Investigating the role of placental microbiome in the pathogenesis of early- and late-onset pre-eclampsia

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Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Discipline of Human Physiology), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

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Co-Supervisor: Professor Jagidesa Moodley

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PREFACE

The experimental and research work described in this thesis was conducted by the candidate at the University of KwaZulu-Natal, Westville, Durban, South Africa, under the supervision of Professor Irene Mackraj and the co-supervision of Professor Jagidesa Moodley.

The contents of this work have not been submitted in any form for any degree to another tertiary institution, and where use has been made of the work of others, it is duly acknowledged in the text. The results reported are from investigations by the candidate.

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DECLARATION 1: PLAGIARISM

I, Kehinde Samuel Olaniyi, declare that:

1. The research reported in this thesis, except where otherwise indicated or acknowledged, is my original work.

2. This thesis has not been submitted in part or in full for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.

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a. Their words have been rewritten, but the general information attributed to them has been referenced.

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5. This thesis does not contain text, graphics, or tables copied and pasted from the internet, unless specifically acknowledged, and the source is detailed in the thesis and the references sections.

The PhD candidate performed the experimental work described in this thesis; others made contributions, which are duly acknowledged in the text. The candidate drafted this publication in full, and it has been reviewed by co-authors.

14th February, 2026

Kehinde Samuel Olaniyi Date

DECLARATION 2: MANUSCRIPTSManuscript 1

Olaniyi KS, Moodley J, Mahabeer Y, Mackraj I. Placental microbial colonization and its association with pre-eclampsia. *Frontiers in Cellular and Infection Microbiology,* 2020;10:413. Link to this article: https://doi.org/10.3389/fcimb.2020.00413. **(Chapter Two of this thesis).** Cited by 41.

Manuscript 2

Olaniyi KS, Mackraj I, Moodley J, Moodley R. Evaluation of the Human Placental Microbiota in Early-and Late-Onset Pre-Eclampsia. *High Blood Pressure & Cardiovascular Prevention,* 2024; 31:677-685. Link to this article: https://doi.org/10.1007/s40292-024-00679-5. **(Chapter Three of this thesis).** Cited by 1.

Manuscript 3

Olaniyi KS, Mackraj I, Moodley J, Moodley R. Assessment of human placental microbial signatures in pre-eclampsia using shotgun metagenomics. *Canadian Journal of Physiology and Pharmacology.* **(Chapter Four of this thesis)**. Accepted for publication.

Manuscript 4

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For the preparation of the above manuscripts, the candidate performed all the experimental work and interpreted the data. The co-authors contributed to editing and verifying the scientific content.

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14th February, 2026

Kehinde Samuel Olaniyi Date

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LIST OF ABBREVIATIONS

ADAM12	Disintegrin and metalloprotease 12
BMI	Body Mass Index
CD8+	Cytotoxic T Cells
CpG	cytosine-phosphate-guanine
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ENVTs	Endovascular Trophoblasts
EOPE	Early-Onset Pre-eclampsia
EVTs	Extravillous Trophoblasts
FISH	Fluorescence in situ hybridisation
HDAC2	Histone deacetylase-2
HELLP	Hemolysis Elevated Liver Enzymes, Low Platelets
HIF1α	Hypoxia-Inducible Factor 1-Alpha
HUVECs	Human Umbilical Vein Endothelial Cells
IL-6	Interleukin-6

ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	Intrauterine Growth Factor
IVF	In vitro fertilisation
LOPE	Late-Onset Pre-eclampsia
LPS	Lipopolysaccharide
miRNAs	MicroRNAs
MMP	Metalloproteinase
NGS	Next Generation Sequencing
NICE	National Institute for Health and Care Excellence
NICU	Neonatal Intensive Care Unit
NO	Nitric Oxide
OUT	Operational Taxonomic Unit
PAPP-A	Pregnancy Associated Plasma Protein A
PCR	Polymerase Chain Reaction
PE	Pre-eclampsia
PIGF	Placental Growth Factor
PIGF	Placental Growth Factor
PP13	Placental Protein 13
RAS	Renin-Angiotensin System

RCTs	Randomized Controlled Trials
sEng	Soluble Endoglin
sFlt-1	Soluble fms-like tyrosine kinase-1
SGA	Small for Gestational Age
SBP	Systolic Blood Pressure
SNP	Single Nucleotide Polymorphism
STBEVs	Syncytio-trophoblast Extracellular Vesicles
TGF-β	Transforming Growth Factor Beta
TIMPs	Tissue Inhibitor of Metalloproteinase
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor-Alpha
Tregs	T Regulatory Cells
uNK	Uterine Natural Killer
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation

THESIS OUTLINE

The principal findings of this PhD research study have been compiled into an article format and presented as a thesis by manuscript. The chapters present the following:

Chapter One: Provide background information with a brief review of selected topics relevant to the study. The study's aim and objectives, as well as hypotheses and potential benefits, are also highlighted.

Chapter Two: Forms part of the literature review and describes the placental microbial colonisation and its association with pre-eclampsia. This manuscript has been published in the journal of Frontiers in Cellular and Infection Microbiology, 2020;10:413. Link to this article: https://doi.org/10.3389/fcimb.2020.00413.

Chapter Three: Reports on the evaluation of the human placental microbiota in early- and late-onset pre-eclampsia. This manuscript has been published in the journal of High Blood Pressure & Cardiovascular Prevention 2024; 31:677-685. Link to this article: https://doi.org/10.1007/s40292-024-00679-5.

Chapter Four: Reports on the assessment of human placenta microbial signature in pre-eclampsia using shotgun metagenomics. This manuscript has been accepted for publication in the Canadian Journal of Physiology and Pharmacology.

Chapter Five: Reports on the preliminary evaluation of the serum level of HDAC-2, HLA-DRA and acetate in pre-eclampsia. This manuscript has been submitted for consideration in Comparative Clinical Pathology Journal.

Chapter Six: Provides general conclusions and synthesis of the research findings. It also highlights the limitations and makes recommendations for future studies.

ABSTRACT

Pre-eclampsia (PE) is a multifactorial pregnancy disorder characterised by new-onset hypertension and proteinuria, often accompanied by end-organ damage and foetal growth restriction. The removal of proteinuria from the diagnostic criteria has further complicated clinical differentiation. Despite extensive research, the precise pathophysiology of PE remains unclear, limiting effective diagnostic and therapeutic strategies. Recent advances suggest that the placental microbiome plays a crucial role in pregnancy outcomes, influencing immune modulation, angiogenesis, and systemic maternal endothelial function. This study explores the potential contributions of the placental microbiome in PE pathogenesis, focusing on microbial profiles, inflammatory mediators, immune response, and epigenetic influences. Thirty age-matched normotensive and early-onset as well as age-matched normotensive and late-onset pre-eclamptic women were recruited. After informed consent was obtained, blood samples were collected through venipuncture, while the placental tissues were obtained at the end of the pregnancy through cesarean section with sterile and standardised clinical procedures. DNA was extracted, and microbiome analysis was conducted using targeted 16S rRNA gene sequencing and shotgun metagenomic sequencing (NGS). The reads were analysed using bioinformatics. In addition, serum was obtained from blood samples, and RNA was extracted. ELISA and qPCR were used to determine histone deacetylase-2 (HDAC-2) and acetate levels, and human leucocyte antigen-DRA (HLA-DRA) levels, respectively. Findings from 16S analysis revealed low biomass, classified as *Actinobacteria, Firmicutes, Bacteroidetes,* and *Proteobacteria, with Proteobacteria* dominated by the classes *Pseudomonadales and Gammaproteobacteria*, and smaller amounts of *Actinobacteria* and *Bacteroidetes*. NGS revealed low biomass, classified as Cutibacterium acnes, Staphylococcus epidermidis, and Bradyrhizobium species. Further analysis found no significant difference in bacterial species between placental samples from women with early- or late-onset pre-eclamptic women and age-matched normotensive controls. Similarly, there was no significant difference in bacterial species between placental samples from EOPE and LOPE. Additionally, there were significant differences in HDAC-2 and acetate levels between the EOPE and NORM groups. Similarly, HLA-DRA levels were significantly higher in EOPE than in the NORM group. The study therefore demonstrates a low number of reads, which might further indicate that the placental samples had very low levels of bacteria, and there is no correlation between placental samples from normotensive individuals and those with early- or late-onset PE. The data also highlight epigenetic dysregulation**,** immune activation, and metabolic alterations associated with PE, particularly in EOPE cases. The findings provide preliminary evidence supporting the role of placental epigenetics, microbial metabolites, and immune dysregulation in PE pathophysiology. Further large-scale approaches are warranted to determine whether HDAC2, HLA-DRA, and acetate could serve as potential diagnostic biomarkers or therapeutic targets for PE management.

Keywords: Bacteria; Epigenetics; Inflammation; Placental Microbiome; Pre-eclampsia; Pregnancy.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background of the Study

Pre-eclampsia (PE) is a pregnancy-specific disorder that is usually characterised by new-onset hypertension and proteinuria. Diagnosis may occur without proteinuria, but with indications of thrombocytopenia and elevated liver enzymes. This multi-systemic syndrome affects 5-8% of pregnancies worldwide, accounting for more than 70,000 maternal and 500,000 fetal deaths annually [1]. The global prevalence of PE is increasing rapidly, and the increase is particularly pronounced in low- and middle-income countries [2]. For instance, a recent report from Africa revealed that 5-18% of pregnant women are affected, owing to low resource settings, poor access to quality prenatal care, poor lifestyle, and dietary habits [3]. Pulmonary embolism is the primary cause of maternal-perinatal morbidity and mortality globally [4]. Evidence exists that PE may be associated with pulmonary oedema and end-organ damage, including renal, liver, cerebral, and visual dysfunctions [1]. Additionally, PE frequently results in fetal growth restriction and predisposes both the foetus and mother to cardiovascular complications later in life [5].

Pre-eclampsia usually develops after 20 weeks of gestation in a previously normotensive individual, and its pathogenesis has been extensively linked to maternal/fetal and placental factors [1, 5], with the placenta recognised as the central causative agent. The pathogenic processes, therefore, include abnormal placentation and the maternal syndrome. The former is characterised by impaired trophoblast invasion and incomplete transformation of spiral arteries, resulting in uteroplacental ischemia, while the latter is characterised by inappropriate levels of pro-angiogenic (VEGF, PLGF, and TGF-β) and anti-angiogenic (sEng and sFlt-1) factors, as well as generalised maternal endothelial dysfunction [6]. Similarly, genetic and epigenetic factors have been reported to contribute to inadequate placentation, which may result in placental oxidative stress and the release of syncytiotrophoblast extracellular vesicles (STBEVs), which can trigger cellular apoptosis and necrosis and fetal growth restriction [6]. However, the precise cause of PE remains unclear despite decades of

research/medical advances. Consequently, diagnostic strategies and treatments, including reducing blood pressure or delivering the foetus and placenta, are suboptimal.

The human microbiome consists of over 100 trillion microbes that live in different parts of the body, including the gut, oral cavity, lungs, genitourinary tract, amniotic fluid, and the placenta [7]. It is an essential part of human life, carrying approximately 150 times as many genes as the entire human genome [8]. Importantly, advances have shown that the human microbiome is involved in basic human biological processes, including modulating the metabolic phenotype, regulating epithelial development, and influencing innate immunity [8].

Chronic diseases such as immune-related diseases (inflammatory bowel disease, hepatocellular carcinoma, etc.), and cardiometabolic-linked diseases (obesity, diabetes mellitus, and atherosclerosis) have been associated with dysbiosis of the human microbiome [8], establishing its crucial role in human health. Recently, evidence suggests the existence of the placental microbiome [9]. Nevertheless, earlier cultivation-dependent studies significantly underestimated its presence, likely because of bacteria that are difficult to culture, as they prefer anaerobic environments or require specific unidentified nutrients [10]. However, more recent DNA-based studies provide evidence of an endogenous microbial community in the placenta [9, 10].

The placental microbiome encompasses a number of microbes, including *Lactobacillus sp.*, *Clostridium sp.*, *Proteobacterium sp.*, *Propionibacterium sp.*, *Enterobacteriaceae sp.*, *Collinsella sp.*, *Enterobacter*, *Gardnerella sp.*, *Bacillus cereus, Listeria, Salmonella, Escherichia sp., Klebsiella pneumoniae, and Anoxybacillus*, among others [9]. Many of these microbes have previously been linked to periodontitis and chorioamnionitis [8], indicating that the placenta is not a sterile organ and that it has an endogenous microbiome, raising questions about the potential role of intrauterine microbes in placental function and fetal development/growth. Recent studies have indicated that placental tissue from healthy-term deliveries contains higher levels of *Lactobacillus spp.*, *Propionibacterium spp.*, and *Enterobacteriaceae spp.* compared to those from preterm deliveries. This finding may support the role of the placental microbiome in normal pregnancy [9, 10].

The placental microbiome is important in immune function, fetal growth, and development [6, 11]. Earlier studies in pregnant women have shown that the gut microbiota significantly contributes to metabolic changes in the mother, and interactions between the human host and her microbes, such as the intraamniotic immune response, also affect pregnancy outcomes [11]. The placental microbiome drives early immune development and emerges as a source of antigenic determinants in neonates or foetuses [11]. However, its contributions to adverse pregnancy outcomes, particularly pre-eclampsia, have not been documented. Nonetheless, the production of pro-inflammatory mediators is a significant factor underlying pregnancy complications, including pre-eclampsia [6]. As a result, understanding the role of the placental microbiome in the pathophysiology of preeclampsia could lead to the development of novel diagnostic tools and therapeutic targets for the prevention and treatment of pre-eclampsia.

1.2 Definition and Classification of Pre-eclampsia

The complex illness known as PE affects between 5–8% of pregnancies. The main indicator of this condition is the development of hypertension after the 20^{th} week of pregnancy, and typically accompanied by disorders of the kidneys, liver, blood, brain, and placenta, among other organs [1]. From moderate to severe, PE can present with a wide range of clinical symptoms, with severe instances potentially posing a threat to the mother's and the foetus's lives [2]. Women who have had PE are more likely to have long-term health issues, including end-stage renal disease, metabolic syndrome, stroke, cardiovascular disease, chronic hypertension, and cognitive impairment. Pre-eclampsia also presents serious dangers to the foetus and the baby [5]. Children whose mothers have PE are more likely to face short-term and long-term health concerns [1, 5]. Short-term consequences from intrauterine growth restriction and premature delivery may affect these babies. Over time, they are more vulnerable to diabetes, hypertension, coronary heart disease, and neurodevelopmental abnormalities [5].

Despite significant research on the pathophysiology of PE, including its relationship with placental malfunction, angiogenic imbalance, and maternal endothelial injury, the possible role of the placental microbiota in the pathogenesis or progression of PE remains largely unknown. Although recent data reveal

that the placenta maintains a unique microbiota that may alter immunological regulation, placental function, and embryonic development [9, 10, 11], the debate whether these findings represent true colonisation or 'contamination' due to sampling or inflammatory conditions still remains. In addition, the molecular linkages between placental microbial dysbiosis and the onset of PE remain poorly understood, highlighting a crucial gap in current understanding. Elucidating this link might reveal unique insights into PE pathogenesis and open up new pathways for early diagnosis, prevention, and therapeutic intervention.

1.2.1 Epidemiology of Pre-eclampsia

Pre-eclampsia is a major hypertensive disorder of pregnancy with significant implications for both maternal and fetal health, contributing substantially to global maternal and perinatal morbidity and mortality [13]. Epidemiological data indicate notable geographic variations in prevalence and outcomes. A study on pregnant women in Nigeria estimated a pooled PE prevalence of 4.51%, with an associated maternal mortality rate of 6.04% and a fetal mortality rate of 16.73% [14]. In contrast, in high-income countries, hypertensive disorders, including PE, account for approximately 9%of maternal deaths, while the rate in Africa and Asia is 16%, reaching as high as 26% in Latin America and the Caribbean [15]. Notably, where maternal mortality is high, most deaths are attributable to eclampsia rather than the other types of PE, suggesting gaps in timely intervention and management [16].

Epidemiological trends over time reveal a complex and evolving pattern in the incidence of PE. In the United States, the incidence of PE during childbirth and delivery increased by approximately 25%, with a continued upward trajectory likely attributable to rising maternal age, obesity, and the growing prevalence of metabolic disorders. In contrast, the incidence of eclampsia has declined globally, a trend that may reflect advancements in antenatal care, improved surveillance, and earlier clinical interventions [16]. Despite advances in obstetric care, severe maternal complications remain a critical concern [17]. Pre-eclampsia is associated with a spectrum of life-threatening conditions, including acute kidney injury (6.37%), cerebrovascular accidents (3%), aspiration pneumonia (5.26%), and puerperal sepsis (3.98%) [13]. Additionally, cardiovascular dysfunction, stroke, respiratory failure, coagulopathy, and liver failure further contribute to maternal morbidity and the

need for admission into intensive care. In a study of hospitals managed by Health Care America Corporation, PE was identified as the second leading cause of pregnancy-related ICU admissions after obstetric haemorrhage, underscoring its clinical severity [18].

The impact of PE extends beyond maternal health to fetal and neonatal outcomes. Globally, PE is implicated in 12–25% of cases of fetal growth restriction and small-for-gestational-age infants, as well as 15–20% of all preterm births [19]. Prematurity-related complications, including neonatal death and long-term morbidity such as respiratory distress syndrome, asphyxia, and neurodevelopmental impairments, are substantial. The burden is particularly pronounced in low-income countries, where a quarter of all stillbirths and neonatal deaths are linked to PE/eclampsia [20]. Infant mortality rates due to PE are three times higher in low-resource settings than in high-income nations, largely due to the lack of neonatal intensive care facilities. The recurrence of PE in subsequent pregnancies is another important epidemiological consideration, with studies reporting recurrence rates between 7%–20%, depending on gestational age at its onset and prior history of the disorder [20]. A study in Iceland that applied strict diagnostic criteria found a 13% recurrence rate for PE or superimposed PE in second pregnancies, emphasizing the importance of early risk assessment and preventive strategies [21].

Sociodemographic factors play a crucial role in the risk of PE. Younger maternal age (<24 years), low socioeconomic status, and limited educational attainment are associated with a higher prevalence of PE, as seen in a study in which 44.4% of PE patients belonged to lower socioeconomic classes and 81.1% had low educational attainment [22]. Family history is another strong risk factor, with 36.67% of PE patients having a family history of the condition. Additionally, comorbid conditions such as diabetes (15.5%) and chronic hypertension (5.55%) significantly increase the risk of PE, indicating the need for the targeted screening and management of high-risk populations [22].

1.2.2 Evidence for Pre-eclampsia Subtypes

Various data sources suggest that PE may not be a single condition. Distinct clinical presentations highlight this complexity, with some women experiencing a slow-progressing disorder that changes minimally during

pregnancy, while others have sudden and severe manifestations [23]. The syndrome includes new-onset gestational hypertension and proteinuria, yet only about one-third of newborns from mothers with PE show fetal growth restriction, despite expectations of decreased placental perfusion. Additionally, PE shows varying primary organ involvement among women, particularly in variants such as HELLP (Hemolysis, Elevated Liver Enzymes, Low Platelets), which primarily affects the liver. The analytical findings associated with PE vary significantly across clinical and research settings and involve both angiogenic and antiangiogenic factors, as well as oxidative stress markers [24]. The long-term maternal health effects of PE also differ, suggesting that various subtypes of PE may lead to distinct risks for chronic diseases postpartum. For instance, PE is associated with an increased cardiovascular risk for mothers, which can double with term PE without small for gestational age (SGA) babies, more than triple with term PE with SGA, and increase by over five times with preterm PE [23, 24]. Notably, the risk can increase up to 10-fold if PE occurs before 34 weeks of gestation, indicating that PE should not be viewed as a uniform condition.

1.2.3 Clinical Impediments to the Recognition of Subtypes

The notion that PE is a single disease with a common pathogenesis persists despite its marked clinical diversity. This perspective is partly influenced by Occam's Razor, which suggests that the simplest explanation is often the most likely [24]. While this reasoning may be appropriate in some contexts, it is insufficient for understanding complex conditions such as PE, particularly in the era of personalised medicine. Importantly, the identification of gestational hypertension and proteinuria as diagnostic features of pre-eclampsia was not based on their pathophysiological relevance. Rather, these were coincidental clinical observations made in the late nineteenth century that preceded the onset of eclamptic seizures. This historical approach encouraged a reduction mindset that sought to unify all clinical presentations into a single disease category, often ignoring clear exceptions. Traditional methods of data presentation have further reinforced this simplification by obscuring variability. Weissgerber and colleagues [25] have demonstrated how presenting continuous data as bar charts with standard deviation or, even more so, standard error effectively conceals individual variation [25]. As a result, there is increasing advocacy for data visualisation approaches that highlight, rather than

mask, variability, which is especially important in a heterogeneous disorder such as pre-eclampsia. This criticism also applies to other data presentation formats that omit indicators of variability.

1.2.4 Current Subtypes of Pre-eclampsia

Several PE subtypes have been identified. There are data illustrating the difficulties in differentiating subtypes and taking into account these hypothesized categories as early targets for investigating the concept of PE variations [23, 24]

1.2.4.1 Early- and Late-onset Pre-eclampsia and Severe and Mild Pre-eclampsia

In clinical practice, PE has traditionally been classified as mild or severe based on blood pressure, clinical findings, and proteinuria [5, 6, 25]. It is also categorised as early-onset, defined as occurring before 34 weeks of gestation, or late-onset, defined as occurring at or after 34 weeks (Figure 1). Although these terms suggest differences in the timing of onset, they more accurately reflect the timing of delivery, as the actual onset is often unknown. Early-onset PE (EOPE) is typically distinct from late-onset PE (LOPE), but severity classification remains largely subjective and can be difficult to reliably distinguish between severe and less severe presentations of the disease. Notably, EOPE is associated with fetal growth restriction and histological evidence of placental malperfusion [26]. These features are generally absent in late-onset cases, which also have a lower predictive value for future maternal cardiovascular disease. Furthermore, current biomarker-based predictors, such as those used in clinical screening, show higher sensitivity for EOPE than the LOPE form [27]. Consequently, there is growing consensus that early-and late-onset PE represent distinct clinical subtypes (Table 1) and (Table 2).

The various known risk factors for PE, including obesity, chronic hypertension, pre-existing diabetes, multifetal gestation, and a history of pre-eclampsia, are also believed to operate through different genetic, metabolic, and molecular pathways. Despite this, conventional laboratory biomarkers such as placental growth factor (PlGF) are unable to distinguish between early- and late-onset forms or between individuals with differing risk profiles. Other biomarkers, including disintegrin and metalloprotease 12 (ADAM12), placental protein 13 (PP13), soluble fms-like tyrosine kinase 1 (sFlt-1), and pappalysin A (PAPP-A), also face similar

limitations. This suggests that the multiple pathogenic pathways leading to pre-eclampsia may converge, making clinical differentiation challenging [27].

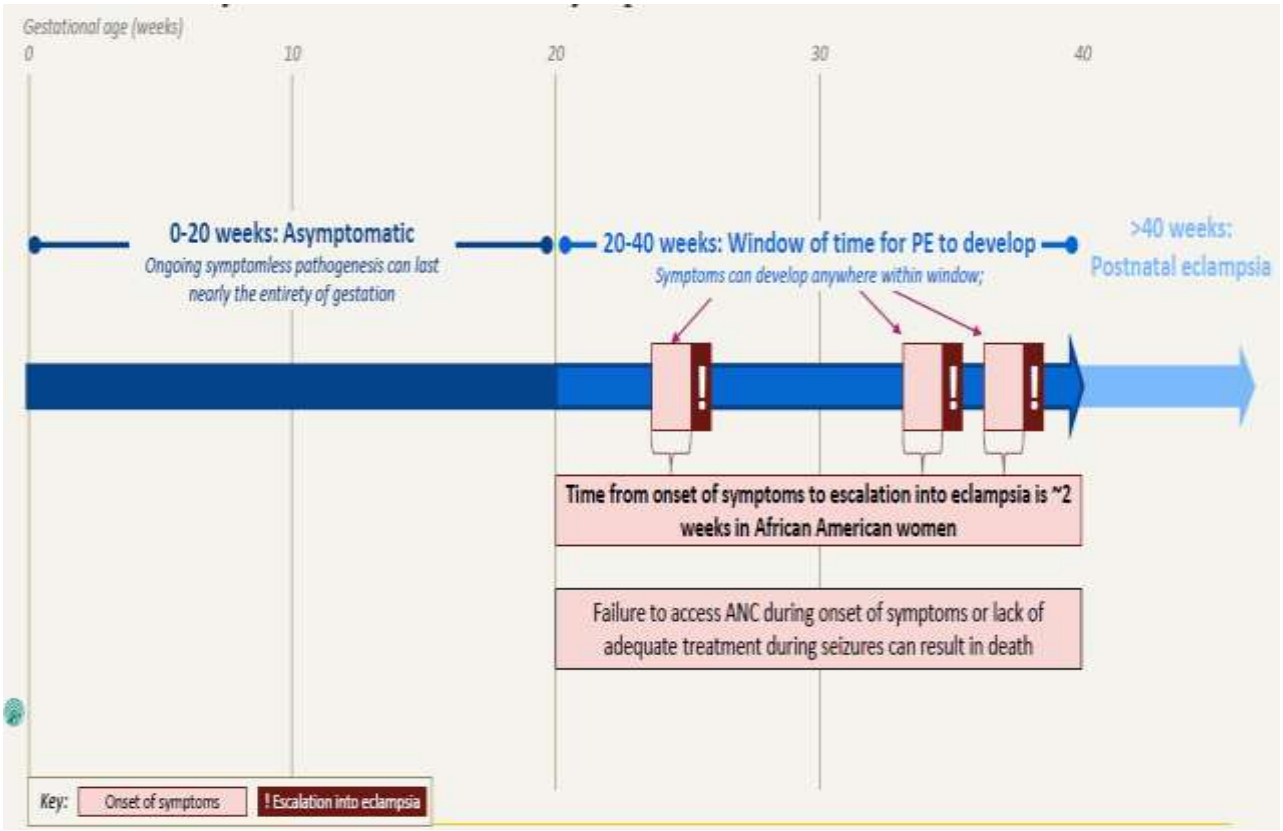

Figure 1: Onset of Pre-eclampsia. The image illustrates the timeline of PE development across gestation. It shows an asymptomatic phase from 0–20 weeks, followed by a 20–40-week window when PE symptoms can appear, and possible progression to postnatal eclampsia after 40 weeks. It notes that symptom escalation to eclampsia occurs within about two weeks in African American women, and emphasises that delayed antenatal care or inadequate treatment during seizures can be fatal [25].

Table 1: Common Features of Early-Onset and Late-Onset Pre-eclampsia

Feature	Early-Onset Pre-eclampsia	Late-Onset Pre-eclampsia
Gestational age of onset	≤34 weeks (before 34 weeks)	>34 weeks (often ≥37 weeks)
Primary cause	Poor placentation, placental dysfunction, ischemia, and incomplete spiral artery remodelling	Maternal vascular/endothelial dysfunction; placental malperfusion due to ageing placenta
Underlying pathophysiology	Extrinsic placental dysfunction with oxidative stress, inflammation, and systemic endothelial injury	Intrinsic placental ageing with maternal metabolic/vascular dysfunction
Angiogenic factors/biomarkers	Marked imbalance: ↓PlGF, ↑sFlt-1; biomarkers useful for diagnosis	Mild imbalance; angiogenic biomarkers are less predictive
Placental lesions	Severe trophoblast apoptosis, infarctions, and placental pathology	Milder and less consistent placental lesions
Clinical severity	More severe; higher risk of complications (HELLP, eclampsia)	Generally milder but still clinically significant
Fetal impact	Severe fetal growth restriction (IUGR), prematurity, high neonatal risk	Minimal or less consistent growth restriction
Maternal risk factors	More immune-related, high anti-angiogenic factors	More metabolic and inflammatory associations
Short-term maternal outcomes	Higher risk of stroke, pulmonary oedema, renal failure, HELLP syndrome	Lower immediate maternal risk, but requires monitoring
Long-term maternal outcomes	Greater long-term risk of CVD, chronic hypertension, metabolic syndrome, and renal disease	Increased risk of hypertension/CVD, but lower than early-onset
Response to management	Often requires early delivery; may need NICU support	Usually managed expectantly until term delivery
Recurrence risk	Higher recurrence risk in future pregnancies	Lower recurrence risk
Overall characterization	Placental-driven disease with severe maternal-fetal consequences	More maternal/placental ageing-related and typically milder

1.3 Risk Factors for Pre-eclampsia

The 2019 National Institute for Health and Care Excellence (NICE) guidelines identify women as being at high risk of developing PE if they have maternal comorbidities such as diabetes, autoimmune disorders, chronic kidney disease, or chronic hypertension [30]. Brown et al. further classify women as being at intermediate risk if they are nulliparous, older than forty years, have a body mass index above 35 kg/m², possess a family history of PE, are carrying multiple foetuses, or have had an interpregnancy interval exceeding ten years [30]. These classifications are supported by the meta-analysis conducted by Bartsch et al., which evaluated over 25 million pregnancies across 92 studies, making it the most extensive study of clinical risk factors to date [31].

Based on these findings, aspirin prophylaxis is recommended when one high-risk factor or two or more intermediate-risk factors are present, particularly when initiated before 16 weeks of gestation. Additional clinical risk factors for PE include sleep disordered breathing, polycystic ovarian syndrome, elevated mean arterial pressure before 15 weeks of gestation, and infections [32]. Certain obstetric histories, including prolonged vaginal bleeding and conception through oocyte donation, also significantly increase the risk of PE [33].

1.4 Pathogenesis of Pre-eclampsia

1.4.1 Pathogenesis of Early-Onset Pre-eclampsia

The pathogenesis of EOPE is primarily associated with placental dysfunction, poor placental perfusion, and significant alterations in the maternal immune response. Key pathogenic factors include the following:

1.4.1.1 Defective Trophoblast Invasion and Spiral Artery Remodelling

Early-onset PE is linked to inadequate trophoblast invasion, leading to shallow remodelling of the maternal spiral arteries. This results in poor placental perfusion, ischemia, and oxidative stress. The failure of spiral artery remodelling leads to high-resistance, low-flow circulation, restricting oxygen and nutrient delivery to the foetus [34].

