Bl/6J mice are primarily utilized as models for obesity and T2DM. These models are instrumental in studying metabolic disorders and associated placental changes rather than directly modelling PE (Aye *et al.*, 2015; Mahany *et al.*, 2018; Lang *et al.*, 2019). Studies on C57BL6 pregnant mice have shown that these pregnant mice when exposed to a high fat or a high fructose diet present with hypertension, proteinuria, endothelial dysfunction, placental inefficiency and fetal growth restriction which are features of PE (Jones *et al.*, 2009; Aye *et al.*, 2015; Asghar *et al.*, 2016). C57BL6 female mice on a high-fat diet in pregnancy is associated with fetal overgrowth due to high fat diet causing the up-regulation of placental nutrient transporters such as GLUT 1 & SNAT2 in placental barrier (Ge *et al.*, 2013). Placenta from mice fed a high-fat diet during

pregnancy have shown elevated expression of HIF-1 α and sFlt-1 and reduced VEGF which suggests that vascular development is delayed in pregnant mice on a high fat diet (Kretschmer *et al.*, 2020).

C57BL6 female mice on a high-fructose diet in pregnancy is associated with placental inefficiency and fetal growth restriction due to placental uric acid which contributes to fetal growth restriction through inhibiting the placental amino acid transport and oxidative stress (Asghar *et al.*, 2016). Placenta from mice fed a high-fructose diet during pregnancy have been shown to have reduced vascularity (Asghar *et al.*, 2016). These alterations that occur in the placenta of mice on a high fructose diet account for the lower weights of the placenta and the pups that have been observed in mice that were on a high fructose (Asghar *et al.*, 2016). High-fat diet has been extensively investigated in animal models, particularly in mice, to assess their effects on pregnancy and fetal development (Sanches *et al.*, 2022). Research has shown that a maternal high-fat diet, both before and during pregnancy can adversely affect fetal growth, leading to intrauterine growth restriction (IUGR) (Ge *et al.*, 2013; Sanches *et al.*, 2022). A high-fat diet can result in obesity, insulin resistance, and systemic inflammation in the mother. These metabolic alterations have been shown to negatively impact placental function, thereby diminishing nutrient and oxygen transfer to the fetus (Ge *et al.*, 2013). A High-fat diet has been shown to disrupt placental structure and function, reduced vascularization and altered expression of nutrient transporters in the placenta are observed, contributing to fetal nutrient deprivation (Zhu *et al.*, 2023). A maternal high-fat diet increases oxidative stress and inflammation in the uterine and placental environment. Elevated levels of pro-inflammatory cytokines such as TNF- α , IL-6 interfere with fetal growth signalling pathways (Ge *et al.*, 2013). Chronic high-fat diet consumption before pregnancy establishes a baseline of metabolic dysfunction. Adipose tissue inflammation and insulin resistance are exacerbated once pregnancy begins which further compromises fetal growth (Zhu *et al.*, 2023). A continued high-

fat diet intake during gestation worsens maternal metabolic health. It has been associated with increased lipid deposition in the placenta and reduced efficiency of nutrient transport to the fetus (Kannan *et al.*, 2021).

There are no animal studies linking diet-induced PD on pregnant female rats as a risk factor for PE. Furthermore, the mechanisms involved are not fully outlined. In our laboratory we have developed a diet induced PD model using a HCHF+ fructose diet, that mimics the western styled diets. On male Sprague rats, the HCHF+ fructose diet has been shown to cause renal and cardiovascular complications (Khathi A *et al.*, 2019; Mluleki Luvuno *et al.*, 2021). We have also established a female animal model of PD using a HCHF+ fructose diet (Nkosi *et al.*, 2024). However, there are no studies that have investigated whether PD pregnant rats are at risk of developing PE. Therefore, in this study, HCHF+ fructose induced prediabetic rats will be used to investigate pregestational PD as a risk factor for PE and the adverse effects of maternal PD on the placenta, liver and kidney.

3.1 The prevalence of PD

PD is defined by moderate elevation in blood glucose levels, where glucose concentrations exceed the normal range but do not reach the threshold for diagnosing T2DM (Edwards & Cusi, 2016; Beulens *et al.*, 2019). PD can be diagnosed through the presence of impaired fasting glucose (5.6-6.9 mmol/L), impaired glucose tolerance (7.8-11 mmol/L) and elevated levels of glycated haemoglobin (HbA1c) (Tabák *et al.*, 2012). PD predisposes individuals to the onset of T2DM as well as the macrovascular and microvascular complications of T2DM (Palladino *et al.*, 2020). In 2019, the global prevalence of PD was approximated at 7.5%, equating to 374 million individuals, and is anticipated to rise to 8.0%, encompassing 454 million people, by 2030 (Saeedi *et al.*, 2019). A recent meta-analysis on people in the ages of 25-45 years indicated that the overall prevalence of PD was 15,56% in the South African population, which translates to over 9 million people (Sosibo *et al.*, 2022). This study highlighted that there is burden of PD

in people of reproductive ages suggesting that there is a high likelihood that when a female gets pregnant, they are at risk of developing pregestational PD.

4 . Justification of the study

T2DM is characterized by chronic hyper glycaemia, hyperlipidaemia which are linked to endothelial dysfunction due to reduced NO level and bioavailability which leads to vasodilation dysfunction and vascular dysfunction (Galicia-Garcia *et al.*, 2020). Additionally, lipid accumulation and insulin resistance further contribute to endothelial dysfunction, thereby playing a role in the development of atherosclerosis which can lead to reduced blood flow and oxygen delivery to tissues, potentially causing local hypoxia (Huynh *et al.*, 2015). T2DM is associated with the reduction in expression of VEGF and HIF1 α which are associated with local hypoxia and are implicated in the onset PE (Kapustin *et al.*, 2021). The onset of T2DM is preceded by PD and the incidence of PD is on the rise. This means more women are at a risk of developing pregestational PD. There are no studies that have looked at how PD affects placental, renal and liver function and whether it predisposes individuals to developing PE. In this study, we sought to investigate the effects pregestational PD as a risk factor of PE and the adverse effects of the pregestational PD on placental, renal and hepatic function.

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Chapter 3: Methodology

Introduction

Chapter 3 gives a general description of how the study was conducted. The sub-sections of this chapter include, study design, sample size calculation of the various manuscripts, data collection, criteria for diagnosis of PD, experimental work and the statistical analysis.

Chemicals/reagents

Drugs and chemicals of analytical grade were sourced from standard pharmaceutical suppliers.

Ethical considerations

The presented study was approved by the University of Kwazulu-Natal ethics committee (AREC) as the procedures involving animals and their care were conducted in conformity with the institutional guidelines. Approved ethics number: AREC/025/19D. upon ethical clearance approval the study was conducting in the Biomedical Research Unit of the University of Kwazulu-Natal.

Study site, population, and design

Study site

The study was conducted in the Biomedical Research Unit of the University of Kwazulu-Natal in Durban, South Africa upon approval by AREC.

Study Design

The role of pregestational PD as a risk factor of PE was studied in the following groups. Group 1: normal pregnant control rats (ND) which received normal standard chow and normal drinking water throughout the study (n = 6/group); Group 2: PD pregnant rats which received the HCHF diet+ 15% fructose throughout the study (n = 6/group); Group3: PE pregnant control rats which received normal standard chow and 0.3g/l LNAME in drinking water from GND 8 to GND 14 (n = 6/group). The PE group served as a standard group, On GND 0, 9,18 body

weight, blood glucose, TGs, blood pressure, 24 hr fluid intake, food intake and urine output were measured.

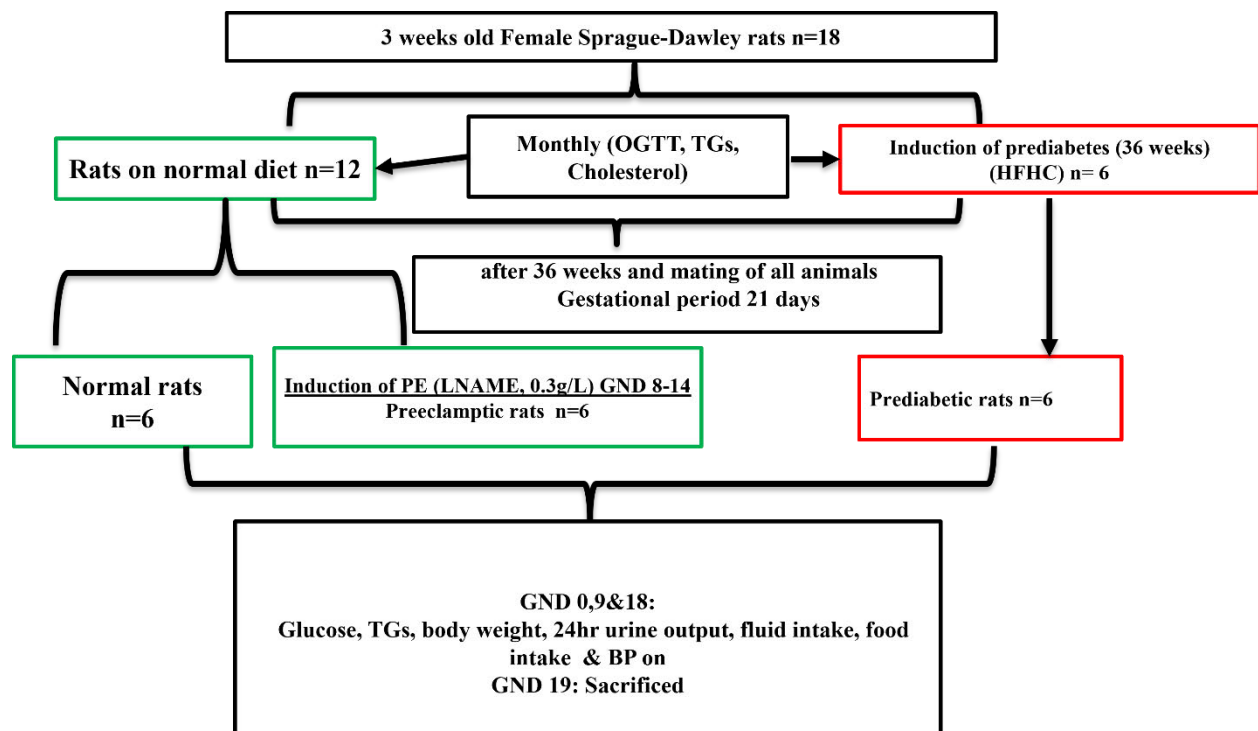


Figure 1: Study design

Sample size calculation

Sample size (n) for the study was calculated using G*Power (version 3.1.9.7), a statistical power analysis software, this was performed to ensure that the sample size was adequate to detect statistically significant differences between the groups while maintaining ethical considerations for animal use.

Induction of PD

After 1 week of the acclimatization to new environment, PD was induced in 4-week-old female Sprague-Dawley rats (n=6) using an established protocol from our laboratory with slight modification. Briefly, the experimental animals in the prediabetic group (n=6) were exposed to a high carbohydrates, high fat diet and 15 % fructose (HCHF diet+ 15% fructose) in drinking water for 39 weeks. The rest of the animals were assigned into the following groups the control

group (n=6) and preeclamptic group (n=6) which received standard chow diet and normal water for 39 weeks. During the PD induction period all the rats were also acclimatized for blood pressure monitoring once a month. The blood pressure monitoring is described later in this section.

Criteria for PD

The American Diabetes Federation criteria was used to diagnose PD in all animals. Animals that had prediabetic symptoms including fasting blood glucose concentration of 6.1-7.1 mmol/L, a two-hour glucose concentration of 7.1-8.1 mmol/L in the oral glucose tolerance test, and plasma triglycerides concentration of greater than 2 mmol/L were considered prediabetic. Blood samples were collected from the tail vein through tail pricking method. Glucose was determined using the Elite® glucometer and blood TGs were determined using the accutrend plus cholesterol meter.

Mating

At the end of the 39th week of induction period, the female Sprague-Dawley rats from Normal (n=6), PE (n=6) and PD (n=6) group were mated with normal males of the same strain. Briefly two females were housed in one cage to allow their oestrus cycle to become synchronised. The ovarian cycle of rats was between four to five days long. Daily vaginal smears were taken and when the female rats were in the pro-oestrus stage of their cycle, a male rat was introduced into the cage. The following day, vaginal smears were taken from the female rats and the presence of sperm indicated successful mating and cytology was used to confirm positive pregnancy. The day of fertilisation was regarded as Gestational Day (GND) 0. The male rats were then be removed from the cage and male rats were sacrificed after successful mating.

Induction of preeclampsia (PE)

The pregnant dams (n=6) from the PE group were given 0.3g/l a day of N^ω-nitro-L-arginine methyl ester (L-NAME) in drinking water from GND 8-GND 18. Blood pressure and proteinuria were determined to confirm successful induction of PE on GND 9. Blood pressures were monitored using a non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA). The animals warmed at $\pm 30^{\circ}\text{C}$ in an enclosed chamber (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Wood land Hills, California, USA) for 30 minutes before taking blood pressure readings.

Plasma and Tissue collection

On GND 19, pregnant rats were sacrificed by decapitation for collection of the placenta, pups by C section and blood. Plasma was obtained by collecting blood into single precooled heparinized containers and the blood was centrifuged at $1000 \times g$ for 10 min in a Hermle Laborechnic GmbH centrifuge (Wehingen, Germany). The placentas were removed, weighed, and then snap frozen in liquid nitrogen. Afterwards, the placenta and plasma were stored in an Ultra Bio Freezer (Snijers Scientific, Tilburg, Netherlands) at -80°C until biochemical

Experimental design of all 3 studies

Study 1

In study 1, the concentration of nitric oxide of all 3 groups were measured from the plasma using a spectrophotometer (Spectrostarnano, BMG LABTECH, Ortenberg Germany). MDA concentration of the 3 groups was measured from the placenta tissue using the Biotek mQuant spectrophotometer (Biotek, Johannesburg, South Africa. Antioxidant enzyme (SOD 1) of all 3 groups were measured on the placenta tissue using an ELISA kit. as per manufacturer's instructions. More information on the methodology for study 1 is given in manuscript 1 (Chapter 4). Angiogenic and antiangiogenic factors expression (VEGF, PlGF and sFlt respectively) of all 3 group was measured on the placenta tissue using Real time-PCR,

Study 2

In study 2, analysis of electrolytes (Na⁺ and K⁺) and kidney function parameters (creatinine, urea, albumin) of all 3 groups were measured from the plasma and urine samples by using IDEXX instrument (IDEXX laboratories (PTY) Ltd. Pretoria, South Africa), antioxidant enzyme (TSOD) of all 3 group were measured on the kidney tissue using an ELISA kit. as per manufacturer's instructions. More information on the methodology for study 2 is given in manuscript 2 (Chapter 5). Aldosterone and KIM 1 concentration of all 3 groups was measured in plasma using an ELISA kit, as per manufacturer's instructions. More information on the methodology for study 2 is given in manuscript 2 (Chapter 5). Expression of Podocyte markers (podocin and nephrin) of all 3 groups was measured in urine Real-time quantitative PCR.

Study 3

In study 3, hepatic triglycerides of all the 3 groups were measured from liver tissue using a triglyceride assay kit, as per manufacturer's instructions. More information on the methodology for study 3 is given in manuscript 3 (Chapter 6). Inflammatory markers (IL 6 and TNF α) of all 3 groups were measured in liver tissue using an ELISA kit, as per manufacturer's instructions. More information on the methodology for study 3 is given in manuscript 3 (Chapter 6). Liver enzymes (ALT and AST) and total bilirubin of all 3 groups were measured in plasma using the Catalyst One Chemistry Analyzer (IDEXX Laboratories, Westbrook, ME, USA). Red blood cell (RBC) count, haemoglobin (Hb), and platelets concentration of all 3 groups were measured from fresh blood haematocytometer (Beckman Coulter, Indianapolis, United States). MDA concentration of all the 3 groups was measured from the liver tissue according to an established protocol. More information on the methodology for study 3 is given in manuscript 3 (Chapter 6). SOD concentration of all 3 groups was measured on the liver using an ELISA kit as per manufacturer's instructions. More information on the methodology for study 3 is given in manuscript 3 (Chapter 6)

Statistical data analysis

Statistical analysis was conducted using GraphPad Prism Software (version 9.00, GraphPad Software, San Diego, California, USA). A normality test (Shapiro-Wilk test) was done followed by a parametric one-way ANOVA. To analyse differences between the controls and the experimental group, Bonferroni's multiple comparisons comparison test was performed. Values of $p < 0.05$ was used to indicate statistical significance.

Chapter 4

Prologue 1

The diagnostic criteria for PE includes hypertension after 20 weeks of gestation and the coexistence of new-onset proteinuria, maternal organ dysfunction, and uteroplacental dysfunction. The definite pathogenesis of PE is still to be fully understood, however, studies indicate that uteroplacental dysfunction plays a significant role in the development of the maternal disorder. Uteroplacental dysfunction is associated with the imbalance between angiogenic factors like vascular endothelial growth factor (VEGF) or placental growth factor (PlGF) and anti-angiogenic factors like soluble fms-like tyrosine kinase 1 (sFlt-1). This imbalance causes endothelial dysfunction. T2DM has been implicated in the pathogenesis of endothelial dysfunction due to the adverse effects of hyperglycaemia on endothelial cells. Prolonged exposure of endothelial cells to hyperglycaemia has been shown to increase the production of ROS causing oxidative stress. Oxidative stress damages endothelial cells and disrupts the delicate balance of vasodilation and vasoconstriction which ultimately leads to endothelial dysfunction. Pregnant mothers with T2DM/GDM have been shown to have compromised endothelial function rendering them susceptible to uteroplacental dysfunction and the risk of developing PE. PD is condition that precedes T2DM and is characterized by intermediate hyperglycaemia, moderate increase in ROS in PD may also be a risk factor for the development of PE however this has not been shown. The present study investigated the effects of pregestational PD on parameters associated with placental dysfunction in a diet induced pregnant prediabetic rat model.

Details of Manuscript

The manuscript is titled “ **Maternal prediabetes as a risk factor of preeclampsia and placental dysfunction in pregnant female Sprague-Dawley rats**” and is authored by Siboto A, Ludidi A, Xulu N, Nkosi A, Sibiya N Khathi A, Ngubane PS and has been formatted according to journal’s guidelines for author(<https://doi.org/10.1080/01443615.2024.2379498>) This journal is accredited by Department of Higher Education and Training South Africa and appears in Scopus accredited list (2021).

Author Contribution: A Siboto was responsible for study conceptualization, study design, conducted experimental work, data analysis first draft writing, and manuscript editing.



Manuscript 1

Pregestational PD mellitus as a risk factor of PE: the adverse effects of pregestational PD on placental function.

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Abstract

PD is associated with intermediate hyperglycaemia dyslipidemia, reduced nitric oxide bioavailability and moderate hypertension. All these factors are risk factors for preeclampsia (PE). However, the effects of the PD during pregnancy on placental dysfunction has not been shown. Accordingly, this study assessed the possible link between maternal PD and the risk of developing PE by investigating the effects of pregestational PD on placental function.

Pregnant female Sprague-Dawley were divided into normal, preeclamptic and prediabetic groups to study the effects of pregestational PD over 19 days of gestation. Blood glucose concentration, and blood pressure were measured on GND 0,9 and 18. Placental vascular endothelial growth factor (VEGF), placenta growth factor and (PlGF) and soluble fms-like tyrosine kinase 1 (sFlt-1) mRNA expression were measured terminally.

Pregestational PD and PE increased blood glucose concentrations. These observations were associated with a decrease in plasma NO concentration and an increase in MAP by comparison to the normal pregnant control group. Maternal PD significantly decreased placental VEGF, PlGF mRNA expression with a slight increase in sFlt-1 mRNA expression.

The results suggest that impaired glucose handling along with an imbalance in angiogenic and antiangiogenic factors in the PD pregnant group is associated with endothelial dysfunction which predispose women to PE. The findings also suggest that pregestational PD is associated with placental dysfunction.

Keywords: pregestational PD, intermediate hyperglycaemia, placenta, vasculogenesis and angiogenesis

1. Introduction

The etiology of PE is unclear, however previous studies have shown that the placenta is the principal contributor as the pathology begins with inadequate cytotrophoblast invasion in early pregnancy (Chiarello *et al.*, 2020). PE is characterized by hypertension and either proteinuria or other systemic symptoms after 20 weeks of gestation (Rana *et al.*, 2019). The risk factors of PE can arise from trophoblastic and non-trophoblastic maternal factors (Weissgerber & Mudd, 2015; Staff, 2019). Trophoblastic maternal risk factors include conditions associated with inadequate placentation observed in PE and IUGR (Magee *et al.*, 2022). At about 10 weeks of gestation, when the placental villi is developed, the cytotrophoblast from placental villi invades the space around the spiral artery (Whitley & Cartwright, 2009; Wang, 2017). This process causes the loss of muscle layer and the endothelium is replaced by extravillous cytotrophoblast. This remodelling of the spiral arteries increases the diameter of the vessels about 10-fold which results in increased blood volume to the intervillous space. This system ensures that adequate nutrients and respiratory gases are provided for fetal growth (Whitley & Cartwright, 2009; Wang, 2017; Shaheen *et al.*, 2020). Failure of the above-mentioned changes leads to reduced placental perfusion and endothelial dysfunction (George, 2010; Chiarello *et al.*, 2020).

A hypoxic uteroplacental environment favours an increase in inflammation, an imbalance in antioxidant defence mechanism and an increased formation of reactive oxygen species (ROS) resulting in oxidative stress. A hypoxic uteroplacental environment also favours an imbalance in angiogenic (VEGF and PLGF) and antiangiogenic proteins (sFlt-1 and sENG) resulting in reduced NO production causing vasoconstriction effect on the uteroplacental blood flow and endothelial dysfunction (Baijnath *et al.*, 2014; Sanchez-Aranguren *et al.*, 2014; Nikuei *et al.*, 2020).

Non-trophoblastic maternal risk factors of PE include chronic hypertension, GDM, T2DM (Vieira *et al.*, 2018; Košir Pogačnik *et al.*, 2020). However, pregestational PD has not been outlined as a risk factor of PE. During pregnancy complicated by T2DM, the human placenta undergoes a number of functional and structural pathologic changes, such as increased placental weight and increased incidence of placental lesions including villous maturational defects and fibrinoid necrosis (Nteeba *et al.*, 2020; Tumminia *et al.*, 2021).. Furthermore, T2DM has been associated with a reduction in NO bioavailability (Kumar *et al.*, 2018; Bahadoran *et al.*, 2020; Vargas *et al.*, 2021). NO plays an important role in the maintenance of normal pregnancy by influencing cytotrophoblast invasion and mediating spiral artery transformation (Zullino *et al.*, 2018; Cyr *et al.*, 2020). Therefore, a reduction in NO is associated with impaired spiral artery remodelling. Endothelial dysfunction and lipid accumulation in T2DM have been shown to lead to atherosclerosis which results in abnormally narrow spiral arterioles and subsequent impaired placental perfusion which results in the onset of PE (Dhananjayan *et al.*, 2016; Yadav *et al.*, 2018).

