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Placental microbiome and preeclampsia: from basic research to clinical practice

Mariana Vieites Branco Fonseca

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Placental microbiome and preeclampsia: from basic research to clinical practice

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Mariana Vieites Branco Fonseca

Aluna do 6º ano profissionalizante de Mestrado Integrado em Medicina

Afiliação: Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto

Endereço: Rua de Jorge Viterbo Ferreira nº228, 4050-313 Porto

Endereço eletrónico: marianafonseca1698@gmail.com; up201604957@edu.icbas.up.pt

Orientador: Dra. Maria Luísa Santos Ferreira Vieira

Docente convidada do Instituto de Ciências Biomédicas Abel Salazar

Assistente Hospitalar Graduada de Ginecologia e Obstetrícia no Centro Materno Infantil do Norte - Centro Hospitalar Universitário do Porto

luisa.sfv@gmail.com

Coorientador: Professor Doutor Luís Guedes Martins

Professor Auxiliar Convidado do Instituto de Ciências Biomédicas Abel Salazar

Assistente Hospitalar de Ginecologia e Obstetrícia no Centro Materno Infantil do Norte - Centro Hospitalar Universitário do Porto

luís.guedes.martins@gmail.com

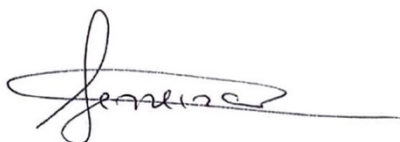
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Autora



(Mariana Vieites Branco Fonseca)

Orientadora



(Dra. Maria Luísa Santos Ferreira Vieira)

Coorientador



(Prof. Doutor Luís Guedes Martins)

Porto, Junho de 2022

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Resumo

Introdução: A pré-eclampsia é uma síndrome multissistêmica, uma das principais causas de mortalidade e morbidade materno-fetal, no entanto não existe uma etiologia comprovada para o desenvolvimento da mesma. A esterilidade da placenta foi anteriormente considerada como uma condição necessária para um desfecho obstétrico favorável, porém atualmente a colonização microbiana é considerada. Estudos recentes destacam a capacidade do microbioma interagir com condições fisiológicas e patológicas incluindo a tolerância imune, a homeostasia energética, diabetes, obesidade, distúrbios do neuro desenvolvimento como o autismo e doenças cardiovasculares.

Objetivo: Revisão da literatura atual sobre a possível identificação de um microbioma placentário e o seu papel no desenvolvimento da pré-eclampsia, com intuito de sugerir potenciais medidas preventivas e, em última instância, diminuir a morbidade materna e fetal associada a esta patologia.

Metodologia: Revisão bibliográfica com base em pesquisa entre Janeiro de 2000 e Dezembro de 2021 na PUBMED, sobre o microbioma materno na pré-eclampsia, particularmente o microbioma placentário, incluindo artigos originais, de revisão bibliográfica e documentos emitidos por sociedades científicas.

Discussão: Em comparação com o microbioma oral, vaginal e intestinal, o microbioma da placenta demonstrou elevada semelhança em termos de composição microbiana com o oral. Relacionando a constituição do microbioma placentário, a sua associação com outros microbiomas, e a patofisiologia da pré-eclâmpsia, possíveis fatores contribuintes para esta patologia hipertensiva foram identificados e incluem os lipopolissacarídeos, os recetores do tipo Toll e o seu efeito inflamatório em cascata, a citocina interleucina-10, a predominância de linfócitos T-auxiliares do tipo 1/tipo 2 e moléculas de ácido gordo de cadeia curta. Dependendo da composição microbiana, observou-se a disrupção na remodelação das artérias espiraladas e aterosclerose das mesmas, com um papel no desenvolvimento desta patologia. Com a descoberta de uma comunidade de microrganismos e fatores envolvidos na pré-eclâmpsia, soluções terapêuticas como probióticos, pré-bióticos e agentes anti-oxidativos foram discutidas. No entanto, vários estudos recentemente refutam a presença de um microbioma placentário e consideram que a interface materno-fetal é um ambiente amicrobiano realçando as limitações dos estudos realizados à data.