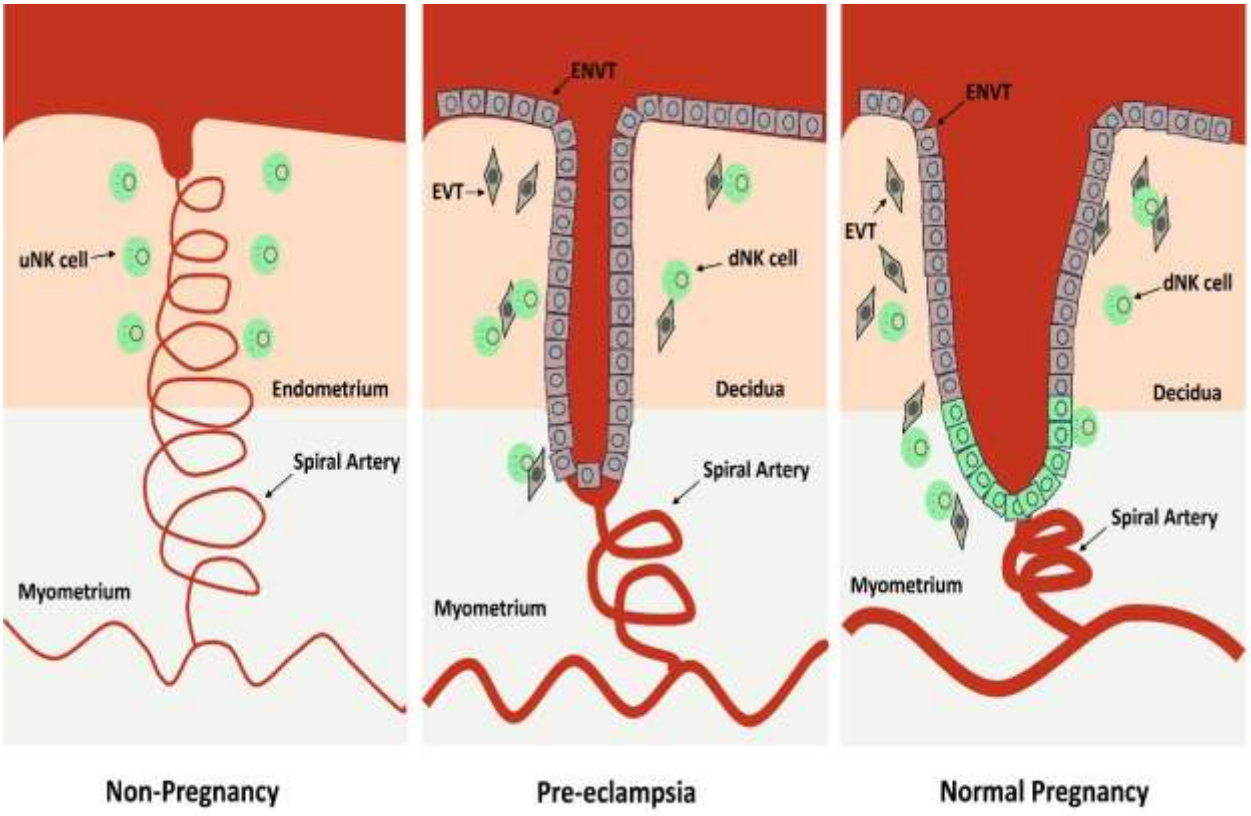

Figure 2: Schematic illustration depicting the differential remodelling of uterine spiral arteries under three physiological states: non-pregnancy, normal pregnancy, and pre-eclampsia. In the non-pregnant state, spiral arteries remain narrow and coiled, embedded within the endometrium and myometrium, with uterine natural killer (uNK) cells present. In normal pregnancy, successful trophoblast invasion, mediated by extravillous trophoblasts (EVTs) and endovascular trophoblasts (ENVTs), leads to extensive spiral artery remodeling. Pre-eclampsia is characterised by impaired trophoblast invasion and inadequate arterial remodelling, resulting in retained narrow, high-resistance vessels that compromise placental perfusion [34].

1.4.1.2 Immune Dysregulation

Early-onset PE also involves defective immunoregulation at the maternal-fetal interface. Studies show a significant reduction in decidual T regulatory cells (Tregs), which normally suppress maternal immune responses against fetal antigens. A lower proportion of Tregs correlates with increased cytotoxic T cells (CD8+), leading to placental inflammation and trophoblast damage [35].

1.4.1.3 Imbalance in Angiogenic and Anti-Angiogenic Factors

Pre-eclampsia is characterised by increased levels of soluble fms-like tyrosine kinase-1 (sFlt-1), which antagonises vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), leading to endothelial dysfunction. Elevated sFlt-1 and decreased PlGF contribute to systemic vasoconstriction, hypertension, and placental ischemia [36].

1.4.1.4 Placental Oxidative Stress and Inflammation

Hypoxia-induced oxidative stress increases trophoblast apoptosis and syncytiotrophoblast shedding, exacerbating systemic inflammation. Elevated levels of tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are observed in EOPE, contributing to vascular dysfunction [37].

1.4.2 Pathogenesis of Late-Onset Pre-eclampsia

Late-onset PE differs significantly from EOPE, as it is more closely associated with maternal vascular dysfunction than with primary placental pathology. Key factors include the following:

1.4.2.1 Systemic Endothelial Dysfunction

Unlike EOPE, late-onset PE is not primarily caused by placental ischemia but rather by maternal factors such as obesity, diabetes, and preexisting cardiovascular disease. The maternal vascular system fails to adapt to pregnancy-induced volume expansion, leading to hypertension [38].

1.4.2.2 Imbalance in Angiogenic Factors

This form of PE (LOPE) is associated with lower levels of PIGF and sVEGFR-2 (vascular endothelial growth factor receptor-2) but higher levels of soluble endoglin (sEng) and sVEGFR-1, contributing to an anti-angiogenic state. Unlike EOPE, hypoxia does not play a major role, and placental lesions are less severe [37].

1.4.2.3 Placental Lesions and Under-perfusion

While placental lesions are more common in EOPE, late-onset PE patients with placental underperfusion exhibit similar angiogenic imbalances. The placental pathology in LOPE is less severe and occurs later in gestation, often with minimal fetal growth restriction [39].

1.4.2.4 Maternal Inflammation and Oxidative Stress

Late-onset PE is linked to maternal systemic inflammation, which may contribute to endothelial dysfunction without primary placental involvement. Elevated inflammatory markers (e.g., TNF-α, IL-6) may contribute to the exacerbation of vascular dysfunction (Figure 3) [39, 40].

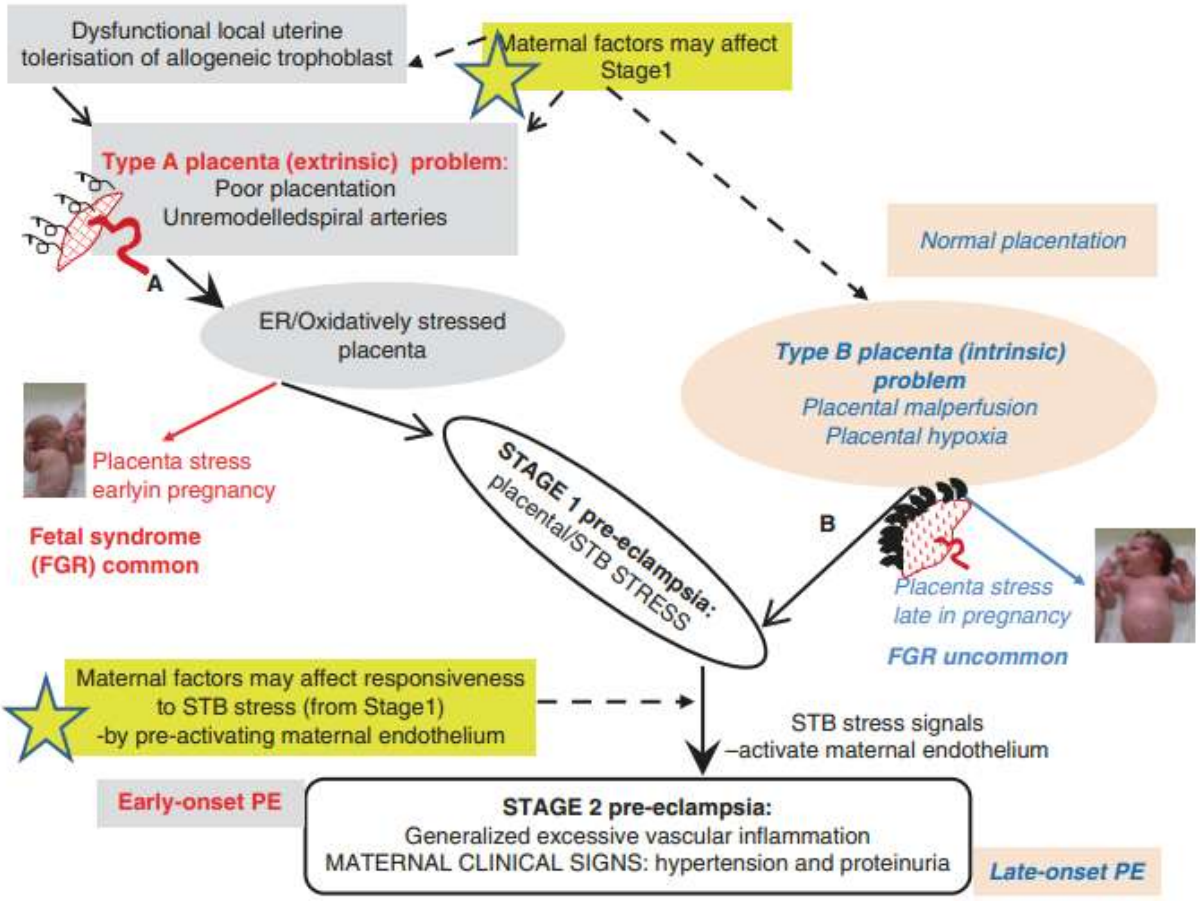

Figure 3: Two-stage model of PE pathogenesis illustrating distinct placental and maternal contributions in early- and late-onset PE. This schematic diagram depicts the dual-stage mechanism underlying PE, differentiating between Type A (extrinsic) and Type B (intrinsic) placental pathologies and their relationship with maternal and fetal outcomes [40].

1.5 Genetic and Epigenetic Link to Pre-eclampsia

The genetic and epigenetic factors underlying PE play a critical role in its pathogenesis, providing insight into the complex mechanisms that lead to placental dysfunction and systemic maternal effects [41]. Pre-eclampsia is a multifactorial disorder, and epigenetic processes such as DNA methylation, histone modifications, and non-coding RNA regulation are pivotal in modulating gene expression during placental development and in the manifestation of PE.

1.5.1 DNA Methylation

DNA methylation is a well-characterized epigenetic mechanism that adds methyl groups to cytosine residues in cytosine-phosphate-guanine (CpG) islands, typically silencing gene expression [42]. In PE, aberrant DNA methylation affects genes essential for placental development, particularly those involved in trophoblast invasion and vascular remodelling [43]. For instance, hypermethylation of the Wnt2 promoter is associated with reduced expression of genes critical for spiral artery remodelling, leading to shallow trophoblast invasion, a hallmark of PE [44]. Similarly, altered methylation of metalloproteinase (MMP) genes, including MMP9, suppresses extracellular matrix remodelling, which is necessary for proper spiral artery transformation [45]. Concurrently, hypomethylation of tissue inhibitors of metalloproteinases (TIMPs), such as TIMP3, disrupts angiogenesis by blocking vascular endothelial growth factor (VEGF) signalling, further impairing placental vascularization [46]. Collectively, these changes result in poor placental perfusion, contributing to ischemia and hypoxia [47].

The role of DNA methylation extends beyond immediate placental dysfunction. Oxidative stress, a characteristic feature of PE, influences methylation patterns, accelerating placental ageing and altering gene expression [48]. Studies have identified differential methylation at CpG sites in genes regulating inflammation, apoptosis, and oxidative stress, underscoring the interaction between environmental stressors and epigenetic regulation in PE [49]. Moreover, methylation changes in imprinted genes, such as IGF-1 and PHLDA2, influence placental and fetal growth, linking epigenetic dysregulation to intrauterine growth restriction (IUGR) and long-term health risks in offspring [50].

1.5.2 Histone Modifications

Histone modifications are another critical epigenetic process contributing to PE. Post-translational modifications, such as acetylation, methylation, and phosphorylation of histones, regulate chromatin structure and gene accessibility [51]. In PE, hypoxia, which is a central feature of the condition, alters histone modifications through pathways involving hypoxia-inducible factor 1-alpha (HIF1α) [52]. For example, reduced acetylation of histone H3 is associated with decreased expression of genes involved in trophoblast differentiation and vascular remodelling [52]. Similarly, increased methylation of repressive histone marks (e.g., H3K9me3) contributes to the transcriptional silencing of angiogenic and trophoblast-invasion-related genes, thereby exacerbating placental insufficiency [51].

1.5.3 MicroRNAs (miRNAs)

MiRNAs are small non-coding RNAs that modulate gene expression post-transcriptionally by degrading mRNA or inhibiting its translation [52, 53]. In PE, dysregulation of miRNAs plays a significant role in trophoblast invasion, angiogenesis, and oxidative stress responses [54]. Upregulation of miRNAs such as miR-155 and miR-29b has been linked to the inhibition of critical pathways. MiR-155 suppresses endothelial nitric oxide synthase (eNOS) activity, reducing nitric oxide (NO) production, a key regulator of vascular tone and blood flow [55]. MiR-29b inhibits VEGF expression and MMP activity, impairing angiogenesis and trophoblast invasion. Conversely, the downregulation of miR-126, a pro-angiogenic miRNA, disrupts VEGF signalling pathways and vascular cell adhesion, further contributing to inadequate placental perfusion [56].

Other miRNAs, such as miR-20a, miR-20b, and miR-17, are overexpressed in PE and target genes critical for angiogenesis and trophoblast function, including HIF1α, VEGF, and Ephrin-B2. These alterations lead to reduced angiogenic capacity, contributing to placental ischemia and fetal growth restriction. MiRNAs also regulate the renin-angiotensin system (RAS) and inflammatory responses in PE. For instance, miR-181a enhances the expression of inflammatory cytokines such as IL-6, which are linked to increased levels of soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sENG), both of which are anti-angiogenic factors implicated in PE pathogenesis [56].

1.6 Complications Associated with Pre-eclampsia

Accumulating evidence exists that exposure to hypertensive disorders of pregnancy has long-term cardiovascular consequences in both the mother and the child, independent of any concurrent pregnancy issues [57]. According to a meta-analysis using data from more than 45,000 people, children and young adults born to pre-eclamptic pregnancies had higher systolic and diastolic blood pressures by 2.39 and 1.35 mmHg, respectively. This disparity would be linked to a 12% higher risk of stroke and an 8% higher risk of death from ischaemic heart disease if it continued into adulthood [58].

A 20-year prospective follow-up birth cohort study of 2868 young people supports these findings by showing that exposure to hypertensive diseases of pregnancy in utero increases the clinical incidence of hypertension. According to this study, approximately 30% of 20-year-olds with hypertension were born to mothers who experienced hypertensive disorders during pregnancy. These individuals exhibited a 2.5-fold higher likelihood of having global lifetime cardiovascular risk (QRISK) scores above the 75^{th} percentile compared to those born from normotensive pregnancies [58]. Evidence suggests that these elevated blood pressure levels persist into later life; a study reported that individuals born following pre-eclamptic pregnancies were more likely to require antihypertensive medication by age 50, while those born after severe PE had a 1.5-fold increased risk of developing hypertension in adulthood [59].

The development of later-life hypertension in the offspring also seems to be significantly influenced by the date of PE onset. Blood pressure differences were observed only in the offspring of early-onset pre-eclamptic pregnancies at ages 6 and 13 in one study that compared offspring born to normotensive pregnancies, early-onset (<34 weeks' gestation), and late-onset (≥34 weeks' gestation) pre-eclamptic pregnancies [57]. The peripheral and central systolic blood pressures of children delivered to women with EOPE increased by 6 mmHg, which was significantly higher than in trials that did not distinguish between early- and late-onset PE [58]. It might be challenging to distinguish between the effects of intrauterine growth restriction (IUGR) and preterm births on the development of later hypertension since both conditions frequently cause early-onset PE. However, it has been demonstrated that there are particular vascular changes in people born preterm to

pre-eclamptic pregnancies. Furthermore, even if the mother was not hypertensive throughout the pregnancy, siblings of children born from pre-eclamptic pregnancies are at a greater risk of developing hypertension later in life, according to recent research including 15,000 young adults [60]. In order to explain variations in the cardiovascular phenotypes of the offspring, it may be necessary to investigate genetic and epigenetic factors in addition to maternal cardiovascular remodelling. This implies that the conventional explanation of in utero stress and developmental reprogramming may not be adequate to explain the risk of hypertension in children born to a mother with PE [61]. Evidence also suggests that the increased risk of hypertension among individuals born to pre-eclamptic pregnancies may be mediated by their distinct vascular profile [58]. Other studies have demonstrated structural and functional vascular alterations in these offspring, including arterial wall thickening and impaired endothelial-dependent vasodilation [62, 63].

Although the exact mechanisms remain unclear, derangements in maternal angiogenic factors, inherited polymorphisms, epigenetic factors, and altered microRNA expression are possible mediators of the abnormalities in vascular development in the offspring of pre-eclamptic pregnancies [58]. Yu et al. [64] investigated maternal angiogenic profiles, neonatal endothelial cell function, and postnatal vascular development in hypertensive and normotensive pregnancies. They found that human umbilical vein endothelial cells (HUVECs) from offspring of hypertensive pregnancies exhibited reduced vasculogenic capacity at birth, corresponding with in vivo findings of approximately twofold lower microvascular density at three months of age compared to controls [58]. Furthermore, the extent of impaired microvascular development correlated with maternal peripartum levels of soluble fms-like tyrosine kinase-1 (sFlt-1), suggesting that elevated maternal antiangiogenic factors may disrupt fetal vascular development in hypertensive pregnancies [64]. Additionally, alterations in neonatal microRNA profiles contribute to endothelial dysfunction in offspring of hypertensive pregnancies [64]. In particular, miR-146a, which regulates endothelial gene expression, was significantly elevated in HUVECs from these offspring compared with those from normotensive pregnancies. Increased miR-146a expression was associated with reduced endothelial proliferation and impaired vascular tube formation in vitro, effects that were reversed by miR-146a inhibition. Conversely, overexpression of miR-146a in HUVECs from normotensive pregnancies

similarly suppressed vascular tube formation, indicating a mechanistic role of this microRNA in mediating vascular dysfunction following hypertensive pregnancy.

Along with these vascular alterations, a recent study found that teenagers exposed to pregnancy-related hypertension illnesses had altered heart anatomy, with signs of unfavourable cardiac remodelling [65]. Compared to controls, those exposed to maternal hypertension had thicker left ventricles, and those exposed to PE also had smaller left ventricular end-diastolic volumes. In a similar vein, Lewandowski et al.'s study of young adults born before their due dates revealed that preterm children of hypertensive pregnancies had a further decrease in left ventricular global peak systolic longitudinal strain than preterm children born to normotensive pregnancies [66]. It will be of interest to track these changes earlier in life and examine whether they affect the risk of cardiovascular disease in these groups in the future. Prematurity, but not hypertensive pregnancies in general, seems to be linked to the highest cardiac remodelling alterations in the early postnatal period and abnormalities in the offspring's autonomic function of the heart, another predictor of cardiac sequelae [67].

1.7 Diagnosis of Pre-eclampsia

Pre-eclampsia is defined globally as new-onset gestational hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg) accompanied by at least one of the following: proteinuria, maternal organ dysfunction (liver, neurological, haematological, or renal involvement), or uteroplacental dysfunction at or after 20 weeks of gestation [30]. It is noteworthy that PE may manifest for the first time during or after delivery; superimposed PE can also be diagnosed in women with chronic hypertension who develop new-onset proteinuria, maternal organ, or uteroplacental dysfunction consistent with PE [30]. Eclampsia happens when there are convulsions in the context of PE.

1.7.1 Blood Pressure Measurements

A suitably sized cuff and validated equipment for use in women with PE should be used to measure blood pressure at least twice, four hours apart, to confirm the presence of hypertension [68]. Although there is no set

frequency, recommendations suggest that high-risk women have their blood pressure checked more frequently in prenatal clinics. Recent research has examined how women might better detect hypertension during pregnancy, especially in high-risk women, by self-monitoring their blood pressure levels at home. Self-monitoring, which seems possible and acceptable to expectant mothers, may reduce the number of clinic visits and is useful for identifying pregnancy-related hypertension and differentiating between white coat hypertension [30, 68, 69].

1.7.2 Proteinuria

Traditionally, dipstick testing has been used to screen for proteinuria. Additional laboratory tests utilising 24-hour urine or, more recently, spot urine samples have been used to confirm the presence of proteinuria. An automated reagent-strip reading system is preferable to visual analysis for screening evaluations using dipstick testing [70]. In the past, 24-hour urine collection was considered the gold standard for confirming proteinuria. However, it has several drawbacks, including being time-consuming, requiring freezing, producing partial samples, and being rarely used in hospitals. Consequently, it is currently advised to measure proteinuria using either spot urine albumin to creatinine (A: Cr) or protein to creatinine (P: Cr) ratios following a positive dipstick test (one protein or more) [70]. According to Waugh et al., there is a substantial correlation between proteinuria measured by 24-hour urine collection and both the P: Cr and A: Cr tests. High sensitivity and specificity have been achieved with diagnostic thresholds of 30 mg/mmol and 8 mg/mmol, respectively [71].

1.7.3 Laboratory and Imaging Tests

To identify maternal organ dysfunction and diagnose PE, the International Society for the Study of Hypertension in Pregnancy (ISSHP) advises that pregnant women with de novo hypertension undergo laboratory testing to measure haemoglobin, platelet count, serum creatinine, liver enzymes, and serum uric acid [30]. Placental growth factor or sFlt-1:PlGF ratio testing has also been introduced in new guidelines for the diagnosis of PE under certain conditions [30]. Numerous studies suggest that circulating angiogenic factors, including PlGF and sFlt-1, play a part in the pathophysiology of PE. Before the onset of the condition, women with PE have lower PlGF levels and higher circulating sFlt-1 levels [5]. The angiogenic proteins PlGF

and VEGF are antagonistic to the anti-angiogenic protein SFlt-1. Vasoconstriction and endothelial dysfunction are caused by sFlt-1's modification of downstream signalling pathways through the inhibition of VEGF and PlGF. In animal models, elevated sFlt-1 levels have been demonstrated to cause a pre-eclamptic-like condition [5]. Additionally, in endothelial cell culture experiments, sFlt-1 removal can restore endothelial dysfunction; thus, overexpression appears to be a significant mechanistic link between placental dysfunction and altered maternal vascular function [5]. Research has demonstrated that low PlGF has a negative predictive value and high sensitivity in identifying pre-eclampsia that requires delivery within 14 days [72]. According to a major stepped-wedge cluster-randomised controlled study conducted in the UK, individuals who underwent PlGF testing were diagnosed with PE considerably more quickly, experienced a substantial reduction in adverse maternal events, and showed no change in unfavourable newborn outcomes [73]. A sFlt-1:PlGF ratio of less than 38 can rule out PE during the next seven days, according to another large clinical study [74]. Based on this research, NICE recommends using PlGF or the sFlt-1:PlGF ratio to assist in ruling out PE in women who are suspected of having it, are between the ages of 20 and 34±6 weeks of gestation [74].

1.8 Treatment and Management of Pre-eclampsia

There are few interventions available for the treatment and avoidance of PE fetal problems. Management options to optimise the foetus's condition, such as antenatal corticosteroids and magnesium sulphate infusions, are primarily intended to prevent adverse outcomes associated with prematurity, as pre-eclampsia accounts for approximately 20% to 30% of all preterm births [75]. Optimising delivery timing is a critical strategy for minimising adverse fetal outcomes in PE. However, determining the appropriate time for birth requires a careful balance between safeguarding maternal health and ensuring adequate fetal maturity. Maternal stabilisation through antihypertensive therapy and, when indicated, magnesium sulphate remains an essential component of management that also benefits fetal well-being [75]. In cases where preterm delivery (between 26 and 36 weeks of gestation) is anticipated within seven days, antenatal corticosteroids, such as betamethasone or dexamethasone, are recommended. Evidence shows that a single course of corticosteroids significantly reduces neonatal morbidity and mortality, including risks of respiratory distress syndrome

(RDS), necrotising enterocolitis, and the need for intensive care admission in pregnancies at risk of preterm birth [76]. Subgroup analysis shows that there is no evidence to imply any difference in the effect of hypertensive illness on preterm birth, even if studies include women who are at risk of preterm delivery regardless of the aetiology [75, 76]. Less is known about the best way to use corticosteroids, when they are most effective in gestations, and whether repeat doses are advantageous. Likewise, the effectiveness of a 12- or 24-hour delay between corticosteroid dosages is unknown, although two doses are known to be necessary [77].

When there is a high risk of adverse events for the foetus and newborn, including low birth weight, prolonged intensive care unit stays, intraventricular haemorrhage, and heightened need for respiratory support, the need for postponing birth in women who have PE before 34 weeks of pregnancy is likely to benefit the unborn child [78]. Although an early birth seems advantageous for a mother between 34 and 37 weeks of pregnancy, it can raise the risk of negative newborn outcomes, such as RDS, particularly if it occurs before 36 weeks [79]. It might be challenging to decide whether to continue expectant care at these gestations or to deliver the foetus right away. Generally speaking, expectant management should be continued unless there are signs that an early delivery is necessary [79]. There is a large-scale study currently evaluating the optimal timing for delivery in pre-eclamptic pregnancies between 34 and 37 weeks of gestation, which should yield further data [80]. Expectant treatment and interventional management do not appear to affect newborn outcomes after 37 weeks of gestation; however, interventional management is recommended because it benefits the mother [80]. If there are maternal or fetal complications that necessitate an earlier delivery, these gestational recommendations might not be applicable. Severe IUGR or signs of increasing fetal impairment on monitoring modalities, which indicate fetal hypoxia, are examples of fetal indications for delivery. A recent multicenter randomized study of pregnancies complicated by early-onset IUGR further evaluated this, with around 50% of the pregnancies being pre-eclamptic [81]. Reduced fetal heart rate on CTG, early ductus venosus changes (as indicated by a high pulsatility index), or late ductus venosus changes (as indicated by the lack of an A wave) were the three antenatal monitoring strategies used in this study to time delivery. The neonatal outcomes were compared. They found that after two years of age, neonates assigned to the group in which delivery

prediction was based on late ductus venosus changes showed a substantial reduction in neurodevelopmental impairment, after adjusting for prematurity. This suggests that a more cautious scheduling strategy, by waiting for late ductus venosus alterations, may improve perinatal outcomes.

1.8.1 Preventive Strategies of Pre-eclampsia

Biochemical and ultrasonographic indicators are increasingly being investigated in reports to improve early prediction of PE. Fetal factors, such as genotype and circulating cell-free fetal DNA in maternal blood, are being examined for their predictive value. A genome-wide association study involving 4,380 PE cases and over 310,000 controls revealed a strong association between PE and alterations in the fetal genome near the Flt-1 locus [82]. Elevated levels of cell-free fetal DNA have also been observed in maternal circulation prior to the onset of symptoms [82]. Among the most promising biomarkers are soluble Flt-1 (sFlt-1) and PlGF, both of which reflect placental dysfunction. Pre-eclampsia has also been linked to elevated lipid profiles, cholesterol, and inflammatory mediators such as TNFα, IL-6, IL-8, and C-reactive protein, many of which appear prior to clinical symptoms [83]. Although uterine artery Doppler studies have yielded inconsistent predictive accuracy, recent meta-analyses indicate that PE can be predicted with accuracy comparable to clinical risk factors during the first trimester, particularly between 11 and 14 weeks of gestation [84]. The integration of markers such as the uterine artery pulsatility index and pregnancy-associated plasma protein A (PAPP-A) into prediction models significantly improves their positive predictive value [85]. Aspirin is currently considered the most evidence-supported pharmacological intervention for reducing the risk of PE in women identified as high risk. Clinical guidelines recommend a daily dose of 75 to 150 milligrams prior to 16 weeks of gestation and continuing until delivery [86]. When initiated early, low-dose aspirin is associated with a modest yet reliable 10 percent reduction in the risk of PE [86].

Various other preventive strategies have been explored, including dietary supplementation and lifestyle interventions. Although vitamin D deficiency is thought to increase the risk of PE, evidence supporting the effectiveness of vitamin D supplementation remains inconclusive, and further randomised controlled trials are needed to establish efficacy [87, 88]. A large randomised trial conducted by the World Health Organisation

(WHO) found that calcium supplementation significantly reduced the severity and adverse outcomes of PE but did not prevent its onset among women with low dietary calcium intake [89]. Current recommendations advise calcium supplementation in pregnant women with low dietary intake [90].

Antioxidant supplements, particularly vitamins C and E, once showed promise but have since been shown to be ineffective in preventing PE in recent trials [91]. Similarly, high-dose folic acid has not demonstrated preventive benefits, although the use of 5-methyl-tetrahydrofolate, a more bioavailable form, may reduce the risk of recurrent PE in select cases [92, 93].

1.8.2 Pharmacological and Lifestyle Approaches

Antithrombotic agents, such as low-molecular-weight heparin, may reduce the risk of PE in women predisposed to placental insufficiency, as previously suggested [93]. However, the variability across studies limits the conclusiveness of this evidence [94]. Small-scale trials have also evaluated the effectiveness of other pharmacologic agents, including L-arginine, pravastatin, ketanserin, and coenzyme Q10, each of which has shown potential in reducing the risk of PE. Nonetheless, comprehensive studies are required to confirm their safety and efficacy [94].

Lifestyle interventions have been proposed as non-pharmacologic strategies for reducing the risk of PE. Diets rich in fruits and vegetables may be protective, although findings are mixed [95]. A recent randomized controlled trial revealed that prenatal counselling on diet and exercise did not significantly affect PE incidence among overweight and obese women. While exercise appears to offer limited preventive benefits, dietary quality may play a more significant role, although conclusive data remain lacking [95].

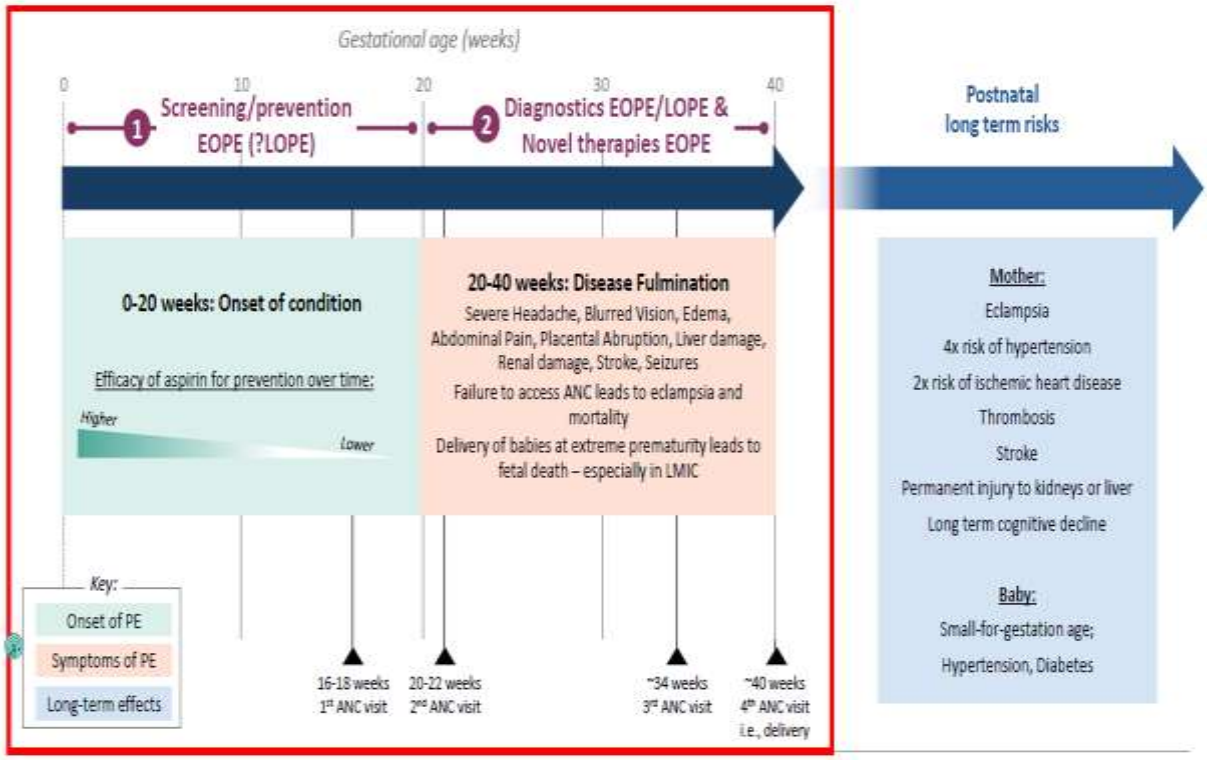

Figure 4: Prevention and Diagnostic Strategies for Pre-eclampsia. The image illustrates the progression, prevention, and long-term impact of PE across gestation. Between 0 and 20 weeks, the condition begins without symptoms. Between 20 and 40 weeks, PE progresses to the symptomatic stage [94].