T2DM is also associated with macrosomia due to hyperglycaemia and hyperlipidaemia which provides an oversupply of glucose to the fetus (Ehrenberg *et al.*, 2004; Kuc *et al.*, 2011; Ornoy, 2011). However, some studies have indicated that T2DM is associated with excessive reactive oxygen species (ROS) production, which has been shown to increase trophoblast apoptosis and deportation, alter placental vascular reactivity and cause a reduction of glucose transporter 1 (GLUT1) expression in the placenta, causing IUGR (Rashid *et al.*, 2018). The onset of T2DM is often preceded by PD which is characterized by moderate hyperglycaemia, moderate hypertension and moderate increases in ROS (Luvuno *et al.*, 2018; Gamede *et al.*, 2019). The effect of chronic hyperglycaemia in T2DM has been shown on placental morphology and function (Vambergue & Fajardy, 2011; Aldahmash *et al.*, 2022). Despite our current understanding, PD has been associated with the early onset of DM complications, however the

relationship between PD and pre-eclampsia remains obscure. Considering the prevalence of PD and cases that remains undiagnosed, understanding the PD as a risk factor for pre-eclampsia is of importance. Therefore, it is of significant importance that we also outline the effect of intermediate hyperglycaemia on placental morphology and function. The aim of the study therefore, is to investigate pregestational PD as a risk factor of PE by evaluating the effects of pregestational PD on placental function. We envisage that investigating No, selected angiogenic and antiangiogenic factors gene expression, and oxidative stress status may reveal functional changes demonstrating PD as risk factor for pre-eclampsia.

2. Materials and methods

2.1 Drugs and chemicals

Drugs and chemicals of analytical grade were sourced from standard pharmaceutical suppliers.

2.2 Animals

The presented study was approved by the University of Kwazulu-Natal ethics committee as the procedures involving animals and their care were conducted in conformity with the institutional guidelines. Approved ethics number: AREC/025/19D. 3 weeks old female Sprague-Dawley rats (n = 18) bred and housed in the Biomedical Research Unit of the University of Kwazulu-Natal were used in the present study. The animals were maintained under standard housing conditions of constant temperature (22 ± 2 °C), CO₂ content of < 5000 pm, relative humidity of $55 \pm 5\%$, illumination (12 h light/dark cycles), noise levels of less than 65 decibels, and ad libitum access to standard rat chow (29% protein, 55% carbohydrate, and 16% fat) and water.

2.2.1 Experimental design

The role of pregestational PD as a risk factor of PE was studied in the following groups. Group 1: normal pregnant control rats (ND) which received normal standard chow and normal drinking water throughout the study (n = 6/group); Group 2: PD pregnant rats which received the HCHF diet+ 15% fructose throughout the study (n = 6/group); Group3: PE pregnant control rats which received normal standard chow and 0.3g/l LNAME in drinking water from GND 8 to GND 14 (n = 6/group). The PE group served as a standard group, On GND 0, 9,18 body weight, blood glucose, TGs, blood pressure was measured.

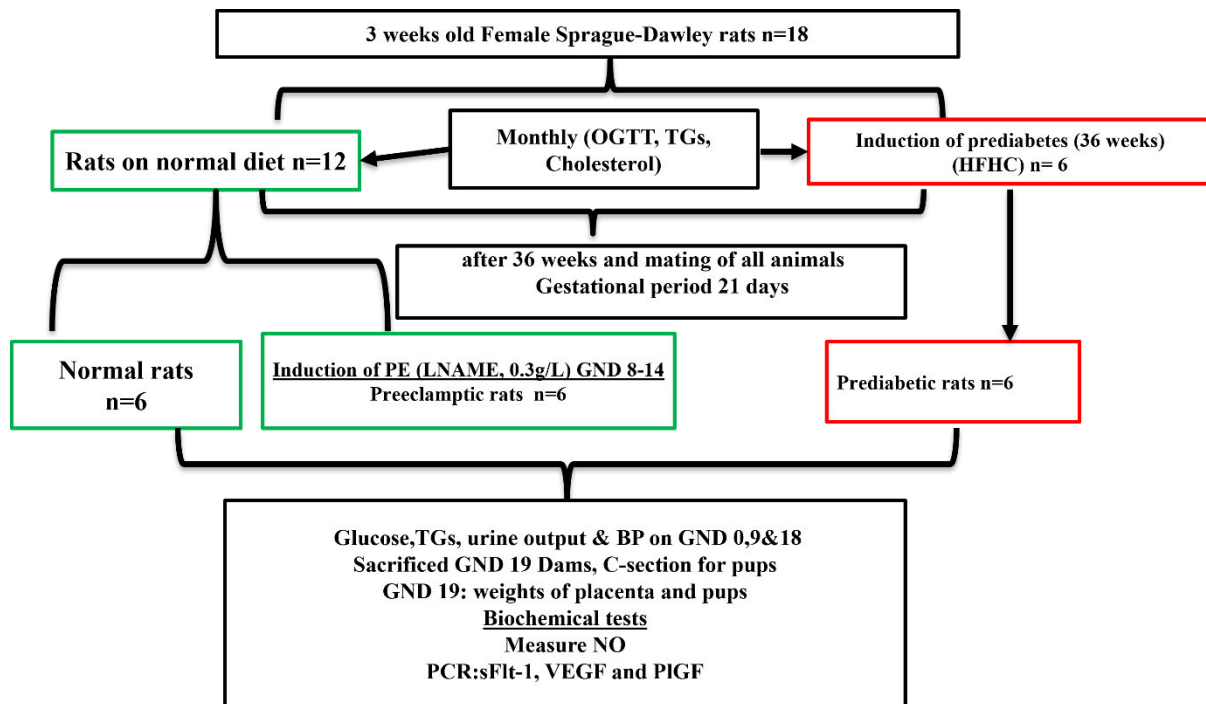


Figure 1: experimental protocol

2.2.2 Induction of PD

After 1 week of the acclimatization to new environment, PD was induced in 4 week old female Sprague-Dawley rats (n=6) using an established protocol from our laboratory with slight modification (Jackson *et al.*, 2012; Luvuno *et al.*, 2018). Briefly, the experimental animals in the prediabetic group (n=6) were exposed to a high carbohydrates, high fat diet and 15 % fructose (HCHF diet+ 15% fructose) in drinking water for 39 weeks (Luvuno *et al.*, 2018; Ludidi *et al.*, 2023). The rest of the animals were assigned into the following groups the control group (n=6) and preeclamptic group (n=6) which received standard chow diet and normal water for 39 weeks. During the PD induction period all the rats were also acclimatized for blood pressure monitoring once a month. The blood pressure monitoring is described later in this section.

2.2.2.1 Criteria for PD

The American Diabetes Federation criteria was used to diagnose PD in all animals. Animals that had prediabetic symptoms including fasting blood glucose concentration of 6.1-7.1 mmol/L, a two-hour glucose concentration of 7.1-8.1mmol/L in the oral glucose tolerance test, and plasma triglycerides concentration of greater than 2 mmol/L were considered prediabetic. Blood samples were collected from the tail vein through tail pricking method. Glucose was determined using the Elite® glucometer and blood TGs were determined using the accutrend plus cholesterol meter

2.2.3 Experimental diet

The composition of a high-fat high-carbohydrate diet was customized as shown in table 1. The experimental diet formula is made up of carbohydrate (55 % Kcal/g), fats (30 % Kcal/g), and proteins (15 % Kcal/g). The drinking water of the experimental animals was supplemented with 15% fructose.

Table 1. Composition of ingredients in the high-fat high-carbohydrate diet

Nutrient	Units	Actual	Nutrient	Units	Actual
Dry Matter	g/kg	919.93	ASTSAA	g/kg	6.79
Metabolizable Energy	MJ/kg	15.86	Asvaline	g/kg	5.80
Crude Protein	g/kg	151.27	Fat	g/kg	250.46
Threonine	g/kg	4.51	Carbohydrate	g/kg	427.29
Isoleucine	g/kg	5.24	Fibre	g/kg	22.08
Lysine	g/kg	6.54	Ash	g/kg	26.31
Methionine	g/kg	4.86	Avl-P	g/kg	1.66
Tryptophan	g/kg	1.30	Calcium	g/kg	5.47
Astatine	g/kg	3.30	Total Phosphorus	g/kg	3.60

2.3 Mating

At the end of the 39th week of induction period, the female Sprague-Dawley rats from Normal (n=6), PE (n=6) and PD (n=6) group were mated with normal males of the same strain. Briefly two females were housed in one cage to allow their oestrus cycle to become synchronised. The ovarian cycle of rats was between four to five days long. Daily vaginal smears were taken and when the female rats were in the pro-oestrus stage of their cycle, a male rat was introduced into the cage. The following day, vaginal smears were taken from the female rats and the presence of sperm indicated successful mating and cytology was used to confirm positive pregnancy. The day of

fertilisation was regarded as GND 0 (Qulu *et al.*, 2015). The male rats were then be removed from the cage and male rats were sacrificed after successful mating.

2.4 Induction of PE

The pregnant dams (n=6) from the PE group were given 0.3g/l a day of N^o-nitro-L-arginine methyl ester (L-NAME) in drinking water from GND 8-GND 18 (Soobryan *et al.*, 2017). Blood pressure and proteinuria were determined to confirm successful induction of PE on GND 9. Blood pressure were monitored using a non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA). The animals warmed at $\pm 30^{\circ}\text{C}$ in an enclosed chamber (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Wood land Hills, California, USA) for 30 minutes before taking blood pressure readings.

2.5 Plasma and Tissue collection

On GND 19, pregnant rats were sacrificed by decapitation for collection of the placenta, pups by C section and blood. Plasma was obtained by collecting blood into single precooled heparinized containers and the blood was centrifuged at $1000 \times g$ for 10 min in a Hermle Laborechnic GmbH centrifuge (Wehingen, Germany). The placentas were removed, weighed, and then snap frozen in liquid nitrogen. Afterwards, the placenta and plasma were stored in an Ultra Bio Freezer (Snijers Scientific, Tilburg, Netherlands) at -80°C until biochemical tests.

2.6 Biochemical tests

2.6.1 Nitric Oxide levels

Plasma NO concentration was measured using the Griess assay. The Griess assay is used for the detection of nitrites and nitrates in a sample, as an indirect measure of levels of NO an indirect indicator of vasodilation in plasma (Tsikas, 2007). To perform this assay, 50 µl of plasma was added in triplicate to the wells of a 96-well microtitre plate. Sodium nitrite (0–200 µM) was used as standard in this assay and 50 µl of sodium nitrite concentration was added in triplicate to the wells. This was followed by the rapid addition of 50 µl vanadium chloride (VCl₃), 25 µl 2% sulphanilamide (SULF) and 50 µl 0.1% N-1-naphthylethylenediamine dihydrochloride (NEDD) into each well. The plate was then incubated (37 °C) for 45 min under dark conditions and the optical density was read at 540/690 nm using a spectrophotometer (Spectrostar nano, BMG LABTECH, Ortenberg Germany). A standard curve was constructed using the results obtained from the sodium nitrite standard and the resultant NO concentration for each sample was determined by extrapolation.

2.6.2 Placental Malondialdehyde (MDA) Measurements.

Placenta tissue was harvested, then tissue was washed with cold PBS (0.01 M, pH 7.4). 20 mg placenta tissue was homogenized in 180 µL homogenate medium with a dounce homogenizer at 4°C. The sample was centrifuged at 12000×g for 10 min at 4°C to remove insoluble material. The supernatant was collected and kept on ice for detection and the protein concentration of the supernatant was determined using BCA Protein Colorimetric Assay Kit (E-BC-K318-M). Placental tissue (50mg) was homogenized in 500 µL of 0.2% phosphoric acid. The homogenate was centrifuged for 10 minutes at 400 x g. Thereafter, 400 µL of the homogenate was supplemented with 400 µL of 2% phosphoric acid. The solution was aliquoted into two separate tubes. Then, 200 µL of 7% phosphoric acid was added into both glass tubes while adding 400 µL of 3mM hydrochloric acid (HCl) into one glass test tube (blank) and 400 µL thiobarbituric

acid (TBA)/butylated hydroxytoluene (BHT) into the other test tube (sample test). To achieve an acidic pH of 1.5, 200 μ L of 1M HCl was added to both the blank and sample test tubes. Both solutions were heated for 15 minutes at 100 °C and allowed to cool to room temperature. After, 1.5 mL butanol was added to the cooled solution; the sample was vortexed for 1 minute to achieve rigorous mixing and allowed to settle until two phases can be observed. The butanol phase/top layer was transferred to Eppendorf tubes and centrifuged for 6 minutes at 13,200xg. The samples were aliquoted into a 96 well microtiter plate in triplicate and the Biotek mQuant spectrophotometer (Biotek, Johannesburg, South Africa) was used to determine the absorbance at 532nm reading. MDA concentration was calculated using absorbance wavelengths by Beers Law:

$$[\text{MDA}] = \frac{\text{Absorbance Final}}{\text{Absorption coefficient (156mmol-1)}}$$

The MDA concentration was normalized to the protein content for each sample:

$$\text{MDA per Protein} = \frac{\text{MDA concentration (nmol or } \mu\text{mol)}}{\text{Protein content (mg or } \mu\text{g)}}$$

2.6.3 Antioxidant enzyme activities

Placenta tissue was harvested, then tissue was washed with cold PBS (0.01 M, pH 7.4). 20 mg placenta tissue was homogenized in 180 μ L homogenate medium with a dounce homogenizer at 4°C. The sample was centrifuged at 12000xg for 10 min at 4°C to remove insoluble material. The supernatant was collected and kept on ice for detection and the protein concentration of the supernatant was determined using BCA Protein Colorimetric Assay Kit (E-BC-K318-M). Placental superoxide dismutase 1 (SOD 1) concentrations was determined using their specific ELISA kit as instructed by the manufacturer (Catalog No: E-EL-R1424; Elabscience Biotechnology Co., Ltd., Houston, TX, USA) via the microplate reader, SPECTROstar Nano

spectrophotometer (BMG LABTECH, Ortenburg, LGBW, Germany). Briefly for the SOD 1 ELISA kits. 50 μ L of each dilution of standard, blank and sample were added into the appropriate wells thereafter 50 μ L of biotinylated detection Ab working solution was added to each well immediately. The plate was covered with the sealer and incubated for 45 min at 37 °C. the plate was washed 3x with 350 μ L of was buffer in each well. After washing step, 100 μ L of HRP conjugate working solution was added to each well and the plate was incubated for 30 min at 37 °C. after the incubation the solution in each well was decanted and the was process was repeated 5x. 90 μ L of substrate reagent was added to each well and the plate was incubated for 15 min at 37 °C. 50 μ L of the stop solution was added and the optical density (OD) of each well was determined using a microplate reader at 450nm.

2.6.5 Total RNA extraction and cDNA synthesis for the analysis of placenta VEGF, PlGF and sFlt expression levels by Real time-PCR

The placenta tissues were collected on day 19 and the tissues were homogenised, and total RNA was isolated using a ReliaPrep miRNA Cell and Tissue Miniprep System (Promega, USA) according to the manufacturer's protocol. The purity and concentration of RNA were determined by Nanodrop 2000 (Termo Scientific, Roche, South Africa). A purity ratio (A260/A280) of 1.7–2.1 was considered acceptable for conversion of RNA to cDNA. Total RNA was reverse transcribed to cDNA using a GoTaq® 2-Step RT-Qpcr System as a cDNA synthesis kit (Promega, USA) according to the manufacturer's instructions. Te BIO-RAD iTaq Universal SYBR Green I master mix was used to perform the PCR amplification on ROCHE LightCycler96 (Roche, South Africa). Primer sequences (Metabion, Germany) used in this study can be found in Table 1 below. PCR was performed using the following cycling conditions: an initial denaturation cycle at 95 °C for 10 min followed by PCR which consisted of 45 cycles at 95 °C for the 30 s, 65 °C for 30 s and a final elongation at 72 °C for 30 s with a single fluorescence measurement. Melting curve analysis will be performed at 95 °C for 30 s;

65 °C for 20 s and 95 °C at a ramp rate of 0.05 °C/s and a continuous fluorescence measurement, followed by a final cooling step at 40 °C for 60 s. The RT-qPCR results were analysed using the $2^{-\Delta\Delta C_q}$ comparative method relative to the control groups (Schmittgen & Livak, 2008). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house keeping gene. Primer sequences were adopted from (Soobryan *et al.*, 2017). Then the Rattus primer sequences were blasted on <http://www.ncbi.nlm.nih.gov/tools/primer-blast/> to verify the primer sequence and their accession number.

2.6.5.1 Table 2 List of primers used in the study.

Gene of interest	Sequence	Accession number
sFlt-1 (soluble fms-like tyrosine kinase 1)	Forward: 5'-AAGGTCTACAGCACCAAG-3' Reverse: 5'-ACATCATCAGAGCTTCC-3'	NM_019306.2
VEGF (vascular endothelial growth factor)	Forward: 5'- GACCCTGGTGGACATCTTCCAGGA -3' Reverse: 5'- GGTGAGAGGTCTAGTTCCCGA -3'	NR_105011.1
PlGF (placental growth factor)	Forward: 5' - CAGCCAACATCACTATGCAG -3' Reverse: 5'- TCCTCTGAGTGGCTGGTTA -3'	L_40030.1
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	Forward: 5'-AGTGCCAGCCTCGTCTCATA- 3' Reverse: 5'-GATGGTGATGGGTTTCCCGT- 3'	NM_017008.2

2.7 Statistical analysis

Sample size (n) for the study was calculated using G*Power (version 3.1.9.7), a statistical power analysis software. This was performed to ensure that the sample size was adequate to detect statistically significant differences between the groups while maintaining ethical considerations for animal use. Statistical analysis was conducted using GraphPad Prism Software (version 9.00, GraphPad Software, San Diego, California, USA). A normality test (Shapiro-Wilk test) was done followed by a parametric one-way ANOVA. To analyse differences between the controls and the experimental group, Bonferroni's multiple comparisons test was performed. Values of $p < 0.05$ was used to indicate statistical significance.

3. Results

3.1 Non-fasting blood glucose and triglyceride concentration

Non-fasting blood glucose and Triglyceride concentrations of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats over the period of 18 gestational days. The prediabetic pregnant group presented with significantly higher glucose concentration throughout gestation when compared to the normal pregnant control group ($p<0.05$; Fig 1). The preeclamptic pregnant group showed significantly increased blood glucose concentration on GND18 when compared to the normal pregnant control group ($p<0.05$; Fig 1) over the experimental period. The prediabetic pregnant group showed to have significantly elevated TG content throughout the period of 18 gestational days when compared to normal pregnant control group ($p<0.05$; Fig 1). The preeclamptic pregnant group had significantly increased TG concentration on GND18 when compared to the normal pregnant control group ($p<0.05$; Fig 1).

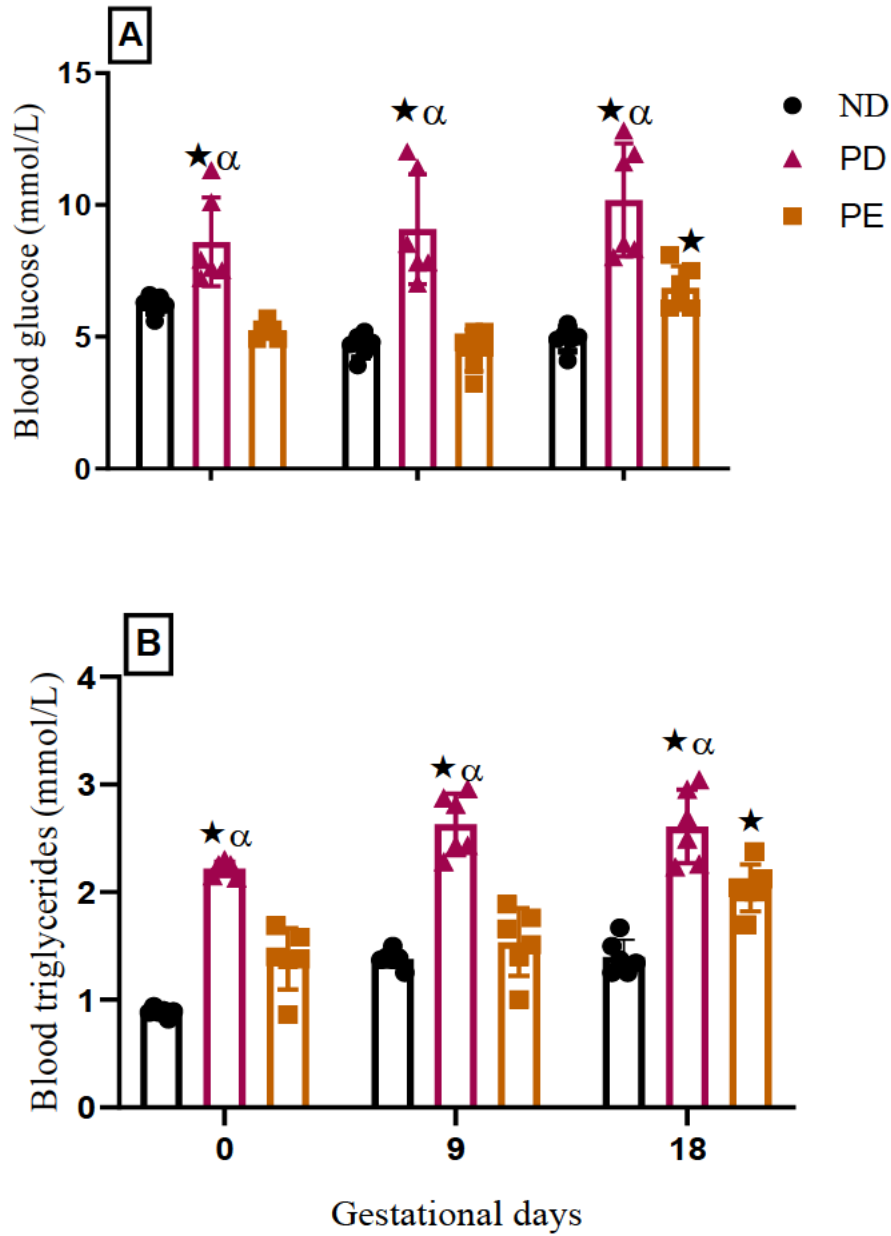


Figure 1: (a) Non-fasting blood glucose and (b) Triglyceride concentration of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats tested over 18 days of gestation measured at GND 0, 9 and 18. Values are presented as means, and SD (n=6 in each group). ★p<0.05 by comparison with normal pregnant group. α p<0.05 by comparison with the preeclamptic group.

3.2 Body weight, food intake and water intake

Body weight, food intake and water intake of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats were recorded over a period of 18 gestational days. The prediabetic pregnant group presented with significantly increased body weight, food intake and water intake throughout gestation when compared to the normal pregnant control group ($p < 0.05$; Fig 2). The preeclamptic pregnant group showed consistent body weight through out gestation, However the preeclamptic group showed a significant increase in food intake and water intake on GND18 when compared to the normal pregnant control group ($p < 0.05$; Fig 2).