1.9 Placental Microbiome

The presence of microbes in the placentas of both healthy term and complicated pregnancies was greatly underestimated in the results of previous cultivation-dependent studies; however, this was probably because bacteria prefer anaerobic environments, which made them difficult to cultivate [9]. However, a thorough characterisation of the placental microbiome in more than 300 healthy, term, and preterm pregnancies was made possible by the use of more recent methods, such as Illumina sequencing [9]. The microbiome, which included *Cutibacterium acne, Neisseria lactamica, Fusobacterium sp., Rhodococcus erythropolis, Prevotella tannerae, Escherichia sp., Neisseria polysaccharea, Streptomyces overmitilis, Bacteroides sp.,* and *Escherichia coli,* was found to be lowly abundant, metabolically enriched, and contained 0.002 mg of bacterial DNA per 1 g of placental tissue isolated [9].

Additionally, a recent study found that the communities of placental microorganisms of healthy term births had significant abundances of *Lactobacillus sp., Cutibacterium acne sp., and Enterobacteriaceae sp.* [96]. On the other hand, preterm placental tissues contain fewer *Lactobacilli spp.* than other microorganisms, suggesting that *Lactobacilli* play a role in favourable pregnancy outcomes. It was reported that 80% of preterm deliveries that occurred after fewer than 30 weeks of gestation revealed signs of bacterial invasion [96], while other studies have demonstrated bacterial colonisation in preterm pregnancies linked to chorioamnionitis and intrauterine inflammation [97]. By triggering innate immune responses against the bacterium, microorganisms may cause pregnancy issues, including preterm delivery, by generating excessive inflammation and/or apoptosis at the maternal-fetal interface, according to growing research [97]. The idea that bacterial colonisation is a normal part of the uterus during and outside pregnancy is currently accepted [96]. In vivo animal models (rodents) have also demonstrated that loading animals with bacteria or bacterial products can cause preterm delivery.

Furthermore, research is underway to characterise the placental microbiota. Nonetheless, the available information indicates that the placental microbiome in a typical pregnancy comprises both gram-positive and gram-negative bacteria, with *Lactobacillus* predominating [98]. On the other hand, dysbiosis, a situation in which Bacteroides predominates and *Lactobacillus* is less prevalent, is characterised by changes or an unnatural shift (imbalance) in the makeup of the placental microbiota [99]. Diet and extrinsic stressors (such as environmental stressors, antibiotic exposure, sleep disturbance, physical activity, psychological stress, and genetics) can all cause this imbalance in the placental microbiome. These have been shown to alter the host immune response, leading to microbial-driven inflammation that causes pregnancy complications, such as PE and preterm birth [99].

1.9.1 The Sterile Womb Paradigm Shift

For almost a century, it was widely believed that the human placenta in the uterus is sterile and that newborns acquire microbiome both during and after delivery [100]. The topic of in utero/placental microbial colonisation remains controversial to this day. The human placenta is devoid of microorganisms, and the low bacterial

counts noted there have been attributed to either acquisition during labour and delivery or contamination of laboratory reagents with bacterial DNA [100]. The presence of placental microbiome was refuted by successful generations of germ-free animals, which further reinforced this theory [101]. Over the past 10 years, however, there has been growing evidence that the fetal-uterine environment is not sterile and that microbial colonisation occurs in the uterus and placenta [101]. The bacterial populations found in the placenta and amniotic fluid of term pregnancies may differ from those in preterm pregnancies, according to recent research using molecular methods [102]. Studies have shown that the chorioamniotic membrane of term pregnancies contains *lactobacilli* and *fastidious* bacteria. Additionally, not all microbial colonisation is associated with infection, chorioamnionitis, or placental inflammation [103]. Crucially, some studies have not only confirmed the existence of the placental microbiome but also identified variations in the microbiota, with a unique genetic profile for each placental area from which they were extracted [13]. All of these studies point to the need to move away from the idea of a sterile uterus and towards in utero microbial colonisation.

However, using culture, qPCR, and 16S rRNA gene sequencing to profile the potential bacterial niches in the placental and fetal tissue of mice compared with the maternal mouth, lung, liver, uterus, cervix, and vagina. Theis et al. recently reported inconsistent results on the presence of microbial niches in placental and fetal tissues [104]. But in the fetal brain sample, the scientists identified a single bacterial isolate with a bacterial load exceeding contamination controls [104]. These reports contradicted some early findings in mice, such as those by Younge et al., who also reported the presence of *Lactobacillus, Bacteroides, Enterococcus, Escherichia,* and *Bacillus* in the placenta and fetal tissues of the mice and confirmed fetal exposure to microbial niches from the placental and extraplacental membranes in utero [105]. Martinez et al. found a diverse bacterial profile in the placenta and concluded that foetuses are exposed to microbial DNA in utero [106]. The lack of multiple fluorescence in situ hybridisation (FISH) to visualise the potential bacterial communities in the placental and fetal tissues of mice, as well as the lack of effective control measures like the use of germfree and wild-type mice, may have limited Theis et al.'s observation, even though it was in agreement with other studies [107]. The low biomass of bacterial communities in the placenta may affect how well current technologies detect them. The knowledge of the potential health benefits and diseases associated

with the placental microbiome should not be undermined by the need for more research using DNA-based methods and microscopy with appropriate controls.

1.9.2 Seeding of the Placental Microbiome

In existing research, the mechanism of placental microbiome seeding is not well understood. According to current evidence, microbes either enter through the mother's circulation to establish themselves during early vascularisation and placentation, or they ascend from the vagina and enter the mother's intestinal lumen and oral cavity, where they are internalised and spread hematogenously to the placenta [108]. Strong evidence supports the existence of vaginal-derived bacteria, and there is a significant correlation between gestational age and *Lactobacilli sp.* from the vagina [109]. Low concentrations of bacteria might seed the placenta, as pregnancy and lactation increase bacterial translocation from the intestinal and oral mucosal epithelial gaps into the maternal bloodstream [109]. The placental microbiome is believed to be mostly derived from the oral microbiota [102]. Detection of bacterial DNA from the oral microbiota has been linked to preterm membrane rupture, preterm birth, miscarriage, and foetal death in relation to the pathogenic genera *Streptococcus* and *Fusobacterium.* This suggests that oral microbiota plays a role in the seeding of placental microbiome [102].

1.9.3 Determination of Placental Microbiome

1.9.3.1 Real-Time PCR for Targeted Detection of Placental Microbial DNA

The application of DNA-based methods, particularly real-time polymerase chain reaction (qPCR), has improved the detection of placental microbiota. qPCR enables amplification of specific bacterial DNA sequences with high sensitivity and specificity [9]. Despite its advantages, the method has a low detection limit and may miss low-abundance microbial populations, especially in low-biomass environments such as the placenta. Therefore, while useful for detecting known bacteria, it may underestimate the true microbial diversity.

1.9.3.2 16S rRNA Gene Sequencing for Broad Microbial Community Profiling

The 16S rRNA gene sequencing targets conserved regions of the bacterial ribosomal RNA gene, enabling researchers to identify and classify bacteria at various taxonomic levels [110]. This technique has become widely used to investigate the overall composition of microbial communities within the placenta. By allowing simultaneous detection of multiple bacterial taxa in a single run, it provides a more comprehensive understanding of the microbiome than culture- or PCR-based methods. It is particularly valuable for exploring microbial shifts associated with pregnancy disorders like pre-eclampsia.

1.9.3.3 Shotgun Metagenomic Sequencing for High-Resolution Microbiome and Functional Analysis

Unlike targeted 16S sequencing, shotgun metagenomics involves random sequencing of all DNA present in a sample, capturing the entire microbial genome landscape [110]. This method provides higher taxonomic resolution and allows for the identification of bacterial, viral, fungal, and archaeal communities. Moreover, it enables functional analysis by identifying genes involved in metabolism, virulence, and host–microbe interactions. This makes it a powerful tool for understanding both the diversity and potential biological roles of microorganisms in placental health and disease.

1.9.3.4 Multiplexing and Library Preparation Strategies in Next-Generation Sequencing

Next-generation sequencing (NGS) technologies have made it possible to analyze large numbers of samples simultaneously through multiplexing. This is achieved by attaching unique barcode sequences or indexed reads to each sample, allowing them to be pooled in a single sequencing run [111]. Universal primers targeting several variable regions of the 16S rRNA gene are used to prepare sequencing libraries, which are further modified with adapter and barcode sequences. The quality and interpretability of NGS data depend heavily on robust bioinformatics pipelines that process raw reads into taxonomic and functional profiles [111].

1.9.3.5 Multi-Omics Approaches for Investigating Microbial Activity and Function in the Placenta

To move beyond identifying microbial presence, advanced "omics" techniques are required to understand the activity and physiological impact of placental microbes. Metatranscriptomics analyses microbial RNA to reveal active gene expression; metaproteomics identifies proteins to assess microbial function; and

metabolomics profiles metabolites produced by microbes [100]. These methods may provide dynamic and functional insights into how microbial communities may influence placental development, immune modulation, and pregnancy outcomes such as fetal growth restriction or pre-eclampsia.

1.9.4 Biological Relevance of the Placental Microbiome

Numerous studies attest to the placental microbiome's role in a typical, healthy pregnancy, particularly in the dominance of non-pathogenic commensals [102]. The following roles of in utero or placental microbial colonization are hypothesized:

(i) Early innate immune development in the foetus and maternal immune regulation: The host's bacterial genetic diversity has produced valuable insights into the placental microbiota's capacity to modulate the immune system. According to some research, there is a favourable correlation between maternal-fetal immunological tolerance and several placental microbial genes, including non-pathogenic commensals [96]. In order to preserve tissue homeostasis and maternal-foetal immunological tolerance, microbiota regulate the host immune response by averting an undesirable inflammatory response during pregnancy [96]. Commensal bacteria have been shown to suppress the NF-κB pathway, a classical inflammatory mediator, by stimulating toll-like receptors (TLRs), specifically TLR4 and TLR2, expressed on trophoblasts. This promotes the production of regulatory cytokines, including interleukin 10 and type 1 interferon-associated chemokines. Therefore, it is conceivable that bacteria might induce regulatory cytokines via the trophoblast, thereby promoting the maternal-fetal interface in a tolerogenic milieu [96]. It remains unclear how trophoblasts integrate microbial signals; however, new research has proposed alternative mechanisms involving epigenetic and histone changes that mediate TLR responsiveness [6]. Furthermore, the placental microbiome is increasingly recognised as a source of antigenic determinants in the infant and may promote early innate immune development [6].

(ii) Modulation of metabolic function: It appears that genes linked to the metabolism of fatty acids, especially short-chain fatty acids, as well as benzoate and tryptophan, are metabolically enriched in the placenta's current bacterial niches. Fetal brain development depends on the placenta's ability to metabolize

tryptophan, and fetal neurodevelopmental abnormalities have been associated with reduced placental tryptophan metabolism [9]. Tryptophan catabolism in the placenta improves placental circulation, growth, and antibacterial action against infections, as well as the development and maintenance of maternal-fetal immunological tolerance [102]. Additionally, placental bacteria have pathways for fatty acid metabolism that may help extract energy from circulating fatty acids such as acetate, butyrate, and propionate. These are essential for providing the foetus with substrates that generate energy. Furthermore, bacterial genes linked to benzoate metabolic pathways have also been shown to be abundant in the placenta and aromatic compounds. For example, many microbes use benzoates as a carbon source [9, 102].

(iii) **Preparing the infant for host-microbial symbiosis:** The initiation of microbial colonization and molecular transfer in the newborn are greatly influenced by the interactions between the bacteria and the human host. Neonatal immune priming and development without exposure to ambient microbes are also supported by animal research [9, 102].

1.10 Placental Microbiome Variations in Pre-eclampsia and Normotensive Pregnancies

1.10.1 Placental Microbiome in Normotensive Pregnancies

In healthy pregnancies, the placenta is reported to harbour a low-biomass microbiome composed of commensal and beneficial bacteria that contribute to immune regulation and fetal development [57]. This has been shown to be dominated by *Lactobacillus, Bifidobacterium,* and *Firmicutes*, with *Proteobacteria* and *Actinobacteria* present in smaller proportions. Their functions include modulating maternal-foetal immune interactions and supporting normal fetal growth. In addition, a stable microbial composition has been suggested to promote angiogenesis and prevent inflammation. Likewise, a balanced microbiome protects against infections and inflammatory responses. Healthy normotensive pregnancies exhibit lower levels of pathogenic bacteria and a well-regulated immune tolerance system at the maternal-fetal interface.

1.10.2 Placental Microbiome in Pre-eclampsia

Pre-eclampsia, when associated with intrauterine growth restriction (IUGR), represents the most severe form of placental dysfunction and is marked by significant alterations in the maternal microbiome, excessive inflammation, and severe oxidative stress. Key microbial changes include a marked reduction in protective species such as Lactobacillus and Bifidobacterium, alongside an overgrowth of pathogenic bacteria, including Fusobacterium nucleatum, Escherichia coli, Enterococcus, and Ureaplasma urealyticum [57]. These changes are accompanied by increased bacterial diversity and abundance, indicating a disruption of the placental barrier. Additionally, there are elevated levels of microbial-derived endotoxins, such as lipopolysaccharide (LPS), and inflammatory cytokines, which further exacerbate the condition. The combined effects of these microbial and immune disturbances contribute to severe oxidative stress, driven by bacterial metabolites and inflammatory mediators.

In pregnancies complicated by IUGR alone, the placental microbiome shows a shift toward dysbiosis, without the intense inflammatory response characteristic of preeclampsia. This microbial imbalance is marked by a reduction in beneficial species such as Lactobacillus and Bifidobacterium, alongside an increase in pathogenic bacteria, including Escherichia coli, Ureaplasma, and Staphylococcus [106]. Elevated levels of bacterial lipopolysaccharide usually trigger mild inflammation and contribute to vascular dysfunction. Additionally, decreased angiogenic support leads to impaired placental perfusion and restricted fetal nutrient supply. Although oxidative stress is less pronounced compared to cases involving both PE and IUGR, it remains sufficient to hinder trophoblast invasion. Overall, IUGR alone is still associated with a compromised placental barrier, increased bacterial translocation, and disruptions in metabolic pathways.

Table 2: Comparison of Placenta Microbiome among Groups

Feature	Normotensive Pregnancies	IUGR without PE	PE+IUGR
Microbial Diversity	Low, stable	Increased, moderate dysbiosis	Highly diverse, severe dysbiosis
Dominant Bacteria	Lactobacillus, Bifidobacterium	Escherichia, Staphylococcus	Fusobacterium, Enterococcus, Ureaplasma
Inflammation	Low	Moderate	Severe
Oxidative Stress	Minimal	Elevated	Extreme
Angiogenic Factors	Balanced	Decreased VEGF/PIGF	VEGF/PIGF depletion
Immune Dysregulation	Minimal	Mild Treg reduction	Severe Treg depletion, CD8+/NK activation
Fetal Outcome	Normal	Mild growth restriction	Severe IUGR, high stillbirth risk

1.11 Rationale of the Study

Despite extensive research, the precise aetiology of pre-eclampsia remains poorly understood, and its multifactorial pathogenesis continues to limit the development of effective diagnostic and therapeutic strategies. Emerging evidence suggests that the placental microbiome possibly play a pivotal role in modulating key physiological processes during pregnancy, including immune tolerance, angiogenesis, and maternal endothelial function, which are pathways that are central to the development of PE. However, the contribution of the placental microbiome to the pathogenesis of PE, particularly in distinguishing between early- and late-onset forms, remains controversial and underexplored. Investigating this relationship may provide novel insights into disease mechanisms and facilitate the identification of potential microbial biomarkers or therapeutic targets to improve the prevention and management of PE.

1.12 Aim of the Study

The aim of this study is to profile the microbes in the placenta of normotensive and pre-eclamptic (early- and late-onset) pregnancies using a targeted 16S analysis and NGS.

1.13 Objectives of the Study

(i) To identify different microbes, present in the placentas of normotensive and early- and late-onset pre-eclamptic pregnancies using a targeted 16S analysis.

(ii) To identify different bacterial species, present in the placentas of normotensive and pre-eclamptic pregnancies using NGS.

(iii) To determine the serum levels of microbial metabolite (acetate), epigenetic regulator (HDAC2) and immune response (HLA-DRA) in normotensive and pre-eclamptic pregnancies.

1.14 Brief Overview of Methodology and Study Design

A multidisciplinary approach was employed to investigate the placental microbiome and its role in PE. Microbial profiling was conducted using targeted 16S analysis and NGS, with taxonomic classification performed through bioinformatics pipelines such as Kraken2, Kaiju, and MetaPhlAn2. Biochemical analyses quantified epigenetic factor (HDAC2) using enzyme-linked immunosorbent assay (ELISA), while metabolomic studies focused on short-chain fatty acids (acetate) as indicators of microbial activity. Molecular studies assessed HLA-DRA gene expression to explore immune pathways involved in PE, using RNA extracted from samples and quantifying expression by qPCR. The study included placental tissue obtained during sterile collection from normotensive pregnancies and those affected by EOPE and LOPE, at cesarean sections performed at a regional hospital in Durban, South Africa. Statistical and bioinformatics tools were used to correlate microbiome composition, metabolic profiles, and inflammatory mediators with PE, providing a comprehensive framework for understanding the condition's pathogenesis.

1.15 Significance of the Study

The study aimed to improve the understanding of the role of the placental microbiome in pre-eclampsia, a significant pregnancy-related disorder that continues to increase maternal and foetal mortality globally. By elucidating microbial involvement in placental function and fetal development, the findings would possibly provide novel diagnostic biomarkers and therapeutic targets for early detection and management of PE.

CHAPTER 2: MANUSCRIPT ONE

Placental Microbial Colonization and Its Association with Pre-eclampsia (Published: Frontiers in Cellular and Infection Microbiology, 2020:1-11).

This publication analytically appraises the role of the placental microbiome in the aetiology of pre-eclampsia (PE), a pregnancy-specific hypertensive disorder that remains a leading cause of maternal-foetal morbidity and mortality. The human placenta, once considered sterile, is now recognized as being colonised with microbes, with its microbiome resembling the oral microbiome more closely than the vaginal or gut microbiome. The distinct composition of the placental microbiome and its influence on physiological and pathophysiological conditions, including metabolism, immune tolerance, obesity, diabetes, and cardiovascular diseases, has garnered significant interest. Despite extensive research, the precise aetiology of PE remains unclear, contributing to suboptimal diagnostic strategies and treatment approaches. Given the emerging understanding of the placental microbiome, we critically evaluated its potential role in PE pathogenesis. Furthermore, based on its implications in disease progression, we explored the therapeutic prospects of modulating the placental microbiome as a possible strategy for preventing and managing PE.

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REVIEW
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Placental Microbial Colonization and Its Association With Pre-eclampsia

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The existence and role of the microbiome in regulating physiological and pathophysiological conditions including metabolism, energy homeostasis, immune tolerance, behavior, obesity, diabetes, and cardiovascular-related diseases is of immense interest. It is now clear that the human placenta is not sterile, but rather colonized with microbes. The placental and vaginal microbiomes are distinct however, the placental microbiome is comparable with the oral microbiome, with a limited variation when compared with the gut microbiome. Pre-eclampsia (PE), a pregnancy-specific hypertensive disorder, remains the leading cause of maternal-fetal morbidity and mortality. This is largely due to the lack of a clear etiology of PE and consequently, diagnostic strategies, and treatment are sub-optimal. The present review focuses on the current understanding of the placental microbiome and its implication in the etiology of PE. It provides a perspective on the alteration of placental microbiome as a possible therapeutic approach in the prevention and management of PE.

Keywords: metabolism, microbe, immune tolerance, placental microbiome, pre-eclampsia, pregnancy

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INTRODUCTION

The microbiome refers to trillions of microbes that reside in different parts of the human body including the oral cavity, nasal cavity, gut, lungs, genitourinary tract, amniotic fluid, and the placenta (Chierico et al., 2014; Amarasekara et al., 2015; Zhang et al., 2015; Kumar and Chordia, 2017; Pelzer et al., 2017). The composition of microbes in an individual host depends on the genetic constitution, dietary intake, disease state, geographical location, and the dominant microbial species (Bull and Plummer, 2014; Chierico et al., 2014; Gilbert et al., 2015), and is genetically diverse as no two microbiota are the same (Kumar and Chordia, 2017). It is an essential component of immunity, capable of influencing metabolism and modulating drug interactions (Tancrede, 1992; Bull and Plummer, 2014; Kumar and Chordia, 2017; Wang et al., 2018). Therefore, the microbiome plays a crucial role in maintaining human health. In fact, dysbiosis (microbial imbalance) through diet, excessive use of antibiotics, obsession with cleanliness, cesarean deliveries etc. have been documented to contribute to some common diseases such as autism, diabetes, obesity, cancer, autoimmune diseases, and asthma (Constante et al., 2017; Postler and Ghosh, 2017).

The uterus and placenta were thought to be germ-free and sterile, maintained by cervical mucus (plug) to keep the baby safe from infection until recently that the existence of the placental microbiome is emerging, specifically in 2014 when the historical view of the sterile uterus and placenta was challenged by Aagaard et al. (2014) who reported detection of bacterial DNA

sequences in the placenta samples of term and preterm pregnancies. Evidence exists that a distinct community of microbes colonize the placenta and include *Lactobacillus* sp., *Propionibacterium* sp., *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria phyla*, *Enterobacter* sp., *Salmonella* sp., *Porphyromonas* sp., *Klebsiella pneumoniae*, *Bacillus cereus*, *Gardnerella* sp., *Variovorax* sp., *Clostridium* sp., *Prevotella* sp., *Listeria* sp., *Escherichia* sp., *Anoxybacillus* sp., among others (Amarasekara et al., 2015; Gomez-Arango et al., 2017), many of which are usually associated with periodontitis and chorioamnionitis (Amarasekara et al., 2015; Gomez-Arango et al., 2017). Histological analyses have also revealed that intracellular bacteria are present in the basal plate of a term placental biopsy (Stout et al., 2013), and that bacteria home and replicate within the placental explants from a term pregnancy (Cao and Mysorekar, 2014). Similarly, a low-abundance of microbiome has been identified from the placentae of a healthy pregnancy using culture-dependent and -independent methods. Likewise, previous studies have suggested oral mucosa as a possible source of the placental microbiome based on the similarities between microbial communities in the placental and oral niches (Aagaard et al., 2014). Additionally, evidence of phyla-specific similarities of microbes in the placenta, infant meconium and amniotic fluid, it has been proposed that microbiota from the placenta *in utero* is the source of the complement of developing fetus (Collado et al., 2016), and detectable bacteria in the umbilical cord blood could also originate from the placenta (Jiménez et al., 2005; Goeden et al., 2013; Cox et al., 2014; Sedlmayr et al., 2014; Gomez-Arango et al., 2017). Collectively, these studies corroborate the existence of microbial communities in the placenta and that the fetal environment is not sterile. However, the involvement of placental microbiome in pregnancy complications particularly pre-eclampsia (PE) is still under investigation.

Pre-eclampsia, the leading cause of maternal-fetal morbidity and mortality (Maebayashi et al., 2014; Rana et al., 2019), is a multi-systemic syndrome that affects 5–8% of pregnancies worldwide, resulting in over 70,000 maternal and 500,000 fetal deaths annually (Wanderer et al., 2013; Goel et al., 2015). It is a pregnancy-specific disorder that is usually characterized by new-onset hypertension and proteinuria. It should be noted that PE can also be diagnosed without the presence of proteinuria but with evidence of thrombocytopenia and elevated liver enzymes. The precise cause of PE remains unknown and as a result, there are no clear screening tools or preventative measures for early diagnosis of PE (Amarasekara et al., 2015; Rana et al., 2019). Although the pathogenesis of PE is well-documented and maternal and fetal/placental factors have been studied extensively (Wanderer et al., 2013; Gathiram and Moodley, 2016; Karumanchi, 2018; Pillay et al., 2019; Rana et al., 2019), with placenta recognized as the central causative agent.

Abnormal placentation has been associated with uteroplacental ischemia early in the first trimester and followed by maternal syndrome in the later second and third trimesters. It is characterized by altered anti-angiogenic and pro-angiogenic factors, shedding of syncytiotrophoblast microparticles, and nanovesicles into the maternal circulation. These changes collectively drive the hypertensive, multi-organ failure response observed in the maternal pre-eclamptic syndrome (Palei et al., 2013; Romero and Chaiworapongsa, 2013; Verma et al., 2018; Pillay et al., 2019). A number of theories have been proposed for the placental dysfunction observed and include oxidative stress, abnormal natural killer cells (NKs) at the maternal-fetal interface, and genetic and environmental factors. Although none of these theories have conclusive evidence, substantive evidence supports the idea that the diseased placenta triggers the release of soluble toxic factors in the maternal circulation and these result in inflammation, impaired endothelial function and maternal systemic disease (Wanderer et al., 2013; Verlohren et al., 2014; Erez et al., 2017; Karumanchi, 2018). It is therefore suggested that the presence of bacteria in the placenta may also alter anti-angiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1) and pro-angiogenic factors, like placental growth factors (PIGF), and vascular endothelial growth factor (VEGF). This results in an antiangiogenic state causing impaired maternal endothelial function that leads to the clinical manifestation of PE (Govender et al., 2012; Baijnath et al., 2014; Pillay et al., 2017). Because the exact cause of PE is unknown, its treatment has been limited to reduction of high blood pressure and delivery of placenta and fetus. The present review is focused on the current understanding of the placental microbiome and its implication in the etiology of PE and provides a perspective that placental microbiome-targeted therapy may be beneficial in the treatment of PE.

THE STERILE *IN UTERO*-PLACENTAL ENVIRONMENTAL PARADIGM SHIFT

The notion that the human *in utero*-environmental placenta is sterile, and that neonates acquire microbiome during and after birth was accepted dogma for more than a century (Escherich, 1885; Funkhouser and Bordenstein, 2013). Even to date, there are still controversies regarding *in utero*/placental microbial colonization. A recent study by Theis et al. (2019) and de Goffau et al. (2019) revealed that the human placenta has no potential microbes and these authors attributed the limited bacterial presence in the placenta to the contamination of used laboratory reagents with bacterial DNA or acquisition during labor and delivery. This view was also supported by successful generations of animals that are germ-free through aseptic transfer of the entire uterus reinforcing evidence against the existence of the placental microbiome (Hedrich and Hardy, 2012). However, increasing evidence in the last decade has revealed that the fetal environment is not sterile, and proof of the existence of *in utero*/placental microbial colonization is being reported (Jiménez et al., 2005; Aagaard et al., 2014; Parnell et al., 2017; Perez-Muñoz et al., 2017). Interestingly, using molecular techniques, recent studies confirmed that the bacterial communities which exist in the placenta and amniotic fluid from term pregnancies may vary from preterm pregnancies (Collado et al., 2016; Gomez-de Aguero et al., 2016). Studies also reveal *lactobacilli* and fastidious bacteria in the chorioamniotic membrane of term pregnancies, and that not all microbial colonization was linked with placental

inflammation, chorioamnionitis, and infection (Lannon et al., 2019). This is consistent with earlier reports that the placenta of a healthy term pregnancy consists of non-pathogenic commensal bacteria (Aagaard et al., 2014). Importantly, others not only support the presence of placental microbiome but also found variance in microbiota with distinct genomic profiles based on the region of placenta they were obtained (Kumar and Chordia, 2017; Parnell et al., 2017). Collectively, these studies support a shift from a sterile uterus paradigm to *in utero* microbial colonization.

Nevertheless, Theis et al. recently reported an inconsistent results in the existence of microbial communities in the placental and fetal tissues using culture, qPCR, and 16S rRNA gene sequencing to profile the possible bacterial communities in the placental and fetal tissue of mice compared with maternal mouth, lung, liver, uterus, cervix, and vagina. Although the authors found a single bacterial isolate in the fetal brain sample having a bacterial load higher than that of contamination controls (Theis et al., 2020). These reports contradicted several early results in mice, including reports by Martinez et al., who found a diverse profile of bacteria in the placenta and concluded that fetuses are exposed to bacterial DNA *in utero* (Martinez et al., 2018), and Younge et al. who also reported the presence of *Lactobacillus*, *Escherichia*, *Enterococcus*, *Bacteroides*, and *Bacillus* in the placental and fetal tissues of the mice and confirmed fetal exposure to microbial communities from the placental and the extraplacental membranes *in utero* (Younge et al., 2019). Although Leiby et al. (2018) and de Goffau et al. (2019), was in agreement with other studies, but despite the use of multiple modes of microbiological inquiry, its observation might possibly be limited by the absence of multiple fluorescent *in situ* hybridization (FISH) to visualize the potential bacterial communities in the placental and fetal tissues of mice, and also by the absence of effective control such as the use of germ-free and wild-type mice. It is possible that the low biomass of bacterial communities in the placenta affects its detection by the existing technologies. Therefore, more studies using DNA-based techniques and microscopy with appropriate controls are required, but this should not dispel the understanding of the potential health benefits and diseases associated with placental microbiome.

THE PLACENTAL MICROBIOME—THE ENDOGENOUS MICROBIAL COMMUNITY

Although findings from earlier cultivation-dependent studies significantly underestimated the existence of microbes in the placenta of healthy term and complicated pregnancies, it was likely due to difficulty to culture bacteria because of their preference for anaerobic environments (DiGiulio et al., 2010). However, using newer techniques such as Illumina sequencing, a comprehensive characterization of the placental microbiome in over 300 healthy, term, and preterm pregnancies was possible (Aagaard et al., 2014). This study detected a lowly abundant but "metabolically enriched" microbiome in 0.002 mg of bacterial DNA per 1 g of placental tissue isolated and it included *Cutibacterium acne*, *Neisseria lactamica*, *Fusobacterium* sp., *Rhodococcus erythropolis*, *Prevotella tannerae*, *Escherichia* sp., *Neisseria polysaccharea*, *Streptomyces avermitilis*, *Bacteroides* sp., and *Escherichia coli* in both normal healthy and complicated pregnancies (Aagaard et al., 2014).

Besides, a recent study found a high abundance of *Enterobacteriaceae* sp., *Cutibacterium acne* sp., and *Lactobacillus* sp. in the communities of placental microbes of healthy term deliveries (Gomez-de Aguero et al., 2016). In contrast, *Lactobacilli* sp. are less than other microbes in the placental tissues of preterm deliveries, supporting the role of *Lactobacilli* in positive pregnancy outcomes. Various studies have confirmed bacterial colonization in preterm pregnancies associated with chorioamnionitis and intrauterine inflammation (Aagaard et al., 2014; Romero et al., 2014; Prince et al., 2015). In one study, 80% of preterm births that occurred <30 weeks of gestation, had evidence of bacterial invasion (Mor and Kwon, 2015). Growing literature suggests that microbes possibly induce pregnancy complications, such as preterm birth through innate immune responses against the bacteria, causing excessive inflammation, and/or apoptosis at the maternal-fetal interface (Romero et al., 2014; Mor and Kwon, 2015). Likewise, *in vivo* models of experimental animals have shown that loading of bacteria or bacterial products in animals triggers preterm delivery (Romero et al., 2014), and currently the notion that bacterial colonization is a normal component of the uterus in pregnancy and non-pregnancy is being accepted (Mor and Kwon, 2015).

Moreover, the characterization of placental microbiome is currently under investigation. However, available data reveals that placental microbiome in normal pregnancy is characterized by both gram-positive and gram-negative bacteria with *Lactobacillus* dominance, which has largely been reported to confer a protective benefit (Gomez-de Aguero et al., 2016; Moreno et al., 2016). Whereas, alterations or unnatural shift (imbalance) in the composition of placental microbiota, known as dysbiosis, a condition that is highly dominated with *Bacteroides* and less of *Lactobacillus* (Bardos et al., 2019). This imbalance in placental microbiome, which can be triggered by diet, extrinsic stressors, including environmental stressors, antibiotic exposure, sleep disturbance, physical activity, and psychological stress and genetics has been reported to alter host immune response, leading to microbial-driven inflammation that underlie pregnancy complications, including preterm birth and PE among others (Gomez-de Aguero et al., 2016; Bardos et al., 2019).

SEEDING OF PLACENTAL MICROBIOME

The process of seeding of placenta microbiome in the current literature is not entirely clear. The present evidence is that: (i) microbes ascend from the vagina, and (ii) maternal intestinal lumen and oral cavity which are internalized and translocated by hematogenous spread to the placenta (**Figure 1**) or enter via maternal circulation to take up residence during early vascularization and placentation (Doyle et al., 2014; Antony et al., 2015; Gomez-de Aguero et al., 2016; Pelzer et al., 2017). The

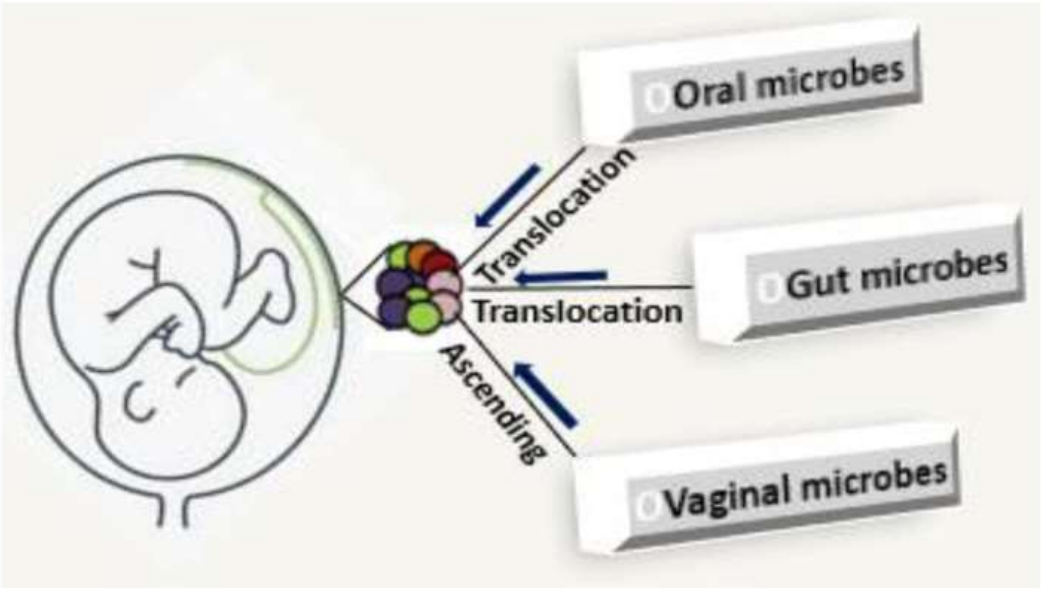

FIGURE 1 | Seeding of placental microbiome; microbes ascend from the vagina, gut, and oral cavity which are internalized and translocated by hematogenous spread to the placenta.

evidence for vaginal-derived bacteria is strong and *Lactobacilli* sp. from the vagina, are positively correlated with gestational age (Romero et al., 2015; Zheng et al., 2015; Pelzer et al., 2017). Translocation of bacteria from the epithelial gaps of the intestine and oral mucosa through the maternal circulation is enhanced during pregnancy and lactation, and this enables the transfer of low numbers of bacteria to possibly seed the placenta (Racicot et al., 2013; Prince et al., 2015; Pelzer et al., 2017; Lannon et al., 2019). Oral microbiota is thought to be a key source of the placental microbiome (Gomez-de Aguero et al., 2016). Bacterial DNA detection from oral microbiota has been associated with preterm rupture of membranes, preterm birth, miscarriage, and death of developing fetus for the genera pathogenic *Streptococcus* and *Fusobacterium* (Amarasekara et al., 2015), suggesting that oral microbiota is a contributor to the seeding of placental microbiome.

DETERMINATION OF PLACENTAL MICROBIOME

During the pre-sequencing era, culture was the most popular method used to determine the presence of microbes in the body, including placental microbes. This method however fails to detect viable but non-culturable microbes, supporting the sterile uterus paradigm. In contrast, the use of DNA-based techniques has upheld the existence of placental microbiome (Aagaard et al., 2014; Gomez-Arango et al., 2017). In addition, the use of real time PCR has successfully been used to profile placental microbiome. However, it has a low detection limit, therefore some viable bacteria may be undetectable. Community-wide composition profiling has been made possible by developmental approaches targeting the 16S ribosomal RNA (rRNA) molecule and its encoding gene. This is extremely useful to characterize the placental microbiota composition and its dynamics. In the last few years, 16S rRNA gene sequencing has become a popular approach to investigate the composition of the placental microbiota. Even more can be revealed at the DNA level, especially by the metagenomics approach in which a maximally representative DNA sample from the entire community of an environmental sample is isolated and subsequently, often in a random fashion, sequenced. Both the targeted 16S rRNA gene sequencing and shotgun metagenomic sequencing have been made possible with the onset of the next generation sequencing (NGS) techniques (Said et al., 2014; Yoneda et al., 2016; Urushiyama et al., 2017). Multiplexing is achieved via two strategies: adding a barcode sequence and through separate indexing reads, which are located outside the primer region, requiring separate sequencing runs (that read "away" from the target DNA molecule). For throughput, libraries are prepared for each sample with universal primers targeting different regions of the 16S rRNA gene. Primers are adapted for high-throughput sequencing with the addition of adapter sequences and barcoded dual-index forward and reverse sequences taken from previous studies (Aagaard et al., 2014; Zheng et al., 2015, 2017; Parnell et al., 2017). The processing of the data generated by NGS depends on the available bioinformatics capacities. Therefore, the DNA-based technique has greatly expanded our current knowledge of the placental microbiome. Other methods are still required to better understand the actual function and activity of microbes (metatranscriptomics, metaproteomics, and metabolomics) in the placenta.

SIGNIFICANCE OF PLACENTAL MICROBIOME

Several reports support the fact that the placental microbiome, particularly with non-pathogenic commensal dominance, is part of normal healthy pregnancy (Romero and Chaiworapongsa, 2013; Aagaard et al., 2014; Mor and Kwon, 2015; Zheng et al., 2017). *In utero* or placental microbial colonization is postulated to be involved in the following functions:

(i) Maternal immune modulation and initiation of early innate immune development in the fetus. The bacterial genetic diversity that is present in the host has generated important information regarding the immune-modulatory potential of the placental microbiota. A number of placental microbial genes particularly non-pathogenic commensals has been suggested to positively correlate with maternal-fetal immune tolerance (Racicot et al., 2013; Mor and Kwon, 2015). Microbiota modulate the host immune response by preventing an undesired inflammatory response during pregnancy thereby maintaining tissue homeostasis and maternal-fetal immune tolerance (Belkaid and Hand, 2014; Mor and Kwon, 2015; Gomez-de Aguero et al., 2016). Stimulation of toll-like receptors (TLRs) particularly TLR4 or TLR2 expressed on the trophoblast, by commensal bacteria, has been reported to suppress the NF-kB pathway (a classical inflammatory mediator), promoting the production of regulatory cytokines, such as type 1 interferon-associated chemokines and interleukin 10 (**Figure 2**). It is therefore plausible that bacteria might stimulate the maternal-fetal interface to a tolerogenic microenvironment through the induction of regulatory cytokines by the trophoblast (Mor and Kwon, 2015). Although the integration of microbial-derived signals by trophoblast remains unclear, epigenetic, and histone modifications or alternative pathways mediating response to TLRs have been recently suggested (Pelzer et al., 2017). In addition, the placental microbiota might be involved in driving early innate immune development and is emerging as a source of antigenic determinants in the newborn (Pelzer et al., 2017; Giessen et al., 2020).

(ii) Modulation of metabolic function. The existing bacterial communities in the placenta seems to be metabolically enriched with genes associated with fatty acid (particularly short-chain fatty acid), tryptophan, and benzoate metabolism. Placental tryptophan metabolism is essential for fetal neural development and impaired placental tryptophan metabolism has been associated with neurodevelopmental defects in the fetus (Gomez-de Aguero et al., 2016). In effect, catabolism of tryptophan in the

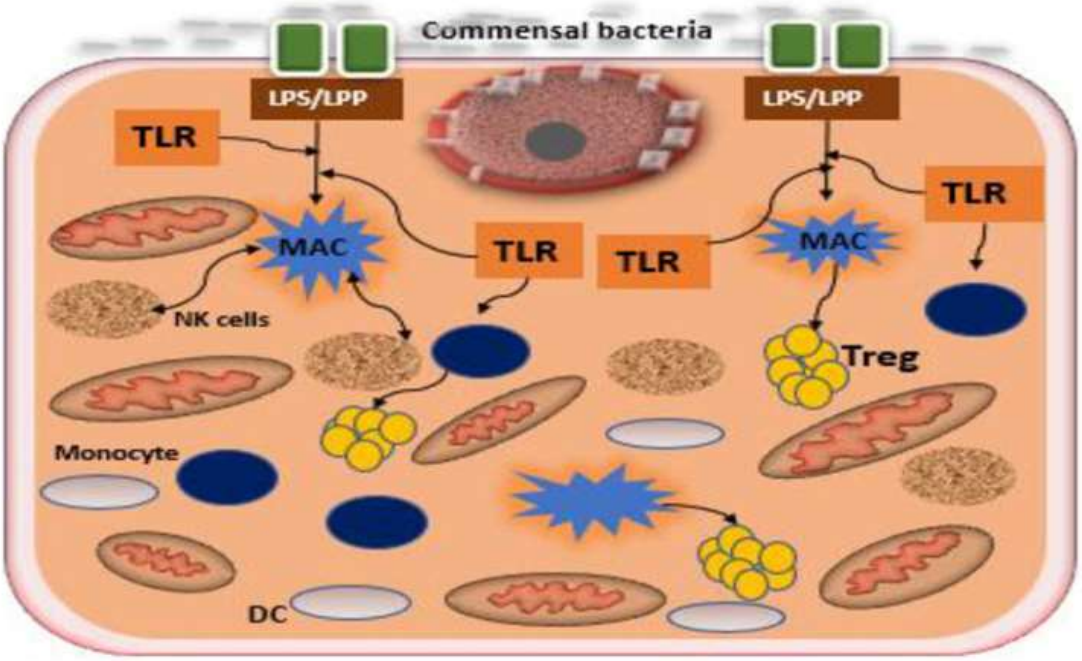

FIGURE 2 | Interaction between commensal bacteria and toll like receptors of the trophoblast in the formation of regulatory cytokines. Commensal bacteria present at the epithelium of the uterus promote the induction of regulatory cytokines by trophoblast and macrophages. Macrophages secrete antimicrobial products that mitigate commensal overgrowth and prevent invasion of pathogenic bacteria. Recognition of bacterial products such as LPS or LPP by trophoblast potentiates the expression of anti-inflammatory factors, increasing T regulatory cells (Tregs), and promoting tolerance. TLR, toll-like receptor; DC, decidual cell; LPS, lipopolysaccharide; MAC, lipoprotein macrophage.

placenta enhances the establishment and maintenance of maternal-fetal immune tolerance (Sedlmayr et al., 2014), placental circulation, growth, and modulation of antimicrobial activity against infections (Sedlmayr et al., 2014). Placental bacteria are also enriched with pathways encoding fatty acid metabolism, which may aid energy extraction from circulating fatty acids and play an important role in supplying energy-yielding substrates to the fetus. Likewise, bacterial genes attributable to pathways involved in benzoate metabolism were also enriched in the placenta and aromatic compounds, for instance, benzoates are used as a carbon source for many microorganisms (Gomez-de Aguero et al., 2016).

(iii) Preparation of the newborn for host-microbial symbiosis. Interaction of the microbes with human host is a significant contributor to pregnancy outcome and seeding of microbial colonization and molecular transfer in the neonates. Animal studies also support a role for neonatal immune priming and development without environmental microbial exposure (Gomez-de Aguero et al., 2016).

THE POSSIBLE INVOLVEMENT OF PLACENTAL MICROBIOME IN PRE-ECLAMPSIA

In order to understand the possible involvement of placental microbiome in PE, it is important to reiterate its spectrum and pathogenesis. Pre-eclampsia is one of the categories of the hypertensive disorder of pregnancy, which includes gestational hypertension, chronic hypertension, PE without severe features, severe PE, eclampsia, and the HELLP syndrome (USAID/Africa Bureau, 2012; Tranquilli et al., 2014). Besides, PE is clinically subtyped based on gestational age into early- and late-onset PE (Tranquilli et al., 2013; Magee et al., 2014; Mayrink et al., 2018). Early-onset PE is defined by new-onset hypertension before or at 33 weeks plus 6 days of gestation while late-onset PE is defined by new-onset hypertension at or after 34 weeks of gestation (Poon et al., 2010; Magee et al., 2014; Mayrink et al., 2019). Severe clinical manifestations of PE results in increased admission to intensive care units and may warrant early fetal-placental delivery to prevent complications that might result in fetal or maternal death (Jeyabalan, 2013; Magee et al., 2014).

To date the precise cause of PE is elusive and there is no screening test that has a high specificity and sensitivity to predict PE (Mayrink et al., 2019). However, several studies including earlier studies from our group have associated the pathogenesis of PE to maternal-fetal/placental factors (Ramesar et al., 2012; Gathiram and Moodley, 2016; Pillay et al., 2017; Karumanchi, 2018). The placenta seems to be a central agent in the etiology of PE and adequate research in this direction is essential. Nevertheless, the pathophysiological processes underlying PE have been primarily described in two stages. The first involves defective placental perfusion, possibly due to impaired placentation with abnormal trophoblast invasion and inadequate remodeling of the uterine spiral arteries, with placental hypoperfusion, hypoxia, and ischaemia resulting in decidual pathology. The second stage, referred to as the maternal syndrome, is characterized by systemic manifestations of inflammatory, metabolic, and thrombotic responses converging to promote vascular dysfunction which may lead to multi-organ damage (Ramesar et al., 2012; Baijnath et al., 2014; Mayrink et al., 2019). Cumulative evidence identified clinical risk factors (blood pressure, proteinuria, uterine-artery Doppler velocimetry, low platelet count, hemolysis, and elevated liver enzymes) and biochemical markers (pro-angiogenic and anti-angiogenic factors, cell free fetal DNA, cytokines, high-temperature-requirement A^3 enzyme, placental proteins, elevated lipid profile among others) as predictive markers of PE (Gathiram and Moodley, 2016; Pillay et al., 2017, 2019; Karumanchi, 2018; Mayrink et al., 2019). Current data has recognized that placental-derived exosomes are potential biomarkers of PE (Pillay et al., 2017, 2019). Nevertheless, the etiology and an accurate screening test for early diagnosis, treatment and prevention, particularly in the nulliparous group that is at high risk of PE, remains elusive.

Immunological and metabolic maladaptations are the critical pathophysiological conditions associated with PE (Mor et al., 2011, 2017; Perez-Sepulveda et al., 2014; Bounds et al., 2015). Several studies have reported that early-onset PE is predominantly associated with an abnormal immune response (Laresgoiti-Servitje, 2013; Bounds et al., 2015; Mor et al., 2017; Lv et al., 2019), while late-onset is largely associated with metabolic perturbation owing to an imbalance between the metabolic demands of the developing fetus and maternal supply (Racicot et al., 2014; Verlohren et al., 2014; Erez et al., 2017; Lokki et al., 2018; Lv et al., 2019). However, considering the possible role of placental microbiome in the modulation of immune response and metabolic function in normal pregnancy (Belkaid and Hand, 2014; Mor and Kwon, 2015; Parnell et al., 2017; Pelzer et al., 2017; Giessen et al., 2020), we postulate that alterations in placental microbiome status contribute to PE through immunological and metabolic disruption. However, a growing body of data corroborates the concept of both uterine and placental communities of low biomass as discussed in the earlier part of the present review, but to predict and understand the contribution of the placental microbiome to PE, knowing the bacteria that are present matters less than knowing how the bacteria are capable of interacting with the host (Perez-Sepulveda et al., 2014; Mor et al., 2017). Interestingly, a recent analysis of placental gene expression in PE also implicates alteration in the expression of receptors on myeloid cells-1, metalloprotease INHA and lactotransferrin which correlate with changes obtainable during infection (Vigliani and Bakardjiev, 2014; Brew et al., 2016; Di Simone et al., 2017; Fillerova et al., 2017). These form the basis for the recent suggestion of antibiotics as a potential preventative measure for PE (Fillerova et al., 2017; Kenny and Kell, 2018).

ROLE OF PLACENTAL MICROBIOME IN TOLERANCE AND PLACENTAL ADAPTATION

The placental microbiome has been reported to exert metabolic and immune regulatory functions in normal pregnancy as reported previously (Aagaard et al., 2014; Belkaid and Hand,

2014; Mor et al., 2017; Pelzer et al., 2017). Any perturbation in the composition or local balance of placental microbiome can lead to an unhealthy dysbiotic state, that may be associated with impaired immunoresponse/metabolic function resulting in adverse pregnancy outcomes particularly PE (Schoenmakers et al., 2018). Amarasekara et al., reported communities of pathogenic commensals such as *Variovorax* sp., *Anoxybacillus* sp., *Prevotella* sp., *Bacillus cereus*, *Escherichia* sp., *Klebsiella pneumoniae*, *Porphyromonas* spp., *Listeria* sp., *Salmonella* sp., and *Dialister* sp. in PE (Amarasekara et al., 2015) are in contrast distinct from the non-pathogenic commensals present in the placentae of normal pregnancies (Aagaard et al., 2014). The former was associated with infection, which possibly triggers inflammatory cells (**Figure 3**) and an imbalance between pro-angiogenic and anti-angiogenic factors. This could result in a series of events, such as impaired trophoblast function leading to endothelial dysfunction and placental hypoxia/ischaemia, which elevates maternal blood pressure causing PE (Amarasekara et al., 2015). In the light of this premise, it seems that the enrichment and diversity of commensals that are present in normal and pre-eclamptic pregnancies differ. The cause of this variation is not clear. Previous studies have linked adverse pregnancy outcomes, particularly preterm deliveries, to gut microbial remodeling and dysbiosis that precedes resultant loss of protective and homeostatic modulation (Shiozaki et al., 2014; Yoneda et al., 2016). Likewise, microbial products including bacterial DNA, which do not necessarily reflect live bacteria, reported to be sufficient to initiate adverse outcomes in pregnancy (Pelzer et al., 2017). Nevertheless, further studies are required to understand the cause of the variation in diversity and enrichment of the placental microbiome between normotensive and pre-eclamptic pregnancies, as it is currently unclear. This may lead to clinical predictive success and early diagnosis of PE.

In addition, the microbial products such as lipopolysaccharide (inflammagenic molecules) from the commensal bacteria including placental commensals have been found to play a major role in PE naturally or *in vivo* (Lin et al., 2012; Cotechini et al., 2014; Kell and Kenny, 2016). Lipopolysaccharide from gram-negative bacteria possibly activates TLRs particularly TLR3, TLR4, TLR7, and TLR8, inducing NF-kB (inflammatory mediator), while lipoproteins (LPP) or peptidoglycans from gram-positive bacteria activates TLR2, which leads through a cascade of intermediary steps to NF-kB activation (**Figure 3**) and thus, collectively initiating the pathogenesis of PE, including abnormal placentation and the maternal syndrome (Cotechini et al., 2014; Kell and Kenny, 2016). Although reports indicate that only humans are afflicted by PE (McCarthy et al., 2011; Xue et al., 2015), evidence from experimental animals, using a low dose infusion of lipopolysaccharide, show a pre-eclamptic-like syndrome, which includes hypertension, proteinuria,

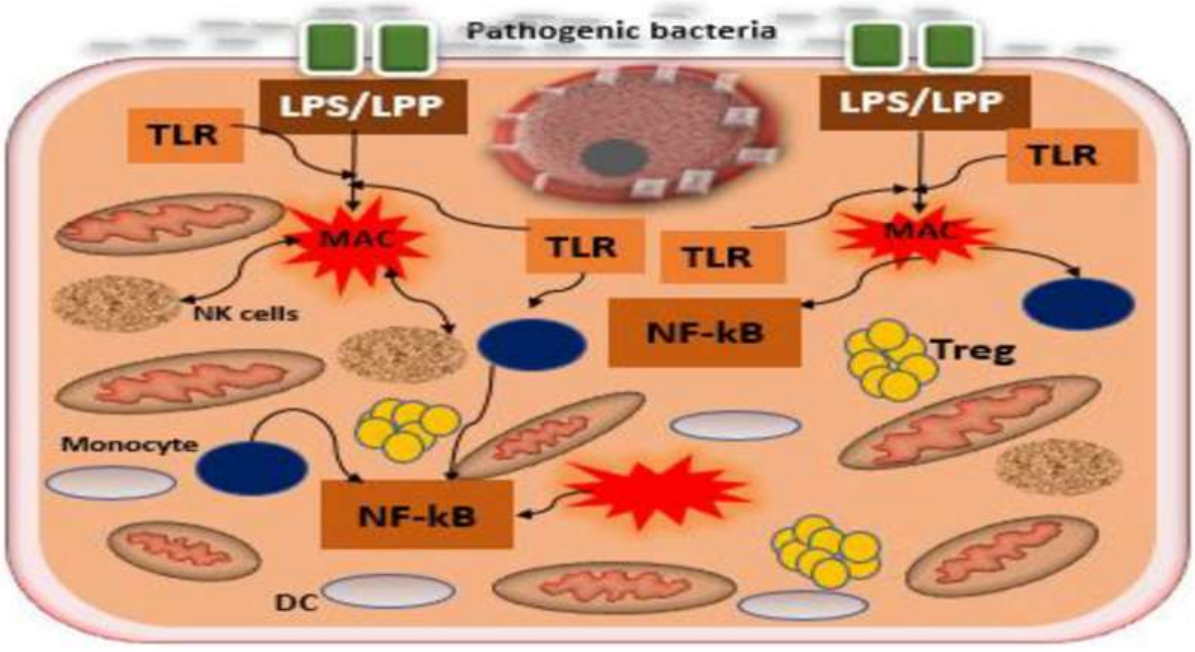

FIGURE 3 | Interaction between pathogenic bacteria and toll-like receptors of the trophoblast in the formation of inflammatory cascades. Pathogenic bacteria, including the Gram-positive and Gram-negative present at the epithelium of the uterus promote the induction of inflammatory cascades by the interactions between the bacterial products LPP/LPS and toll-like receptors, TLR2/TLR4 expressed by trophoblast. Dysbiosis of placental microbiome increases the growth of pathogenic bacteria disrupting symbiosis among microbiota, trophoblast, and immune cells in the uterus, leading to inflammatory cascades that have been recognized in the pathogenesis of PE. Tregs, T regulatory cells; TLR, toll-like receptor; DC, decidual cell; LPS, lipopolysaccharide; LPP, lipoproteins; MAC, macrophage.

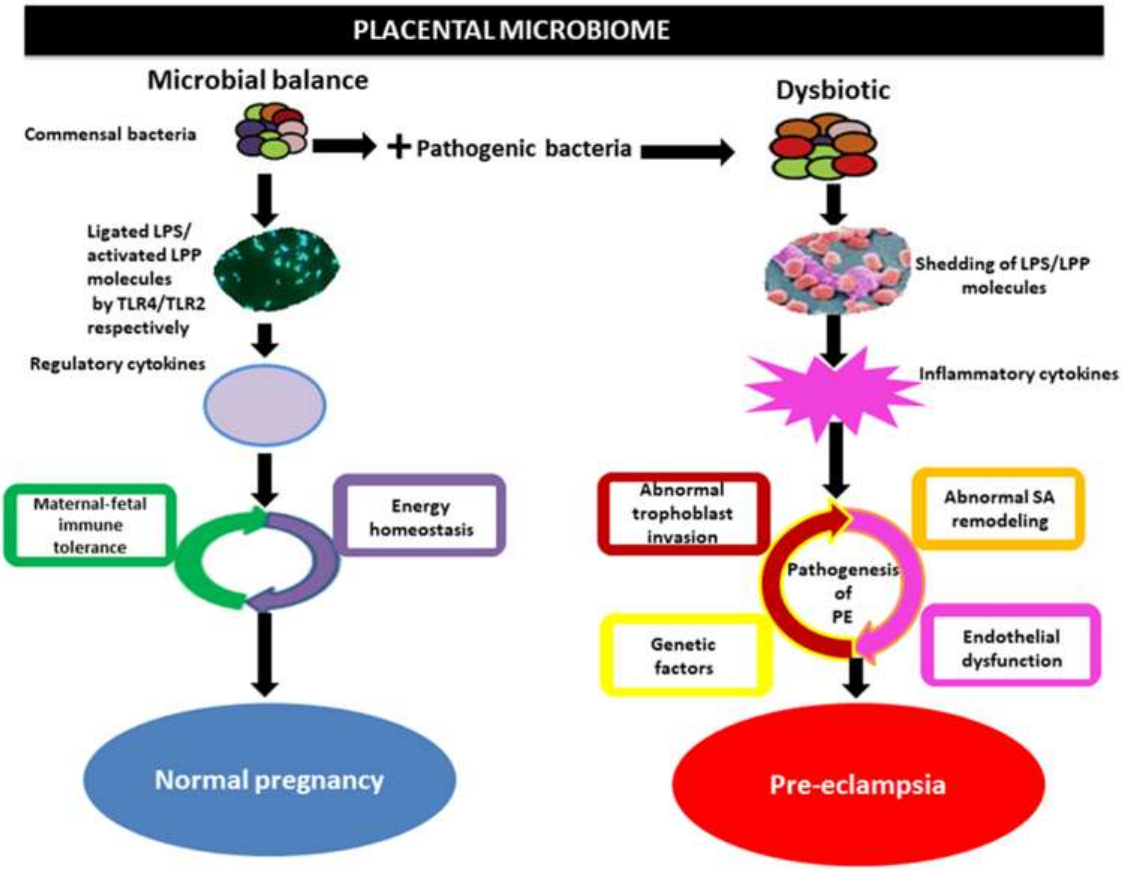

FIGURE 4 | Implication of the placental microbiome in pre-eclampsia. Alteration of placental structures could result from the direct action of pathogenic bacteria through the release of lipopolysaccharide (LPS) or lipoproteins (LPP), which activates inflammatory cytokines via interactions with toll-like receptors (TLRs especially TLR4/TLR2). This invokes the pathogenic process of pre-eclampsia. Whereas, under physiological condition commensal bacteria release bacterial products such as LPS/LPP from gram-negative and positive bacteria, respectively, which is ligated by toll-like receptors (TLR4) and activated by TLR2 located on the surface of trophoblast. This in turns activates regulatory cytokines to promote tolerogenic microenvironment (maternal-fetal immune tolerance) and maintains energy homeostasis in normal pregnancy. SA, spiral artery.

thrombocytopenia, increased anti-angiogenic factors, endothelial dysfunction, and elevated liver enzymes among others (Cotechini et al., 2014; Lip et al., 2017; Li et al., 2019). This implies that the lipopolysaccharide molecule is among the main mediators of PE (**Figure 4**). Moreover, placental microbial dysbiosis may also disrupt the metabolism of tryptophan and fatty acids, causing impaired maternal-fetal energy homeostasis, which possibly initiate or exaggerate pre-eclamptic events, especially during late gestation, hence, causing severe PE.

CONCLUSION

An extensive review of the literature on the placental microbiome has been explored, including studies that have linked pregnancy complications especially PE to microbial alterations in the placenta of the host. The present review demonstrates that placental microbes play an important etiological role in PE by possibly shedding inflammagenic molecules such as lipopolysaccharide or lipoproteins, with a resultant inflammatory cascade that accompanies abnormal placentation and maternal endothelial cell activation.

FUTURE RESEARCH

Further studies with DNA-based techniques and well-controlled procedures are required to identify specific commensal bacteria that are possibly responsible for PE. Besides, the assessment of the clinical utility of early or pre-emptive antibiotics to reduce PE needs to be explored. We also like to propose a study that will determine the role of placental microbiome in early- and late-onset PE. It is also essential to combine the microbiome research with whole-genome sequencing, as well as metagenomics and metabolomics in order to adequately understand and interpret

data regarding the potential effects of the microbiome on maternal immune response, metabolism, and epigenetics.

AUTHOR CONTRIBUTIONS

KO and IM conceived the project. KO drafted the review article under the supervision of IM and JM. KO, IM, JM, and YM read, revised, and approved the final review article. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER 3: MANUSCRIPT TWO

Evaluation of the Human Placental Microbiota in Early- and Late-Onset Pre-eclampsia (Published: High Blood Pressure and Cardiovascular Prevention, 2024:1-9).

This publication assesses the role of the placental microbiota in the aetiology of pre-eclampsia (PE), a pregnancy-specific hypertensive disorder with an unclear pathogenesis. Despite decades of research, the precise mechanisms underlying PE remain elusive, and the contribution of the placental microbiota to pregnancy complications, particularly PE, remains controversial. To address this, we investigated the microbial composition of placental tissues from women with early- and late-onset PE compared to age-matched normotensive controls. Using standardized sterile clinical procedures, placental tissues were collected via cesarean section, followed by DNA extraction and microbiome analysis through targeted 16S sequencing and bioinformatics. The findings revealed a significant difference in blood pressure between early-/late-onset PE and normotensive controls. Microbial classification identified bacterial phyla including *Actinobacteria, Firmicutes*, *Bacteroidetes*, *Proteobacteria,* with *Proteobacteria* dominated by the classes *Pseudomonadales* and *Gammaproteobacteria* with smaller amounts of *Actinobacteria* and *Bacteroidetes.* However, no significant differences were observed in bacterial composition between early-/late-onset PE and normotensive controls, and no correlation was found between specific bacterial species and PE onset. These results suggest a low biomass of bacterial species in placental samples, indicating that bacterial presence in the placenta is minimal and does not correlate with early- or late-onset PE. This study contributes to the ongoing debate on the existence and role of the placental microbiota in pregnancy complications and highlights the need for further research into alternative mechanisms underlying PE pathogenesis.