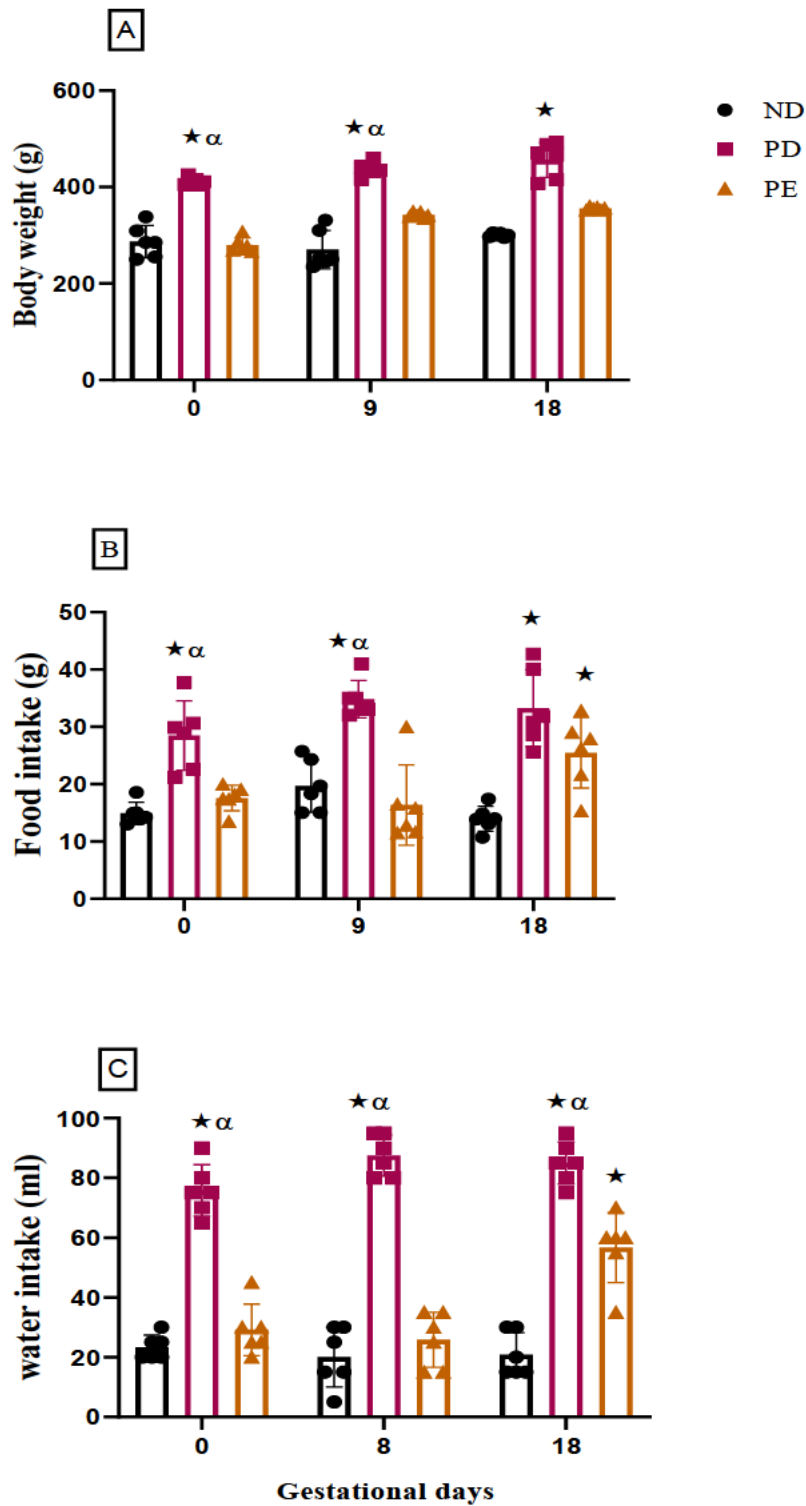


Figure 2: (a) Body weight, (b) food intake and (c) water intake of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The values are presented as means, and SD (n=6 in each group). ★p < 0.05 by comparison with normal pregnant group. α p < 0.05 by comparison to pregnant preeclamptic group.

3.3 Mean arterial pressure.

Mean arterial pressure measured on GND 0, 9 and 18 days of gestation of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The prediabetic group showed significantly increased mean arterial blood pressure throughout gestation when compared to the normal pregnant group ($p<0.05$; Fig 3). The pregnant preeclamptic rats presented with normal blood pressure on GND 0 however on GND 9 and GND 18 the pregnant preeclamptic rats showed significantly high blood pressure when compared to the normal pregnant rats ($p<0.05$; Fig 3).

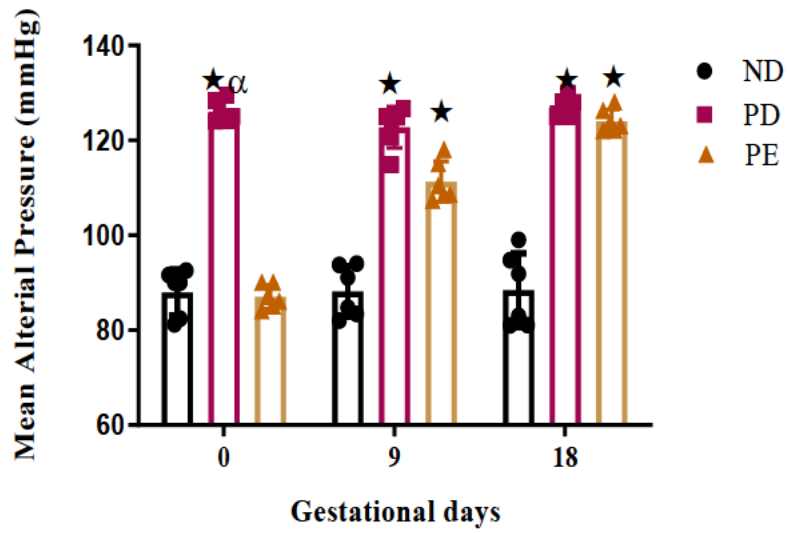


Figure 3: Mean arterial blood pressure of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The values are presented as means, and SD (n=6 in each group).

★p < 0.05 by comparison with normal pregnant group. α p < 0.05 by comparison to pregnant preeclamptic group

3.4 Proteinuria

Urinary albumin concentration of normal pregnant, pregnant prediabetic, pregnant preeclamptic rats measured on GND 0, 9 and 18 (Figure 4). The PE and PD pregnant group showed significant increase urinary albumin levels on GND 9 and 18 when compared to the ND group ($p < 0.05$, Fig 4).

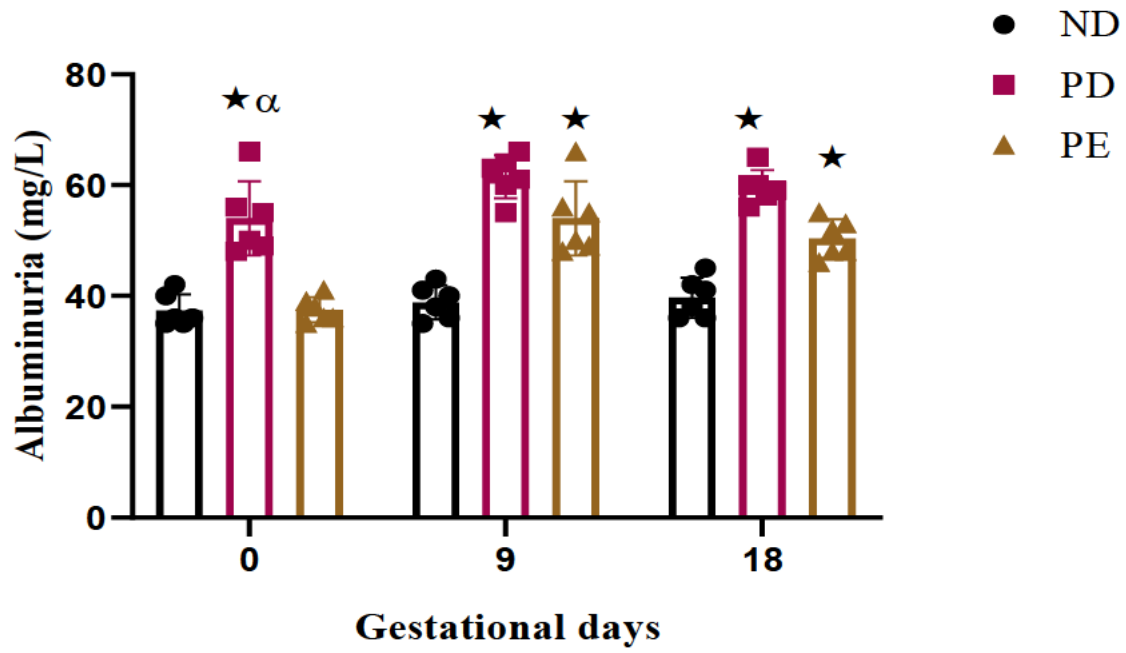


Figure 4: Albuminuria of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats measured on GND 0, 9 and 18. The values are presented as means, and SD (n=6 in each group). ★ $p < 0.05$ by comparison with normal pregnant group. α $p < 0.05$ by comparison to pregnant preeclamptic group.

3.5 Average litter size per group, placenta weight, fetal weights and F:P ratio

Placenta, fetal weights and average litter size of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats over the period of 19 gestational days. The pregnant prediabetic rats had significantly high placenta weights when compared to the preeclamptic group and the fetal weights of pups born from prediabetic rats were greater than those born from the preeclamptic rats, however, the pups born from prediabetic rats had significantly low birth weight when compared to pups born from normal rats ($p < 0.05$; Table 3). The pregnant preeclamptic group which had significantly low placenta weights and pup weight when compared to the normal pregnant rats ($p < 0.05$; Table 3).

Table 3: Average litter size, placenta, fetal weights and F:P ratio on GND 19 of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats. Values are represented as means, and SD (n=6 in each group).

Experimental groups	Average litter size per group	Fetal weight (g)	Placenta weights (g)	F:P ratio
ND	14.7±0.2	4.628±0.465	0.663±0.040	7.368±1.76
PD	10.1±1.4*	2.807±0.471*	0.747±0.073*	4.000±2.15
PE	12.8±0.8*	2.176±0.330*	0.467±0.033*	3.416±17

* $p < 0.05$ by comparison with normal pregnant group. $\alpha p < 0.05$ by comparison to pregnant preeclamptic group

3.6 Placental angiogenic and anti-angiogenic status

Placental mRNA expression of (a) sFlt-1, (b) VEGF and (c) PlGF in the placenta of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats over the period of 19 gestational days. The pregnant prediabetic group presented with a significantly high mRNA expression of sFlt-1 and low mRNA expression of VEGF and PlGF when compared to the normal pregnant group ($p < 0.05$; Fig 5). The pregnant preeclamptic group had significantly high mRNA expression of sFlt-1 and low mRNA expression of VEGF and PlGF when compared to the normal pregnant group ($p < 0.05$; Fig 5).

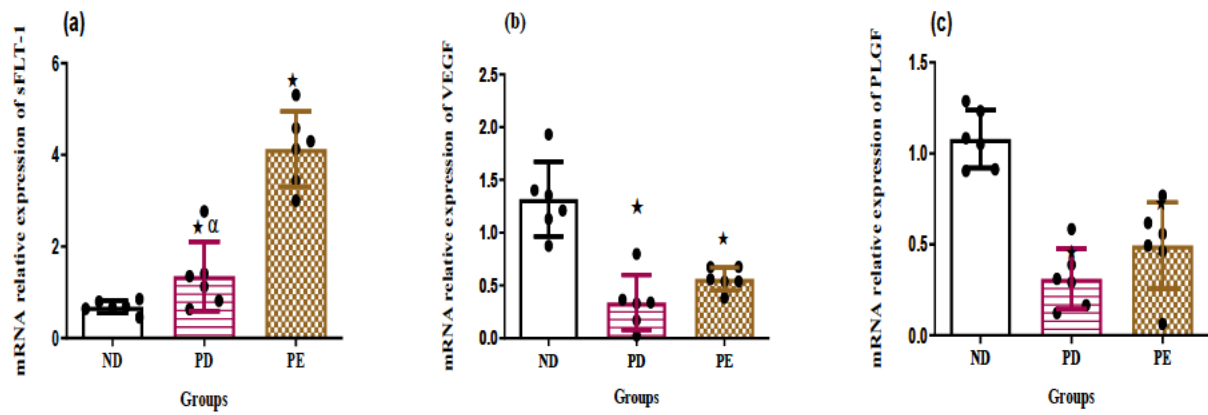


Figure 5: Placental mRNA expression of (a) sFlt-1, (b) VEGF and (c) PlGF of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats and over the period of 19 gestational days. The values are presented as means, and SD (n=6 in each group). ★ $p < 0.05$ by comparison with normal pregnant group. α $p < 0.05$ by comparison to pregnant preeclamptic group.

3.7 Plasma NO concentration

The plasma NO concentration after 19 days of gestation of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The pregnant prediabetic group showed significantly low nitric oxide concentration when compared to the normal pregnant group ($p<0.05$; Fig 6). The pregnant preeclamptic group showed significantly low nitric oxide concentration when compared to the normal pregnant group ($p<0.05$; Fig 6).

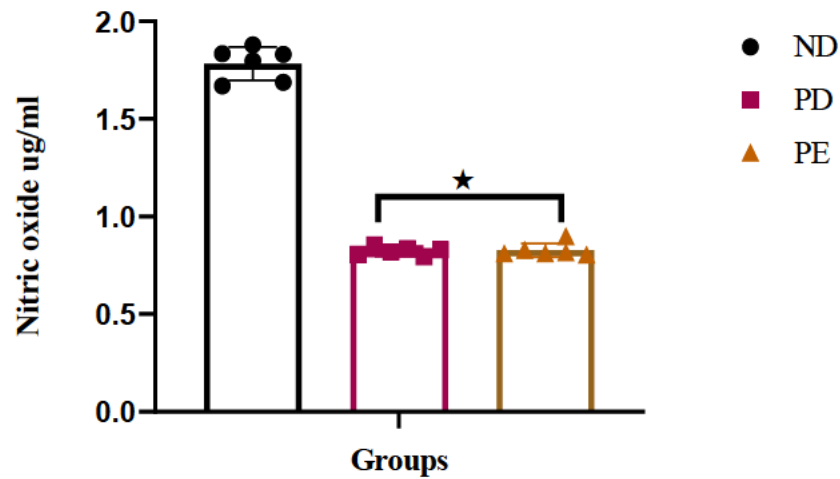


Figure 6 : plasma nitric oxide concentration of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The values are presented as means, and vertical bars represent SEM (n=6 in each group). ★ $p < 0.05$ by comparison with normal pregnant group. $\alpha p < 0.05$ by comparison to pregnant preeclamptic group.

3.8 Oxidative stress status

MDA concentration, SOD concentration placental tissue of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats and after 19 day of gestation. The pregnant prediabetic group showed significantly increased MDA concentration and a decreased activity of SOD in comparison with the normal pregnant group which were similar results to the normal pregnant group ($p < 0.05$; Table 2). The pregnant preeclamptic group showed significantly increased MDA concentration and a decreased activity of SOD in comparison with the normal pregnant group ($p < 0.05$; Table 2).

Table 4: The effects of PE and pregestational PD on MDA concentrations and SOD in the placental tissue over the of 19 days of gestation. Values are represented as means and SD (n=6 in each group)

Groups	Placenta MDA ($\mu\text{mol/mg}\cdot\text{prot}$)	SOD (pg/mg protein)
ND	0.320 $\pm$ 0.0108	3.684 $\pm$ 0.253
PD	0.462 $\pm$ 0.018*	2.428 $\pm$ 0.106*
PE	0.476 $\pm$ 0.025*	2.485 $\pm$ 0.348*

* $p < 0.05$ by comparison with normal pregnant group.

4. Discussion

The aim of the study was to investigate the effects of pregestational PD as a risk factor of PE by assessing the adverse effects of pregestational PD on placental function. We investigated the effects of pregestational PD on glucose metabolism, lipid profile during pregnancy and effects of pregestational PD on placenta angiogenic and anti-angiogenic status. Furthermore, we also assessed for the development of oxidative stress and antioxidant status in the placenta as well as placental and pup weights after 19 days of gestation.

During pregnancy, maternal adaptations occur in multiple systems. In the context of glucose and lipid metabolism, these adaptations occur to ensure adequate shunting of glucose to promote fetal development while maintaining adequate maternal nutrition (Newbern & Freemark, 2011; Angueira *et al.*, 2015). In this study, the PE pregnant group, presented with an increase in blood glucose and TGs concentration on GND 18 in comparison to the normal pregnant control group. PE is associated with an exacerbation of physiological changes associated with pregnancy such as insulin resistance (Scioscia *et al.*, 2009; Sinha *et al.*, 2014). The underlying pathological process of PE lies in the placental tissue, therefore, altered placental lipid metabolism could result in maternal lipid perturbations, thereby affecting fetal growth (Yadav *et al.*, 2018; Khair *et al.*, 2021).

In the PD pregnant group, we observed impaired glucose and lipid homeostasis which was shown by an increased blood glucose and TG concentration throughout gestational period in comparison to the normal and PE pregnant group. In this study, the female rats in the prediabetic group were prediabetic prior to pregnancy due to possible insulin resistance and decreased insulin sensitivity present in PD (Luvuno *et al.*, 2018). PD is defined by elevated blood glucose levels that are higher than normal but not high enough to be classified as T2DM (Herman, 2023). According to the American Diabetes Association (ADA) PD is typically identified by fasting blood glucose concentration of 6.1-7.1 mmol/L, a two-hour glucose

concentration of 7.1-8.1mmol/L in the oral glucose tolerance test, and plasma triglycerides concentration of greater than 2 mmol/L (Herman, 2023). In this study, we observed that both non-fasting blood glucose and triglyceride levels were elevated to levels consistent with PD classification, in line with the criteria set by the ADA which reflect an increased metabolic risk, and are in line with other findings from previous research that suggests these markers are reliable indicators of PD and insulin resistance (Li *et al.*, 2018). According to current health guidelines, PD is typically diagnosed using fasting glucose and HbA1c levels, as study by Nkosi *et al* 2024 showed the pregnant prediabetic female rats had impaired fasting blood glucose and elevated levels of HbA1c (Nkosi *et al.*, 2024). PD is associated with insulin resistance which can increase peripheral lipolysis and TG synthesis leading to abnormal lipid metabolism (Butte, 2000; Luvuno *et al.*, 2018). These lipid/lipoprotein abnormalities are known to enhance endothelial dysfunction, and act as major risk factors for PE (Khaire *et al.*, 2021). Endothelial damage and lipid accumulation can lead to atherosclerosis, and vasoconstriction which result in abnormally narrow spiral arterioles and subsequent impaired placental perfusion (Yadav *et al.*, 2018).

Hyperglycaemia is associated with alterations in insulin signalling and leptin sensitivity. These changes can impair appetite regulation, increasing food intake (Ancu *et al.*, 2021). The PD group had significantly higher food consumption compared to normal pregnant controls. This increased food intake is thought to compensate for the inefficient energy utilization caused by hyperglycaemia. Hyperphagia (Mogensen *et al.*, 2020; Cui *et al.*, 2023). Pregnancy itself naturally increases energy demands due to the metabolic requirements of the growing fetus, placenta, and maternal tissue (Cui *et al.*, 2023). When combined with hyperglycaemia, the dysregulated metabolic state can exacerbate hyperphagia, as the body attempts to meet perceived energy deficits (Raghavan *et al.*, 2019). Hyperglycaemia and insulin resistance during pregnancy can impair leptin signaling in the hypothalamus, leading to reduced satiety

and increased food consumption (Raghavan *et al.*, 2019; Cui *et al.*, 2023). Elevated ghrelin has been observed in hyperglycaemic states, further driving hyperphagia. Fluctuations in insulin levels affect hunger regulation and glucose availability, contributing to altered feeding behaviour (Cui *et al.*, 2023).

The assessment of placenta dysfunction in this study was through observing angiogenic and anti-angiogenic status. Placental vasculogenesis and angiogenesis are an important processes that establishes maternal and fetal circulation and plays a key mechanistic role in vascular development of the placenta (Bardin *et al.*, 2015). Studies have shown a close relationship between embryonic development and the degree of placental vasculogenesis and angiogenesis. Failure in vasculogenesis and angiogenesis is tightly linked to the development of placental pathologies such as PE (PE), early pregnancy loss, and intrauterine growth restriction (IUGR) (Arroyo & Winn, 2008; Bardin *et al.*, 2015). Placental vasculogenesis and angiogenesis is regulated by angiogenic growth factors mainly the vascular endothelial growth factor (VEGF) family. (Arroyo & Winn, 2008). The VEGF family consists of VEGF, Placenta Growth Factor (PlGF) and their membrane bound receptors. When these growth factors bind to their transmembrane receptors, a series of downstream proteins are activated, which induce endothelial cell proliferation, migration, and tube formation (Ahmed *et al.*, 2000). VEGF is a potent endothelial survival factor with the following functions. VEGF induce vasodilation by increasing nitric oxide (NO) production and facilitates blood flow. PlGF controls trophoblast proliferation and differentiation by binding to membrane bound receptors (Arroyo & Winn, 2008; Jardim *et al.*, 2015). In this study, the normal pregnant control group had significantly higher VEGF and PlGF mRNA expression suggesting normal placental angiogenic status.

The PE pregnant group showed a significantly lower mRNA expression of VEGF and PlGF with high mRNA expression of sFlt-1 in comparison to the normal pregnant control group showing an imbalance in placental angiogenic and antiangiogenic status. Improper

implantation to the maternal uterine wall results in reduced placental perfusion or a hypoxic uteroplacental environment in PE which favours the release antiangiogenic proteins such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) into the maternal circulation that contribute to vascular dysfunction (Rana *et al.*, 2019; Nikuei *et al.*, 2020). sFlt-1 lowers the effect of vascular VEGF and PlGF by acting as a VEGF and PlGF antagonist thus causing complications such as endothelial dysfunction, vasoconstriction and the production of ROS (Rana *et al.*, 2019). Therefore, excessive sFlt-1 impair pregnancy-induced adaptation in maternal placental bed vessels and contribute to systemic maternal endothelial dysfunction and ischemia in women with PE (Magee *et al.*, 2022).

Insulin resistance and chronic hyperglycaemia have been associated with the down-regulation of pro-angiogenic factors and upregulation of anti-angiogenic factors, sFlt1 hence, poor placental development has been reported in T2DM (Miele *et al.*, 2000; Byford *et al.*, 2021). The effects of intermediate hyperglycaemia, such as seen in pregestational PD, on placental development is however not clearly outlined. An imbalance in placenta angiogenic and antiangiogenic status together with atherosclerosis and vasoconstriction by pregestational PD is associated with reduced blood flow in the placenta which causes hypoxia environment in the placenta. This may result in the development of microcytic anemia often observed in hypoxic conditions (Chaudhry & Kasarla, 2017). The stressed placenta observed in the PD pregnant group and PE pregnant group promotes the production of ROS which play a role in IUGR.

In normal pregnancy, the high levels of estrogen helps the placenta adapts to the increasing levels of ROS by modulation of hypoxia-inducible factor 1 α (HIF-1 α) and by increasing cellular antioxidant defenses (Bardin *et al.*, 2015). This explains the low MDA concentration and high antioxidants enzymes, SOD and CAT in the normal pregnant control group. However, in cases of possible incomplete placentation observed in PE and PD, oxidative stress may occur at an excessive level, leading to endothelial dysfunction.

In this study, PE pregnant group, presented with increased lipid peroxidation as indicated by an increase in MDA and low levels of antioxidant enzymes SOD and CAT in comparison to the normal pregnant control group. Similarly, to the PE group, the PD pregnant group also showed increased MDA concentrations and low levels of antioxidant enzymes in comparison to the normal pregnant control group. These results suggest that intermediate hyperglycaemia plays a role in the development of oxidative stress in the placenta, thus affecting placental development. Hyperglycaemic conditions favour the production of ROS through mitochondrial glucose oxidation, protein glycation, advanced glycation end product formation and the polyol pathway (Gamede *et al.*, 2019). A decrease in antioxidants enzymes, could also be attributable to a defective NRF2/KEAP1 pathway, which is responsible for the expression of antioxidants, however, further studies may be pertinent to support this speculation.

The progression of oxidative stress causes vascular dysfunction which negatively affects NO bioavailability leading to vasoconstriction and ultimately increased blood pressure. Nitric oxide (NO) synthesis and released by endothelial cells plays an important role in vascular relaxation effect contributing to the modulation of vascular tone (Zullino *et al.*, 2018). Importantly NO also plays a role in the maintenance of normal pregnancy by influencing cytotrophoblast invasion and mediating spiral artery transformation (Sutton *et al.*, 2020). In this study the PE pregnant group had low NO concentration and high blood pressure when compared to the normal pregnant control group. This was expected as L-names suppresses NO production through inhibition of NOS leading to high blood pressure. Interestingly, the PD pregnant group, similarly to the PE pregnant group showed reduced NO bioavailability and elevated blood pressure in comparison to normal pregnant controls. Insulin insensitivity plays a role in endothelial dysfunction by disrupting not only NO release but also NO synthesis. Therefore resulting in vasoconstriction and high blood pressure (Cyr *et al.*, 2020). The observation may also be ascribed to oxidative damage in the placenta leading to inflammation,

apoptosis, and the release of several anti-angiogenic factors, such as sFlt1 and Seng, and cytokines (Nikuei *et al.*, 2020). These placental derived factors act on the maternal vascular endothelium, inducing oxidative stress and stimulating the production and secretion of pro-inflammatory cytokines, as well as vasoconstrictors such as endothelin 1.

Elevated oxidative stress and endothelial dysfunction causes IUGR which ultimately affects fetal growth and development (Kautzky-Willer *et al.*, 2016; Zhao *et al.*, 2021) Furthermore, PE is associated with structural abnormalities such as poor vascularization with reduced vascular branching and fewer capillaries and increased apoptosis which is associated with reduced nutrient and oxygen transport to the fetus which causes IUGR. In this study, we assessed the effects of pregestational PD on placental weight and birthweights since placental weights are strongly correlated with birthweight. In our study there was a strong correlation between small placentas and low birthweight in the PE group when compared to the normal pregnant control group. Interestingly, the PD group presented with significantly higher placental weights and lower birthweights when compared to the PE group. This suggests that an intermediate hyperglycaemia may lead to IUGR. The results in this study differ from those reported in other studies that used similar rat model. Ludidi *et al* 2023 reported macrosomia in pups born from prediabetic animals (Ludidi *et al.*, 2023). Our results are contradicting this study because in our study the PD group large placenta which attributed to IUGR. Small weight and large placenta attributed to dysfunction to the vessels that feed the growing fetus. Ludidi *et al* 2023 reported that lipogenesis in placenta and fetuses that may have induced macrosomia and IUGR. The severe fetal growth restriction observed in our study, particularly in the PE model, exceeded the levels reported in similar L-NAME-induced PE models using younger rats (Yang *et al.*, 2019; Nakamura *et al.*, 2023). This discrepancy may be attributable, in part, to the advanced maternal age of the rats in our study, as advanced age is associated with increased oxidative stress, heightened inflammation reduced placental efficiency, and impaired

uteroplacental perfusion (Diaz *et al.*, 2005; Care *et al.*, 2015). These age-related changes could exacerbate the pathological mechanisms of PE and PD, amplifying their impact on fetus. Advanced maternal age is recognized as a risk factor for pregnancy complications, including PE (Pasha *et al.*, 2021). In a study by Pasha *et al.* (2021), a rat model of advanced maternal age (9.5 months old, approximately equivalent to 35 human years) was used to investigate vascular adaptations during pregnancy (Pasha *et al.*, 2021). The findings revealed that older pregnant rats exhibited vascular dysfunction, characterized by impaired endothelial function and increased vascular stiffness, which are associated with the development of PE (Pasha *et al.*, 2021). This study provides evidence that advanced maternal age in rats can exacerbate conditions leading to PE, suggesting a similar risk may be present in humans (Pasha *et al.*, 2021). However there is a need for animal studies that will focus on the effects of advanced maternal age PD.

Fetal weight to placental weight ratio (FW:PW) represents the efficiency of the placenta in supporting fetal growth. A lower F:P Ratio usually indicates reduced placental efficiency, often seen in conditions like PE or IUGR. The PE group and PD group had significantly low FW:PW ratio in comparison to the ND group reflecting poor placental efficiency. Placental inefficiency can result in restricted fetal growth, fetal loss, or even reduced litter sizes in severe cases (Lean *et al.*, 2017; Babah *et al.*, 2018). In rodent models, placental inefficiency is often observed in conditions such as advanced maternal age, PE, or PD, all of which are associated with impaired placental vascularization, reduced nutrient transport capacity, and heightened oxidative stress (Babah *et al.*, 2018). In this study, we used 9-month-old rats to study the effects of pregestational PD on pregnancy and pregnancy outcomes. It is important to note that these animals are classified as being of advanced maternal age, which, according to the literature, has been associated with reduced litter size and fetal growth restriction (Care *et al.*, 2015; Lean *et al.*, 2017). In this study the PD pregnant group and the PE pregnant group had a significantly

reduced litter size compared to the normal pregnant control. To mitigate this potential confounding factor, all experimental groups were age-matched, ensuring that observed differences in pregnancy outcomes are primarily attributable to dietary effects. However, a limitation of our study was the absence of a young control group. We exclusively used 9-month-old rats to investigate the effects of pregestational PD on the placenta function, litter size, and fetal growth. Although studies have shown that maternal age can influence pregnancy outcomes independently of metabolic conditions (Morton *et al.*, 2017; Napso *et al.*, 2019; Correa-de-Araujo & Yoon, 2021). The lack of a younger comparison group in our study limits our ability to distinguish the specific effects of pregestational PD from potential age-related changes. Future studies incorporating younger control groups could help clarify these interactions and provide a more comprehensive understanding of how maternal age and metabolic status interact to affect pregnancy outcomes.

5. Conclusion

The PD pregnant group exhibited moderate hyperglycaemia, endothelial dysfunction, reduced NO, high blood pressure, proteinuria and a decrease in placental angiogenic factors and increase in antiangiogenic factors. This suggests that PD may be a risk factor for PE since PD induced preeclamptic like placental dysfunction. Considering the prevalence of PD, further studies are warranted to support our observations. This could pave way to consider PD diagnosis and management during pregnancy to minimize risk of developing pre-eclamptic-like disorders.

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7. Declaration of interest statement

The authors declare no conflict of interest.

8. Contribution to authorship

The authors confirm contribution to the paper as follows: study conception and design: A.Siboto, P.S Ngubane; data collection: A.Siboto, A.Ludidi ; analysis and interpretation of results: A.Siboto; draft manuscript preparation: A.Siboto; writing—review and editing A.Siboto, A Ludidi, P.S Ngubane, A Khathi; visualization, A.Siboto; supervision, A.Khathi, P.S Ngubane.; project administration P.S Ngubane; funding acquisition, P.S Ngubane . All authors reviewed the results and approved the final version of the manuscript.

9. Data availability statement

All the data related to the manuscript is presented in figures and tables and extra raw data is available.

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Chapter 5

Prologue 2

In manuscript 1 we investigated the effects of pregestational PD on uteroplacental dysfunction associated with the upregulation of sFlt-1 and down regulation of VEGF and PlGF. The results in manuscript 1 demonstrated that pregestational PD is associated with increase blood glucose, TGs, food intake, water intake, blood pressure and proteinuria, may be a risk for the development of PE since pregestational PD induced PE like placental dysfunction. Previous studies have demonstrated that placental dysfunction is associated with poor kidney function. Therefore, in this study we sought to investigate whether pregestational PD will exhibit PE like associated renal dysfunction in pregnant female Sprague-Dawley rats.

Renal dysfunction in PE is associated with hypertension and proteinuria which are observed after 20 weeks of gestation. Renal abnormalities observed in PE include loss of podocyte, diffuse endothelial swelling, and mesangial expansion. These renal abnormalities are precipitated by hypoxic condition due to elevation in antiangiogenic factor such as sFlt-1 in the maternal circulation. Therefore, in the present study, we investigated the effects of pregestational PD on selected markers of renal dysfunction in a prediabetic rat model. The manuscript is titled: **Preeclamptic like adverse effects of pregestational PD on renal function** and is authored by Siboto A, Ludidi A, Xulu N, Nkosi A, Sibiya N, Khathi A and Ngubane PS. The manuscript is under review in the journal **Frontiers in Physiology, section Renal Physiology and Pathophysiology (Manuscript ID: 1333944)** and has been formatted according to the journal's guidelines to authors. This journal is accredited by Department of Higher Education and Training South Africa and appears in Scopus accredited list (2021)

Author Contribution: A Siboto was responsible for study conceptualization, study design, conducted experimental work, data analysis first draft writing, and manuscript editing.

Manuscript 2

Preeclamptic like adverse effects of pregestational PD on renal dysfunction

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Abstract

Background: (PD and PE are both characterized by endothelial dysfunction due to increased circulating antiangiogenic factors secreted by a poorly perfused placenta such as soluble fms-like tyrosine kinase 1 (sFlt-1) which have been shown to have adverse effects on different maternal organs, including the liver and kidney. However, we have not shown whether the upregulation of sFlt-1 and down regulation of vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) in pregestational PD is associated with PE like kidney dysfunction. Therefore, the aim of the study was to investigate whether pregestational PD exhibits PE like kidney dysfunction in pregnant female Sprague-Dawley rats.

Materials and methods: The animals were divided into 3 groups: the normal pregnant group, the preeclamptic pregnant group (PE) and prediabetic pregnant group (PD) (n = 6 in each group). On GND 0, 9, 18, blood glucose, blood pressure, 24-h fluid intake and 24-h output were measured. Following sacrifice on GND 19, blood and kidney tissues were collected for the measurement of electrolytes, lipid peroxidation (MDA), total superoxide dismutase (TSOD), plasma aldosterone and kidney injury molecule 1 (KIM-1).

Results: The results showed significant increases in plasma electrolyte, aldosterone, KIM-1, , urinary albumin, podocin and nephrin, MDA and blood pressure, in the PD pregnant group as compared to ND group.

Conclusion: pregestational PD exhibits pe like effects on kidney function as demonstrated by increases in urinary albumin, podcin and nephrin and KIM-1.

Keywords : Pregestational PD, podocytes, KIM-1, Kidney dysfunction, preeclampsia

1. Introduction

The kidney's involvement in PE is attributed to glomerular endothelial damage and an imbalance between pro-angiogenic and antiangiogenic factors (Karumanchi SA, 2005; Tomimatsu T, 2019). The upregulation of antiangiogenic factors has been shown to occur in hypoxic conditions. A hypoxic condition on the kidneys causes acute tubular necrosis (Verlohren S, 2020.). The upregulation of soluble fms-like tyrosine kinase 1 (sFlt-1) in PE induces hypertension, proteinuria and glomerular endotheliosis by antagonizing vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) (Magee LA, 2022; Tomimatsu T, 2019). Therefore, antagonism of VEGF and PlGF by sFlt-1 plays a role in development of kidney dysfunction in PE (Brennan LJ, 2014 .; Eremina V, 2008). The health of the fenestrated endothelium is maintained by the VEGF expression in organs such as the kidney (Ali LE, 2019; Jourde-Chiche N, 2019). Thus, studies have shown that tissues with, especially the renal glomerulus have fenestrated endothelium which makes the kidney more susceptible to damage by sFlt-1–mediated VEGF blockage (Holmes VA, 2013; Majumder & Advani, 2017; Wewers *et al.*, 2021). Therefore, the absence of VEGF induces proteinuria and glomerular endotheliosis (Sircar M, 2015).

The renal complication in PE is characterized by glomerular endotheliosis, a characteristic of renal lesion shown by glomerular endothelial swelling, occlusion of capillary lumens and loss of fenestration due to the injury of podocytes leading to proteinuria (Sircar M, 2015). Both podocyte and podocyte specific proteins that is podocin and nephrin are found in the urine of preeclamptic women thereby showing that podocyte loss and injury is the pathological defect that can be used to explain proteinuria in PE (Henao DE, 2013; Gilani SI, 2017). Women with PE have been shown to have elevated nephrinuria, suggesting that nephrin shedding occurs in PE and increased nephrinuria is correlated with the degree of proteinuria and also shows the disruption of the slit diaphragm. Urinary podocin and nephrin mRNA expression are direct

indicators of podocyte health and damage to the slit diaphragm, as they are specific components of these structures (Johnstone & Holzman, 2006; Armelloni *et al.*, 2021). Changes in their expression levels reflect cellular damage and/or detachment of podocytes into the urine (Chebotareva *et al.*, 2021). This provides mechanistic insights that protein biomarkers alone cannot offer. Proteinuria is a hallmark of kidney disease, however proteinuria is a downstream consequence of podocyte injury (Chebotareva *et al.*, 2021). Measuring mRNA of podocin and nephrin levels allows researchers to pinpoint the upstream processes affecting the podocyte and slit diaphragm (Wang *et al.*, 2015; Chebotareva *et al.*, 2021). Studies have shown that in PE the expression of urinary mRNA podocin and mRNA nephrin was elevated due to podocyte injury, early glomerular dysfunction (Armelloni *et al.*, 2021). When podocyte get injured, their intracellular constituents could be excreted into the urine. Therefore, high urinary mRNA of these of podocin and nephrin could in a way infer to the extent of damage of the podocyte (Armelloni *et al.*, 2021). The cause of proximal tubule injury in PE is unclear, however, it has been shown to be associated with urinary excretion of kidney injury molecule 1 (KIM -1) which is a marker for proximal tubule injury (Kamel *et al.*, 2020).

PE and T2DM are both characterized by endothelial dysfunction due to increased circulating antiangiogenic factors secreted by a poorly perfused placenta such as sFlt-1 which brings about adverse effects on different maternal organs (Jung *et al.*, 2022; Ludidi *et al.*, 2023). Recent studies have shown that kidney dysfunction observed in T2DM render pregnant women more susceptible to PE due to the adverse effects of hyperglycaemia- hyperglycaemia on the blood vessels (Ottanelli *et al.*, 2020). Chronic hyperglycaemia has been shown induced vasoconstriction of the small blood vessels (Marcovecchio, 2017). These changes contribute to high blood pressure and endothelial dysfunction (Kattah AG, 2017). The onset of T2DM is preceded by PD. We have recently shown that PD exhibits kidney disfunction in male prediabetic animals (Mabuza LP, 2020). This study showed that PD is associated with increased

urinary mRNA of podocin and nephrin in urine (Mabuza *et al.*, 2020). As the podocyte get injured, their intracellular constituents could be excreted into the urine. Therefore, high mRNA of these podocin and nephrin, could in a way infer to the extent of damage of the podocyte (do Nascimento *et al.*, 2013; C. Li & Szeto, 2024). With these observations, they may suggest risks of kidney dysfunction in pregestational PD, thus further makes women more susceptible to PE. Furthermore, recent studies in our laboratory have shown that intermediate hyperglycaemia is associated with slight upregulation of sFlt-1 and down regulation of VEGF and PlGF which makes pregestational PD a risk factor for PE (Ludidi *et al.*, 2023). However, we have not shown whether the upregulation of sFlt-1 and down regulation of VEGF and PlGF in pregestational PD is associated with PE like kidney dysfunction. Therefore, the aim of the study was to investigate whether pregestational PD exhibits PE associated kidney dysfunction in pregnant

2. Materials and methods

Standard pharmaceutical suppliers were used to source drugs used in this study and commercial suppliers were used to purchase all other chemicals which were of analytical grade quality.

2.1 Animals

The approved ethics number for the study is AREC/025/19D which was approved by the University of Kwazulu-Natal ethics committee and all procedures involving animals and their care were conducted in accordance with the institutional guidelines. Female Sprague-Dawley rats (n = 18) were bred and housed in the Biomedical Research Unit of the University of Kwazulu-Natal and the animals were maintained under standard housing conditions,

2.2 Experimental protocol

After the acclimatization to the new environment, the animals were divided into 3 groups: the normal pregnant group (n = 6), the preeclamptic pregnant group (n = 6) and prediabetic pregnant group (n = 6). The normal pregnant group (H. Li *et al.*) were given standard rat chow and water ad libitum for 36 weeks. The preeclamptic pregnant group (PE) received normal standard chow and 0.3g/l LNAME in drinking water from GND 8 to GND 14. The prediabetic pregnant group (PD) were given high fat, high carbohydrate diet and drinking water was supplemented with fructose (15%) for 36 weeks to induce PD (Ludidi *et al.*, 2023). On GND 0, 9, 18 blood glucose, blood pressure, 24-h fluid intake and 24-h output were measured. The 24-h urine samples of the pregnant rats were collected on GND 0, 9, 18. Urinary protein concentration were determined using an automated biochemical analyser.

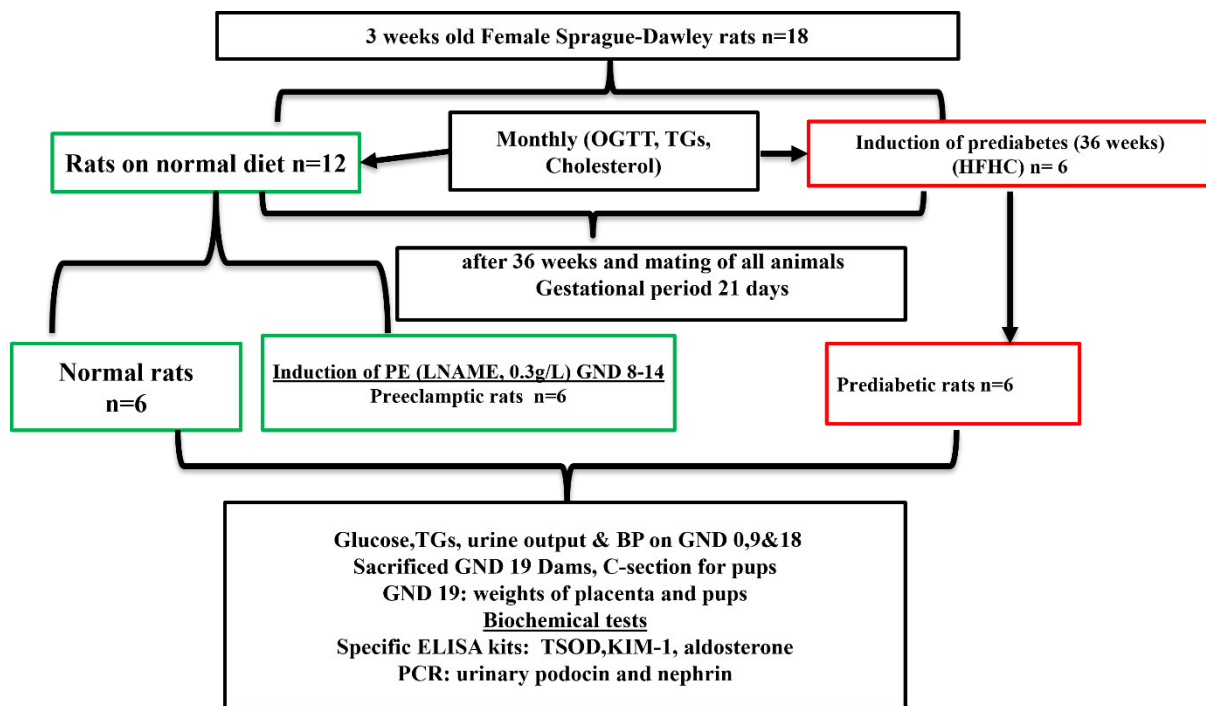


Figure 1: experimental protocol

2.3 Induction of PD

After 1 week of the acclimatization to new environment, PD was induced in 4 week old female Sprague-Dawley rats (n=6) using an established protocol from our laboratory with slight modification (Jackson *et al.*, 2012; Luvuno *et al.*, 2018). Briefly, the experimental animals in the prediabetic group (n=6) were exposed to a high carbohydrates, high fat diet and 15 % fructose (HCHF diet+ 15% fructose) in drinking water for 39 weeks (Luvuno *et al.*, 2018; Ludidi *et al.*, 2023).

2.3.1 Criteria for PD

The American Diabetes Federation criteria was used to diagnose PD in all animals. Animals that had prediabetic symptoms including fasting blood glucose concentration of 6.1-7.1 mmol/L, a two-hour glucose concentration of 7.1-8.1mmol/L in the oral glucose tolerance test, and plasma triglycerides concentration of greater than 2 mmol/L were considered prediabetic. Blood samples were collected from the tail vein through tail pricking method. Glucose was

determined using the Elite® glucometer and blood TGs were determined using the accutrend plus cholesterol meter

2.3.1 Experimental diet

The composition of a high-fat high-carbohydrate diet was customized as shown in table 1. The experimental diet formula is made up of carbohydrate (55 % Kcal/g), fats (30 % Kcal/g), and proteins (15 % Kcal/g). The drinking water of the experimental animals was supplemented with 15% fructose.

Table 1. Composition of ingredients in the high-fat high-carbohydrate diet

Nutrient	Units	Actual	Nutrient	Units	Actual
Dry Matter	g/kg	919.93	ASTSAA	g/kg	6.79
Metabolizable Energy	MJ/kg	15.86	Asvaline	g/kg	5.80
Crude Protein	g/kg	151.27	Fat	g/kg	250.46
Threonine	g/kg	4.51	Carbohydrate	g/kg	427.29
Isoleucine	g/kg	5.24	Fibre	g/kg	22.08
Lysine	g/kg	6.54	Ash	g/kg	26.31
Methionine	g/kg	4.86	Avl-P	g/kg	1.66
Tryptophan	g/kg	1.30	Calcium	g/kg	5.47
Astatine	g/kg	3.30	Total Phosphorus	g/kg	3.60

2.4 Mating

At the end of the 39th week of induction period, the female Sprague-Dawley rats from normal (n=6), PE (n=6) and PD (n=6) group were mated with normal males of the same strain. Briefly two females were housed in one cage to allow their oestrus cycle to become synchronised. The ovarian cycle of rats was between four to five days long. Daily vaginal smears were taken and when the female rats were in the pro-oestrus stage of their cycle, a male rat was introduced into the cage. The following day, vaginal smears were taken from the female rats and the presence of sperm indicated successful mating and cytology was used to confirm positive pregnancy. The day of fertilisation was regarded as GND 0 (Qulu *et al.*, 2015). The male rats were then be removed from the cage and male rats were sacrificed after successful mating.

2.5 Induction of PE

The pregnant dams (n=6) from the PE group were given 0.3g/l a day of N^ω-nitro-L-arginine methyl ester (L-NAME) in drinking water from GND 8-GND 18 (Soobryan *et al.*, 2017). Blood pressure and proteinuria were determined to confirm successful induction of PE on GND 9. Blood pressure were monitored using a non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA). The animals warmed at $\pm 30^{\circ}\text{C}$ in an enclosed chamber (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Wood land Hills, California, USA) for 30 minutes before taking blood pressure readings.

2.6 24 hr fluid and urinary output

Urine sample will be collected over night, each rat was housed in a metabolic cage which offer 99% separation efficiency of urine and faeces, Animal waste is directed through a collection funnel and onto a linear diffuser. The diffuser allows solid matter to travel down into a fecal collection vessel. Liquid waste flows along grooves down the inclined diffuser, passing through the urine port, and into a urine collection vessel. A urine deflector prevents excess urine from

splashing and contaminating faecal specimen. Separate urine and faeces samples are collected in two standard 50 ml centrifuge vessels.

2.7 Plasma and Tissue collection

On GND 19, pregnant rats were sacrificed by decapitation for collection of the kidney tissues and plasma which was obtained by collecting blood into heparinized containers and the blood was centrifuged at 1000 ×g for 10 min in a Hermle Laborechnik GmBH centrifuge (Wehingen, Germany). The kidney tissues and plasma were stored in an Ultra Bio Freezer (Snijers Scientific, Tilburg, Netherlands) at –80°C until biochemical tests.