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ORIGINAL ARTICLE

Evaluation of the Human Placental Microbiota in Early- and Late-Onset Pre-Eclampsia

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Abstract

Introduction Despite many decades of research, the exact etiology of pre-eclampsia (PE) remains unknown. Several etiopathologies have been suggested, including the role of the placental microbiota. However, the existence of placental microbiota and its possible contribution to pregnancy complications, particularly PE has remained controversial.

Aim The present study was designed to identify different microbes that co-exist the placenta of women with early- and late-onset PE.

Methods Thirty age-matched normotensive and early-onset as well as age-matched normotensive and late-onset pre-eclamptic women respectively, were recruited. After obtaining an informed consent, the placental tissues were obtained through caesarian section with sterile and standardized clinical procedures. DNA was extracted from each tissue and microbiome analysis was conducted using a targeted 16 S analysis and the reads were analyzed with bioinformatics.

Results There was a significance difference between the blood pressure of early-/late-onset PE compared with age-matched normotensive controls, respectively. In addition, the reads from placencental samples were classified as belonging to the phyla, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, with *Proteobacteria* dominated by the classes *Pseudomonadales* and *Gammaproteobacteria* with smaller amounts of *Actinobacteria* and *Bacteroidetes*. There was no significant difference between the placental bacterial species of early-/late-onset PE compared with age-matched normotensive controls, respectively. Further analysis found no correlation between bacterial species and early- or late-onset PE.

Conclusion The present results demonstrate a low biomass of bacterial species, which might further indicate that the placental samples had very low levels of bacteria species and there is no correlation between the bacterial composition and early- or late-onset PE.

Keywords Hypertension · Microbiome · Placenta · Pre-eclampsia · Pregnancy

1 Introduction

Pre-eclampsia (PE) is a pregnancy-specific hypertensive disorder that is independently the major cause of maternal-fetal morbidity and mortality. It is a multisystem syndrome that is commonly characterized but not always by proteinuria [1, 2]. Pre-eclampsia develops after 20 weeks of pregnancy and usually near pregnancy term de novo or superimposed to chronic hypertension [3, 4]. In addition, PE is a global burdened complication that affects 2–10% pregnancies worldwide and results in 50 000–60 000 deaths annually [5, 6]. Studies have shown that PE may be associated with pulmonary edema, impaired renal, liver, cerebral and visual functions in the mother [7, 8]. It may also be accompanied by fetal growth restriction and predisposes maternal and fetus to the risks of development of cardiovascular complications

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and diabetes mellitus later in life [3, 9]. Based on its pathogenesis and clinical manifestations, PE is divided into early- and late-onset which occurs before and after 34 weeks of gestation, respectively.

The pathophysiological mechanism has been linked to incomplete transformation of spiral arteries resulting in hypoperfusion of the placenta which leads to impaired nutrient supply to the fetus. In addition, inappropriate levels of anti-angiogenic factors (sEng and sFlt-1)/pro-angiogenic factors (VEGF, PlGF and TGFβ) and generalized maternal endothelial dysfunctions has been associated with the pathophysiology [10–12]. Similarly, PE also shares common pathophysiologic pathways with cardiovascular diseases such as complement components dysregulation like CD93 [13]. However, the etiology of PE remains unclear despite decades of research, which restricts its management to the reduction of blood pressure or delivery of the fetus and placenta.

It is now apparent from recent scientific evidence that the microbiome modulates immune function, metabolism and behavior in humans and animals. It is likewise evident that placenta as a driver of fetal destiny is not just a sterile organ but rather has its own endogenous microbiome [14]. Although earlier cultivation-dependent studies significantly underestimate the incidence of microbial presence within the placenta, which is likely due to the presence of bacteria that are hard to culture due to their preference for anaerobic environments or requirement for specific unidentified nutrients [15]. However, DNA-based studies provide evidence for the presence of a low biomass endogenous microbial community within the placenta [16–18]. The placental microbiome from healthy term deliveries has a high abundance of Lactobacillus spp., Propionibacterium spp. and members of the Enterobacteriaceae family [16, 17]. Lactobacilli spps are less abundant in placental tissues of preterm deliveries, possibly supporting a role for these genera in positive pregnancy outcomes. In addition, gut and vaginal microbiota have been revealed as the primary sources of placental microbiome [17, 19]. Reports also show that gut microbiota has a strong relationship with cardiovascular disease and hypertension [20, 21].

Placental microbiome is important in fetal growth, immune function or development [14, 22]. Earlier studies in pregnancies have shown that gut microbiota significantly contributes to the metabolic changes observed in the mother and interaction between the human host and her microbes such as intraamniotic immune response also affects pregnancy outcome [23]. Placental microbiome drives early immune development and is emerging as a source for antigenic determinants in neonates or fetus [17]. Recently, La et al. reported that the placental samples of women with gestational diabetes were enriched with abundance of genera *Bifidobacterium*, *Duncaniella* and *Ruminococcus* and possibly contributing to adverse pregnancy outcomes [24]. However, the contribution of placental microbiota to adverse pregnancy particularly pre-eclampsia is inconclusive. The present study was designed to identify different microbes that co-exist the placenta of women with early- and late-onset PE, and the possible involvement of these microbes in the pathogenesis of PE.

2 Methods

2.1 Study Population

One hundred and twenty (120) pregnant women who are monitored and met eligibility criteria as well as signed a written informed consent at the Maternity Clinic of Prince Mshiyeni Memorial Hospital in the Durban South, South Africa were recruited for the study. The study was performed in accordance with applicable guidelines and regulations and ethical approval was obtained from the Biomedical Review Ethical Committee of the University of Kwazulu-Natal, Durban, South Africa (BE253/19). Criteria for inclusion as early- and late-onset PE were gestational age of 24–33 and > 34 weeks, respectively with hypertension (defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg) and accompanied by proteinuria (defined as protein in a 24-hour urine collection ≥ 300 mg) and/or evidence of thrombocytopenia (low blood platelet count of ≤ 50 000 per microliter). While criteria for inclusion for age-matched normotensive pregnancies are normal blood pressure (values under 140/90 mmHg during pregnancy) and blood platelet count. Exclusion criteria were chronic hypertension, cardiovascular or renal disease, diabetes mellitus, bleeding disorders, HIV infection and previous medical conditions as well as non-consenting pregnant women. A questionnaire survey was administered to collect women's demographic information and information about mode of delivery was extracted from the medical records.

2.2 Sample Collection

A total of 120 placental samples were tested in this study. The placental samples were collected through a caesarian section and transferred to a trained theater nurse in a sterile environment of the operating and delivery room. Sterile scissors were used to take a segment of the placenta (1 × 1 cm) from the basal plate (maternal side) and the center point of the placenta. These pieces were immediately put in a sterile cryopreservation tube and placed in a −80◦C biofreezer.

2.3 Sample Preparation and DNA Extraction

Placental tissue samples (100 mg each) were homogenized using a tissue homogenizer. Total DNA was extracted by using the QIAamp DNA body fluid and tissue kit (Qiagen) in accordance to the manufacturer's instructions, with an additional bead-beating step with MPBio Lysing Matrix B beads to minimize Gram-negative extraction bias. NanoDrop NC2000 spectrophotometer and agarose gel electrophoresis were used to determine the quantity and quality of extracted DNAs respectively. All DNAs were stored at -80 °C until it is required for further analysis.

2.4 16 S rRNA gene Amplicon Sequencing and Analysis

Metagenomic analysis was performed as previously described (Huang et al., 2021). In brief the The V3-V4 region of the 16 S rRNA gene was PCR-amplified from microbial genomic DNA using primers (forward primer, 5′-GTG CCA GCM GCC GCG GTA A-3′; reverse primer, 5′-GGA CTA CHV GGG TWT CTA AT-3′). The PCR products were detected using dual-indexing amplification and sequencing approaches on the Illumina MiSeq plat-form. Mean quality value across each base position in all the reads were high with the expected read length of 300 bp for both forward and reverse reads.

2.5 Bioinformatic Analysis

For metagenomics analysis, a R script (R v.4.1.2 (2021-11-01)) was developed in RStudio that implemented several packages specific for microbiome analysis. Sequencing data was filtered to remove low-quality sequencing reads and to trim the reads to a consistent length. Most Illumina sequencing data shows a trend of decreasing average quality towards the end of sequencing reads. Based on previous studies [25]. We trimmed forward reads at position 280, and reverse reads at position 200. The first 10 nucleotides of each read were also removed based on empirical observations across many Illumina datasets that these base positions are particularly likely to contain errors. After filtering, the typical amplicon bioinformatics workflow clusters sequencing reads into operational taxonomic units (OTUs): groups of sequencing read's that differ by less than a fixed dissimilarity threshold. In this analysis high-resolution DADA2 (v.1.22.0) method [26] was used to infer amplicon sequence variants (ASVs) exactly, without imposing any arbitrary threshold, and thereby resolving variants that differ by as little as one nucleotide. The error model in the sequence inference algorithm does not include a chimera component, and therefore we expect the sequence table to include many chimeric sequences. Chimeric sequences were removed by comparing each inferred sequence to the others in the table and removing those that can be reproduced by stitching together two or more abundant sequences. Taxonomic assignments were done with the DECIPHER package [27] with the Silva reference database version 138 based on previous study [25]. After amplicon processing with DADA2, the typical standard outputs are a fasta file, a count table, and a taxonomy table. This data was converted to the Phyloseq data structure for subsequent statistical analysis [28].

2.6 Statistical Analysis

GraphPad Prism 9 was used to analyze the clinical characteristic data and the data were represented as mean ± SEM. Comparison between mean values was performed using one-way ANOVA and followed by Bonferroni *post hoc* test with a p-value less than 0.05 considered statistically significant. For metagenomic data, a R script (R v.4.1.2 (2021-11-01) was developed with bioinformatics method and permutational analysis of variance (PERMANOVA) was used to compared non-parametric data.

3 Results

3.1 Clinical Characteristics of Participants

There was a significant increase in SBP and DBP of early-onset PE and late-onset PE compared with early normotensive pregnancy and late normotensive pregnancy, respectively. Where as there was no significant difference between the SBP and DBP of late-onset PE compared with early-onset PE. There was no significant difference in age, gestational age, weight, hemoglobin concentration, parity and gravidity of early-onset PE and late-onset PE compared with early normotensive pregnancy and late normotensive pregnancy respectively. Where as the gestational age of late-onset PE was significantly higher than early-onset PE (Table 1).

3.2 Microbial Profile

Analysis of the 16 S rRNA metagenomics dataset from placentae of women with early- or late-onset PE and gestational age-matched normotensive with the DADA2 microbiome analysis pipeline. DADA2 describes microbial communities using unique sequence variants present in the data, known as amplicon sequence variants (ASVs), rather than clustering groups of similar sequences into operational taxonomic units (OTUs) used by many other pipelines. Sequencing data was of sufficient quality and quantity for the study (Table 2),

Table 1 Clinical characteristics of participants

	N_E	N_L	PE_E	PE_L
SBP (mmHg)	108.8 ± 1.4	106 ± 4.3	140.3 ± 2.6*	146.5 ± 2.0^
DBP (mmHg)	71.1 ± 1.1	74.2 ± 1.4	91.0 ± 1.6*	95.5 ± 1.3^
Age (years)	29.4 ± 1.4	28.9 ± 1.0	31.7 ± 1.1	31.5 ± 1.0
Gestational age (weeks)	24.4 ± 0.2	37.8 ± 0.3	25.7 ± 0.4	35.8 ± 0.9#
Hemoglobin (g/mol)	10.9 ± 0.3	10.5 ± 0.2	10.9 ± 0.3	10.7 ± 0.2
Weight (kg)	88.6 ± 2.1	91.6 ± 1.6	100.0 ± 2.9	92.7 ± 3.8
Parity	1.3 ± 0.2	1.8 ± 0.3	1.4 ± 0.1	1.5 ± 0.1
Gravidity	2.2 ± 0.2	2.8 ± 0.3	2.5 ± 0.1	2.7 ± 0.1

Values are expressed as mean ± SEM (*$p < 0.05$ vs. N_E ^$p < 0.05$ vs. N_L, #$p < 0.05$ vs. PE_E). Early normotensive pregnancy (N_E); Late normotensive pregnancy (N_L); Early-onset pre-eclampsia (PE_E); Late-onset pre-eclampsia (PE_L);

Table 2 Summary of the data after Dada2 analysis

Metric	Results
Min. number of reads	3560
Max. number of reads	2.9157×10^4
Total number of reads	7.43319×10^5
Average number of reads	1.6518×10^4
Median number of reads	1.662×10^4
Min. number of ASVs	124
Max. number of ASVs	674
Total number of ASVs	8272
Average number of ASVs	426
Median number of ASVs	414
Number of ASVs = NA	858
Sparsity	0.949
Any ASVs sum to 1 or less?	TRUE
Number of singleton ASVs	1327
Percent of ASVs that are singletons	16.042
Number of sample variables are:	6 (SampleID, Condition, Time, SampleNo, SampleType, Group)

and the data was analyzed using analysis pipeline. Majority of amplicons were classified as belonging to the phyla, *Actinobacteriota*, *Firmicutes*, *Bacteroidetes*, *Cyanobacteria* and *Proteobacteria*, with *Proteobacteria* dominated by the classes *Alphaproteobacteria* and *Gammaproteobacteria* (Figs. 1, 2, 3, 4 and 5). PERMANOVA evaluates the hypothesis that the centroids and dispersion of the community are equivalent between the compared groups. We compared the sample groups early- and late-onset PE with age-matched normotensive controls and there was no significant difference. Further analysis found no correlation between bacterial species and early- or late-onset PE (Figs. 6, 7, 8, 9 and 10).

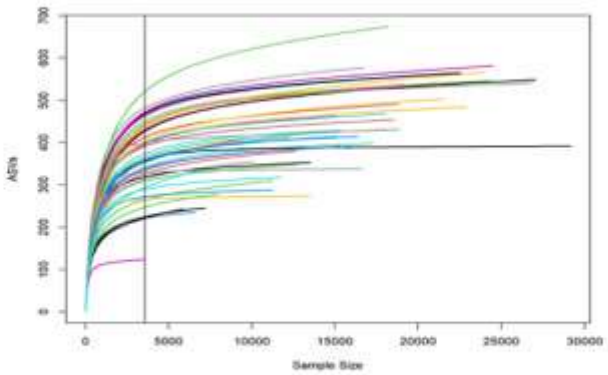

Fig. 1 Rarefaction curve of amplicon sequence variants. The rarefaction curve is a plot of the number of species against the sequence of the samples. This curve is created by randomly re-sampling the pool of N samples several times and then plotting the average number of species found in each sample. Generally, it initially grows rapidly (as the most common species are found) and then slightly flattens (as the rarest species remain to be sampled). The x-axis indicates the number of valid sequences and the y-axis shows the observed species in ASVs. Each curve represents a different monitoring sample and the vertical straight line indicates the minimum number of sequences found in a sample. Amplicon sequence variants (ASVs)

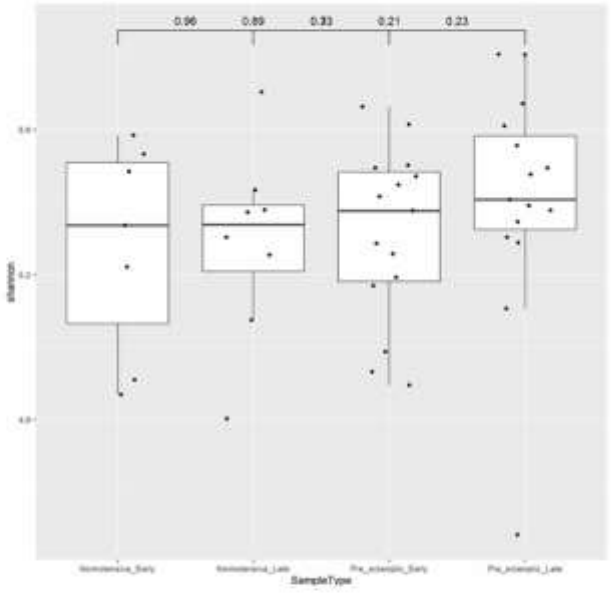

Fig. 2 Shannon index. This diversity index is a quantitative indicator of the number of different bacteria that are present in each group. Shannon index is compared between different groups with p-values > 0.05 (PE_E vs. N_E), (PE_L vs. N_L) and (PE_L vs. PE_E). Sample groups are: Normotensive early (N_E), Normotensive late (N_L), Pre-eclamptic early- and Pre-eclamptic late-onset (PE_E and PE_L)

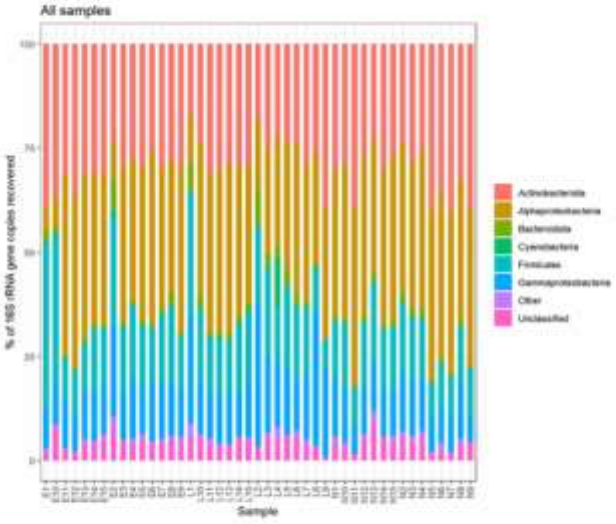

Fig. 3 Taxonomic summary. Table of proportions of each Phylum, and breakdown of the Proteobacteria to the class level. Only taxa that make up greater than 5% in any individual sample were used

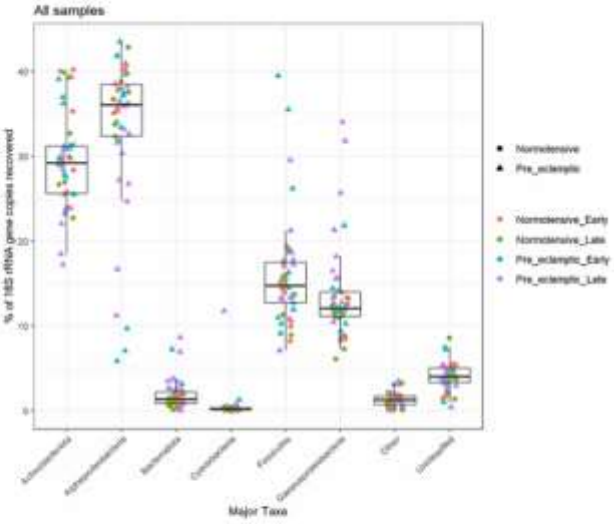

Fig. 5 Taxonomic summaries of all samples. Sample groups are: Normotensive early (N_E), Normotensive late (N_L), Pre-eclamptic early- and Pre-eclamptic late-onset (PE_E and PE_L)

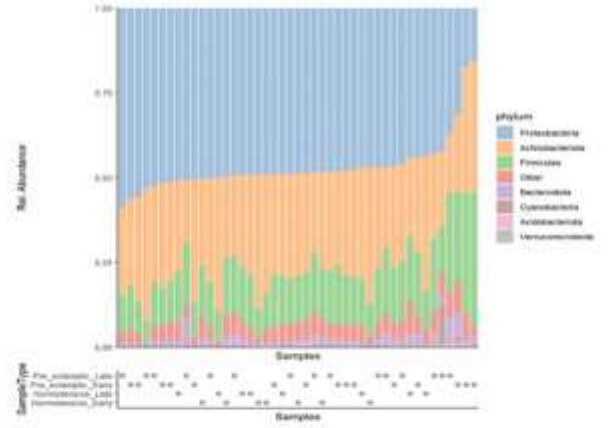

Fig. 4 Composition bar plot. Relative abundance is calculated, top taxa are retrieved for the Phylum rank and the barplot is visually ordered by rank by abundance. Sample groups are: Normotensive early (N_E), Normotensive late (N_L), Pre-eclamptic early- and Pre-eclamptic late-onset (PE_E and PE_L)

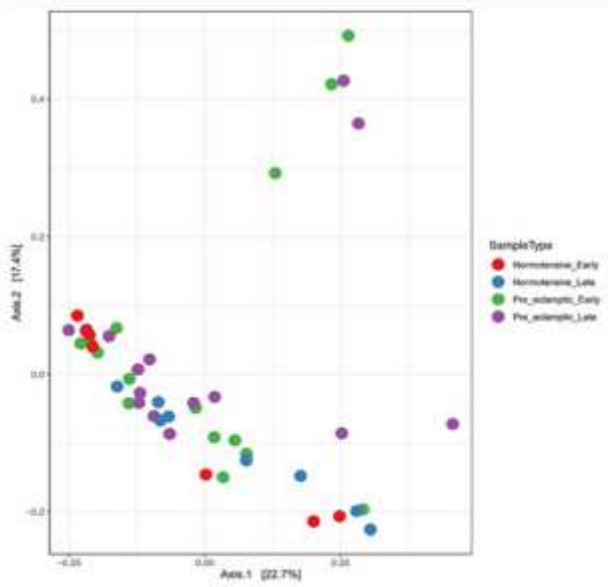

Fig. 6 Nonmetric multidimensional scaling (MDS) plot. Sample groups are: Normotensive early (N_E), Normotensive late (N_L), Pre-eclamptic early- and Pre-eclamptic late-onset (PE_E and PE_L)

4 Discussion

Regardless of decades of research, the etiology of PE remains unclear, limiting the management to suboptimal level [29, 30]. In addition, the association of pregnancy complications and adverse pregnancy outcomes with disruption of gut or placental microbiota [14, 20, 21] formed the basis for futher investigation. The presnt study investigated whether there are reproducible differences in the placental microbiota of women that are normotensive, or with early- or late-onset PE, and assessed the potential functional impact of the differences in microbiota, inso far as they are detected. Similarly the study produced an overall view of the base composition and the diversity of placental microbiota in normotensive controls and early- or late-onset PE. The results of the study therefore demonstrate a significance difference between the blood pressure of early- and late-onset PE compared with age-matched normotensive controls, respectively. Additionally, the reads from placencental

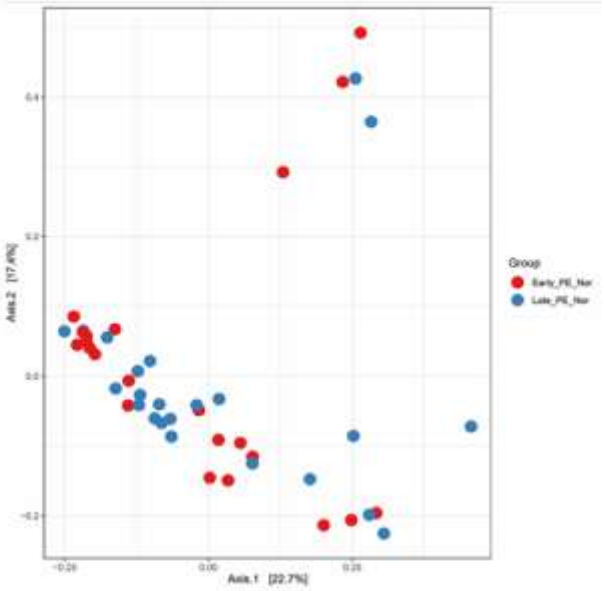

Fig. 7 Nonmetric multidimensional scaling (MDS) plot. Sample groups are: Normotensive early (N_E), Normotensive late (N_L), Pre-eclamptic early- and Pre-eclamptic late-onset (PE_E and PE_L). x-axis is MDS1 and y-axis is MDS2

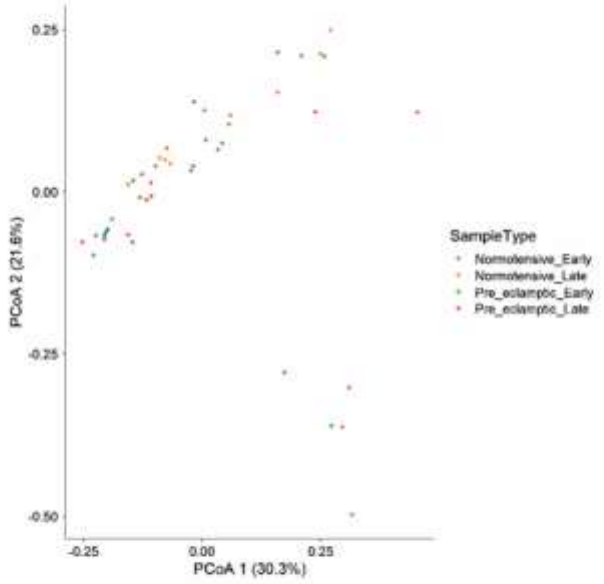

Fig. 8 Principal coordinates analysis plot. PCoA was performed, and the first two principal coordinates, PCoA1 and PCoA2 plotted. Sample groups are: Normotensive early (N_E), Normotensive late (N_L), Pre-eclamptic early- and Pre-eclamptic late-onset (PE_E and PE_L). x-axis is PCoA1 and y-axis is PCoA2

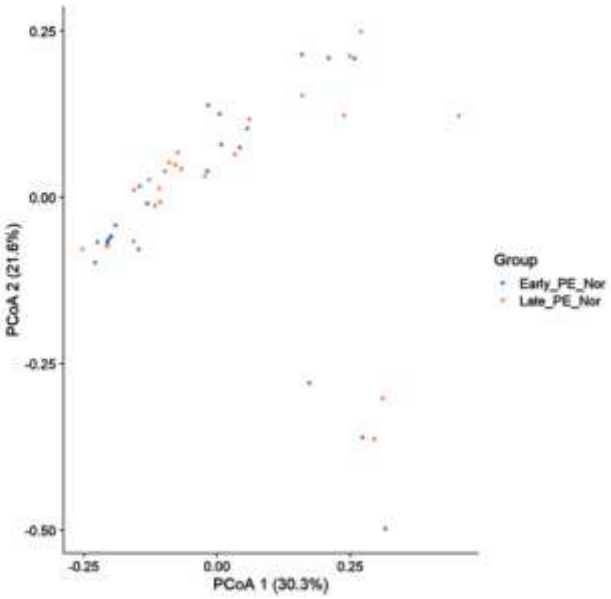

Fig. 9 Principal coordinates analysis plot. PCoA was performed, and the first two principal coordinates, PCoA1 and PCoA2 plotted. Sample groups are: Normotensive early (N_E), Normotensive late (N_L), Pre-eclamptic early- and Pre-eclamptic late-onset (PE_E and PE_L). x-axis is PCoA1 and y-axis is PCoA2

samples were classified as belonging to the phyla, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, with *Proteobacteria* dominated by the classes *Pseudomonadales* and *Gammaproteobacteria* with smaller amounts of *Actinobacteria* and *Bacteroidetes*. There was no significant difference between the placental bacterial species of early- and late-onset PE compared with age-matched normotensive controls, respectively. Further analysis found no correlation between bacterial species and early- or late-onset PE.

The refraction curve of amplicon sequencing variants show a deflection in all the groups' samples, which indicate the number of species found in each of the groups' samples. The results show that the placental samples from age-matched normotensive controls and early- or late-onset PE contain a number of species (Fig. 1). This is also confirmed by Shannon index, which quantitatively indicate the diversity of bacterial species present in all the groups (Fig. 2). There is slight variation in diversity of bacterial species found in early- or late-onset PE and age-matched normotensive controls but not significant following comparison. The above observation was in consonance with earlier studies that reported the presence of bacterial species or RNA in the placental tissue [24, 31], though without significant differences between early- or late-onset PE and age-matched normotensive controls. In this study, taxonomic summaries reveal that majority of bacteria is classified as belonging to the phyla, *Actinobacteriota*, *Firmicutes*, *Bacteroidetes*,

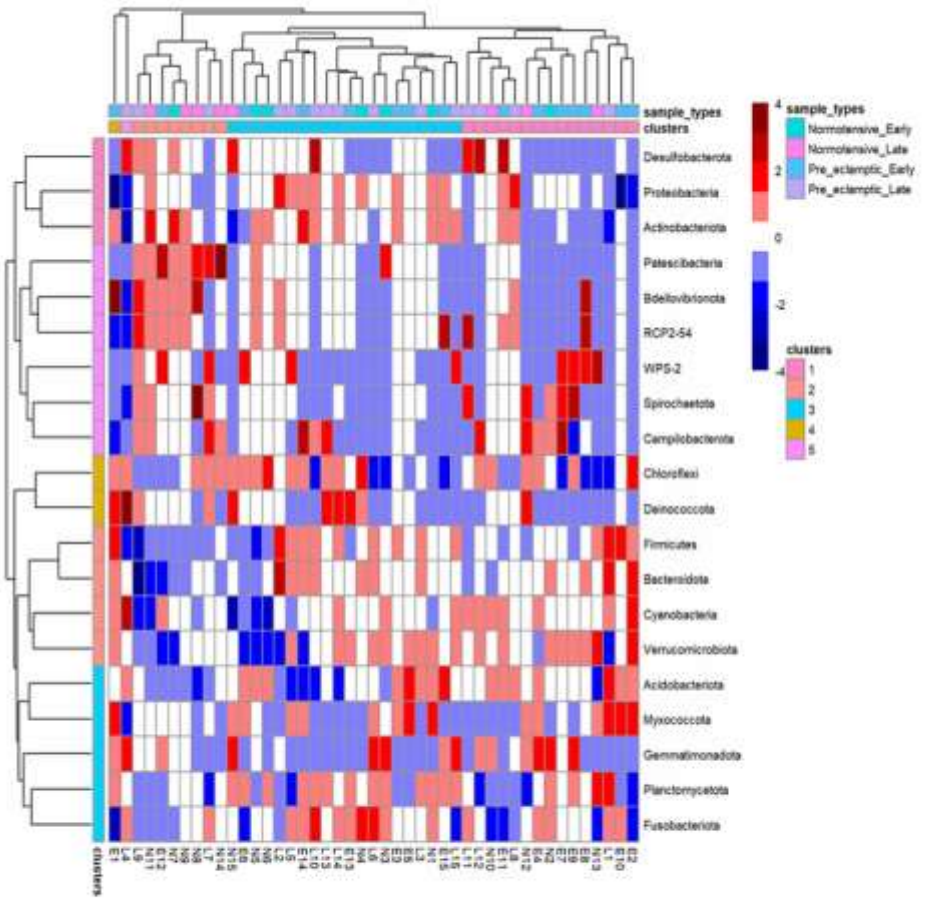

Fig. 10 Composition heatmap. Community composition can be visualized with a heatmap, where the horizontal axis represents samples and the vertical axis the taxa. Color of each intersection point represents abundance of a taxon in sample representative from the groups. Here, abundances are first CLR (centered log-ratio) transformed to remove compositionality bias. Then Z transformation is applied to CLR-transformed data. This shifts all taxa to zero mean and unit variance, allowing visual comparison between taxa that have different absolute abundance levels. After these rough visual exploration techniques, we can visualize the abundances at Phylum level. Sample groups are: Normotensive early (N_E), Normotensive late (N_L), Pre-eclamptic early- and Pre-eclamptic late-onset (PE_E and PE_L)

Cyanobacteria and *Proteobacteria* (Figs. 3, 4 and 5). The *Proteobacteria* is further dominated by the classes *Alphaproteobacteria* and *Gammaproteobacteria*. Aagaard and co-authors first demonstrated the consistent presence of a microbiome in placentas from healthy pregnancies composed of non-pathogenic commensal microbiota from the *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria* phyla [32]. However, this is disputed by several studies claiming that the human placenta is sterile and does not have a microbiome [18, 33–35].