2.8 Biochemical analysis.

The biochemical analysis of electrolytes (Na⁺ and K⁺) and kidney function parameters (such as creatinine, urea, albumin) were determined after 19 days of gestation in the plasma and urine samples by using IDEXX instrument (IDEXX laboratories (PTY) Ltd. Pretoria, South Africa) as instructed by the manufacturer. Albumin excretion rate (AER), albumin creatinine ratio (ACR), creatinine clearance (CC) and glomerular filtration rate (GFR) were calculated according to Batenburg *et al.*,2012 (Batenburg W.W). The calculations were done as shown below:

hr urine albumin excretion (mg/ 24 hr)=24 hr urine volume×albumin

AER (µg/min) = Urinary Albumin (mg/dl) × Urine Volume (dl) ×
1000 / Period of urine collected (min)

CC (ml/min) = [(Urinary Creatinine in mg/ dl) × (Urinary volume in
ml)]/[(Serum Creatinine in mg/ dl) × 1440 min]

ACR (mg/g) = [Urinary Albumin (mg/dl)/Urinary Creatinine (mg/dl)]
× 1000

$\text{GFR mL/min} = \text{Urine creatinine mg/dL} \times 24 \text{ hr urine volume mL}$

$\text{Plasma creatinine mg/dL} \times 60 \text{ min} \times 24 \text{ hr}$

2.8.1 ELISAs

Specific ELISA kits were used for measurements of the following parameters: kidney tissue total superoxide dismutase (TSOD) (catalog No: E-BC-K020-M; manufacturer: Elabscience), plasma aldosterone (catalog No: E-EL-0070; manufacturer: Elabscience) and kidney injury molecule (KIM-1) concentrations (catalog No: E-EL-R3019; manufacturer: Elabscience). Briefly, Kidney tissue was harvested, then tissue was washed with cold PBS (0.01 M, pH 7.4). 20 mg kidney tissue was homogenized in 180 μL homogenate medium with a dounce homogenizer at 4°C. The sample was centrifuged at 12000 $\times g$ for 10 min at 4°C to remove insoluble material. The supernatant was collected and kept on ice for detection and the protein concentration of the supernatant was determined using BCA Protein Colorimetric Assay Kit (E-BC-K318-M). For the TSOD and aldosterone ELISA kits. 50 μL of each dilution of standard, blank and sample were added into the appropriate wells thereafter 50 μL of biotinylated detection Ab working solution was added to each well immediately. The plate was covered with the sealer and incubated for 45 min at 37 °C. the plate was washed 3x with 350 μL of was buffer in each well. After washing step, 100 μL of HRP conjugate working solution was added to each well and the plate was incubated for 30 min at 37 °C. after the incubation the solution in each well was decanted and the was process was repeated 5x. 90 μL of substrate reagent was added to each well and the plate was incubated for 15 min at 37 °C. 50 μL of the stop solution was added and the optical density (OD) of each well was determined using a microplate reader at 450nm

For a KIM 1 ELISA kits, Briefly, 100 μL standard or sample was added to each well and the plate was incubated for 90 min at 37 °C. After incubation the liquid was removed and 100 μL

of biotinylated detection Ab was added and incubated for 1 hour at 37°C, the plate was washed 3x. 100µL HRP conjugate was added and incubated for 30 min at 37°C. The plate was washed 5x then 90µL of substrate reagent was added and incubated 15 min at 37 °C and 50µL of stop solution was added and the plate was immediately read at 450nm.

Kidney tissue malondialdehyde (MDA) levels were measured using a previously described protocol (L. Mabuza, 2019). Briefly, Kidney tissue (50mg) was homogenized in 500 µL of 0.2% phosphoric acid. The homogenate was centrifuged for 10 minutes at 400 x g. Thereafter, 400 µL of the homogenate was supplemented with 400 µL of 2% phosphoric acid. The solution was aliquoted into two separate tubes. Then, 200 µL of 7% phosphoric acid was added into both glass tubes while adding 400 µL of 3mM hydrochloric acid (HCl) into one glass test tube (blank) and 400 µL thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into the other test tube (sample test). To achieve an acidic pH of 1.5, 200 µL of 1M HCl was added to both the blank and sample test tubes. Both solutions were heated for 15 minutes at 100 °C and allowed to cool to room temperature. After, 1.5 mL butanol was added to the cooled solution; the sample was vortexed for 1 minute to achieve rigorous mixing and allowed to settle until two phases can be observed. The butanol phase/top layer was transferred to Eppendorf tubes and centrifuged for 6 minutes at 13,200xg. The samples were aliquoted into a 96 well microtiter plate in triplicate and the Biotek mQuant spectrophotometer (Biotek, Johannesburg, South Africa) was used to determine the absorbance at 532nm reading.

$$[\text{MDA}] = \frac{A_{523\text{nm}} - A_{600\text{nm}}}{\text{Absorption coefficient (156mmol}^{-1}\text{)}}$$

Absorption coefficient (156mmol⁻¹)

The MDA concentration was normalized to the protein content for each sample:

$$\text{MDA per Protein} = \frac{\text{MDA concentration (nmol or } \mu\text{mol)}}{\text{Protein content (mg or } \mu\text{g)}}$$

Protein content (mg or µg)

2.8.2 Real-time PCR

Real-time quantitative PCR for urinary podocin and nephrin was carried out according to Mabuza *et al.*, 2020 (Mabuza *et al.*, 2020) briefly RNA was isolated from the urine samples according to the manufacturer's protocol ZR Urine RNA Isolation Kit™ (Zymo Research Corp, Irvine, USA). Urine RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) by using iScript™ cDNA Synthesis Kit (Bio Rad, California, USA) through incubation in a thermal cycler (SimpliAmp Thermal Cycler, Applied biosystems, Life technologies). The urinary mRNA podocin and nephrin was quantified by real-time PCR lightcycler (Roche LightCycler 96, USA). All the samples were run in duplicate and GAPDH mRNA levels were used as a housekeeping gene to normalize the podocin mRNA level. The sequences of the used oligonucleotide primers (Mabuza LP, 2020) (Metabion International AG, Planegg, Germany)

Table 1 List of primers used in the study

Gene of interest	Sequence	Accession number
Podocin	Forward: 5'- TGG AAG CTG AGG CAC AAAGA-3' Reverse: 5'- AGA ATC TCA GCC GCC ATC CT-3'	AY_039651.1
Nephrin	Forward: 5'- CTGCTGCCTGTGCTCTTTGC -3' Reverse: 5'- GGCCATAGGCTCTCTCCACT -3'	AF172255.1
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	Forward: 5'-AGTGCCAGCCTCGTCTCATA-3' Reverse: 5'-GATGGTGATGGGTTTCCCGT-3'	NM_017008.2

2.9 Statistical analysis

Sample size (n) for the study was calculated using G*Power (version 3.1.9.7), a statistical power analysis software, this was performed to ensure that the sample size was adequate to detect statistically significant differences between the groups while maintaining ethical considerations for animal use. Statistical analysis was conducted using GraphPad Prism Software (version 9.00, GraphPad Software, San Diego, California, USA). A normality test(Shapiro-Wilk test) was done followed by a parametric one-way ANOVA. To analyse differences between the controls and the experimental group, a Bonferroni's multiple comparisons test was performed. Values of $p < 0.05$ was used to indicate statistical significance.

3. Results

3.1 24-h urine output

24-h urine output of ND, PE pregnant and PD pregnant groups were monitored on day 0, 9 and 18 during gestation period (Figure 1). The PD pregnant group had a significantly higher urine output throughout gestational period in comparison to the ND group ($p < 0.05$, Fig 1). The PE pregnant group had significantly high blood glucose concentration, water intake on GND 18 and a low urine output in comparison to the ND group ($p < 0.05$; Fig 1).

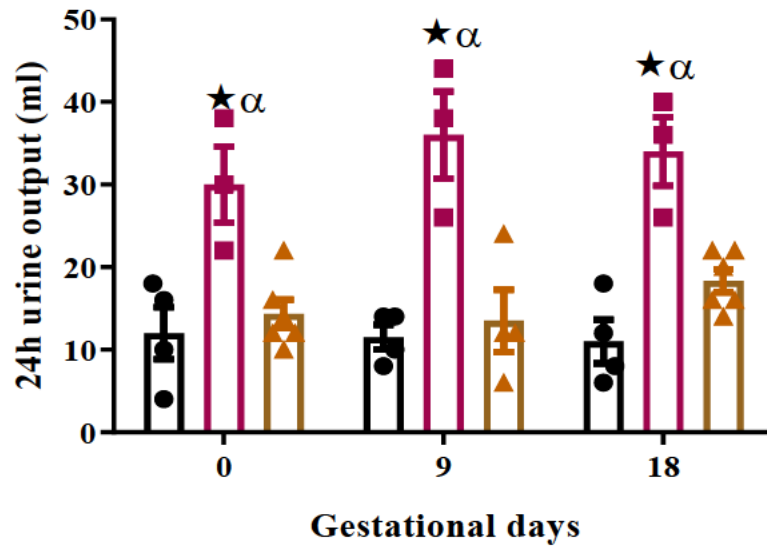


Figure 1: 24 h urine output of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats tested over 18 days of gestation measured on GND 0, 9 and 18. Values are presented as means, and (n=6 in each group).★ p<0.05 by comparison with normal pregnant group. α p<0.05 by comparison with the preeclamptic group.

3.2 Aldosterone concentration

Aldosterone concentration of ND, PD pregnant and PE pregnant groups. plasma aldosterone was measured after 19 days of gestation. the aldosterone concentration were significantly elevated in PD group in comparison to both the ND and PE pregnant group ($p < 0.05$; Fig 2). aldosterone concentration in PE pregnant group was not significantly elevated compared to the ND group ($p < 0.05$; Fig 2).

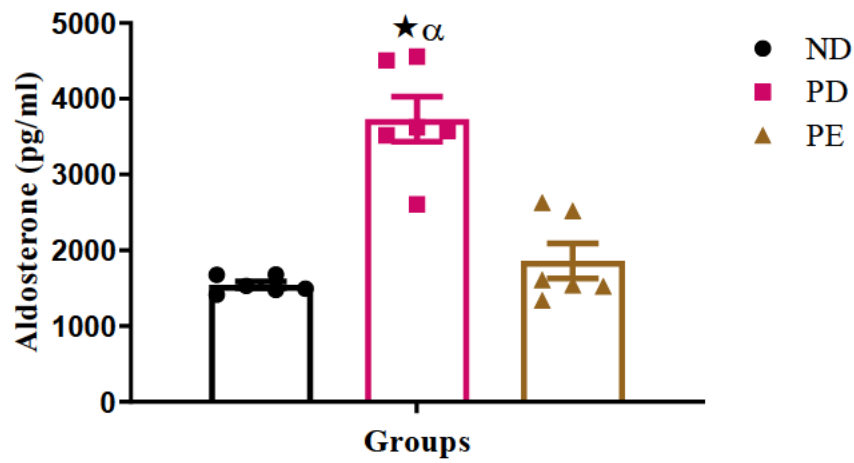


Figure 2: Aldosterone measured after 19 days of gestation of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats measured after 19 days of gestation. Values are presented as means, and SD (n=6 in each group).★ p<0.05 by comparison with normal pregnant group. α p<0.05 by comparison with the preeclamptic group.

3.3 Kidney oxidative stress and antioxidant concentrations

Kidney MDA and TSOD concentrations of ND, PD pregnant groups and PE pregnant measured after 19 days of gestation (Table 1). The results show that the PD pregnant group presented with significantly renal MDA levels and reduced TSOD concentration in comparison to ND group ($p < 0.05$; Table 1). The PE pregnant group had significantly high kidney MDA and reduced TSOD concentration in comparison to ND group ($p < 0.05$; Table 1).

Table 1: Kidney MDA and TSOD concentrations in the placental tissue of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic. Values are represented as means and SD (n=6 in each group).

Groups	MDA (nmol/ g protein)	T-SOD (pg/mg prot)
ND	0.025±0.001	3.000±0.148
PD	0.051±0.004*	1.736±0.180*
PE	0.062±0.005*	1.801± 0.151*

* p < 0.05 by comparison with normal pregnant group

3.4 Real-time quantitative PCR of urinary podocytes specific proteins (nephrin and podocin)

Urinary podocyte-specific proteins (nephrin and podocin) of ND, PD pregnant and PE pregnant groups on measured after 19 days of gestation during gestation period (Figure3). The expression of podocin and nephrin mRNA was significantly higher in the urine of of both PE and PD pregnant group in comparison to with the ND group ($p < 0.05$; Fig 3).

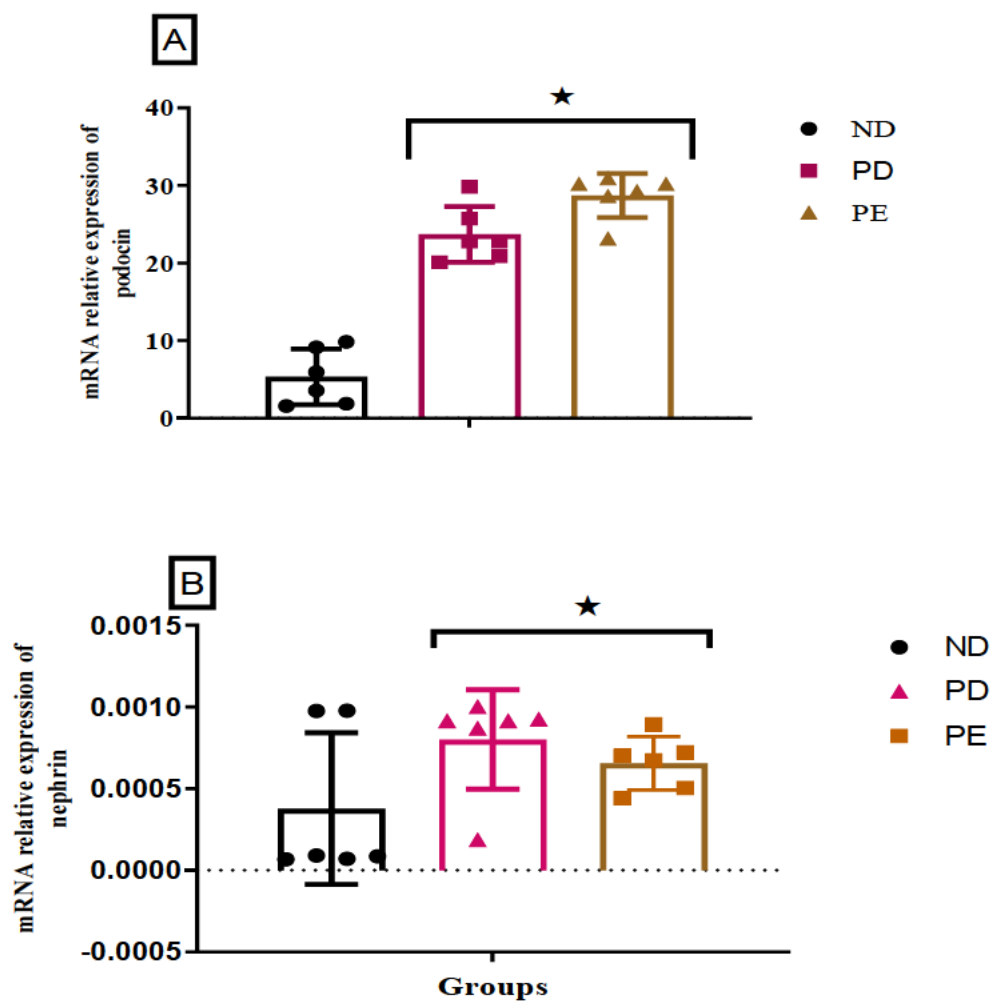


Figure 3: mRNA expression of urinary podocin (A) and urinary nephrin (B) of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats measured after 19 days of gestation. Values are presented as means and SD (n=6 in each group). ★p<0.05 by comparison with normal pregnant group.

3.5 Electrolyte handling, albumin, creatinine and urea levels

Urine and plasma electrolyte handling, albumin, creatinine and urea levels of ND, PD pregnant PE pregnant rats (Table 2). The PE and PD pregnant group showed significant decrease in urinary Na⁺, K⁺, creatinine and urea levels as well as increased urinary albumin levels when compared to the ND group ($p < 0.05$, Table 2). The PE and PD pregnant group showed significant increase in plasma Na⁺, K⁺, creatinine and urea levels as well as decreased plasma albumin levels when compared to the ND group ($p < 0.05$, Table 2).

Table 2: Urine and plasma electrolyte handling, albumin, creatinine and urea levels of normal pregnant rats, preeclamptic pregnant rats and prediabetic pregnant rats. Values are represented as means; and SD (n=6 in each group).

Groups	Urinary parameters				
	Na ⁺ (mmol/L)	K ⁺ (mmol/L)	Albumin (g/L)	Urea (mmol/L)	Creatinine (μmol/L)
ND	82.200±2.672	30.200±1.530	40.400±1.568	20.000±1.732	51.600±2.205
PD	35.800±2.458*	5.080±0.208*	59.600±1.503*	39.600±2.663*	36.800±1.985*
PE	45.650±2.011*	17.643±1.941*	56.040±1.479*	32.600±1.288*	33.88±2.344*
Groups	Plasma parameters				
	Na ⁺	K ⁺	Albumin	Urea	Creatinine
ND	122.400±1.400	4.540±0.264	45.000±1.225	5.860±0.681	0.688±0.104
PD	147.200±2.939*	10.680±0.063*	28.600±2.135*	2.800±0.130*	1.622±0.069*
PE	135.600±1.945*	9.760±0.432*	25.400±1.637*	3.750±0.513*	2.034±0.037*

* p < 0.05 by comparison with normal pregnant group

3.6 Kidney function markers

Markers of kidney function: Albumin excretion rate, albumin creatinine ratio, creatinine clearance and glomerular filtration barrier of ND, PE pregnant and PD pregnant groups (Table 3). The PE and PD pregnant group similarly showed significantly increased AER and ACR with a decreased CC and GFR when compared to the ND group ($p < 0.05$; Table3).

Table 3: Influences of the PE and pregestational PD on kidney function. Values are presented as means $\pm$ SEM, (n=6) in each group. Values are represented as means and SD (n=6 in each group).

Groups	AER ($\mu\text{g}/\text{min}$)	ACR (mg/g)	CC (ml/min)	GFR($\text{ml}/\text{min}/100\text{g}$)
ND	0426.300 $\pm$ 0.349	079.020 $\pm$ 0.511	1.580 $\pm$ 0.110	1.390 $\pm$ 0.230
PD	2138.000 $\pm$ 0.150*	163.800 $\pm$ 0.962*	0.540 $\pm$ 0.040*	0.540 $\pm$ 0.040*
PE	1135.000 $\pm$ 0.106*	167.400 $\pm$ 0.738*	0.290 $\pm$ 0.050*	0.300 $\pm$ 0.060*

* $p < 0.05$ by comparison with normal pregnant group

3.7 KIM-1 concentration

Plasma KIM-1 concentration of ND, PE pregnant and PD pregnant groups after 19 days of gestation (Figure 4). The PE pregnant group showed a slight elevation in KIM-1 concentration when compared to the ND group ($p < 0.05$; Fig 4). The PD pregnant group showed a significant increase in plasma KIM-1 concentration when compared to the ND group ($p < 0.05$; Fig 4).

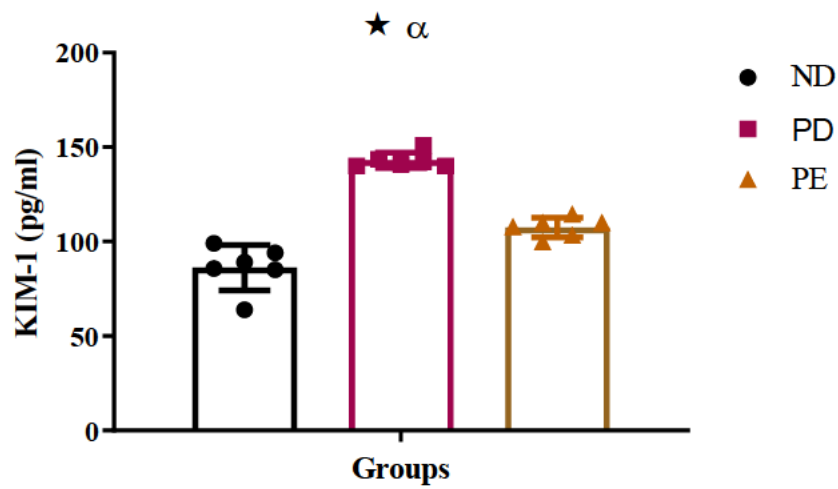


Figure 4: KIM-1 concentration of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats and after 19 days of gestation. Values are presented as means, SD (n=6 in each group).★ p<0.05 by comparison with normal pregnant group. α p<0.05 by comparison with the preeclamptic group.

4. Discussion

The aim of the study was to determine the effects of pregestational PD as a risk of the development of PE with specific interests on the effects of intermediate hyperglycaemia on kidney function during pregnancy. We assessed 24 h fluid intake and urine output, electrolyte handling and the effects of pregestational PD on development of kidney reactive oxygen species (ROS). We also assessed the adverse effects of maternal PD on the activation of RAAS, basement membrane with specific interests on podocyte specific proteins, podocin and nephrin and on the proximal tubule by assessing plasma KIM-1 concentration.

PE has two distinguished stages of development, the first stage consists of poor placentation. The second stage consists of release of anti-angiogenic factor (sFlt-1 and sEng) and increased production of ROS by the stressed placenta (ACJJori, 2019; Tomimatsu T, 2019). In this study the PE pregnant group showed a significant increase in oxidative stress as shown by an increase in lipid peroxidation marker MDA and lower levels of TSOD in comparison with the ND. The PD pregnant group presented with similar results to the PE pregnant group. Intermediate hyperglycaemia-induced oxidative stress on the kidneys promotes endothelial dysfunction (Vodošek Hojs N, 2020). The endothelial dysfunction of afferent arteriole leads to poor nephron perfusion, ultimately kidney dysfunction.

PE as compared with normal pregnancy, is characterized by a reduced intravascular volume (Alex C, 2020). Despite this reduction in circulating volume, most components of the circulating RAAS are down-regulated in PE compared with normal pregnancy (Gennari-Moser C, 2011 ; Batenburg W.W, 2012). However, there is not enough data to explain this phenomenon. However, authors have speculated that this could be due to a physiological response to the increased blood pressure or an increase in antiangiogenic factors (Koen VERDONK, 2014). One of the down regulated components of the RAAS in PE is aldosterone (Lumbers *et al.*, 2019; Ives *et al.*, 2020; Koen VERDONK, 2014). Low levels of aldosterone

have been shown to be responsible for the decreased volume expansion in PE and which causes poor placental perfusion (Premjith G, 2020). In this study we observed slightly low aldosterone levels and significantly elevated blood pressure in the PE pregnant group in comparison to the ND group. The elevation in blood pressure in PE while aldosterone is suppressed shows that hypertension in PE is due to other factors such as sFlt-1, AT1-AA and ROS that are released by a stressed placenta (iddiqui AH, 2010; Yang J, 2015).

In this study, the PD pregnant group presented with a significant increase in aldosterone and increased blood pressure. This corresponds with previous studies that show that PD in male Sprague rats is associated with the activation of the intrarenal renin aldosterone system which is thought to increase Na^+ retention (Mabuza LP, 2020). Na^+ retention, as assisted by increased aldosterone levels, has been shown to increase blood pressure (Mabuza LP, 2020). Electrolyte imbalance can be attributed to basement membrane alterations which include basement membrane thickening and changes in the slit diaphragm (Sterns, 2015). Electrolytes such as Na^+ and K^+ play an important role in the pathogenesis of hypertension. They contribute significantly to the functioning of the vascular smooth muscles which is responsible for regulation of blood pressure (AK Sayyed, 2013; Craici IM, 2014). PE is associated with sodium retention and substantial alterations in intracellular water and electrolyte concentration (Hinrichs *et al.*, 2020). In the current study, decreased urinary and increased plasma Na^+ and K^+ levels were observed in PD pregnant group which are similar results to the preeclamptic pregnant group in comparison to ND group.

Kidney dysfunction in PE characterized by glomerular endotheliosis and proteinuria (Tomimatsu T, 2019). The PE pregnant group had significant increased urinary albumin and decreased urinary creatinine and urea plasma in comparison ND group. The development of albuminuria is due to the loss of both size and charge selectivity of the glomerular barrier. PE is associated with a characteristic glomerular lesion which is glomerular capillary

endotheliosis, mesangial cell swelling, hypertrophy and loss of endothelial fenestrations(Wewers *et al.*, 2021).

Markers for renal dysfunction and altered GFR in chronic hyperglycaemia are elevated plasma urea and creatinine concentration (A. Chutani, 2014). The PD pregnant group showed significantly decreased urinary creatinine and urea concentration with an increased urinary albumin concentration in comparison to ND group. Additionally, these correlated with increased plasma urea and creatine levels with decreased plasma albumin levels.