In a recent study by Olomu et al., 2020, studying the existence of the placental microbiome in gestational diabetes mellitus, they reported that the placental microbiome was dominated by the phyla *Proteobacteria* and *Firmicutes*, with smaller amounts of *Actinobacteria* and *Bacteroidetes*. In their study they included positive vaginal-rectal control samples, and these were dominated by members of the phylum *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, suggesting that the placental microbiome observed might be due to contamination from the vaginal-rectal microbiome. In their study, they promote the use of both negative and positive controls for such studies [36]. In the present data set we found similarly that the phyla *Proteobacteria* and *Firmicute* dominate, but in contrast we have elevated levels of *Actinobacteria* and *Bacteroidetes* (Fig. 3).

Similarly, MDS and PCA plots (Figs. 6, 7, 8 and 9) do not show any separation of the data based on phenotype. We compared the sample groups early- and late-onset PE with age-matched normotensive controls and there was no significant difference. Early or late onset PE therefore do not have a significant effect on overall placental microbiota composition. Community composition displayed in a heatmap, did not show any correlation between sample groups and bacterial composition (Fig. 10). Therefore, the present data suggest that there is no correlation between bacterial species and with early or late onset-PE. Although the study is not without limitation in such that the study lacks both negative and positive controls of known and quantifiable bacteria, making it impossible to verify the accuracy of the taxonomic assignments and to filter out possible contaminations. Similarly, the sample size is low which could have affected the significant power. Nevertheless, the present findings provide basis for the future study in the use

of high-throughput and advance technologies for bacterial RNA/DNA sequencing and detection.

5 Conclusion

The present results demonstrate a low biomass of bacterial species, which might further indicate that the placental samples had very low levels of bacteria species, though without correlation between the bacterial composition and early- or late-onset PE. Nevertheless, there was a variation in the diversity of bacterial species detected in early- or late-onset PE and age-matched normotensive controls but below the threshold of significance.

Author Contribution KSO and IM conceived and designed the experiment. KSO conducted the study and analyzed the results. IM, JM, and RM contributed reagents to the study. KSO drafted the manuscript under the supervision of IM, JM and RM and all the authors approved the manuscript for submission.

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Data Availability The data supporting the present study will be made available from the corresponding author on request.

Declarations

Conflict of Interest The authors declare that there are no conflicts of interest.

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CHAPTER 4: MANUSCRIPT THREE

Assessment of Human Placental Microbiota in Pre-eclampsia Using Shotgun Metagenomic (Accepted for publication: Canadian Journal of Physiology and Pharmacology).

This publication evaluates the role of placental bacterial species in the aetiology of pre-eclampsia (PE), a serious pregnancy complication that significantly contributes to maternal-fetal morbidity and mortality. Despite extensive research, the precise cause of PE remains unknown, and the existence and contribution of placental bacteria to its pathogenesis remain controversial. To investigate this, we evaluated the presence of bacterial species in the placenta of women with PE compared to normotensive controls using an advanced technique, next-generation sequencing. Placental tissues were collected via cesarean section using sterile and standardized clinical procedures from early-/late-onset PE and age-matched normotensive women. DNA was extracted, sequenced using Illumina NextSeq500 with a shotgun metagenomic approach, and analyzed through bioinformatics. The results showed that most reads were classified as belonging to the phyla, *Cutibacterium acnes*, *Staphylococcus epidermidis*, and various *Bradyrhizobium* species. PE samples showed notable presence of *Corynebacterium tuberculostearicum* and *Pseudomonas* species, while normotensive samples were dominated by *Bradyrhizobium* species and *Cutibacterium acnes*. Further analysis showed no significant difference between bacterial species of pre-eclamptic and normotensive placental samples. The results show very low levels of bacteria in the placental samples. In addition, a small difference was observed between the bacterial compositions of pre-eclamptic (early-/late-onset PE) and age-matched normotensive placental tissues, but not statistically significant.

Assessment of human placental microbial signatures in pre-eclampsia using shotgun metagenomics

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Running title: Placental microbiota in pre-eclampsia

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Abstract

This study evaluated the presence of bacterial species in the placenta of women with pre-eclampsia and compared with that of normotensive women. One hundred and twenty participants, comprising 60 pre-eclamptic (30 early- and late-onset, respectively) and 60 age-matched normotensive women (30 early and late-gestation normotensive, respectively) were recruited. After informed consent was obtained, the placenta were obtained through caesarean section with sterile and standardized clinical procedures. DNA was extracted from each tissue, and the samples were pooled into six libraries and sequenced on Illumina NextSeq500 using a shotgun metagenomic approach. Bioinformatics was used to analyse the reads with the implementation of Kraken2/MetaPhlAn classification methods and complemented by multi-layered contamination assessment strategy that included frequency-based decontam filtering. Most reads were classified as belonging to the phyla *Cutibacterium acnes*, *Staphylococcus epidermidis*, and various *Bradyrhizobium* species. PE samples showed notable *Corynebacterium tuberculostearicum* and *Pseudomonas* species, while *Bradyrhizobium and Cutibacterium acnes* dominated normotensive samples. Further analysis showed no significant difference between bacterial species of pre-eclamptic and normotensive placental samples. The results show very low levels of bacteria in the placental samples. In addition, a little difference was observed between the bacterial compositions of pre-eclamptic and age-matched normotensive placental tissues, but not statistically significant.

Keywords: Microbiota; Bioinformatics; Placenta; Pre-eclampsia; Shotgun metagenomic.

Introduction

Pre-eclampsia is a complex, multisystem pregnancy disorder defined by new-onset hypertension arising after 20 weeks of gestation and accompanied by one or more signs of maternal or fetal involvement. According to current international guidelines, including the 2021 International Society for the Study of Hypertension in Pregnancy (ISSHP) recommendations, diagnosis requires systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg on at least two occasions, together with evidence of organ dysfunction such as renal impairment, liver involvement, neurological complications, hematological abnormalities, or uteroplacental dysfunction, including fetal growth restriction (Magee et al. 2022). Affecting approximately 1.8–16.7% of pregnancies in developing countries (Belay et al. 2019), pre-eclampsia remains one of the leading causes of maternal and perinatal morbidity and mortality worldwide (Saxena et al. 2016). In addition to its immediate clinical consequences, including preterm birth, fetal growth restriction, and increased perinatal mortality, pre-eclampsia is associated with long-term cardiovascular and metabolic complications for both mother and child (Nankali et al. 2013; Tesfahun et al. 2023), imposing substantial and enduring burdens on healthcare systems (Li et al. 2017; Hao et al. 2019).

Despite its major clinical significance, the precise pathophysiological mechanisms underpinning pre-eclampsia remain incompletely understood. The disorder is widely regarded as a placental disease, originating from abnormal placentation and maladaptation of the maternal–fetal interface, which subsequently leads to systemic endothelial dysfunction and exaggerated inflammatory responses. These uncertainties have motivated researchers to explore a broad range of biological, environmental, and immunological contributors to disease onset, including the potential role of infectious agents and subclinical maternal infections. Several studies have suggested that bacterial or viral components may trigger aberrant systemic inflammation, endothelial dysfunction, or placental maladaptation, all of which are hallmarks of pre-eclampsia (Ahmadian et al. 2020; Celewicz et al. 2023). Notably, associations between specific pathogens, such as Helicobacter pylori, and an increased risk of pre-eclampsia have

been reported, supporting the hypothesis that chronic or unresolved infection-related immune activation may contribute to disease development (Franceschi et al. 2012). These observations corroborate the importance of exploring infection-related mechanisms, including the potential influence of microbial communities within the placenta, as part of the broader etiopathogenic framework of pre-eclampsia (Gomez de Agüero et al. 2016; Bisson et al. 2023).

The placenta is a vital organ that mediates nutrient, gas, and waste exchange between mother and fetus and serves as a key immunological interface, protecting the fetus from pathogens while promoting maternal immune tolerance to the semi-allogeneic conceptus. Disruption of placental development, perfusion, or immune function can precipitate adverse pregnancy outcomes, including pre-eclampsia, intrauterine growth restriction, and stillbirth (Sõbe et al. 2015; Sovio et al. 2024; Sai et al. 2025). Consequently, understanding the factors that shape placental health, including infectious exposures and microbial interactions, is central to advancing maternal–fetal medicine.

Considering the growing recognition of the importance of the human microbiota in health and disease, increasing attention has been directed towards the possible role of microbial communities in pregnancy. Once thought to be confined to external surfaces and specific mucosal sites, the microbiota is now recognised as an integral component of multiple physiological systems, contributing to immune regulation, metabolism, and host homeostasis (Zheng et al. 2020; Hou et al. 2022). The existence of a placental microbiota, however, remains a topic of ongoing debate. While some studies suggest that the placenta harbours a distinct microbial community (La et al. 2022; Panzer et al. 2023), others argue that it is largely sterile and that detected microbial DNA may reflect contamination during sample collection and processing (De Goffau et al. 2019). Nevertheless, emerging evidence indicates that, if present, microbial communities could influence pregnancy outcomes by modulating immune responses or directly affecting placental function (Gomez de Agüero et al. 2016; Blaser et al. 2021).

Given the substantial clinical burden of pre-eclampsia and its long-term consequences for maternal and offspring health, clarifying the contribution of infection- or microbiota-related mechanisms has important translational relevance. A deeper mechanistic understanding may ultimately support the development of improved diagnostic biomarkers, preventive strategies, and early intervention approaches (Inversetti et al. 2024). Although previous studies have identified microbial taxa potentially associated with adverse pregnancy outcomes, robust investigations of microbial communities directly within the placenta, particularly in relation to pre-eclampsia, remain limited. Moreover, traditional microbiome profiling approaches, such as 16S rRNA gene sequencing, provide only a partial view of microbial diversity and offer limited insight into functional potential (DiGiulio et al. 2015).

Shotgun metagenomic sequencing offers a more comprehensive alternative by enabling unbiased characterisation of the entire genetic material within a sample, thereby providing detailed information on both microbial composition and functional capacity (Durazzi et al. 2021; Kopera et al. 2024). Unlike 16S rRNA sequencing, which is restricted primarily to bacterial identification, shotgun metagenomics permits the detection of a broader range of microorganisms, including viruses and fungi, while also revealing functional genes and metabolic pathways that may be physiologically relevant (Xie et al. 2023). This approach therefore, holds particular promise for uncovering microbial functions and interactions that may contribute to placental health or pathology.

The present study, therefore, aimed to apply shotgun metagenomic analysis to investigate the microbiota of the human placenta in cases of pre-eclampsia. By comparing microbial profiles between pre-eclamptic and healthy placental samples, we sought to determine whether specific microbial taxa or functional genes are associated with this pregnancy complication. Through this integrative approach, this study might advance understanding of the potential microbial contribution to pre-eclampsia and provide justification for future diagnostic or therapeutic strategies.

METHODS

Study Population

One hundred and twenty (120) black South African pregnant women comprising 60 pre-eclamptic women (30 early- and late-onset, respectively) and 60 age-matched normotensive women (30 early- and late-gestation normotensive, respectively), who signed a written informed consent, were recruited for the study at the Maternity Clinic of Prince Mshiyeni Memorial Hospital, Durban South, South Africa between 10/09/2019 and 25/08/2020. For this study, ethical approval was obtained from the Biomedical Review Ethical Committee of the University of Kwazulu-Natal, Durban, South Africa (BE253/19). Criteria for inclusion as early- or late-onset pre-eclampsia were gestational age of 24-33 or >34 weeks, symptoms of proteinuria (≥ 300 mg of protein in a 24-hour urine collection), high blood pressure (systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg) and thrombocytopenia (low blood platelet count of ≤ 50 000/μL). While the criteria for inclusion for age-matched normotensive pregnancies (NT) were normal blood pressure (120/80 mmHg) and blood platelet count. Exclusion criteria were chronic hypertension, cardiovascular or renal disease, diabetes mellitus, bleeding disorders, HIV infection and previous medical conditions, as well as non-consenting pregnant women were excluded. A questionnaire survey was administered to collect women's demographic information and clinical characteristics (Table S1).

Sample collection

In this investigation, 120 placental samples were examined. A caesarean section was used to obtain the placental samples, which were immediately given to a qualified theatre nurse in the sterile operating and delivery room. Within 30 mins of fetal delivery, 1 × 1 cm section of the placenta was cut with sterile scissors from the placenta's central point and the maternal side's basal plate. In order to minimize contamination, the placental fragments were promptly placed in a sterile cryopreservation tube and kept in a biofreezer set at -80°C.

Sample preparation and DNA extraction

A tissue homogenizer was used to homogenize 100 mg of placental tissue samples. Following the manufacturer's recommendations, total DNA was extracted using the QIAamp DNA Body Fluid and Tissue Kit (Qiagen). To reduce Gram-negative extraction bias, an additional bead-beating step was incorporated using MPBio Lysing Matrix B tubes containing 0.1 mm silica beads. Bead beating was performed in a vortex-adapter bead-beating device at an oscillation frequency of 3,000 rpm for 45 seconds, followed by a 30-second cooling interval on ice to minimize heat buildup. The amount and quality of isolated DNAs were assessed using agarose gel electrophoresis and a NanoDrop NC2000 spectrophotometer, respectively. Until they were needed for additional analysis, all DNAs were kept at -80°C.

Shotgun metagenomic analysis

DNA isolated from pre-eclamptic and normal placental samples were quantified, normalised and pooled as previously documented (Gomez-Arango et al. 2017) into six libraries, as shown in Table 1. The pooled samples were sequenced on an Illumina NextSeq500 using a shotgun metagenomics approach, a high-throughput sequencing platform. Shotgun metagenomics offers several advantages for studying microbiomes: it allows for the detection of entire genomes rather than specific regions (such as 16S rRNA) and provides insights into both the taxonomic composition and functional capabilities of microbial communities. DNA from the samples was extracted using the HostZERO MicrobialDNA Kit (Zymo Research). The resulting genomic DNA samples were fragmented using an enzymatic approach (NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina). Resulting DNA fragments were size-selected (>200 bp), using AMPure XP beads. The fragments were end-repaired, and Illumina-specific adapter sequences were ligated to each fragment. Each sample was individually indexed, and a second size selection step was performed. Samples were then quantified, using a fluorometric method (NEBNext Library Quant Kit for Illumina), diluted to a standard concentration (4nM) and then sequenced on Illumina's NextSeq platform, using a NextSeq mid out kit (300 cycle), following a standard protocol as described by the manufacturer. 2Gb of data (2x150bp paired-end reads) were produced for each sample.

Table 1: Pooled DNA samples analysed

Sample ID	Condition
E22_S6	Early-onset pre-eclampsia
L21_S4	Late onset pre-eclampsia
L22_S5	Late onset pre-eclampsia
N22_S1	Late gestation normotensive
N23_S2	Late gestation normotensive
N24_S3	Early gestation normotensive

Bioinformatic analysis

A R script (R v.4.1.2 (2021-11-01) was created in RStudio for metagenomics analysis, implementing multiple packages tailored for microbiome analysis. Quality control and host DNA removal were performed using KneadData (https://github.com/biobakery/kneaddata) with reference to the human genome (GRCh38), including adapter trimming with Trimmomatic (ILLUMINACLIP: adapters.fa:2:30:10 SLIDINGWINDOW:4:20 MINLEN:50). Enhanced human read removal was conducted using Bowtie2 (bowtie2 2.5.4) with very-sensitive alignment parameters to minimize human contamination. Taxonomic classification employed a dual classifier approach: primary classification using Kraken2 (Kraken version 2.1.3) with a confidence threshold of 0.1 and minimum hit groups of 3, followed by abundance estimation with Bracken (Bracken version 3.0.1) for species-level resolution. Additional validation was attempted using MetaPhlAn (MetaPhlAn version 4.2.2) for cross-method comparison.

Kraken2/ Bracken taxonomic profiling

Kraken is a taxonomic classification system using exact k-mer matches to achieve high accuracy and fast classification speeds (Wood et al. 2019; Abellan-Schneyder et al. 2021). This classifier matches each k-mer within a query sequence to the lowest common ancestor (LCA) of all genomes containing the given k-

mer. The k-mer assignments inform the classification algorithm. Reads were matched to a standard database capped at 16 GB archaea, bacteria, viral, plasmid, human1 and UniVec-Core sequences.

Reads-based taxonomic profiling (MetaPhlAn2)

MetaPhlAn2 is a commonly used taxonomic profiling tool that aligns metagenome reads to a predefined marker-gene database to perform taxonomic classification (Truong et al. 2015; Tran and Phan, 2020). Kraken 2 performs exact k-mer matching to sequences within the NCBI non-redundant database and uses lowest common ancestor (LCA) algorithms to perform taxonomic classification (Lu et al. 2022; Blanco-Míguez et al. 2023), while MetaPhlAn2 is a commonly used taxonomic profiling tool and is a medium-confidence taxa identifier (Zhu et al. 2010; Truong et al. 2015; Lu et al. 2022).

Contamination assessment

The dual validation was complemented by a multi-layered contamination assessment strategy that includes frequency-based decontam filtering (Davis et al. 2018), comprehensive databases of known laboratory contaminants (kit reagents, skin, environmental, and water contaminants), and enhanced taxonomic matching algorithms with multiple criteria for species identification.

Statistical analysis

A script that represents a methodologically sound approach to small-sample microbiome analysis was developed. Given the small sample size, we employed robust statistical methods appropriate for limited power studies. Alpha diversity analysis used exact Wilcoxon tests with bootstrap 95% confidence intervals, while beta diversity employed PERMANOVA and ANOSIM tests. Effect sizes were estimated using Cliff's Delta for small samples, with a focus on effect size estimation rather than significance testing. Pearson correlation was used to determine the relationship between microbial detection results and patients' clinical indicators (systolic and diastolic blood pressure). Statistical significance was considered at a p-value less than 0.05.

RESULTS

Quality of reads

Total clean microbial reads obtained after quality control and host-read removal ranged from 3,729 to 27,757 reads per sample, with detected taxa ranging from 113 to 758 species. Pre-eclampsia samples showed variable clean read counts (E22_S6: 3,729; L21_S4: 14,255; L22_S5: 27,757), while normotensive samples showed moderate variability (N22_S1: 26,436; N23_S2: 10,426; N24_S3: 15,190). The quality control and decontamination workflow revealed extremely low microbial content typical of placental tissue samples, with reductions from raw to clean reads across all samples (Table 2 and Figures S1, S2 and S3). Raw sequencing reads ranged from 1.38 to 8.40 million reads per sample, but after KneadData quality control and human genome removal, only 3,729 to 27,757 clean microbial reads remained per sample, representing retention rates of 0.044% to 1.03%. This substantial reduction (>99% of reads removed) reflects the predominantly human genomic content of placental tissue and underscores the ultra-low biomass nature of placental microbiota. Sample E22_S6 showed the lowest retention rate (0.044%), while L21_S4 had the highest (1.03%), with no clear pattern distinguishing pre-eclampsia from normotensive samples.

Table 2: General statistics of reads before and after quality control

Sample_ID	Raw_Reads	Clean_Reads
E22_S6	8400021.0	3729.0
N24_S3	7175597.0	15190.0
L22_S5	5638741.0	27757.0
L21_S4	1384696.0	14255.0
N23_S2	5063852.0	10426.0
N22_S1	8006212.0	26436.0

Only a small percentage (<1%) of paired reads survive the KneadData quality control step for all samples. Early-onset PE (E22_S6); Late-onset PE (L21_S4 and L22-S5); Late gestation normotensive (N22_S1 and N23-S2); Early gestation normotensive (N24_S3).

Contamination assessment results and dual classifier validation

Given the ongoing scientific debate on the existence of a genuine placental microbiota and the critical importance of distinguishing authentic microbial signals from laboratory contamination in ultra-low biomass samples, we implemented an enhanced contamination assessment framework integrated with a dual classifier approach. The contamination database, building upon previous findings (Salter et al. 2014) categorizes potential contaminants into four classes: kit reagent contaminants (Pseudomonas spp., Ralstonia pickettii, Burkholderia cepacia), skin contaminants (Staphylococcus epidermidis, Cutibacterium acnes, Corynebacterium spp.), environmental contaminants (Bacillus spp., Methylobacterium, Bradyrhizobium), and water contaminants (Legionella, Variovorax, Delftia). This assessment was applied to both Kraken2/Bracken and MetaPhlAn outputs, enabling cross-method validation of contamination patterns and generating quantitative contamination fractions (low: <5%, moderate: 5-15%, high: >15% of total reads).

The results were integrated into the confidence classification system, where taxa detected by both methods with minimal contamination signals received "high confidence" designation, MetaPhlAn-only detections were classified as "medium confidence," and Kraken2/Bracken-only detections, particularly those matching known contaminants, were designated "low confidence." (Table 3 and Table S2). Our enhanced contamination assessment revealed a striking methodological discrepancy that validated the dual classifier approach: while Kraken2/Bracken analysis indicated high contamination levels in 5/6 samples (range: 20.9-39.7% of total reads) and moderate contamination in 1/6 samples (6.6%), MetaPhlAn analysis showed 0% contamination across all samples. This difference reflects the distinct detection mechanisms of each method, with Kraken2's comprehensive k-mer database detecting environmental bacteria lacking the specific marker genes required for MetaPhlAn classification. The substantial contamination detected by Kraken2, likely representing kit reagent,

environmental, and water contaminants consistent with ultra-low biomass sample processing, would have severely compromised biological interpretation in a single-method analysis. However, MetaPhlAn's marker-gene specificity provided crucial validation, identifying taxa with robust phylogenetic support while filtering out likely contaminants. This cross-method validation demonstrates that environmental bacteria detected exclusively by Kraken2 (including *Bradyrhizobium, Pseudomonas*, and related genera) represent laboratory artefacts rather than genuine placental microbiome signals. Consequently, we adopted a conservative analytical approach, focusing biological interpretation on high-confidence taxa detected by both methods and classifying single-method detections as low-confidence findings requiring validation in larger studies. This contamination pattern underscores both the critical importance of comprehensive contamination assessment in placental microbiome research and the methodological strength of dual classifier validation for distinguishing authentic biological signals from laboratory artefacts in ultra-low biomass samples.

Table 3: Dual classifier comparison results

Sample	Bracken Contamination Fraction	Bracken Contamination Level	MetaPhlAn Contamination Fraction	MetaPhlAn Contamination Level
L21_S4	0.39725669497060745	High	0.0	Low
N22_S1	0.325426944971537	High	0.0	Low
N23_S2	0.2635013868494045	High	0.0	Low
N24_S3	0.20912505523641184	High	0.0	Low
L22_S5	0.23702736459634383	High	0.0	Low
E22_S6	0.06646862235425176	Moderate	0.0	Low

Early-onset PE (E22_S6); Late-onset PE (L21_S4 and L22-S5); Late gestation normotensive (N22_S1 and N23-S2); Early gestation normotensive (N24_S3).

Taxonomic composition

The most abundant taxa across all samples included *Cutibacterium acnes*, *Staphylococcus epidermidis*, and various *Bradyrhizobium* species. PE samples showed a notable presence of *Corynebacterium tuberculostearicum* and *Pseudomonas* species, while NT samples were dominated by *Bradyrhizobium* species and *Cutibacterium acnes* (Figure 1). Out of 121 taxa tested for differential abundance, no taxa reached statistical significance after multiple testing correction (FDR < 0.05). However, several taxa showed large effect sizes that warrant attention in future studies. *Bradyrhizobium* species ma5 demonstrated the largest effect size (Cohen's d = -5.86, P = 0.077), being substantially more abundant in normotensive samples (mean: 16.0 reads) compared to PE samples (mean: 3.3 reads). Notably, *Variovorax* species. Varisp41 exhibited the fifth-largest effect size (Cohen's d = 2.79, P = 0.059) with a distinctive presence/absence pattern: detected exclusively in PE samples (mean: 29.7 reads, median: 21 reads) while completely absent in all normotensive samples (mean: 0 reads). This represents a particularly compelling finding as it demonstrates a consistent directional difference despite relatively low overall abundance, suggesting potential biomarker significance. The $\log_2$ fold-change of 4.94 indicates a substantial enrichment in PE placentas. Other taxa with notable effect sizes included *Mycobacterium* sp. SMC-16 (Cohen's d = -3.70, P = 0.081, more abundant in NT), *Diaphorobacter* sp. JS3050 (Cohen's d = -3.49, P = 0.064, more abundant in NT), and *Corynebacterium* sp. SCR221107 (Cohen's d = -2.89, P = 0.077, more abundant in NT).

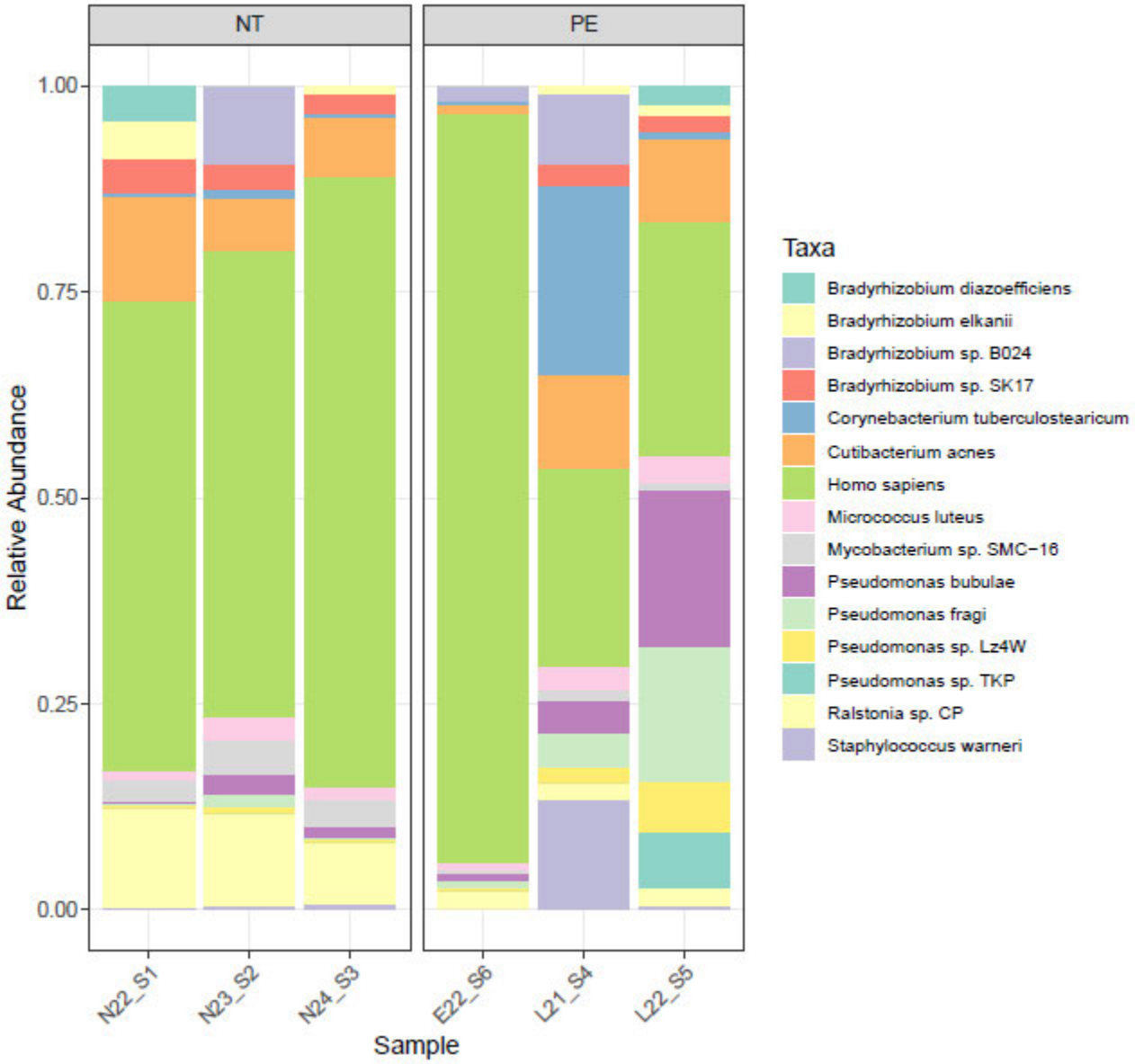

Figure 1: Taxonomic Composition. Top 15 Most Abundant Taxa (Relative Abundance). Early-onset PE (E22_S6); Late-onset PE (L21_S4 and L22-S5); Late gestation normotensive (N22_S1 and N23-S2); Early gestation normotensive (N24_S3); Normotensive (NT) and Pre-eclampsia (PE). Normotensive group includes N22_S1, N23_S2, and N24_S3, while Pre-eclamptic group include E22_S6, L21_S4, and L22_S5.

Alpha diversity analysis

Alpha diversity metrics showed no statistically significant differences between groups, consistent with the limited statistical power. Shannon diversity: PE mean = 3.00 ± 1.63, NT mean = 3.27 ± 0.35 (P = 0.7, Cliff's Delta = 0.33, 95% CI: [-2.02, 0.93]). Simpson diversity: PE mean = 0.73 ± 0.36, NT mean = 0.82 ± 0.07 (P = 0.7, Cliff's Delta

= 0.33, 95% CI: [-0.46, 0.18]). Species richness (Chao1): PE mean = 328.5 ± 202.2, NT mean = 438.2 ± 66.9 (P = 0.7, Cliff's Delta = -0.33, 95% CI: [-317.1, 86.7]) (Figure 2 and Table S3).

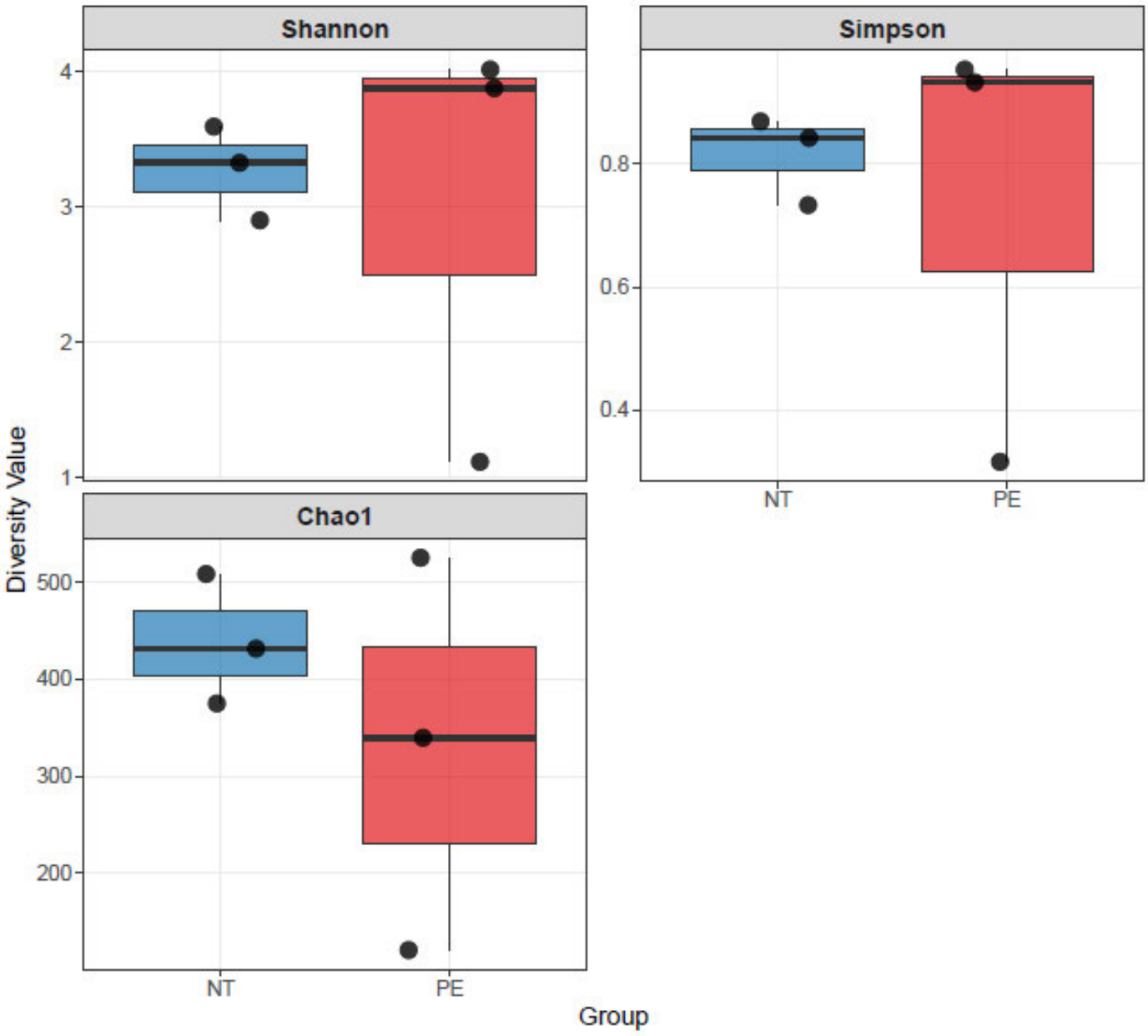

Figure 2: Alpha Diversity Analysis − Enhanced Small Sample Approach. Pre-eclampsia ((PE, which comprises of Early-onset PE (E22_S6) and Late-onset PE (L21_S4 and L22-S5) and Normotensive (NT, which comprises of Early gestation normotensive (N24_S3) and Late gestation normotensive (N22_S1 and N23-S2))

Beta diversity and community structure

Beta diversity analysis revealed modest but non-significant separation between groups. Bray-Curtis PERMANOVA showed $R^2 = 0.195$ ($P = 0.5$), while Jaccard distance yielded $R^2 = 0.213$ ($P = 0.3$). ANOSIM statistics were negative for both metrics ($R = -0.037$, $P = 0.6$), indicating no significant clustering by group membership (Table 4).

Table 4: Beta diversity between pre-eclampsia and normotensive

Distance_method	PERMANOVA R^2	PERMANOVA P	ANOSIM R	ANOSIM P
Bray Curtis	0.19546138209215	0.5	0.0370370370370369	0.6
Jaccard	0.213303885230831	0.3	0.0370370370370369	0.6

Pre-eclampsia comprises of Early-onset PE (E22_S6) and Late-onset PE (L21_S4 and L22-S5) and Normotensive comprises of Early gestation normotensive (N24_S3) and Late gestation normotensive (N22_S1 and N23-S2).

Correlation analysis between clean microbial reads and blood pressure (systolic and diastolic blood pressure) in pre-eclampsia and normotensive

Figure 3 showed a significant correlation between clean microbial read and SBP (p=0.04) as well as DBP (p=0.02) in PE, while no significant correlation was observed between clean microbial read and SBP (p=0.98) as well as DBP (p=0.44) in NT.

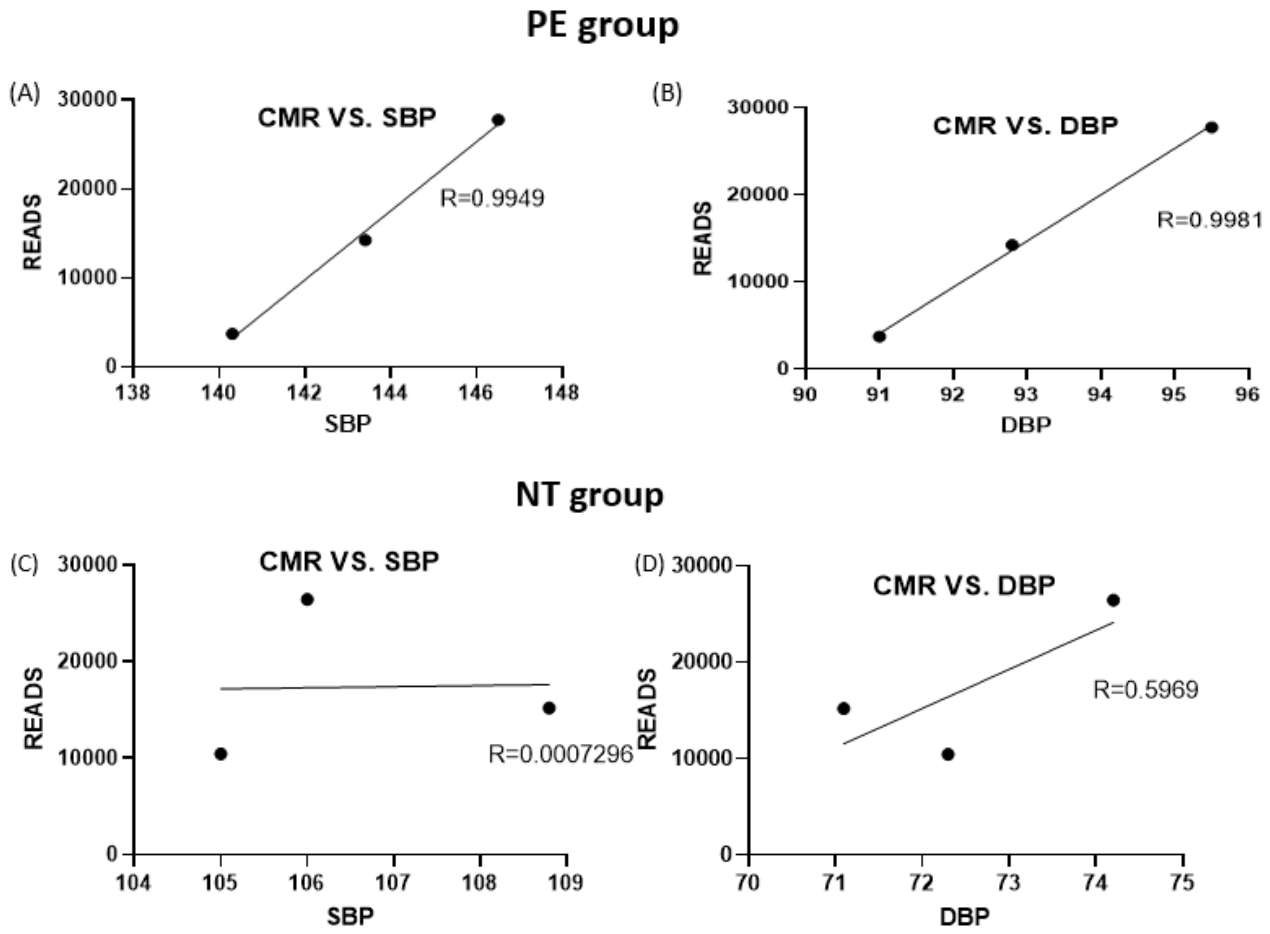

Figure 3: Correlation analysis between clean microbial read (CMR) and blood pressure (SBP and DBP) in pre-eclampsia (a, b) and normotensive (c, d). Systolic blood pressure (SBP; mmHg); Diastolic blood pressure (DBP; mmHg).

DISCUSSION

This study investigated the bacterial composition of placental tissue in women with pre-eclampsia compared to normotensive women, utilizing shotgun metagenomic sequencing to assess bacterial diversity. The findings revealed the presence of bacterial DNA, primarily from the phyla *Cutibacterium acnes*, *Staphylococcus epidermidis*, and various *Bradyrhizobium* species. Pre-eclamptic samples showed a notable presence of *Corynebacterium tuberculostearicum* and *Pseudomonas* species, while normotensive samples were dominated by *Bradyrhizobium* species and *Cutibacterium acnes*. However, further analysis showed no significant difference between the bacterial compositions of pre-eclamptic (early- and late-onset PE) and age-matched normotensive placental tissues. The study further noted a very low abundance of bacterial DNA across samples, corroborating

recent evidence questioning the concept of a distinct placental microbiota (Gschwind et al. 2020; Briana et al. 2021).

The notion of a placental microbiota has been controversial. Earlier studies suggested that the placenta harbours a unique microbial community, which could influence fetal development and pregnancy outcomes (La et al. 2022; Panzer et al. 2023). This study's findings contribute to this ongoing debate by showing that the bacterial DNA found in placental samples was minimal and no significant difference between pre-eclamptic and normotensive cases, despite the notable presence of *Corynebacterium tuberculostearicum* and *Pseudomonas* species in PE. This low bacterial presence supports the idea that the placenta may not have a substantial microbiota, at least in the sense of a functionally active microbial community similar to those in the gut or skin. Corynebacterium species are common constituents of skin and environmental microbiota and are frequently reported as potential laboratory contaminants; however, some members of this genus have been implicated in inflammatory processes and opportunistic infections (Olender et al. 2019). Likewise, Pseudomonas species, although often associated with environmental and reagent contamination, are also known for their ability to modulate host immune responses and contribute to oxidative stress-related pathways [Hu et al. 2019]. Their detection in PE samples may therefore reflect either low-level translocation events from maternal tissues or high sample susceptibility due to disease-related tissue changes. While these observations cannot be interpreted as evidence of a functional placental microbiota, they support the need to distinguish true biological signals from background noise.

Shotgun metagenomic sequencing, as used in this study, is a good approach that provides a broader view of microbial DNA compared to traditional 16S rRNA gene sequencing, which primarily targets bacteria. By sequencing all DNA present in the sample, this method enables the detection of a wider range of organisms and also allows for functional analysis of microbial genes. This technique is especially valuable in a context where microbial biomass is low, such as in the placenta, as it maximizes the likelihood of detecting any bacterial DNA present. However, its sensitivity also raises the risk of contamination, meaning careful handling and rigorous

controls are essential to ensure the validity of the findings. In this study, we implemented an enhanced contamination assessment framework integrated with our dual classifier approach (Kraken2/Bracken and MetaPhlAn). The results were integrated into our confidence classification system, where taxa detected by both methods with minimal contamination signals received "high confidence" designation, MetaPhlAn-only detections were classified as "medium confidence," and Kraken2/Bracken-only detections, particularly those matching known contaminants, were designated "low confidence." This comprehensive contamination framework provides the methodological rigour required for placental microbiome studies and enables transparent reporting of potential contamination sources, addressing at least in part the current limitation for low-biomass microbiome research and strengthening the scientific credibility of the present findings in the contested field. Despite the rigorous methodological approach, the lack of significant microbial differences between pre-eclamptic and normotensive samples after multiple testing correction (FDR < 0.05) suggests that bacterial composition may not play a critical role in the pathogenesis of pre-eclampsia, at least based on the types of bacteria detectable by shotgun metagenomics.

Similarly, the lack of detectable differences in bacterial profiles between pre-eclamptic and normotensive placental samples in this study implies that if bacteria are involved in the development of pre-eclampsia, they may not do so through colonization of the placenta. Alternatively, microbial influences on pre-eclampsia may stem from maternal sites with well-established microbiota, such as the gut, oral cavity, or urogenital tract, where dysbiosis has been associated with various health conditions, including pregnancy complications (Nuriel-Ohayon et al. 2019). For instance, gut microbiota can modulate systemic inflammation and immune responses, which are key aspects of pre-eclampsia. Future studies might explore whether microbial imbalances in these maternal sites correlate with placental changes or systemic markers of pre-eclampsia.

However, it is noteworthy that several taxa demonstrated large effect sizes that merit attention and underscore the potential biological relevance of microbial dysbiosis in PE placentas. *Bradyrhizobium* sp. ma5 emerged as the

taxon with the most substantial difference between PE and normotensive placentas, displaying a markedly reduced abundance in PE samples (mean: 3.3 reads) compared to normotensive (mean: 16.0 reads), with a very large effect size (d = -5.86). The presence of *Bradyrhizobium* species in human tissue, including the placenta, has been increasingly reported in metagenomic studies, and previous studies have attributed their presence in negative controls to environmental contamination (De Goffau et al. 2019). In addition, *Variovorax* species, Varisp41 was uniquely detected in PE samples and completely absent in all normotensive groups. Although the overall abundance (prevalence of the taxon across all samples) was low (mean: 29.7 reads, median: 21), the consistent presence/absence pattern across groups and the high $\log_2$ fold change (4.94) reflect its potential relevance. The effect size (d = 2.79) (the magnitude of difference between groups) further supports a biologically meaningful difference, which is worth investigating in future study with large sample size. *Variovorax* species are environmental bacteria capable of biodegradation and have been associated with oxidative stress responses (Dawson et al. 2020). Their selective presence in PE samples could represent either a genuine biological association with PE pathophysiology, potentially related to the oxidative stress characteristic of this condition, or differential contamination patterns between sample groups. The consistent presence/absence pattern across all samples strengthens the potential biological relevance, though validation in larger cohorts with enhanced contamination controls is essential. Future studies could also combine metagenomic analysis with transcriptomic or proteomic approaches to determine whether any bacterial presence in the placenta is active or merely incidental. The results obtained might be due to the low number of reads which might further indicate that the placental samples had very low or non-existing levels of bacteria. Additionally, the pooling of samples and the small sample size may have contributed to the absence of a significant difference between the bacterial species in pre-eclamptic and normotensive placental samples, and this might also influence the detection resolution. Despite these limitations, incorporating clinical correlations provides additional information for interpreting the biological relevance of the detected microbial signals. In this study, a correlation analysis revealed that, within the pre-eclamptic group, clean microbial reads were significantly associated with both SBP ($p = 0.04$) and DBP ($p =$

0.02). Although the direction and magnitude of these associations should be interpreted cautiously due to the low bacterial abundance, they suggest that even minimal bacterial DNA signatures may relate to clinical severity or underlying inflammatory processes characteristic of pre-eclampsia. In contrast, no significant correlations were observed between clean microbial reads and SBP ($p = 0.98$) or DBP ($p = 0.44$) in normotensive pregnancies, further demonstrating a potential disease-specific pattern. While these findings do not establish causality, they add an important clinical dimension to the microbiological data and support the need for further investigations using larger cohorts and higher-resolution methods. Overall, this study provides valuable data from Sub-Saharan Africa and contributes to the growing body of research on whether microbial signatures, however sparse, may have relevance in the pathogenesis of pre-eclampsia. Future studies might also examine microbial metabolites or components in maternal blood or placental tissues to explore the possibility that microbial effects on pre-eclampsia may be indirect, with maternal microbiota at other sites impacting systemic inflammation, nutrient absorption, or immune regulation in ways that contribute to placental dysfunction. Another avenue for future research could involve longitudinal studies that track changes in the maternal microbiome, immune markers, and placental health over time to better understand the dynamic relationships among these factors. Integrating multi-omics approaches, combining metagenomics, transcriptomics, proteomics, and metabolomics, could offer a more comprehensive picture of how microbial and host factors interact in the context of pre-eclampsia.

CONCLUSION

The present findings demonstrate that placental samples contained a very low number of microbial reads, reinforcing the interpretation that the placenta harbours minimal bacterial DNA. This primary observation aligns with emerging evidence challenging the existence of a substantive placental microbiota. Although no statistically significant differences were observed between pre-eclamptic and normotensive groups after FDR adjustment, this does not preclude potential biological relevance. Notably, several genera displayed moderate effect sizes and consistent directional trends, suggesting that subtle microbial signals, while not detectable with the current sample

size, may warrant further investigation in larger, better-powered studies. These results, therefore, contribute meaningfully to the ongoing debate regarding the presence and functional significance of microbes in the placenta. At the same time, the possibility that maternal microbiota from other body sites, or microbial translocation, may influence placental health and pregnancy outcomes remains an important area for future research. To advance this field, future studies should incorporate larger cohorts, deeper sequencing, improved host DNA depletion methods, and rigorous contamination-aware workflows to enhance detection sensitivity in low-biomass environments. Ultimately, understanding the pathogenesis of pre-eclampsia would require an integrative approach that considers both microbial and non-microbial contributors, including genetic, immunological, and environmental factors. This study provides valuable insight while suggesting the need for continued, methodologically robust investigations to unravel the complex mechanisms underlying this multifaceted condition.

Declarations

Ethical approval and consent to participate

The study was conducted in accordance to the Declaration of Helsinki (World Medical Association, 2013), and ethical approval was obtained from the Biomedical Review Ethical Committee of the University of Kwazulu-Natal, Durban, South Africa (BE253/19). All participants provided informed consent.

Consent for publication

NA

Data Availability

The data supporting the present study are available in the figshare repository:

https://doi.org/10.6084/m9.figshare.30817376

Competing Interests

The authors declare that there are no conflicts of interest.

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Author contribution

KSO and IM conceived and designed the study, KSO conducted the investigation. IM, JM and RM contributed reagents for the study. KSO drafted the original manuscript. All the authors read, reviewed, revised and approved the manuscript for submission.

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Supplemmentary Materials

Clinical characteristics of participants

There was a significant increase in MAP of early-onset PE and late-onset PE compared with early gestation normotensive and late gestation normotensive, respectively. Whereas there was no significant difference between the MAP of late-onset PE compared with early-onset PE. There was no significant difference in age and BMI of

early-onset PE and late-onset PE compared with early gestation normotensive and late gestation normotensive, respectively. Whereas the BMI of late-onset PE was significantly higher than early-onset PE. Early/Late-onset PE showed the presence of proteinuria (+1), which was not detectable in Early/Late gestation normotensive.

Table S1: Clinical characteristics of the participants

	N_{Early}	**N_{Late}**	**PE_{Early}**	**PE_{Late}**
Age (years)	29.4 ±1.41	28.9± 1.02	31.7± 1.11	31.5±1.02
BMI (kg/m^2)	29.97±4.40	33.74±5.22	30.47±6.12	34.09±5.40$^{\#}$
SBP (mmHg)	108.8±1.4	106±4.3	140.3±2.6*	146.5±2.0$^{\$}$
DBP (mmHg)	71.1±1.1	74.2±1.4	91.0±1.6*	95.5±1.3$^{\$}$
MAP (mmHg)	83.67±8.21	84.8±10.53	107.3±12.42*	112.5±10.31$^{\$}$
Proteinuria (dipstick)	ND	ND	+1	+1

Values are expressed as mean±SEM, n=30, Comparison between mean values was performed using one-way ANOVA and followed by Bonferroni *post hoc* test, (*$p<0.05$ vs N_{Early}, $^{\$}p<0.05$ vs N_{Late}, $^{\#}p<0.05$ vs PE_E). Early gestation normotensive (N_{Early}); Late gestation normotensive (N_{Late}); Early-onset pre-eclampsia (PE_{Early}); Late-onset pre-eclampsia (PE_{Late}); Not detectable (ND).

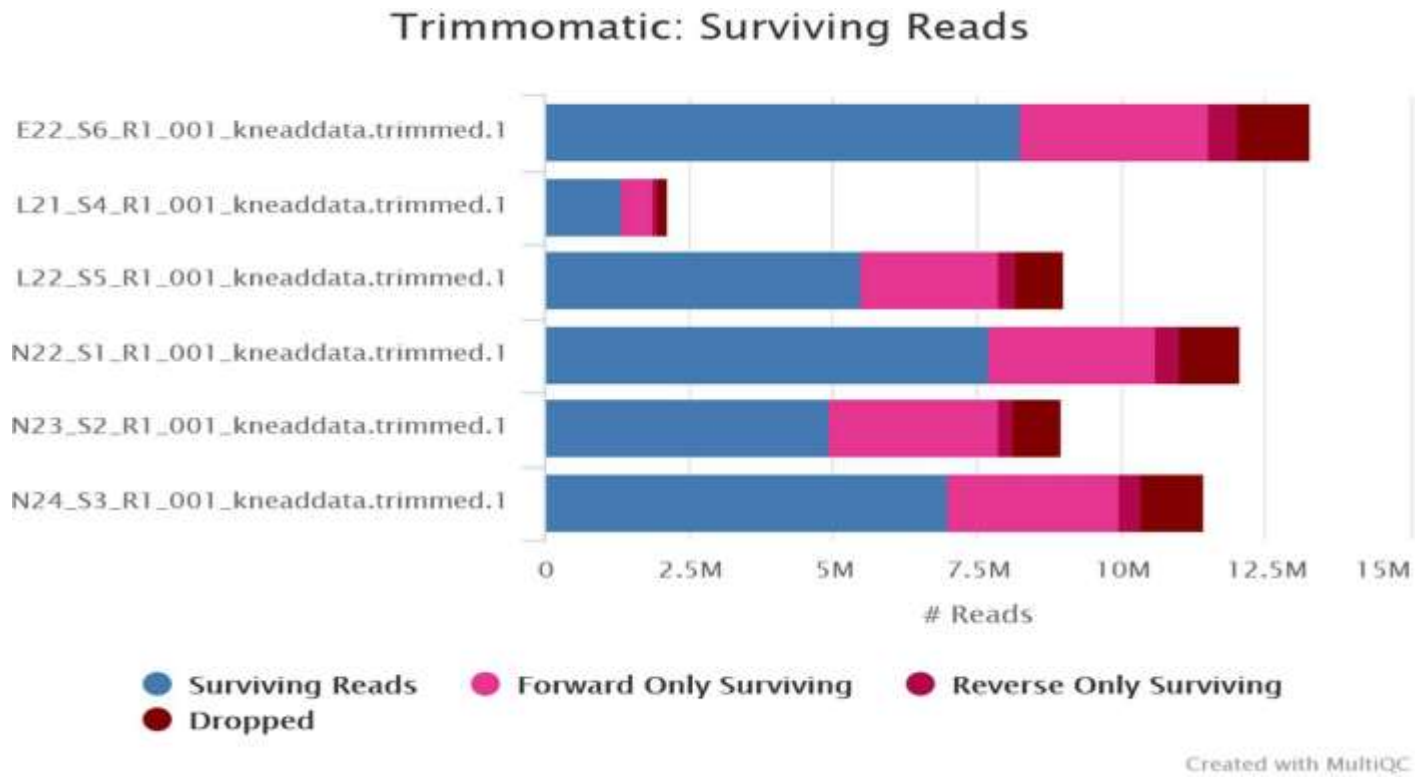

Figure S1: Summary of data after Trimmomatic quality control step.

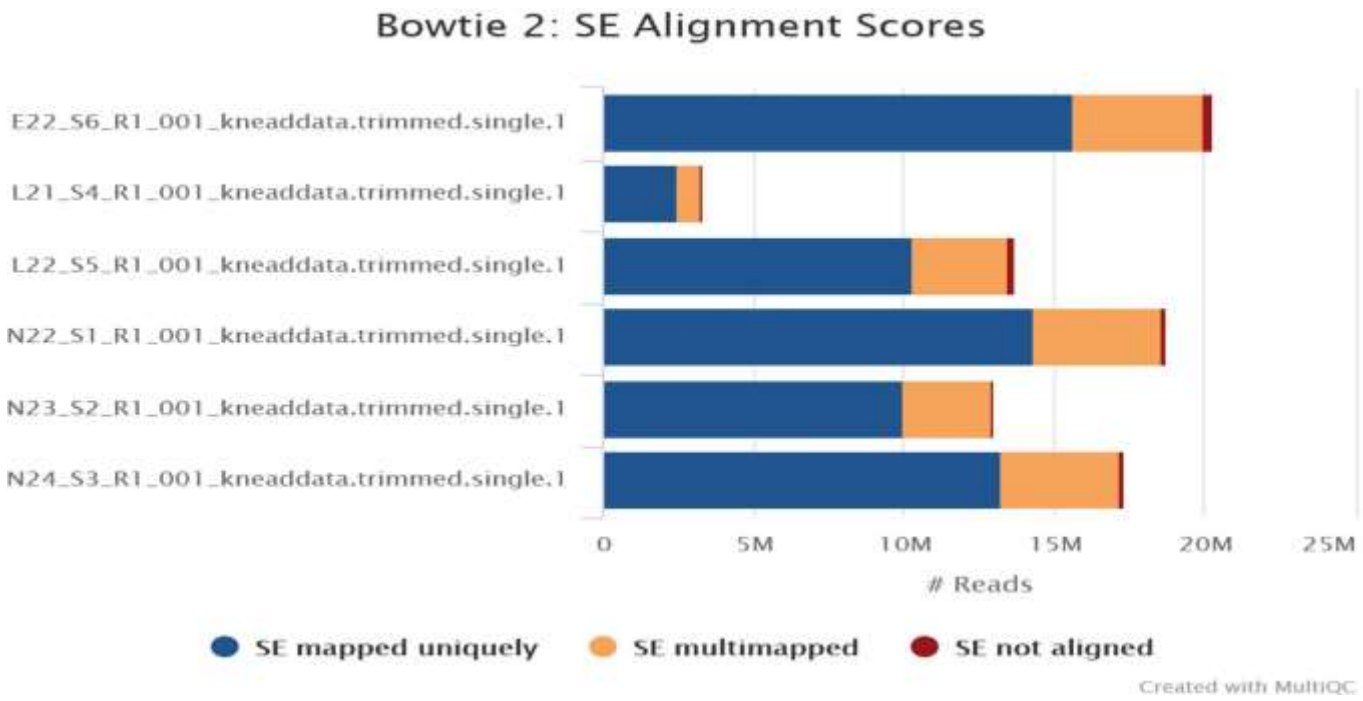

Figure S2: Summary of data after Bowtie2 mapping of sequencing reads to human reference sequences. This plot shows the number of reads aligning to the reference in different ways.

Figure S3: FastQC summary of sequence read quality and length.

Table S2: Contamination assessment dual classifier

Sample	Bracken_Contamination_Fraction	Bracken_Contamination_Level		MetaPhlAn_Contaminat
L21_S4	0.39725669497060745	High	0.0	Low
N22_S1	0.325426944971537	High	0.0	Low
N23_S2	0.2635013868494045	High	0.0	Low
N24_S3	0.20912505523641184	High	0.0	Low
L22_S5	0.23702736459634383	High	0.0	Low
E22_S6	0.06646862235425176	Moderate	0.0	Low

Table S3: Dual classifier quality summary

SampleID		Group	Kraken_Total_Reads	Kraken_Detected_Taxa	Kraken_Shannon_Diversity	Kraken_Simpson_Div	
E22_S6	PE	2693	113	1.27602212639679	0.357516406289966	113	113
L21_S4	PE	7655	423	4.35698575647328	0.963927130186893	423	423
L22_S5	PE	17614	758	4.30426811153403	0.948956090016291	758	758
N22_S1	NT	15810	689	3.87761054447107	0.884089902186824	689	689
N23_S2	NT	6129	390	3.53015656150339	0.855874065188058	390	390
N24_S3	NT	9052	519	3.17545055344666	0.758657642425001	519	519

CHAPTER 5: SHORT REPORT FOUR

Preliminary Evaluation of Serum Level of HDAC-2, HLA-DRA and Acetate in Pre-eclampsia

This preliminary report assesses the role of microbiota-derived metabolites and immune-related epigenetic mechanisms in the pathophysiology of pre-eclampsia (PE), a pregnancy-specific hypertensive disorder with an elusive aetiology. Emerging evidence suggests that microbial metabolites, such as short-chain fatty acids (e.g., acetate), and epigenetic modifications, including histone deacetylation and immune gene regulation, may contribute to PE development. To investigate these molecular alterations, the study evaluated the levels of histone deacetylase-2 (HDAC-2), human leukocyte antigen-DRA (HLA-DRA), and acetate in the blood samples from pregnancies complicated by early-onset pre-eclampsia (EOPE) compared to age-matched normotensive control (NORM). Blood samples were collected via venipuncture and centrifuged for serum collection, RNA was extracted for molecular analysis using qPCR and other biochemical parameters were determined with ELISA. The preliminary findings revealed a significant reduction in HDAC-2 level in EOPE compared to normotensive control. In addition, HLA-DRA level was significantly elevated in EOPE, suggesting enhanced immune system activation in early-onset PE. Similarly, acetate level was significantly reduced in EOPE compared to normotensive controls, indicating potential disruptions in microbial metabolite pathways. These results highlight distinct molecular alterations associated with early-onset PE, particularly in immune regulation and microbial metabolite production. The observed increase in HLA-DRA suggests heightened immune activation, while the decrease in acetate may reflect altered metabolic processes. These preliminary findings warrant further investigation in a larger cohort.

Preliminary evaluation of serum levels of HDAC-2, HLA-DRA and acetate in pre-eclampsia

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Abstract

Background: Despite decades of research, the exact aetiology of pre-eclampsia (PE) remains elusive. Emerging evidence suggests that microbiota may play a role through alterations in microbial metabolites, such as short-chain fatty acids (e.g., acetate), as well as by influencing immune responses and epigenetic mechanisms, including histone modification and DNA methylation. Hence, the present study was designed to evaluate the levels of histone deacetylase-2 (HDAC-2), human leucocyte antigen-DRA (HLA-DRA), and acetate from serum from pregnancies complicated by early-onset pre-eclampsia (EOPE), compared to age-matched normotensive control.

Methods: Thirty age-matched normotensive (NORM) and early-onset pre-eclamptic women were recruited. After obtaining informed consent, blood samples were obtained through venipuncture with standardized clinical procedure. The samples were centrifuged, and serum was extracted. RNA was also extracted from the serum for molecular analysis. ELISA and real-time PCR techniques were used for the determination of HDAC-2 and acetate and HLA-DRA, respectively.

Results: There was a significant decrease when comparing the level of HDAC between the EOPE and NORM group. Similarly, the levels of HLA-DRA showed a significant increase in EOPE compared with the NORM group, while the levels of acetate in EOPE showed a significant decrease when compared with the NORM group.

Conclusion: Overall, this study highlights that these markers of epigenetic dysregulation, immune activation and metabolic alterations are associated with PE, particularly in EOPE cases. The findings provide preliminary evidence supporting the role of epigenetics, microbial metabolites and immune dysregulation in PE pathophysiology. Further research is warranted to determine whether HDAC2, HLA-DRA and acetate could serve as potential diagnostic biomarkers or therapeutic targets for PE management.

Keywords: Acetate; Histone deacetylase-2; HLA-DRA; Placenta; Pre-eclampsia; Pregnancy.

INTRODUCTION

Pre-eclampsia (PE), a complex hypertensive disorder of pregnancy, remains a significant cause of maternal and neonatal morbidity and mortality (1). The disorder is characterized by placental insufficiency and systemic inflammation. Understanding the roles of HDAC-2, HLA-DRA, and acetate in PE, particularly early -onset PE (EOPE), provides insights into its pathophysiology and potential therapeutic effects. In this study, the focus was on EOP due to its strong association with immune dysregulation and inflammation (2, 3, 4).

Histone deacetylases (HDACs) regulate gene expression by removing acetyl groups from histones, leading to chromatin condensation and transcriptional repression (5). HDAC-2, a key member of the HDAC family, modulates inflammation, cellular stress responses, and immune regulation. In normal pregnancy, HDAC-2 supports the immunotolerant environment at the maternal-fetal interface by suppressing pro-inflammatory cytokines (5). Proper HDAC-2 activity ensures balanced gene expression essential for trophoblast function and placental development (6). Aberrant HDAC-2 activity in pre-eclampsia disrupts epigenetic control, leading to excessive inflammation and impaired trophoblast invasion (7). This contributes to defective spiral artery remodelling and placental insufficiency, particularly in EOPE. Early-onset cases exhibit more severe HDAC-2 dysregulation compared to late-onset cases, correlating with the greater placental contribution to disease pathology in the former (8). Late-onset pre-eclampsia, often linked to maternal systemic factors, may involve secondary HDAC-2 alterations (8). Targeting HDAC-2 through selective inhibitors or modulators could restore epigenetic balance and reduce inflammatory pathways. Such interventions hold promise, particularly for early-onset cases, where placental pathology is more pronounced.