The renal glomeruli have fenestrated endothelial which are necessary for the unique permeability characteristics of the glomerular filtration barrier which makes it a target organ in PE. This is because a hypoxic placenta promotes production of sFlt-1 which targets fenestrated endothelium, especially the renal glomerulus and induces proteinuria and glomerular endotheliosis (Petrica L, 2014; Alda Tufro, 2012). Podocytes have been shown to be involved in the production of VEGF (Henao & Saleem, 2013). The loss of podocyte as indicated by urinary podocin and nephrin is associated with the reduction of VEGF production by podocytes which leads to massive proteinuria and glomerular endotheliosis in PE (Bajinath *et al.*, 2017; Gilani SI, 2017 ; Kerley & McCarthy, 2018). In this study, the PE pregnant group showed a significant increase in urinary mRNA of podocin and nephrin which correlated with an increase in albuminuria in comparison with the ND group. In T2DM-induced kidney injury, podocyte loss and injury are associated with marked proteinuria (B. He, 2014). In the PD pregnant group, we observed an increase in urinary mRNA of podocin and nephrin which correlated with an increase in albuminuria. These results were similar to the PE pregnant group in comparison to ND group. Previous studies have demonstrated that the down regulation of podocin and nephrin which are key podocyte slit diaphragm proteins are involved in the development of proteinuria in DN (Petrica L, 2014; Fukuda A, 2012). In this study we measure urinary mRNA expression of podocin and nephrin because urinary mRNA levels of these protein have been shown to

reflect early molecular changes in podocytes prior to the detection of significant protein alterations (Zeng & Szeto, 2021; Inoue, 2022). Furthermore, urinary mRNA of podocin and nephrin originates directly from injured podocytes, providing a specific marker for podocyte damage (Zeng *et al.*, 2024). However, the very low expression of nephrin necessitates a cautious interpretation of the data in both the PD and PE.

The increase in urinary protein excretion during PE and pregestational PD may impair the proximal tubule due to inflammation, therefore, we measured KIM-1 as a marker of renal tubular damage to diagnose renal injury in PE and pregestational PD (Yuping Wang, 2015; Nowak N, 2016) order of references. Studies have shown that KIM-1 is a more accurate marker for kidney injury as compared with the traditional used serum creatinine (Schulz C-A, 2020). In our study, the plasma KIM-1 concentration was significantly elevated in PE and pregestational PD in comparison to the ND group. The increased plasma levels of KIM-1 in PE and pregestational PD provide valuable evidence that not only glomerular cells (endothelial cells and podocytes) but also the function of proximal tubule epithelial cells are disturbed in PE and pregestational PD (Zhao X, 2019; Schmidt IM, 2022).

5. Conclusion

The PD pregnant group exhibited moderate hyperglycaemia, impaired electrolyte handling, high blood pressure, increased plasma KIM-1, proteinuria and an increase in urinary podocin and nephrin may suggest pregestational PD as a risk factor for preeclamptic like kidney dysfunction.

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Chapter 6

Prologue 3

HELLP syndrome is a severe complication of PE which is associated with liver dysfunction. HELLP syndrome is characterized by the presence of hemolysis, elevated liver enzymes, and low platelets. In PE, the uteroplacental ischemia results in endothelial dysfunction which is responsible for hypertension, the release of inflammatory mediators and liver dysfunction. In chapter 3, Manuscript 2 of this study, we observed intermediate hyperglycaemia, increased blood pressure, increased urinary excretion of podocyte specific proteins podocin and nephrin and impaired electrolyte handling which depicted renal dysfunction in the prediabetic pregnant rats. Also, the results showed an increase blood glucose, TGs, food intake, water intake, blood pressure and proteinuria, and results also showed that oxidative stress and plasma concentration of KIM-1 are significantly increased in the prediabetic pregnant rats. However, the effects of pregestational PD on liver function have not been studied. Therefore, in the present study, we investigated the effects pregestational PD on selected parameters of liver dysfunction in a diet induced pregnant prediabetic rat model. The manuscript is titled: **Pregestational PD mellitus exhibits PE associated complications: The adverse effects of pregestational PD on liver dysfunction** and is authored by Siboto A, Ludidi A, Xulu N, Nkosi A, Sibiya N, Khathi A, Ngubane PS. The present manuscript is under review in the journal of diabetes and its complications (manuscript number: JDC-D-24-00090). This journal is accredited by Department of Higher Education and Training South Africa and appears in WoS accredited list (2021)

Author Contribution: A Siboto was responsible for study conceptualization, study design, conducted experimental work, data analysis first draft writing, and manuscript editing.

Manuscript 3

Pregestational PD exhibits PE associated complications: The adverse effects of pregestational PD on liver dysfunction.

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Abstract

Introduction: Both PE and PD are known hypertensive disorders of pregnancy which shows a correlation between these two diseases. The etiology of PE involves inadequate trophoblast invasion of maternal spiral arteries leading to the release of antiangiogenic factors which disrupt the formation of new vessels, in the liver, impaired vessel formation leads to liver dysfunction due to inadequate blood supply. Recent study in our laboratory has shown that pregestational PD is a risk factor for developing PE during pregnancy as pregestational PD increased antiangiogenic factors. However, the pregestational PD antiangiogenic release has not been shown to be associated with liver dysfunction. Therefore, this study seeks to investigate pregestational PD as a risk factor for HELLP syndrome.

Materials and methods: The animals were divided into 3 groups: the normal pregnant group ND, the preeclamptic pregnant group (PE) and prediabetic pregnant group (PD). On GND 19 animals were sacrificed and blood and liver tissues were collected for the measurement of liver TGs, liver enzymes, TNF- α , IL 6 and haematology parameters.

Results: The results showed significant increases in liver TGs , liver enzymes, TNF- α , IL 6, and haematology parameters in the PE and PD pregnant group as compared to ND group.

Conclusion:

pregestational PD exhibited dyslipidemia, an increase in inflammation and liver enzymes which are associated with liver dysfunction.

Keywords: HELLP, pregestational PD, liver function

1. Introduction

Studies have shown that PE complicates about 2-4% of pregnancy globally (Collaborators, 2016), whereas there are no studies that show statistics of pregnancy complicated by PD. Both PE and PD are known hypertensive disorders of pregnancy which shows a correlation between these two diseases (Boucheron *et al.*, 2022; Ludidi *et al.*, 2023). Additionally, both pathologies have adverse effects on both the mother and fetus, such as a significantly increased risk of developing hypertension, heart failure, end-stage chronic kidney disease (CKD), and T2DM (Jiang *et al.*, 2022; Poon *et al.*, 2023). The main mechanism involved in the etiology of PE is inadequate trophoblast invasion of maternal spiral arteries with subsequent maternal global endothelial dysfunction and hypoxia condition due to the antiangiogenic factors produced by a stressed placenta (Magee LA *et al.*, 2022). Recent study by Ludidi *et al.* has shown that PD is a risk factor for developing PE during pregnancy as PD is associated with an increase in antiangiogenic factors (Ludidi *et al.*, 2023). The upregulation of antiangiogenic factors in PE has been shown to exert their adverse effects on the maternal endothelium including all areas of fenestrated endothelium in the liver, thus causing the hepatocytes to become leaky and release liver enzymes to the maternal circulation (Van Lieshout *et al.*, 2019).

One of the complications of severe PE is the HELLP syndrome. The diagnosis of HELLP syndrome requires the presence of microangiopathic hemolysis, thrombocytopenia and abnormalities of liver enzymes (Gardikioti *et al.*, 2022). HELLP syndrome has been shown to impact the liver's synthetic function, potentially affecting albumin production therefore PE decreases with a plasma albumin due to liver and kidney dysfunction (Alese *et al.*, 2021). All of these abnormalities have been observed in prediabetic male Sprague Dawley rats (Siboto *et al.*, 2022). However, these abnormalities have not been shown to occur in prediabetic pregnant female Sprague Dawley rats. Therefore, the aim of the study was to investigate pregestational PD as a risk factor for HELLP syndrome.

2. Materials and methods

2.1 Drugs and chemicals

Drugs were sourced from standard pharmaceutical suppliers. All other chemicals were of analytical grade were purchased from standard commercial suppliers.

2.2 Animals

The study was approved by the University of Kwazulu-Natal ethics committee as the procedures involving animals and their care were conducted in conformity with the institutional guidelines. Approved ethics number: AREC/025/19D. 3 weeks old female Sprague-Dawley rats (n = 18) bred and housed in the Biomedical Research Unit of the University of Kwazulu-Natal were used in the present study. The animals were maintained under standard housing conditions of constant temperature (22 ± 2 °C), CO₂ content of < 5000 pm, relative humidity of $55 \pm 5\%$, illumination (12 h light/dark cycles), noise levels of less than 65 decibels, and ad libitum access to standard rat chow (29% protein, 55% carbohydrate, and 16% fat) and water.

2.2.1 Experimental design

The role of pregestational PD as a risk factor of PE was studied in the following groups. Group 1: normal pregnant control rats (ND) which received normal standard chow and normal drinking water throughout the study (n = 6/group); Group 2: PD pregnant rats which received the HCHF diet+ 15% fructose throughout the study (n = 6/group); Group3: PE pregnant control rats which received normal standard chow and 0.3g/l LNAME in drinking water from GND 8 to GND 14 (n = 6/group). On GND 0, 9,18 body weight was measured.

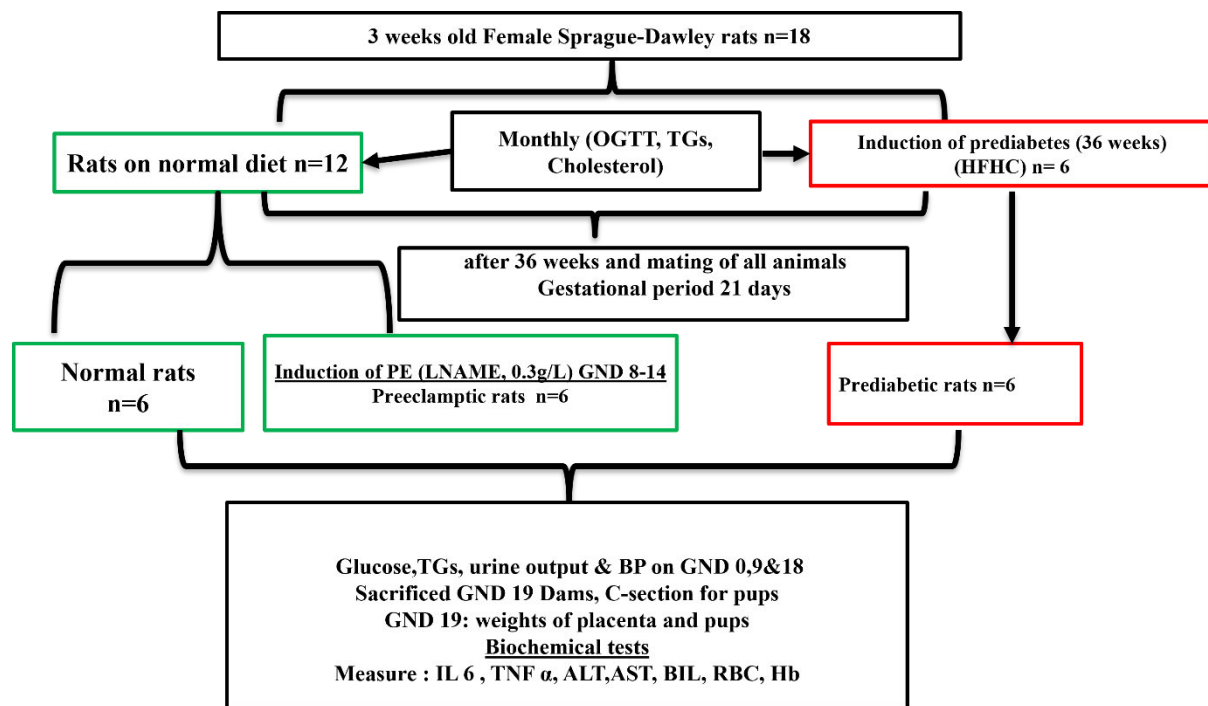


Figure: experimental protocol

2.3 Induction of PD

After 1 week of the acclimatization, PD was induced in 4 week old female Sprague-Dawley rats (n=6) using an established protocol from our laboratory with slight modification (Jackson *et al.*, 2012; Luvuno *et al.*, 2018). Briefly, the experimental animals were exposed to a high carbohydrates, high fat diet and 15 % fructose (HCHF diet+ 15% fructose) in drinking water for 36 weeks (Luvuno *et al.*, 2018). The control group (n=6) and preeclamptic group (n=6) received standard chow diet and normal water for 36 weeks. During the induction period all the rats were also acclimatized to blood pressure machine once a month.

2.3.1 Criteria for PD

The American Diabetes Federation criteria was used to diagnose PD in all animals. Animals that have prediabetic symptoms including fasting blood glucose concentration of 6.1-7.1 mmol/L, a two-hour glucose concentration of 7.1-8.1mmol/L in the oral glucose tolerance test, and plasma triglycerides concentration of greater than 2 mmol/L were considered prediabetic. Blood samples were collected from the tail vein through tail pricking method. Glucose was determined using the Elite® glucometer and blood TGs were determined using the accutrend plus cholesterol meter.

2.3.2 Experimental diet

The composition of a high-fat high-carbohydrate diet was customized as shown in table 1. The experimental diet formula is made up of: carbohydrate (55 % Kcal/g), fats (30 % Kcal/g), and proteins (15 % Kcal/g). The drinking water of the experimental animals was supplemented with 15% fructose.

Table 1. Composition of ingredients in the high-fat high-carbohydrate diet

Nutrient	Units	Actual	Nutrient	Units	Actual
Dry Matter	g/kg	919.93	ASTSAA	g/kg	6.79
Metabolizable Energy	MJ/kg	15.86	Asvaline	g/kg	5.80
Crude Protein	g/kg	151.27	Fat	g/kg	250.46
Threonine	g/kg	4.51	Carbohydrate	g/kg	427.29
Isoleucine	g/kg	5.24	Fibre	g/kg	22.08
Lysine	g/kg	6.54	Ash	g/kg	26.31
Methionine	g/kg	4.86	Avl-P	g/kg	1.66
Tryptophan	g/kg	1.30	Calcium	g/kg	5.47
Astatine	g/kg	3.30	Total Phosphorus	g/kg	3.60

2.4 Mating

36 weeks old Female Sprague-Dawley rats (n=18) were mated with males of the same strain. Briefly two females were housed in one cage to allow their oestrus cycle to become synchronised. The ovarian cycle of rats was between four to five days long. Daily vaginal smears were taken and when the female rats were in the pro-oestrus stage of their cycle, a male rat was introduced into the cage. The following day, vaginal smears were taken from the female rats and the presence of sperm indicated successful mating and cytology was used to confirm positive pregnancy. The day of fertilisation was regarded as GND 0 (Qulu *et al.*, 2015). The male rats were then be removed from the cage and male rats were sacrificed after successful mating.

2.5 Induction of PE

The pregnant dams (n=6) were given 0.3g/l a day of N^o-nitro-L-arginine methyl ester (L-NAME) in drinking water from GND8-GND 14 (Soobryan *et al.*, 2017). Blood pressure and proteinuria were determined to confirm successful induction of PE on GND 9. Blood pressure were monitored using a non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA). The animals warmed at $\pm 30^{\circ}\text{C}$ in an enclosed chamber (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Wood land Hills, California, USA) for 30 minutes before taking blood pressure readings.

2.6 Plasma and Tissue collection

On GND 19, pregnant rats were sacrificed by decapitation for collection of the liver tissue, whole blood and plasma. Plasma was obtained by collecting blood into single precooled heparinized containers and the blood was centrifuged at $1000 \times g$ for 10 min in a Hermle Laborechnic GmbH centrifuge (Wehingen, Germany). The liver tissues were removed, weighed, and then snap frozen in liquid nitrogen. Afterwards, the liver tissues and plasma were stored in an Ultra Bio Freezer (Snijers Scientific, Tilburg, Netherlands) at -80°C for biochemical tests.

Relative liver weight of all the animals in each experimental group were determined from the percentage of the ratio of liver weight to the body weight.

$$\text{relative liver weight} = \frac{\text{liver weight}}{\text{body weight}} \times 100$$

body weight

2.7 Biochemical tests

2.7.1 Lipid profile

The preparation of liver tissue samples and the homogenate medium used to determine hepatic triglyceride were according to the manufacturer's instructions in the triglyceride assay kit (Catalog No: E-BC-K238 ; Manufacturer: Elabscience). Briefly, liver tissue was harvested, then tissue was washed with cold PBS (0.01 M, pH 7.4). 20 mg liver tissue was homogenized in 180 μ L homogenate medium with a dounce homogenizer at 4°C. The sample was centrifuged at 12000 $\times$ *g* for 10 min at 4°C to remove insoluble material. The supernatant was collected and kept on ice for detection and the protein concentration of the supernatant was determined using BCA Protein Colorimetric Assay Kit (E-BC-K318-M). 2.5 μ L of distilled water was added to the blank well, 2.5 μ L of the standard was added to the standard well, 2.5 μ L of the sample was added in each sample well and 250 μ L of a working solution was added in all the wells, and mixed thoroughly and the plate was incubated 37°C for 10 min, the OD value was measured at 510 nm with microplate reader.

calculations for tissue samples:

$$\text{TG (mmol/gprot)} = \Delta A1 / \Delta A2 \times c \times f \div \text{Cpr}$$

$\Delta A1$: ODSample – ODBlack (If Operate with automatic biochemical analyzer, the ODBlack is 0)

$\Delta A2$: ODStandard – ODBlack (If Operate with automatic biochemical analyzer, the ODBlack is 0)

c: Concentration of standard.

f: Dilution factor of sample before test.

Cpr: Concentration of protein in sample (gprot/L).

2.7.2 Liver Inflammatory markers

For IL 6 and TNF α measurements, 50 mg of liver tissue was homogenized, and the concentration was determined using an ELISA kit following the manufacturer's instructions (Catalog no: E-EL-R0015 and catalog no.: E-EL-R2856; manufacturer: Elabscience). IL6 and TNF α ELISA kits, Briefly, 100 μ L standard or sample was added to each well and the plate was incubated for 90 min at 37 °C. After incubation the liquid was removed and 100 μ L of biotinylated detection Ab was added and incubated for 1 hr at 37°C, the plate was washed 3x. 100 μ L HRP conjugate was added and incubated for 30 min at 37°C. The plate was washed 5x then 90 μ L of substrate reagent was added and incubated 15 min at 37 °C and 50 μ L of stop solution was added and the plate was immediately read at 450nm. Protein detection was done as follows liver tissue was harvested, then tissue was washed with cold PBS (0.01 M, pH 7.4). 20 mg liver tissue was homogenized in 180 μ L homogenate medium with a dounce homogenizer at 4°C. The sample was centrifuged at 12000 $\times$ g for 10 min at 4°C to remove insoluble material. The supernatant was collected and kept on ice for detection and the protein concentration of the supernatant was determined using BCA Protein Colorimetric Assay Kit (E-BC-K318-M).

2.7.3 Liver enzymes

Liver damage biomarkers including alanine aminotransferase, aspartate aminotransferase (AST) , total bilirubin (TBIL), albumin were measured in plasma using the Catalyst One Chemistry Analyzer (IDEXX Laboratories, Westbrook, ME, USA).

2.7.4 Haematological Analysis

Red blood cell (RBC) count, haemoglobin (Hb), and platelets concentration were measured in blood collected from all the rat groups using the haematocytometer (Beckman Coulter, Indianapolis, United States).

2.7.5 Lipid Peroxidation and Antioxidant Profile.

The antioxidant profile of the liver was determined by measuring the concentration of SOD 1 according to the manufacturer's instructions (Catalog No: E-EL-R1424; Elabscience Biotechnology Co., Ltd., Houston, TX, USA) via the microplate reader, SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenburg, LGBW, Germany). Briefly for the SOD 1 ELISA kits. 50 μ L of each dilution of standard, blank and sample were added into the appropriate wells thereafter 50 μ L of biotinylated detection Ab working solution was added to each well immediately. The plate was covered with the sealer and incubated for 45 min at 37 $^{\circ}$ C. the plate was washed 3x with 350 μ L of was buffer in each well. After washing step, 100 μ L of HRP conjugate working solution was added to each well and the plate was incubated for 30 min at 37 $^{\circ}$ C. after the incubation the solution in each well was decanted and the was process was repeated 5x. 90 μ L of substrate reagent was added to each well and the plate was incubated for 15 min at 37 $^{\circ}$ C. 50 μ L of the stop solution was added and the optical density (OD) of each well was determined using a microplate reader at 450nm). Furthermore MDA concentration in the liver was determined to estimate the amount of lipid peroxidation according to previously described protocol by (Gamede *et al.*, 2021). Briefly, liver tissue (50mg) was homogenized in 500 μ L of 0.2% phosphoric acid. The homogenate was centrifuged for 10 minutes at 400 x g. Thereafter, 400 μ L of the homogenate was supplemented with 400 μ L of 2% phosphoric acid. The solution was aliquoted into two separate tubes. Then, 200 μ L of 7% phosphoric acid was added into both glass tubes while adding 400 μ L of 3mM hydrochloric acid (HCl) into one glass test tube (blank) and 400 μ L thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into the other test tube (sample test). To achieve an acidic pH of 1.5, 200 μ L of 1M HCl was added to both the blank and sample test tubes. Both solutions were heated for 15 minutes at 100 $^{\circ}$ C and allowed to cool to room temperature. After, 1.5 mL butanol was added to the cooled solution; the sample was vortexed for 1 minute to achieve rigorous mixing and allowed to settle

until two phases can be observed. The butanol phase/top layer was transferred to Eppendorf tubes and centrifuged for 6 minutes at 13,200xg. The samples were aliquoted into a 96 well microtiter plate in triplicate and the Biotek mQuant spectrophotometer (Biotek, Johannesburg, South Africa) was used to determine the absorbance at 532nm reading.

$$[\text{MDA}] = \frac{A_{523\text{nm}} - A_{600\text{nm}}}{\text{Absorption coefficient (156mmol}^{-1}\text{)}}$$

The MDA concentration was normalized to the protein content for each sample:

$$\text{MDA per Protein} = \frac{\text{MDA concentration (nmol or } \mu\text{mol)}}{\text{Protein content (mg or } \mu\text{g)}}$$

2.8 Statistical Analysis.

Sample size (n) for the study was calculated using G*Power (version 3.1.9.7), a statistical power analysis software, this was performed to ensure that the sample size was adequate to detect statistically significant differences between the groups while maintaining ethical considerations for animal use. Statistical analysis was conducted using GraphPad Prism Software (version 9.00, GraphPad Software, San Diego, California, USA). A normality test (Shapiro-Wilk test) was done followed by a parametric one-way ANOVA. To analyse differences between the controls and the experimental group, Bonferroni's multiple comparisons comparison test was performed. Values of $p < 0.05$ were used to indicate statistical significance.

3. Results

3.1 Liver weight and relative liver weights

(A) Liver weights and (B) relative liver weights of normal pregnant rats, pregnant prediabetic rats after and pregnant preeclamptic rats after 19 gestational days. Both preeclamptic pregnant and prediabetic group showed significantly increase in both liver weights and relative liver weights when compared to the normal pregnant control group ($p < 0.05$; Fig 2).