Human leukocyte antigen-DRA (HLA-DRA) belongs to the HLA class II molecule family, critical for antigen presentation and immune system activation (9). While HLA-DRA expression is tightly regulated in normal pregnancy, its aberrant expression in pre-eclampsia disrupts maternal-fetal immune tolerance. Syncytiotrophoblasts and extravillous trophoblasts typically do not express HLA class II molecules, including

HLA-DRA, preventing maternal immune recognition of paternal antigens (10). This immune privilege is vital for successful pregnancy outcomes. Studies have demonstrated that HLA-DRA is aberrantly expressed in the syncytiotrophoblast of pre-eclamptic placentae. This leads to immune activation and inflammation, impairing trophoblast invasion and vascular remodelling (10). The degree of HLA-DRA expression correlates with the severity of placental dysfunction, which is more pronounced in early-onset cases (11). Late-onset pre-eclampsia may involve secondary immune activation. Factors such as oxidative stress, hypoxia, and pro-inflammatory cytokines may upregulate HLA-DRA in the placenta (11). This aberrant expression could be a response to environmental stressors unique to pre-eclampsia. HLA-DRA could serve as a biomarker for early detection of pre-eclampsia and stratification of disease severity. Therapies aimed at modulating microbiota may suppress aberrant HLA-DRA expression, and this might restore immune balance at the maternal-fetal interface (12).

Acetate, a short-chain fatty acid (SCFA) produced by gut microbiota, plays a crucial role in maternal and fetal immune regulation (13). It influences T-cell development and anti-inflammatory pathways, which are critical during pregnancy. Maternal acetate contributes to fetal thymic development and the generation of regulatory T cells (Tregs) (13). Tregs are essential for maintaining immune tolerance to paternal antigens expressed by the foetus (14). Maternal serum acetate levels are significantly reduced in pre-eclampsia. This correlates with impaired fetal thymic development and reduced Treg output, leading to immune dysregulation (15). Decreased acetate may result from altered maternal gut microbiota or reduced dietary fibre intake. These changes compromise the maternal-fetal immune axis (15). Animal studies demonstrate that maternal acetate supplementation rescues fetal thymic Treg development and reduces pre-eclampsia-like symptoms (16). Translating these findings to human pregnancy could offer a novel therapeutic approach. Promoting a high-fibre diet to enhance SCFA production and modulate immune/epigenetic changes during pregnancy may reduce PE risk. Hence, the aim of this preliminary study was examine the levels and expression of these factors in PE.

METHODS

Study Population

Thirty age-matched normotensive (NORM) and early-onset pre-eclamptic women respectively, who are monitored and met eligibility criteria as well as signed a written informed consent at a regional hospital in the Durban, South Africa were recruited for this phase of the study. The study was performed in accordance with applicable guidelines and regulations and ethical approval was obtained from the Biomedical Review Ethical Committee of the University of Kwazulu-Natal, Durban, South Africa (BE253/19). Criteria for inclusion as early-onset PE were gestational age of 24-33 weeks, with hypertension (defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg) and accompanied by proteinuria (defined as protein in a 24-hour urine collection ≥ 300 mg) and/or evidence of thrombocytopenia (low blood platelet count of ≤ 50 000 per microliter). While the criteria for inclusion for age-matched normotensive pregnancies are normal blood pressure (values under 140/90 mmHg during pregnancy) and blood platelet count. Exclusion criteria were chronic hypertension, cardiovascular or renal disease, diabetes mellitus, bleeding disorders, HIV infection and previous medical conditions as well as non-consenting pregnant women.

Sample collection and preparation

Blood samples were collected through venipuncture in plain sample tubes, centrifuged at 3500 rpm for 10 minutes (Heraeus, Megafuge 1.0R, ThermoScientific, Waltham, U.S.A.), and the serum was stored at -80^{0}C until required for biochemical analysis.

Biochemical analysis

Determination of HDAC-2 and acetate levels

In the serum samples, the concentration of HDAC2 and acetate was determined using the quantitative sandwich Enzyme-Linked Immunosorbent Assay (ELISA) technique. A customized ELISA kit specific for human HDAC-2 and acetate, respectively, was purchased commercially (Cloud-Clone Corp, Wuhan), and the analyses were performed according to the manufacturer's instructions.

Each well contained 100 µL of appropriately diluted standards, and homogenized placenta samples were added to each well. The plates were sealed and incubated for 60 minutes at 37°C. The plate contents were removed after 60 minutes, 100 µL of Detection Reagent A was added, and an antibody working solution was added to each well. The plates were sealed, and incubation was followed at 37˚C for 60 minutes. The solution from the wells was aspirated, and 350 µL of wash buffer was added to each well. After that, the plates were washed three times with buffers. 100 µL of Detection Reagent B working solution was added, and the plate was incubated at 37˚C for 30 minutes.

The plate was washed with buffer solutions five times, forming the final washing step. Subsequently, 90 µL of substrate solution was added to each well, and then the plate was sealed and incubated for 10-20 minutes at 37˚C. After the incubation solution in the wells was aspirated, a wash process for a total of 5 times was conducted. Then 50 µL of stop solution was added to each well and mixed thoroughly by tapping the sides of the plate; drops of water and fingerprints on the bottom were removed before the reading was done.

A microplate reader (Spectrostar-Nano BMG, Labtech, Germany), set at 450nm, was used to determine the optical density. The standard curve was prepared and used to extrapolate the concentration of HDAC-2 and acetate in the serum, respectively. The assays were duplicated, and average values were considered for all the calculations.

Determination of HLA-DRA expression

Total RNA was extracted using the QIAamp RNA body fluid kit (Qiagen) following the manufacturer's instructions. Samples with an OD260/OD280 ratio >1.90 were included. All the RNA samples were DNase-treated. One microgram of RNA per sample was reverse-transcribed using the iScript cDNA synthesis kit. Based on PCR efficiency, β-Actin was used as a reference gene. The PCR primers and cycling conditions, and β-Actin RT-PCR were validated. Each PCR reaction was prepared comprising 0.25 pmol/µ primer, 5µ SYBR Green Master mix, 11µ cDNA and water to maintain a volume of 10µ. Reactions were run in duplicate, followed by 45

cycles of denaturation, annealing and extension. Detection of fluorescent products was carried out at the end of the 72^0C °C extension period, and the fold changes were determined.

Table 1: Primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
Actin	GCCTTTGCCGATCCGC	GCCGTAGCCGTTGTCG
HLA-DRA	AAGGCACATGGAGGTGATGG	TACGGAGCAATCGAAGAGGC

Statistical analysis:

GraphPad Prism 10 was used to analyze the data, and the data was represented as mean±SD. Comparisons between mean values was performed using Student's t-test with a p-value less than 0.05 considered statistically significant.

RESULTS

There was a significant decrease ($p<0.02$) in the level of HDAC (0.12±0.01) in EOPE compared to NORM (0.15±0.01) group. Similarly, the levels of HLA-DRA (0.04±0.02) showed a significant increase ($p<0.005$) in EOPE compared with NORM group (0.10±0.01), while the levels of acetate in EOPE (83.33±5.05) showed a significant decrease ($p<0.004$) when compared to NORM group (156.70±10.91).

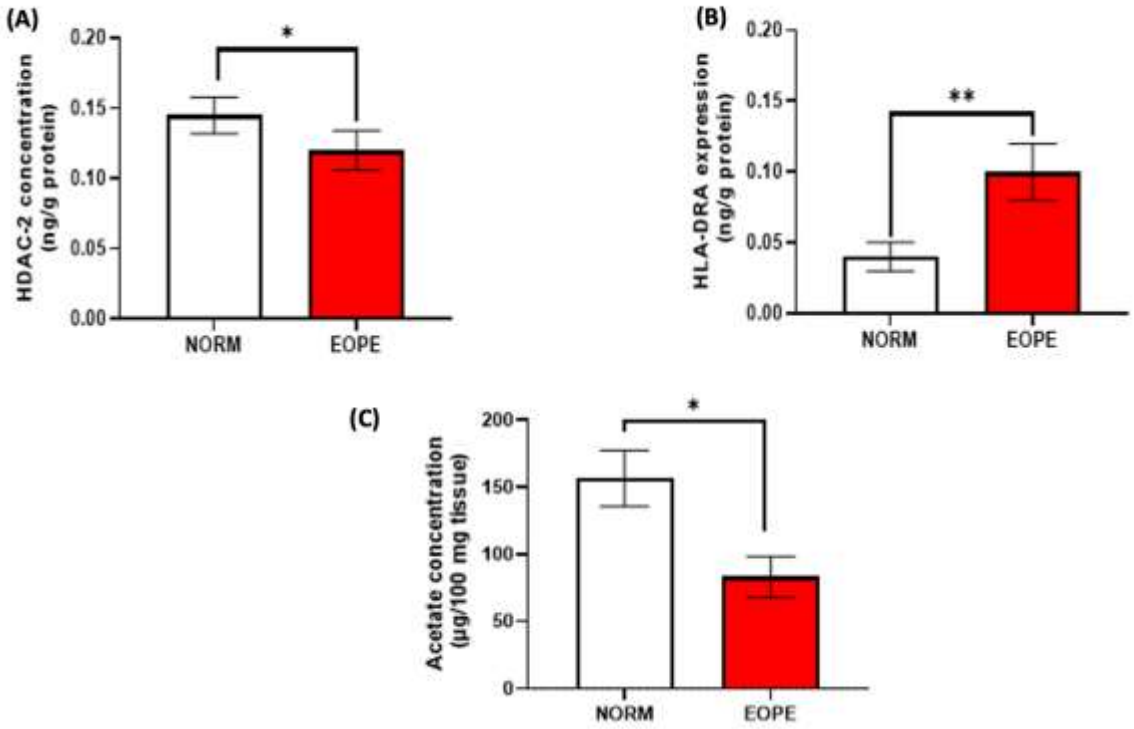

Figure 1: Concentration of HDAC-2 (a), HLA-DRA (b) and acetate (c) in Normotensive (NORM) and early-onset pre-eclampsia (EOPE). Data was analyzed with Student's t-test. Data are expressed as mean ± S.D. ***(*p<0.05 vs NORM).***

DISCUSSION

The findings of this study provide key insights into the potential molecular mechanisms underlying PE, particularly EOPE. The observed alterations in HDAC-2, HLA-DRA, and acetate levels suggest an interplay between epigenetic modifications, immune activation, and microbial metabolite dysregulation, which may contribute to the pathophysiology of PE. HDAC-2 is a key enzyme involved in chromatin remodelling and gene expression regulation. The significant decrease in HDAC-2 levels in EOPE compared to normotensive pregnancies suggests that epigenetic modifications may play a role in the altered placental function observed in

PE. HDAC-2 downregulation is known to influence inflammatory gene expression, leading to an exacerbated immune response, which aligns with the inflammatory nature of PE (7, 17). The restraint of trophoblast proliferation and migration in PE individuals through the suppression of HDAC2 was reported (18, 19). Additionally, previous research has linked HDAC-2 with vascular homeostasis and endothelial function, both of which are commonly impaired in PE (5, 6). The reduction in HDAC-2 suggests that PE may be associated with epigenetic reprogramming that influences immune response, inflammation, and vascular dysfunction.

There was also a significant increase in HLA-DRA levels in EOPE compared to normotensive controls, which further supports the role of immune system dysregulation in PE. HLA-DRA, a key component of the major histocompatibility complex (MHC) class II, is involved in antigen presentation and immune activation (10, 20). Increased expression of HLA-DRA suggests an enhanced maternal immune response against fetal or placental antigens, which could lead to placental inflammation, oxidative stress, and endothelial dysfunction, hallmarks of PE. This finding is consistent with prior studies suggesting that early-onset PE is characterized by a heightened maternal immune response and a failure of immune tolerance mechanisms, leading to restraint trophoblast proliferation and abnormal placental development (11).

In addition, the level of acetate in EOPE decreased when compared to normotensive pregnancy, indicating a potential disruption in microbial-derived metabolic pathways. Acetate is a SCFA primarily produced by gut microbiota, and it plays a critical role in immune regulation, inflammation suppression, and vascular homeostasis (15). Reduced acetate levels suggest that microbiome alterations may contribute to the immune dysregulation and vascular dysfunction, as well as the inflammation observed in PE. SCFAs like acetate have been shown to modulate T regulatory (Treg) cell function and inflammatory cytokine production, which are essential for maintaining maternal-fetal immune tolerance (13). A deficiency in acetate could therefore exacerbate placental inflammation, oxidative stress, and endothelial dysfunction, all of which are implicated in PE pathophysiology.

The combined findings of HDAC-2 downregulation, HLA-DRA upregulation, and acetate depletion suggest a multifaceted pathophysiological mechanism in PE. The interplay between epigenetic modifications, immune system activation, and microbial metabolite alterations could create a pro-inflammatory environment in the placenta, disrupting normal trophoblast function and leading to the vascular and immune dysfunctions characteristic of PE. These findings have significant implications for the understanding and management of PE. The observed molecular alterations suggest that targeting epigenetic regulators, immune modulators, or microbial metabolites could be potential therapeutic strategies for PE.

CONCLUSION

The findings of this study provide further insights into the molecular alterations associated with PE, particularly EOPE. The significant decrease in HDAC-2 levels in EOPE suggests potential epigenetic dysregulation, which may contribute to abnormal gene expression and placental dysfunction. Additionally, the elevated levels of HLA-DRA in EOPE indicate heightened immune system activation, reinforcing the role of immune dysregulation in the pathogenesis of PE. The significant reduction in acetate levels in EOPE further highlights a potential disruption in microbial metabolite production, which may impact immune tolerance and vascular function during pregnancy. These findings underscore the complex interplay between epigenetic regulation, immune response, and microbial metabolism in PE. While this study provides preliminary evidence of these molecular changes in EOPE, further research is required to elucidate the changes that are likely to occur in late-onset PE and their precise mechanistic roles in PE development. Future studies should explore whether acetate supplementation, immune modulation, or epigenetic therapies could serve as potential strategies for PE prevention and management.

LIMITATION AND FUTURE STUDY

The sample size was relatively small, which may limit the generalizability of the findings. Future studies should use a larger, multicenter cohort, which would strengthen the robustness of the observed associations. More

analysis should be performed on epigenetic markers and microbiome-derived metabolites in both early- and late-onset pre-eclamptic cohorts.

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CHAPTER 6

SYNTHESIS AND CONCLUSION

6.1 Synthesis of Key Findings

As well documented, pre-eclampsia (PE) is broadly classified into two primary phenotypes based on the gestational age at onset. Early-onset preeclampsia (EOPE) occurs before 34 weeks of gestation and is predominantly associated with placental insufficiency. This condition is believed to result from the inadequate invasion of extravillous trophoblasts into the maternal spiral arteries, leading to poor placental perfusion and severe consequences for both mother and foetus. While late onset pre-eclampsia (LOPE) typically emerges after 34 weeks and is thought to stem from placental overcrowding and a failure to adequately support the metabolic demands of the rapidly growing foetus [112]. Although the precise mechanisms underpinning PE remain unclear, the distinction between EOPE as a preterm condition and LOPE as a term condition poses a significant clinical challenge. Timely and accurate differentiation between these phenotypes is critical to guiding appropriate management and improving outcomes. Adding to this public health concern is the ongoing debate surrounding the presence of a placental microbiome. While some studies suggest the placenta is a sterile environment, emerging evidence indicates the presence of a unique microbial community that may influence placental development and function [113, 114].

Given the controversies surrounding placental microbial colonisation [115, 116], a comprehensive review of the literature was conducted to examine the role of the placental microbiome in the pathogenesis of PE. The review shows that the microbiome is underrepresented in placental tissue, and disruption of the placental microbiome may trigger immune activation and endothelial dysfunction characteristic of PE. However, causal links and the plausibility of microbial-mediated inflammation contributing to abnormal placentation and endothelial dysfunction remain to be firmly established (Chapter 2). To build on these insights, the study was designed to investigate the potential involvement of the placental microbiome in the aetiology of PE, a multifactorial

hypertensive disorder of pregnancy with systemic implications. Using a combination of real-time PCR, NGS, and molecular analyses, the study evaluated the presence and diversity of microbial communities in placental tissues, performed a preliminary quantification of microbial metabolites, and assessed immunological and epigenetic markers. The data revealed low microbial biomass in placental samples from both pre-eclamptic (early- and late-onset) and age-matched normotensive pregnancies, without significant differences in bacterial species. Additionally, the biochemical analyses demonstrated an interplay between microbial, immune, and epigenetic factors in PE pathogenesis, particularly EOPE. These findings offer preliminary yet valuable insight into a possible microbiota-metabolite-epigenome axis influencing PE development, particularly in EOPE. Hence, this chapter synthesizes the major findings and discusses their implications for future research and clinical practice.

6.2 Objectives of the Study

The study was guided by the following objectives:

(i) To identify different microbes, present in the placentas of normotensive and early- and late-onset pre-eclamptic pregnancies using a targeted 16S analysis.

(ii) To identify different bacterial species, present in the placentas of normotensive and pre-eclamptic pregnancies using NGS.

(iii) To determine the serum levels of microbial metabolite (acetate), epigenetic regulator (HDAC2) and immune response (HLA-DRA) in normotensive and pre-eclamptic pregnancies.

6.3 Interpretation of Findings

6.3.1 Placental Microbiome and Pre-eclampsia Pathogenesis

The findings of this study using a targeted 16S analysis suggest that while bacterial species such as *Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria,* with *Proteobacteria* dominated by the classes *Pseudomonadales* and

Gammaproteobacteria with smaller amounts of *Actinobacteria* and *Bacteroidetes* are present in placental tissues, their overall biomass is low, and their composition did not show a significant difference between EOPE or LOPE and age-matched normotensives (0.1705). This challenges the previous report that placental microbiota plays a direct role in PE pathophysiology or pregnancy complications [117, 118, 119, 120]. However, the observed variation in bacterial diversity, although not statistically significant, suggests that subtle microbial differences may exist and could influence placental function in ways not yet fully understood. The lack of a strong microbial signature in PE supports the notion that the placenta is either a sterile or a low-microbial environment, reinforcing scepticism about the clinical relevance of placental microbiome. If bacterial communities are indeed present, their role in pregnancy complications like PE remains unclear, possibly due to their low abundance (Chapter 3).

In addition, the assessment of bacterial DNA composition in placental tissues of pre-eclamptic (early/late-onset) and normotensive women using shotgun metagenomic sequencing, a comprehensive approach well-suited for low-biomass environments [104, 120] such as the placenta. The analysis revealed that bacterial DNA, primarily from *Cutibacterium acnes*, *Staphylococcus epidermidis*, and *Bradyrhizobium species*, was detected in both pre-eclamptic and normotensive placentas. However, the overall microbial load was low, and no statistically significant differences in bacterial composition were found after adjusting for multiple comparisons. Despite the lack of significance, the study identified notable taxa of potential biological interest, including a reduction in *Bradyrhizobium sp*. ma5 in PE samples and the exclusive presence of *Variovorax species* in PE placental samples, both with large effect sizes. These findings, while not definitive, underscore the potential for subtle microbial shifts that may have biological relevance. However, they also highlight the methodological challenge of distinguishing true microbial signatures from environmental contaminants in low-biomass samples.

Importantly, the study employed a rigorous dual-classifier system (Kraken2/Bracken and MetaPhlAn) combined with an enhanced contamination assessment framework. This methodological robustness strengthens confidence in the findings and contributes meaningfully to the ongoing debate regarding the existence and role of placental

microbiota. The absence of a consistent, functionally distinct microbial community in the placenta aligns the present findings with growing evidence that challenges the concept of a true placental microbiome and its involvement in the pathogenesis of PE (EOPE and LOPE) (Chapter 4). Interestingly, the study provides data from a Sub-Saharan African population, which fills a critical gap in the global understanding of placental microbiota and pre-eclampsia, offering insights that are more representative of diverse populations with disproportionately high PE burdens. It encourages region-specific microbiome research and supports equitable global scientific discourse. Nevertheless, future investigations into the microbial contributions to pre-eclampsia may focus on maternal sites with high microbial biomass (e.g., gut, oral cavity, vagina). Dysbiosis in these sites has been reported to indirectly influence placental health through systemic immune modulation, metabolic changes, or microbial metabolites [121, 122, 123].

6.3.2 Alteration in Microbial Metabolite, Immune Response and Epigenetic Interaction

Although the overall microbial load of placental tissues from pre-eclamptic and age-matched normotensive pregnancies was low, and no statistically significant differences were observed in the bacterial composition of PE (EOPE and LOPE) compared with normotensive controls, hence biochemical analysis of HLA-DRA, HDAC-2 and acetate using qPCR and ELISA techniques provides a molecular insight to the pathogenesis of PE, particularly EOPE, by revealing the interconnected roles of epigenetic regulation, immune activation, and microbial metabolite dysregulation. The significant reduction in HDAC-2 levels in EOPE compared with age-matched normotensive individuals suggests that epigenetic reprogramming may contribute to aberrant placental gene expression. HDAC-2 is critical in chromatin remodelling, immune regulation, and vascular homeostasis. Its suppression may lead to increased inflammatory responses, impaired trophoblast function, and endothelial dysfunction [124, 125], as corroborated by the elevated expression of HLA-DRA in EOPE (Chapter 5), a key component of the MHC class II complex, indicating an exacerbated maternal immune response. This may reflect increased antigen presentation and immune activation at the maternal-fetal interface, contributing to placental

inflammation, immune intolerance, and oxidative stress, which are critical features of PE's pathogenesis. Therefore, the present results align with emerging evidence that epigenetic modifications, including HDAC and DNA methylation, are critical regulators of placental gene expression and may influence pathways involved in trophoblast invasion, angiogenesis, and immune tolerance [6, 126]. Similarly, a significant reduction in serum acetate (a microbiota-derived short-chain fatty acid) level was observed in the EOPE compared with age-matched normotensive control, implicating microbiome-related metabolic disruption in PE. Acetate plays a crucial role in regulating immune responses, maintaining vascular tone, and supporting anti-inflammatory signalling [127, 128, 129]. Its depletion suggests a loss of these protective effects, potentially exacerbating immune dysregulation and vascular dysfunction that characterise the pathogenesis of PE. Collectively, these findings suggest that PE, particularly EOPE, may arise from a convergence of epigenetic alterations, dysregulated maternal immune tolerance, and microbiome-derived metabolic insufficiency, all of which foster a pro-inflammatory and vasculopathic placental environment. Hence, HDAC-2 and HLA-DRA, along with altered acetate levels, may serve as candidate biomarkers for early detection of PE, especially EOPE. Biomarker panels that integrate these molecular changes could improve the risk assessment and diagnosis of PE. It is noteworthy that the alterations in HDAC-2, acetate and HLA-DRA in PE might not be attributable to the low biomass of placental microbiome but the microbiota in other maternal sites, such as the gut, oral cavity, and vagina. These appear consistent with previous studies, which reported that maternal gut microbiota as a key player in modulating placental immunity and pregnancy outcome, and targeting the gut microbiota may offer a strategy for preventing pre-eclampsia [123].

6.4 Conclusion

While the presence of a consistent and clinically relevant placental microbiome remains unsubstantiated due to the low microbial biomass and lack of significant differences in bacterial species between pre-eclamptic and normotensive pregnancies, the study's integrative molecular approach reveals that even in the absence of high microbial community, perturbations in microbial metabolites (acetate), immune markers (HLA-DRA), and

epigenetic regulators (HDAC-2) may drive pathological processes in PE. The observed reduction in HDAC-2 and acetate, coupled with an elevation of HLA-DRA in EOPE, suggests a microbiota–metabolite–epigenome axis that contributes to disrupted immune activation, endothelial dysfunction, and placental maladaptation. These findings imply that PE is less likely caused by direct microbial invasion of the placenta and more plausibly influenced by indirect effects of microbial products and host immune-epigenetic responses. Importantly, the study contributes novel data from a Sub-Saharan African cohort, helping to bridge geographic gaps in microbiome and PE research. Overall, the findings underscore the need for a broader, systemic approach to understanding PE, incorporating host–microbiome interactions at maternal sites with high microbial load and molecular signalling pathways. These insights hold promise for developing early biomarkers and targeted interventions to mitigate the burden of PE, particularly in high-risk populations.

6.5 Limitations of the Study

The present study has few limitations. First, the absence of negative bacterial control during sequencing and downstream analyses represents an important methodological constraint. Without this control, it was difficult to fully rule out background contamination, an issue particularly relevant in low-biomass samples such as placental tissue. This limitation might affect the certainty of taxonomic assignment and the interpretation of low-abundance microbial signals. Although stringent aseptic sampling, standardised DNA extraction procedures, and bioinformatic filtering were applied to minimise contamination risk, the inclusion of comprehensive controls in future studies would strengthen confidence in microbial identification and improve reproducibility. Second, the relatively small sample size limits statistical power and might have reduced the ability to detect subtle but biologically meaningful differences in microbial composition between pre-eclamptic and normotensive placentas. Small cohort sizes can increase the risk of type II error and might limit the generalisability of the findings. Future multicentre studies with larger, more diverse cohorts would enable more robust subgroup analyses, increase statistical power, and enhance the external validity of the results.

Third, although the study identified key microbial, epigenetic, immune and metabolic alterations associated with EOPE, the investigation of LOPE was comparatively limited. Given that EOPE and LOPE may differ in underlying pathophysiology, the molecular pathways identified in EOPE cannot be fully extrapolated to LOPE. Further research is therefore required to characterise microbiome-host interactions across PE subtypes and to clarify whether similar mechanisms operate in LOPE or whether distinct biological pathways predominate. Lastly, while advanced sequencing approaches (targeted 16S rRNA and shotgun metagenomics) were employed, functional validation of identified microbial taxa and associated metabolites was beyond the scope of the present study. As such, inferred functional pathways and mechanistic links between microbial profiles and host responses are interpreted cautiously. Future studies that integrate metatranscriptomics, metabolomics, and in vitro or in vivo functional models would help confirm biological relevance. Despite these limitations, the study provides important preliminary data and justification for future research into the placental microbiome and its interaction with epigenetic and immunometabolic pathways in PE.

6.6 Recommendations for Future Research

Future investigations should use a large sample size and employ advanced high-throughput sequencing and metagenomic approaches to enhance the detection of microbes and clarify their functional roles in the pathogenesis of PE. Additionally, longitudinal studies that track changes in the maternal microbiome, immune markers, and placental health over time to better understand the dynamic relationships among these factors. Integrating multi-omics approaches, combining metagenomics, transcriptomics, proteomics, and metabolomics, could offer a more comprehensive picture of how microbial and host factors interact in the context of pre-eclampsia. Investigation should also explore microbiome-targeted interventions, such as probiotics, prebiotics, or antibiotics, to evaluate their potential in PE prevention and management. Studies are also needed to elucidate the genetic and epigenetic mechanisms linking microbial activity to PE, to identify novel biomarkers and therapeutic targets for early diagnosis and effective treatment strategies.

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APPENDICES

APPENDIX I

Hospital Permission

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Physical Address: 330 Langalibalele Street, Pietermaritzburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3189/ 3122 Fax: 033 394 3782
Email:
www.kznhealth.gov.za

DIRECTORATE:
Health Research & Knowledge Management

NHRD Ref: KZ_201909_001

Dear Mr KE Olaniyi
UKZN

Approval of research

1. The research proposal titled **'The role of placental microbiome in the pathogenesis of preeclampsia in women'** was reviewed by the KwaZulu-Natal Department of Health.

   The proposal is hereby **approved** for research to be undertaken at Prince Mshiyeni Memorial Hospital.

2. You are requested to take note of the following:
   a. Kindly liaise with the facility manager BEFORE your research begins in order to ensure that conditions in the facility are conducive to the conduct of your research. These include, but are not limited to, an assurance that the numbers of patients attending the facility are sufficient to support your sample size requirements, and that the space and physical infrastructure of the facility can accommodate the research team and any additional equipment required for the research.
   b. Please ensure that you provide your letter of ethics re-certification to this unit, when the current approval expires.
   c. Provide an interim progress report and final report (electronic and hard copies) when your research is complete to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Mr X. Xaba on 033-395 2805.

Yours Sincerely

[redacted]

Dr E Lutge
Chairperson, Health Research Committee
Date: 03/10/19.

Fighting Disease, Fighting Poverty, Giving Hope

APPENDIX II

Ethical Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

27 August 2019

Mr KS Olaniyi (218085055)
School of Laboratory Medicine and Medical Sciences
College of health sciences
Kennethnitty2010@gmail.com

Dear Mr Olaniyi

Protocol: The role of placental microbiome in the pathogenesis of pre-eclampsia in women
Degree: PhD
BREC Ref No: BE253/19

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 20 March 2019.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 12 March 2019 to BREC letter dated 23 May 2019 has been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have been met and the study is given full ethics approval and may begin as from 27 August 2019. **Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.**

This approval is valid for one year from **27 August 2019.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (If applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on **08 October 2019.**

Yours sincerely

[redacted]

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

Cc: Postgrad administrator: [redacted] Supervisor: [redacted]

Biomedical Research Ethics Committee
Professor V Rambiritch (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2486 Facsimile: +27 (0) 31 260 4609 Email: [redacted]
Website: [redacted]

APPENDIX III

Ethical Recertification

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

01 November 2022

Mr KS Olaniyi (218085055)
School of Laboratory Medicine and Medical Sciences
College of health sciences
[redacted]

Dear Mr Olaniyi

Protocol: The role of placental microbiome in the pathogenesis of pre-eclampsia in women
Degree: PhD
BREC Ref No: BE253/19

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	27 August 2022
Expiration of Ethical Approval:	26 August 2023

I wish to advise you that your application for Recertification received on 18 October 2022 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

The lapse period of certification has been condoned.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 13 December 2022.

Yours sincerely

[redacted]

Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

APPENDIX IV

Certificate of Editing

LANGUAGE INSTITUTE

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Harwin Road,
Scottsville
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Tel: [redacted]
admin@kznlanguageinstitute.com
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Registration number: 131 804 NPO

Certificate of editing 18 August 2025

Name: Kehinde Samuel Olaniyi

Title: Investigating the role of placental microbiome in the pathogenesis of early- and late-onset pre-eclampsia

This serves to confirm that the above document was edited substantively by members of the KZN Language Institute's professional English language editing team. The document was returned to the author with tracked changes and comments intended to correct errors and to clarify meaning. It was the author's responsibility to attend to these changes.

[redacted]

J. Kerchhoff

Director of the KwaZulu-Natal Language Institute

KZN Language Institute - Transforming Words

APPENDIX V

Notification of Acceptance for Manuscript Three

cjpp-2025-0274.R2 - Notice of Acceptance

Canadian Journal of Physiology and Pharmacology

to me, JMOG, Moodleyrosh, mackraji

29-Jan-2026

Dear Kehinde Olaniyi and co-authors:

We are pleased to inform you that your manuscript is now acceptable for publication in Canadian Journal of Physiology and Pharmacology. Your manuscript will be returned to your Author Center for you to complete your final manuscript steps.