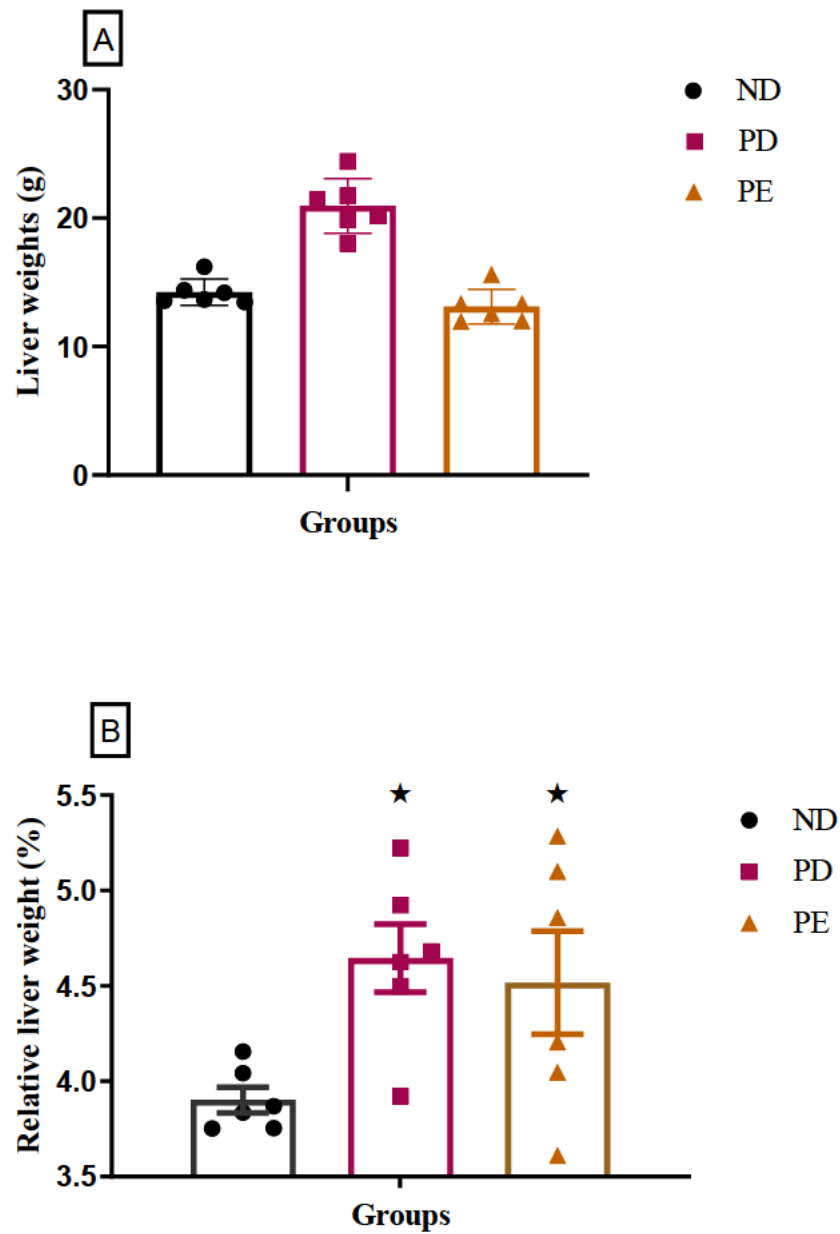


Figure 1: (A)Liver weights and (B) relative liver weights of normal pregnant rats, pregnant prediabetic rats after and pregnant preeclamptic rats measured after 19 days of gestation. Values are presented as means, and SD (n=6 in each group). ★ $p<0.05$ by comparison with normal pregnant group.

3.2 Liver triglyceride (TGs) concentration

Liver TGs concentrations of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats after the period of 19 gestational days. Both prediabetic pregnant and preeclamptic group showed significantly increased liver TGs concentration when compared to the normal pregnant control group ($p<0.05$; Fig 1).

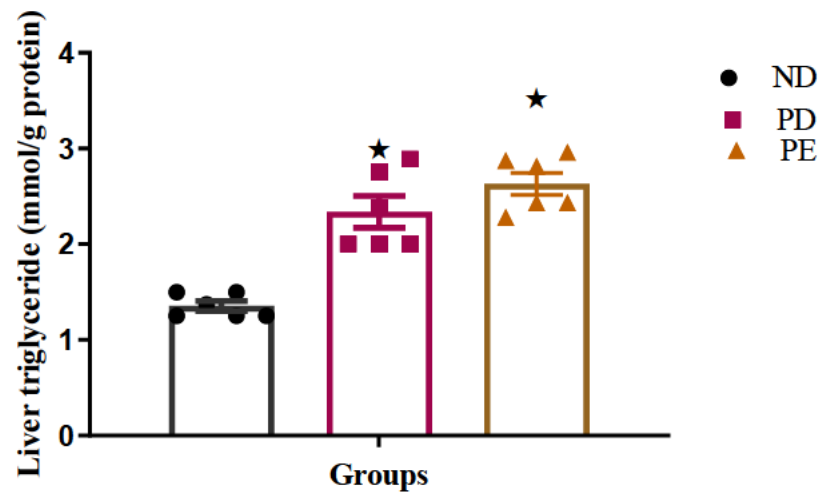


Figure 2: Liver TGs concentrations tested after 19 days of gestation. Values are presented as means and SD (n=6 in each group). ★ p<0.05 by comparison with normal pregnant group.

3.3 Oxidative stress marker

MDA concentration, SOD concentration in the liver tissue of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats after 19 day of gestation. The pregnant preeclamptic group showed significantly increased MDA concentration and a decreased activity of SOD in comparison with the normal pregnant group ($p < 0.05$; Table 1). The pregnant prediabetic group showed significantly increased MDA concentration and a decreased activity of SOD in comparison with the normal pregnant group which were similar results to the pregnant preeclamptic group (Table 1).

Table 1: The effects of PE and pregestational PD on MDA concentrations and SOD activity in the liver tissue over the of 19 days of gestation. Values are represented as means and SD (n=6 in each group)

Groups	MDA (nmol/g protein)	SOD (pg/ mg protein)
ND	0.025±0.001	2.077 ±0.172
PD	0.053 ± 0.005* α	1.134 ± 0.231* α
PE	0.049 ± 0.004*	0.528 ±0.144*

* p < 0.05 by comparison with normal pregnant group. α p < 0.05 by comparison with preeclamptic pregnant group.

3.4 Inflammatory Markers (IL-6 and TNF α)

All experimental groups were sacrificed on GND 19 and the plasma IL-6 and TNF α concentration were measured in normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats. The results showed that both plasma IL-6 and TNF α concentration of preeclamptic pregnant and prediabetic group was significantly higher than that of normal pregnant control ($p < 0.05$; Fig 3).

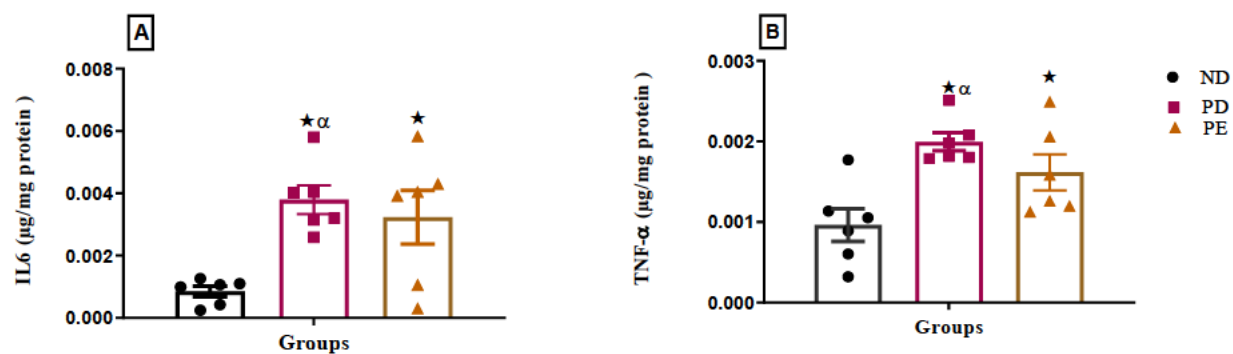


Figure 3: Inflammatory markers (A) IL6 and (B) TNF α on the liver of normal pregnant rats, pregnant preeclamptic and pregnant prediabetic measured after 19 gestational days. The values are presented as means, and SD (n=6 in each group). ★p < 0.05 by comparison with normal pregnant group. α p < 0.05 by comparison to pregnant preeclamptic group

3.5 Plasma Liver Enzymes and bilirubin

All experimental groups were sacrificed on GND19 and plasma AST, ALT and bilirubin concentrations were measured in normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats. The results showed that both plasma AST and ALT concentrations of pregnant prediabetic group and pregnant preeclamptic group was significantly higher than that of normal pregnant control ($p < 0.05$; Fig 4). The concentration of plasma bilirubin was significantly decreased in both of pregnant prediabetic group and pregnant preeclamptic group when compared to the normal pregnant control ($p < 0.05$; Fig 4).

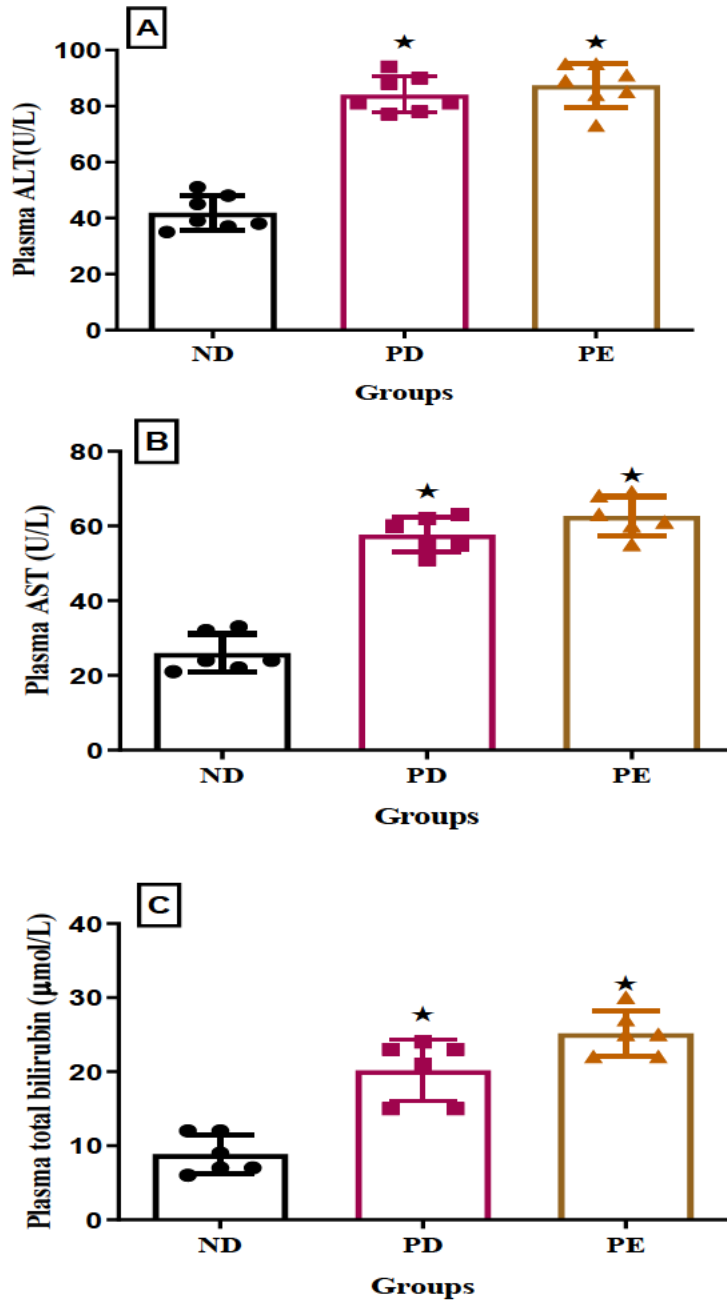


Figure 4: Plasma AST (A), ALT (B) and bilirubin (C) concentrations of normal pregnant rats, pregnant prediabetic and pregnant preeclamptic rats measured after 19 gestational days. The values are presented as means, and SD (n=6 in each group). ★ $p < 0.05$ by comparison with normal pregnant group.

3.6 Plasma albumin concentration

Plasma albumin concentration of normal pregnant rats, pregnant preeclamptic rats and pregnant prediabetic rats measured after the period of 19 gestational days. Both prediabetic pregnant and preeclamptic group showed significantly decreased plasma albumin concentration when compared to the normal pregnant control group ($p<0.05$; Fig 5).

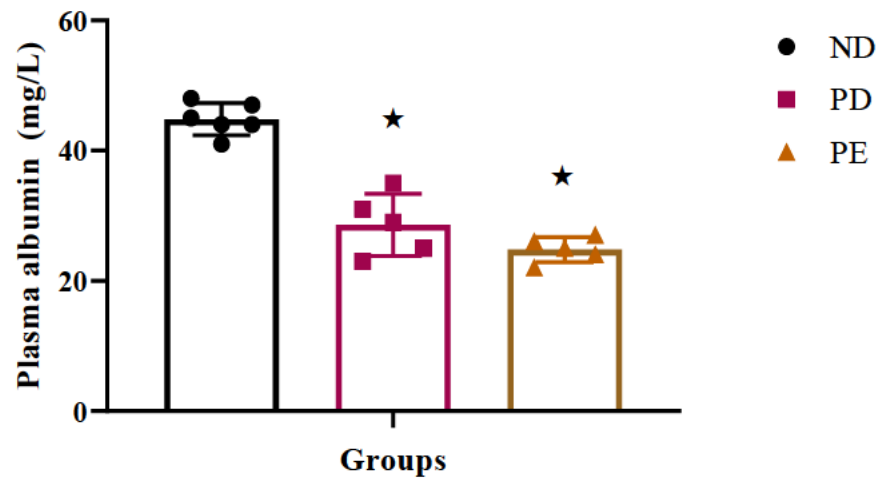


Figure 5: Plasma albumin concentrations of normal pregnant rats, pregnant prediabetic and pregnant preeclamptic rats measured after 19 gestational days. The values are presented as means, and SD (n=6 in each group). ★ $p < 0.05$ by comparison with normal pregnant group.

3.7 Haematological parameters

Haematological parameters of normal pregnant rats, pregnant prediabetic rats and pregnant preeclamptic rats and after 19 days of gestational period. pregnant preeclamptic rats and pregnant prediabetic rats exhibited a decrease in haematological parameters, namely red blood cell count (RBC), haemoglobin (Hb), and platelets concentration in comparison to normal pregnant rats ($p < 0.05$; Fig 6).

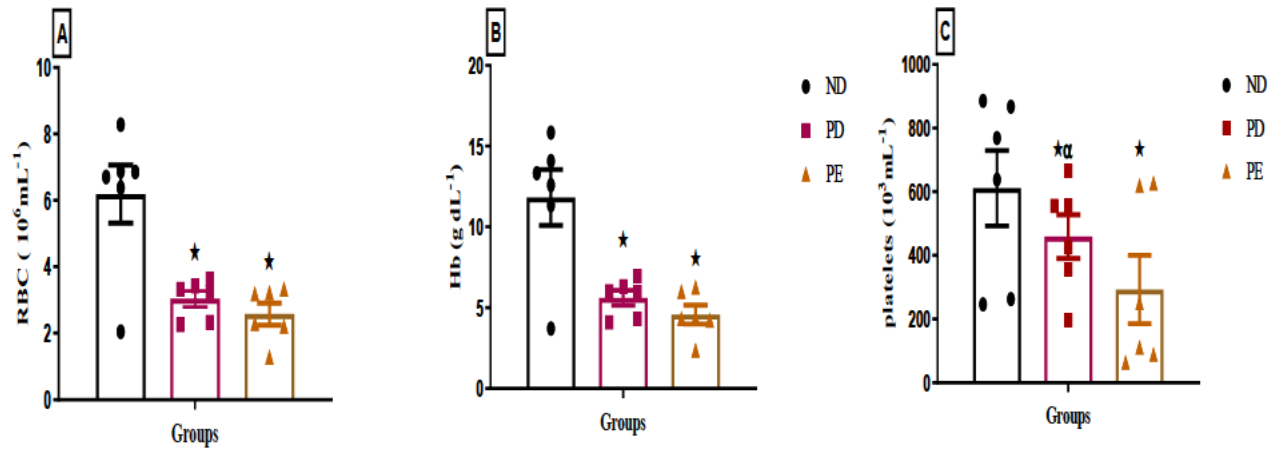


Figure 6: RBC (A), Hb (B) and platelets (C) concentrations of normal pregnant rats, pregnant prediabetic and pregnant preeclamptic rats measured after 19 gestational days. The values are presented as means, and SD (n=6 in each group). ★ $p < 0.05$ by comparison with normal pregnant group. α $p < 0.05$ by comparison to pregnant preeclamptic group.

4. Discussion

The results of the present study showed that pregestational PD is a risk factor for HELLP syndrome due to metabolic complications that begin on the onset of PD. In this study we assessed liver TGs, relative body weight, liver oxidative stress and antioxidant status, inflammatory markers, liver enzymes and hematology markers to investigate pregestational PD as a risk factor for the HELLP syndrome. The Diagnosis of HELLP syndrome requires the presence of microangiopathic hemolysis, thrombocytopenia and abnormalities of liver enzymes (Rimaitis *et al.*, 2019). Pregnant women who do not have all these parameters are considered to have partial HELLP syndrome (Aydin *et al.*, 2014). The aim of the study was to investigate pregestational PD as a risk factor for HELLP syndrome which is one of the complications of severe PE (Aydin *et al.*, 2014).

There is a direct relationship between maternal TGs and newborn weight (Clinck *et al.*, 2022). This has been shown in both animal and human studies (Abdel-Hamid *et al.*, 2020; Chen *et al.*, 2021; Clinck *et al.*, 2022). Lipids have been shown to play a major role in fetal development, however deviations in maternal hyperlipidaemia trigger pathogenic events such as atherosclerosis and cardiovascular disease (CVD) later in life of the child (Mocarzel *et al.*, 2020). In this study we observed an increase in TG concentration in both the pregnant PE and pregnant PD groups in comparison to the normal pregnant control. The symptoms of PE are caused by endothelial dysfunction related to hypoxia in the placenta and abnormal lipid metabolism (Alese *et al.*, 2021). Lipid abnormalities in PE have been shown to be associated with liver steatosis (Haggai *et al.*, 2022). PD is associated with insulin resistance, which can increase peripheral lipolysis, triglyceride synthesis and hepatic uptake of free fatty acids, which ultimately leads to NAFLD (Gamede *et al.*, 2019). NAFLD has been shown to have adverse effects on pregnancy-related outcomes, maternal and child health (Hershman *et al.*, 2019).

These adverse effects include hypertensive complications of pregnancy, PE, preterm birth, and low birth weight (Sarkar *et al.*, 2020).

Accumulation of TGs in the liver of pregnant PE and pregnant PD is associated with increased relative liver weights in both groups and the development of oxidative stress and reduced antioxidants activity in the liver in both the pregnant PE and PD groups in comparison to the normal pregnant group. Oxidative stress causes hepatocellular damage through several different mechanisms, including lipid peroxidation, that can directly stimulate cell necrosis and activation of apoptosis (Tan *et al.*, 2020). Hepatic fat accumulation and oxidative stress can cause an inflammatory response followed by necrosis, fibrosis and cirrhosis, ultimately leading to liver failure (Farzanegi *et al.*, 2019; Arroyave-Ospina *et al.*, 2021). We observed increased inflammatory markers (TNF and IL 6) in both the pregnant PE and PD groups in comparison to the normal pregnant group.

These inflammatory mediators have been shown to activate stellate cells and scar tissue deposition in the liver (Wang *et al.*, 2022). Hepatocyte inflammation and hepatic fat accumulation is associated with hepatocyte injuries which leads to the release of transaminases enzyme such as ALT and AST (Utzschneider *et al.*, 2019; Ziolkowska *et al.*, 2021). In this study, these enzymes were significantly elevated in pregnant PE and pregnant PD group by comparison with normal pregnant group. The liver enzyme levels in plasma reflects the presence of injury in the liver as these enzymes are components of hepatocytes that are released into circulation upon hepatocyte damage. Serum levels of liver enzymes can also be used as a marker of hepatic fat deposition (Lee *et al.*, 2012; Brady, 2020). The decrease in plasma albumin observed in both PD and PE in comparison with normal pregnant group is associated Impaired liver synthetic function.

The stressed placenta in HELLP releases antiangiogenic factors, ROS (oxidative stress) which interact vascular endothelial cells (EC) and induce release of inflammatory cytokines in the maternal circulation (Perez Botero *et al.*, 2021). The Damaged vascular endothelium causes changes to RBCs as the RBCs pass through the vessels which results in microangiopathic hemolytic anemia (Perez Botero *et al.*, 2021). The pregnant PE group presented with significantly reduced platelet count which correlated with low RBC count and low haemoglobin concentration in comparison to the normal pregnant group. Hepatocyte damage in HELLP is enhanced by the microangiopathy which impedes portal blood flow (Ang *et al.*, 2021). In PE, the ratio of prostacyclin-thromboxane production rate is decreased favouring the vasoconstrictive thromboxane and both these substances also play a role in the event of thrombocytopenia (Oe *et al.*, 2018).

Hyperglycaemia induce oxidative damage is associated with reduced function of red blood cell (RBC) due to reduced NADPH which is necessary its antioxidant enzymatic activity (Pernow *et al.*, 2019). T2DM increases RBC concentration causing an increase in blood viscosity and development of high blood pressure (Pernow *et al.*, 2019). IL6 is elevated in T2DM and has been shown to promote apoptosis of immature erythrocytes, thus causing a reduced number of circulating RBCs and in turn causing a reduction of circulating haemoglobin (Randeria *et al.*, 2019). The pregnant PD group had significantly low RBC and low haemoglobin levels in comparison to the normal pregnant group. RBCs in T2DM are stiffed by chronic hyperglycaemia causing them to have a harmful effect on the vasculature (Nada, 2015). These stiff RBCs have been shown to damage endothelium in addition to the damage caused by oxidative stress (Nada, 2015). These deformed RBCs are therefore susceptible to degradation by Kupfer cells, which could partly explain increases in bilirubin in this study. Additionally, the change composition and function of RBCs in T2DM causes endothelium aggregation. This is also considered as an effect that also facilitates adhesion of platelets, which results in

thrombus formation in T2DM. A study in prediabetic male Sprague-Dawley rats showed that RBCs abnormalities are not enough to cause (Mzimela *et al.*, 2021). During the prediabetic state there is a progressive deterioration in structural properties of RBCs (Mzimela *et al.*, 2021). Both PD and HELLP syndrome show similar adverse effects such as elevated liver enzyme, thrombocytopenia and thrombotic microangiopathy, however these occur through different mechanism in both the diseases.

5. Conclusion

The PD pregnant group exhibited increased liver weights, increased liver TGs, liver inflammation increased plasma liver enzymes and decreased RBC, Haemoglobin and increased platelet count which may suggest pregestational PD as a risk factor for HELLP syndrome.

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Chapter 7

Synthesis

Studies have shown that PE complicates about 3-8% of pregnancy, T2DM and PE share similar pathology such as endothelial dysfunction, hypertension and renal dysfunction. However, there are no studies that show how PD can possibly complicate pregnancies similarly to PE given similar etiologies. According to the International Society for the Study of Hypertensive diseases (ISSHP), PE can be diagnosed in the absence of proteinuria, as the presence of a new-onset hypertension after 20 weeks gestation and other symptoms associated with PE such as intrauterine growth restriction (IUGR) and liver dysfunction (Unger *et al.*, 2020). The main mechanism involved in the etiology of PE is inadequate trophoblast invasion of maternal spiral arteries with subsequent maternal global endothelial dysfunction and hypoxia condition due a stressed placenta (Jung *et al.*, 2022; Magee LA *et al.*, 2022). The stress in PE has been shown to produce antiangiogenic factors such as sFlt-1 and sEng which have been shown to bind and antagonize the membrane bound angiogenic factors such as VEGF and PlGF and inhibit their normal function therefore leading to reduced NO, endothelial dysfunction. Ultimately, this results in maternal end organ dysfunction including kidney and liver dysfunction (Guerby *et al.*, 2021; Hu & Zhang, 2021). PE is a multifaceted disorder with different risks factors that can arise from trophoblast and non-trophoblastic risk factors (Ridder *et al.*, 2019; Magee LA *et al.*, 2022).

Non-trophoblastic maternal risk factors of PE include chronic hypertension, GDM, T2DM (Shiozaki & Saito, 2018). However, pregestational PD has not been outlined as risk factor of PE. During pregnancy complicated by T2DM, the human placenta undergoes a number of functional and structural pathologic changes (Kapustin *et al.*, 2021). Furthermore, T2DM has

been associated with the endothelial dysfunction and the production of ROS and antiangiogenic factors, hence is considered a risk factor for PE. T2DM is often preceded by PD (Khathi *et al.*, 2019). PD is defined according to the American diabetes association (ADA), as a state of intermediate hyperglycaemia, hyperinsulinaemia, impaired glucose tolerance, and peripheral and hepatic insulin resistance and moderate hypertension and moderate increase in ROS (Luvuno *et al.*, 2021). PD causes abnormal changes in intracellular metabolism of most body organs including the kidneys and liver (Gamede *et al.*, 2019; Mabuza *et al.*, 2020). However, the adverse effects of PD on placenta function have not been studied. The lack of such data prompted us to investigate pregestational PD as a risk factor to the development of PE and the adverse effects of pregestational PD on PE associated complications such as placenta, liver and kidney dysfunction.

PE is associated with placenta dysfunction due to inadequate trophoblast invasion which results in a hypoxic environment in the placenta. A hypoxic placenta is associated with reduced production and expression of angiogenic factors including VEGF and PLGF and an increase in antiangiogenic factors such as sFlt-1 and sEng (Magee LA *et al.*, 2022). The imbalance between angiogenic and antiangiogenic factors is well studied in PE and T2DM however there were no studies that investigated antiangiogenic factors in pregestational PD. This was investigated in manuscript 1. The measure of placenta dysfunction in this study was measured by looking at angiogenic and anti-angiogenic status. pregestational PD was shown to predispose pregnant prediabetic female Sprague-Dawley rats to PE. This was evidenced by a slight decrease in the slightly increased mRNA expression of sFlt1 and reduced mRNA expression of VEGF observed in the placenta. We also observed low NO concentration indicating endothelial dysfunction and vasoconstriction on the blood vessels which is associated with reduced blood flow. Endothelial dysfunction causes IUGR which affects fetal growth and development (Yzydorczyk *et al.*, 2017). These findings were in agreement with those of a previous study

that showed that PE is associated with the imbalance in angiogenic and antiangiogenic factors and IUGR (Fox *et al.*, 2014). The release of antiangiogenic factors into the maternal circulation from the intervillous space causes antiangiogenic factors to act on the maternal vascular endothelium, inducing oxidative stress and stimulating the production and secretion of pro-inflammatory cytokines (Harmon *et al.*, 2016). However, the effects of these antiangiogenic factors induced by pregestational PD on kidney function had not been studied. This was investigated in manuscript 2. The kidney's involvement in PE is attributed to glomerular endothelial damage and an imbalance between pro-angiogenic and antiangiogenic factors which induce a hypoxic environment in kidneys and causes acute tubular necrosis and injury of podocytes leading to proteinuria (Sani *et al.*, 2019). Interestingly, Pregestational PD was associated with kidney dysfunction which was evident by increased urinary podocin and nephrin indicating podocyte loss and injury to the slit diaphragm. Furthermore, we observed increase in plasma KIM-1 concentration that has been associated with proximal tubule damage leading to proteinuria. These findings were in agreement with those of a previous that show that PE is associated with podocyte loss and proteinuria (Garovic, 2014). Following the novel findings in manuscript 1 and manuscript 2, the next step was to look at liver dysfunction that is associated with the slight elevation of sFlt-1 in pregestational PD. Therefore, manuscript 3 investigated pregestational PD as a risk factor for HELLP syndrome. One of the complications of severe PE is the HELLP syndrome and the diagnosis of HELLP syndrome requires the presence of microangiopathic hemolysis, thrombocytopenia and abnormalities of liver enzymes. The symptoms of PE are caused by endothelial dysfunction related to hypoxia in the placenta and abnormal lipid metabolism (Alese *et al.*, 2021). Lipid abnormalities in PE has been shown to be associated with liver steatosis. hepatic fat accumulation and oxidative stress can cause an inflammatory response followed by necrosis, fibrosis and cirrhosis, ultimately leading to liver failure (Alese *et al.*, 2021). Hepatocyte inflammation and hepatic fat

accumulation is associated with hepatocyte injuries which leads to the release of transaminases enzyme such as ALT and AST (Van Lieshout *et al.*, 2019). Novelty shown by manuscript 3 was that pregestational PD is associated with liver dysfunction because in this study we observed increase in plasma liver enzymes such as ALT and AST which indicates that there is presence of injury in the liver as these enzymes are components of hepatocytes that are released into circulation upon hepatocyte damage. Serum levels of liver enzymes can also be used as a marker of hepatic fat deposition. Additionally, we observed reduced platelet count, low RBC count and low haemoglobin concentration in the pregestational PD group which is similar to what is observed in severe PE. Hepatocyte damage in HELLP is enhanced by the microangiopathy which impedes portal blood flow therefore, pregestational PD is a risk factor for HELLP syndrome due to metabolic complications that begin on the onset of PD.

Taken together, the study showed that diet-induced pregestational PD has adverse effects on the placenta, the kidneys, and the liver due to moderate hyperglycaemia, moderate increase in antiangiogenic factors which act on maternal endothelial and induce endothelial dysfunction which eventually results in dysregulation of placenta, renal and hepatic homeostasis. Therefore, we consider PD as a risk factor for PE and we recommend PD diagnosis during early pregnancy as a measure to prevent pregnancy complications. We also recommend lifestyle modification to all the women in order to prevent pregestational PD and associated complications.

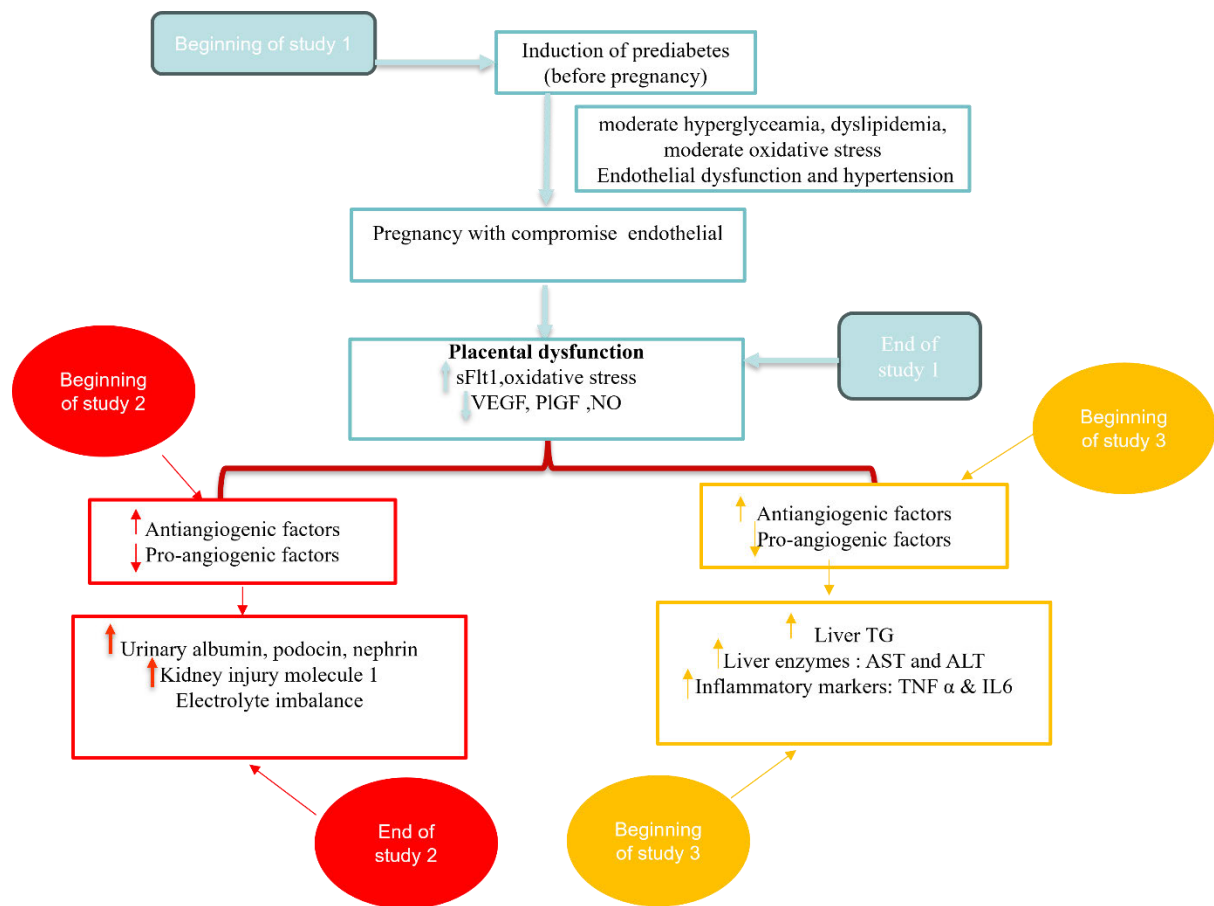


Figure 1: Summary of findings from the manuscripts

Conclusion

The results of this study show that pregestational PD is a risk factor of PE due to metabolic dysfunctions that occur before pregnancy leading to a compromised endothelium, placental, renal and liver dysfunction. The findings of this study further suggest that pregestational PD has adverse effects on the fetus similar to PE.

Shortfalls and future studies

A more reliable indicator of PD is HbA1C, we did not measure it which is one of the shortfalls of the study. In this study it took a long time to induce PD in female Sprague Dawley rats due to the protective effects of oestrogen. It took 39 weeks to successfully induce PD by this time these animal are considered as advanced maternal age. The advanced age of the rats used in this study could act as a confounding factor, making it difficult to isolate the effects of PD and PE from the underlying age-related changes in maternal and placental physiology. While this model provides valuable insights into pregnancy outcomes in older animals, it highlights the need to consider maternal age when interpreting the severity of fetal growth restriction. The animals in this study were age matched however we do not know if the results would have been the same if we have used a younger group. Future studies should include younger control groups to delineate the effects of maternal age from those of the disease models and provide a broader understanding of the interaction between these variables. Also the lower levels of nephrin in the study are considered as a limitation. Not measuring podocin, nephrin protein expression was a shortfall ideally these proteins should have been done using an ELISA or western blot, and quantify how much protein is present in a sample.

Due to ethical reasons, we could not measure sFlt-1 on GND 9 which could have confirmed successful induction of PE. Due to limited funding, hypoxic parameters such as hypoxic inducible factor -1 and sEng were not measured in manuscript 1. HIF-1 and sEng expression could have given insights on oxygen homeostasis within cells. Therefore, a future study could

expand on the hypoxic parameters that were not measured in manuscript 1. Angiotensin II Type 1 Receptor Agonistic Autoantibody (AT1-AA) could have been determined in manuscript 2, AT1-AA have been shown to interact with endogenous angiotensin II, significantly increase endothelin 1 and reactive oxygen species levels, and aggravate pathological changes in renal vascular endothelial cells. Furthermore, an in-depth investigation could be done on human studies investigate the effects of pregestational PD on pregnancy and

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Appendices

Appendix 1: AREC Ethics Approval Letter

14 June 2021

Ms Anelisiwe Siboto (212518629)
School of Laboratory Medicine & Medical Sciences
Westville

Dear Ms Siboto,

Protocol reference number: AREC/025/019D

Project title: The effects of Syzygium aromaticum derived oleanolic acid on female Sprague-Dawley rats with preeclampsia (LNAME model) and prediabetes (HCHF model) co-exist: effects on the pups.

Full Approval –

Research Application With regard to your report received on 04 June 2021, 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.

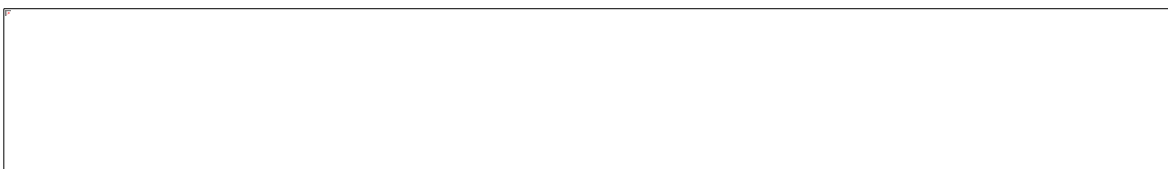
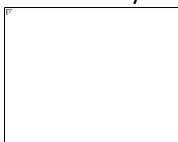
Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

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 13 June 2022.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

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

Sethu Ngubane **cc BRU**

Manager: Dr Jaca

Animal Research Ethics Committee (AREC)

Ms Karen Reinertsen (Administrator)

Westville Campus, Govan Mbeki Building

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Website: <http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx>

Appendix 2 : Published manuscript 1 of this study



Journal of Obstetrics and Gynaecology



ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/ijog20

Maternal prediabetes as a risk factor of preeclampsia and placental dysfunction in pregnant female Sprague-Dawley rats

Aneliswe Siboto, Asiphaphola Ludidi, Ntethelelo Sibiya, Andile Khathi & Phikelelani Ngubane

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Appendix 3: OGTT results of normal pregnant group, pregnant PD, pregnant PE taken on GND 0, 9 and 18.

OGTT	GND 0			GND 9			GND 18		
	NPD	PD	PE	NPD	PD	PE	NPD	PD	PE
0	4,8	7,6	4,5	5,75	7,63	5,3	6,1	6	5,1
15	8,7	9,1	6,8	8,73	9,05	8,3	6,95	7,8	6,1
30	6,8	7,7	5,9	6,75	7,72	6,5	6,3	7,18	5,6
60	6,6	6,9	6	6,55	6,92	6,5	5,8	6,74	5,5
120	5,8	7,3	5	5,85	7,32	5,5	4,9	6,82	5

Appendix 4: Establishing a Female Animal Model of Prediabetes Using a High-Carbohydrate, High-Fat Diet



Article

Establishing a Female Animal Model of Prediabetes Using a High-Carbohydrate, High-Fat Diet

Ayanda Nkosi ¹, Reveshni Pather ², Bongeka Mshengu ¹, Andile Khathi ¹ and Phikelelani Ngubane ^{1,*}

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Abstract: Prediabetes is a condition that often precedes the onset of type 2 diabetes and is characterized by moderate levels of insulin resistance. This condition is well established in male animal models for diabetes; however, few female models exist. There is accumulating evidence that sex variations affect the pathogenesis, treatment, and consequences of numerous diseases, such as type 2 diabetes. Therefore, we sought to develop a diet-induced prediabetic female animal model to better understand prediabetes development and its effects in females. Female Sprague Dawley rats were randomly allocated to one of two groups: the standard diet (SD) group fed a standard diet with normal drinking water, and the high-carbohydrate, high-fat (HCHF) group fed a high-carbohydrate and high-fat diet with drinking water supplemented with fructose. During induction, we measured food intake, body weight, body mass index (BMI), and oral glucose tolerance response (OGT). After the induction period, biochemical analyses were conducted to assess the levels of plasma leptin, ghrelin, insulin, and glycated hemoglobin (HbA1c). Glycogen concentrations were quantified in the liver and skeletal muscles. The HCHF diet-fed group presented higher body weight gain, food intake, and BMI levels, which were accompanied by elevated plasma insulin, ghrelin, and liver and skeletal muscle glycogen levels compared to the SD-fed group. In the HCHF diet-fed group, the HOMA-IR was above 1.9, suggesting the presence of moderate levels of insulin resistance. The OGT response was significantly higher in the HCHF-fed group versus the SD-fed group, suggesting impaired glucose tolerance, thus displaying the signs and symptoms of prediabetes. The HCHF diet with fructose led to the induction of prediabetes in female Sprague Dawley rats. This model could be used to investigate and outline the pathophysiological complications associated with prediabetes in females as a result of the prolonged ingestion of a high carbohydrate, high-fat diet with fructose. The development of this model could also serve as an effort to further bridge the gap regarding the inclusion of females in biomedical research, thus providing advancements in deriving better, specified treatment strategies for women.

Keywords: prediabetes; high-carbohydrate; high-fat diet; insulin resistance; impaired glucose tolerance



Citation: Nkosi, A.; Pather, R.; Mshengu, B.; Khathi, A.; Ngubane, P. Establishing a Female Animal Model of Prediabetes Using a High-Carbohydrate, High-Fat Diet. *Curr. Issues Mol. Biol.* **2024**, *46*, 12397–12416. <https://doi.org/10.3390/cimb46110736>

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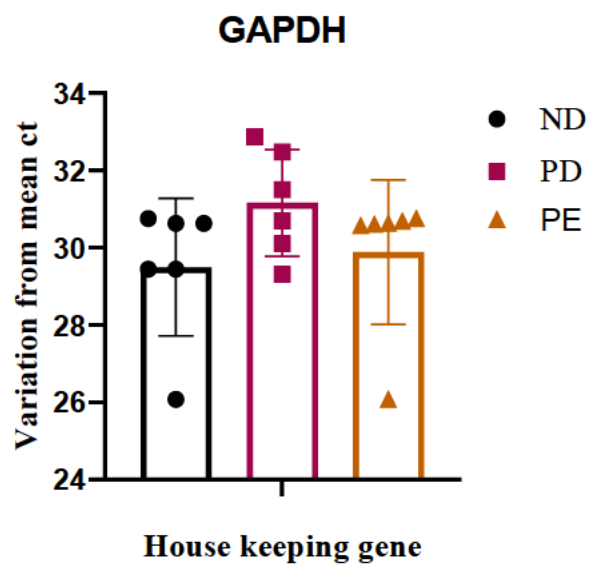


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1. Introduction

The rising prevalence of type 2 diabetes (T2DM) is considered an important global health concern [1]. Five hundred thirty-seven million individuals have been estimated to be living with diabetes, and T2DM represents 98% of global diabetes diagnoses, with greater incidence in men than in women [2,3]. However, at the time of type 2 diabetes diagnosis, women appear to face a larger risk factor burden [4]. The rapid development of the economy and urbanization has led to the growing prevalence of T2DM in many parts of the world, affecting the quality of life of many people and resulting in serious morbidity and premature mortality [5]. This is related to the increases in body mass index and fasting

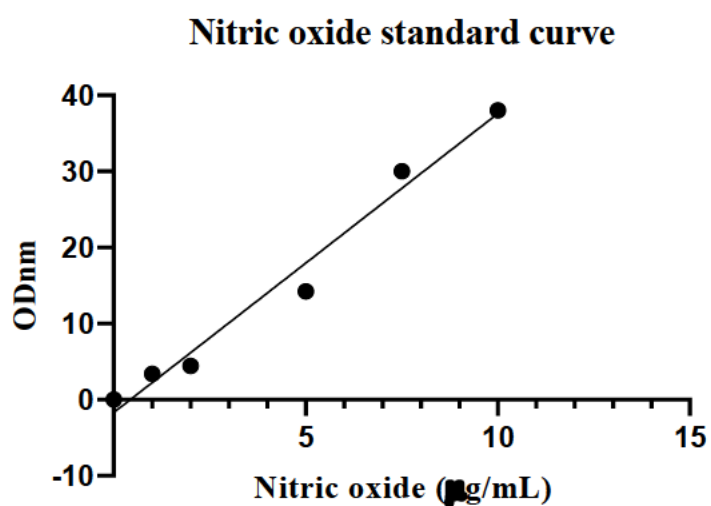
Appendix 5: GAPDH results



Appendix 6: NO standard curve and the graph generated

Nitric oxide standard curve and equation

	540nm	690nm	540/690	Concentration
STD CURVE	2,853	0,075	38,04	10
	3,1825	0,106	30,02358	7.5
	1,5995	0,1125	14,21778	5
	0,759	0,171	4,438596	2
	0,2585	0,0765	3,379085	0



the Standard Curve Equation:

- Substitute the absorbance value of the sample (y) into the standard curve equation to calculate the nitrite concentration (x).

$$X = \frac{y - c}{m}$$

x: Nitrite concentration (µM).

y: Measured absorbance.

m: Slope of the standard curve.

c: Y-intercept of the standard curve

Appendix 7: Abstract of CHS symposium 2023 oral presentation

SCHOOL OF LABORATORY MEDICINE AND MEDICAL SCIENCES RESEARCH DAY

4 OCTOBER 2023

SENATE CHMABER, WESTVILLE CAMPUS

INSTRUCTIONS:

- Please forward your abstracts electronically to dudhrajhp@ukzn.ac.za by **21 AUG 2022**.
- Participants should indicate their preference of presentation type (oral or poster).

CRITERIA FOR THE ACCEPTANCE OF ABSTRACTS:

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

GUIDELINES:

- Use Arial 11 point font throughout.
- The abstract must have a title: IN BOLD TYPE AND UPPER CASE (capitals).
- All authors must be listed: Surname followed by initials; do not include titles. (**bold and underline** the presenter's name).
- Author affiliations must be shown: Department, Centre or Unit; Use * and # symbols to match affiliation with author.

Example: **Taylor, M.**^{*}, Suleman, F.[#]

MATERNAL PREDIABETES AS A RISK FACTOR OF PREECLAMPSIA: THE ADVERSE EFFECTS OF MATERNAL PREDIABETES ON PLACENTAL DYSFUNCTION.

Siboto A^{*}, Ludidi A^{*}, Khathi A^{*}, Ngubane P.S^{*}

^{*}School of Laboratory Medicine and Medical Sciences

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

PLEASE FILL IN ALL PRESENTER DETAILS

Presenter Information

Surname: Siboto	First Name: Anelisiwe
Research Theme: Endocrinology	Tel: [REDACTED]
E-Mail: 212518629@stu.ukzn.ac.za	Date: 16/08/2023

ABSTRACT TEMPLATE

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

250 words max.

**TITLE, AUTHOR AND
AFFILIATION**

ABSTRACT

BODY:

Background/Aim(s): Studies have shown that type 2 diabetes mellitus (T2DM) is a risk factor for preeclampsia (PE). However, the onset of T2DM is often preceded by prediabetes (PD). While there is a well-established link between T2DM and PE, the effects of the intermediate hyperglycaemia observed during prediabetes on placental dysfunction has not been shown. Accordingly, this study sought to investigate a possible link between maternal prediabetes and the risk of developing preeclampsia.

Methods: Pregnant female Sprague-Dawley were divided into normal, and prediabetic groups to study the effects of maternal prediabetes on placental dysfunction over 19 days of gestation. Blood glucose, Triglyceride content (TGs) and blood pressure were measured on GND 0,9 and 18. Placental vascular endothelial growth factor (VEGF), placenta growth factor and (PIGF) and soluble fms-like tyrosine kinase 1 (sFlt-1) mRNA expression and placenta and pup weights were measured terminally.

Results: Maternal prediabetes and preeclampsia increased blood glucose and TGs concentration causing a decrease in NO concentration and an increase in MAP by comparison to the normal pregnant control group. Maternal prediabetes significantly decreased VEGF, PIGF mRNA expression with a slight increase in sFlt-1 mRNA expression and a concomitant increase in birthweights and increased placental weight in comparison the normal pregnant group.

Discussion: The results suggest that impaired glucose handling along with an imbalance in angiogenic and antiangiogenic factors in the PD pregnant group is associated with

Research Theme: Endocrinology

Ethics Number: AREC/025/019D

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George, E. M., & Granger, J. P. (2010). . (2010). Recent insights into the pathophysiology of preeclampsia. *Expert Review of Obstetrics & Gynecology*, 5, 557–566.