Conclusão: Embora uma relação entre a composição microbiana oral, intestinal, vaginal e da placenta tenha sido identificada, não existe evidência conclusiva para determinar um “microbioma disbiótico da placenta” associado à pré-eclâmpsia e o efeito direto entre a composição microbiana e a pré-eclâmpsia. Na esperança de um conhecimento crescente sobre a repercussão microbiana na saúde materna, propõe-se estudos adicionais com protocolos estabelecidos, para minimizar a contaminação, seguidos de análise meta genômica e metabolômica, para aprofundar a evidência de um microbioma placentário assim como a patofisiologia envolvida na pré-eclâmpsia.

Palavras-chave: Preeclampsia, microbiota, pregnancy complications, gastrointestinal microbiome, placenta diseases, placental microbiome, preeclampsia AND placental microbiome, preeclampsia AND gut OR vaginal OR oral microbiome

Abstract

Introduction: Preeclampsia is a multi-systemic syndrome, one of the leading causes for maternal and foetal mortality and morbidity and yet no exact aetiological cause can be identified for its development. The placental sterility was previously considered an obligation for healthy obstetric outcomes, however nowadays the microbial colonization is proposed. Recent research highlights the microbiome's ability to modulate both physiological and pathological conditions including immune tolerance, energy homeostasis, diabetes, obesity, neurodevelopmental disorders such as autism and cardiovascular diseases.

Objectives: Bibliographic review aiming to identify the placental microbiome, it's role in the development of preeclampsia, in hopes of suggesting potential preventive measures, and therefore leading to the possible reduction of foetal and maternal morbidity associated with this disease.

Methods: Bibliographic review based on research made in the PubMed database from January 2000 to December 2021, about maternal microbiome and preeclampsia, particularly placental microbiome, including original articles, bibliographic reviews and guidelines emitted by scientific societies.

Discussion: In comparison to vaginal, oral, and gastrointestinal microbiome the placental microbiome revealed a greater similarity with oral microbial composition. Linking the constitution of placental microbiome, its relationship with other body niche microbiomes and the pathophysiology of preeclampsia, possible contributing factors to this hypertensive disorder were identified as lipopolysaccharide molecules, toll-like receptors and their inflammatory cascade effect, interleukin-10 cytokine, T-helper 1/T-helper 2 cell predominance and short-chain fatty acid molecules. Similarly faulty spiral artery remodelling and atherosclerosis were found in relationship to microbial composition. With the determination of a placental microbial community and involved factors in preeclampsia, therapeutic solutions including probiotics, prebiotics and anti-oxidative measures have been discussed. However, several studies have recently contradicted the presence of a placental microbiome and considered this maternal-foetal interface an amicrobial environment drawing attention to the limitation of studies available to date.

Conclusion: Although a relationship between the microbial composition of oral, gastrointestinal, vaginal, and placental microbiome was revealed, to date there is no conclusive evidence of a "preeclamptic dysbiotic microbiome" and the direct effect of the microbial composition and preeclampsia. In hopes of increasing the knowledge of the microbial repercussion in maternal

health, we propose that further studies are produced with established protocols to minimize contamination and that metagenomic and metabolomic analysis are executed to deepen the evidence of a placental microbiome as well as the pathophysiology involved in preeclampsia.

Key Words: Preeclampsia, microbiota, pregnancy complications, gastrointestinal microbiome, placenta diseases, placental microbiome, preeclampsia AND placental microbiome, preeclampsia, AND gut OR vaginal OR oral microbiome

Abbreviations

BP | Basal plate

CST | Community State Type

DNA | Deoxyribonucleic acid

FM | Foetal membranes

GDM | Gestational diabetes Mellitus

GWG | Gestational weight gain

HDP | Hypertensive disorders of pregnancy

IL | Interleukin

LPP | Lipoproteins

LPS | Lipopolysaccharide

NF- κ B | Nuclear factor - κ B

OTU | Operational taxonomic unit

PCR | Polymerase chain reaction

PE | Preeclampsia

PPROM | Preterm Premature Rupture of Membranes

PTB | Preterm birth

PV | Placental villi

rRNA | Ribosomal ribonucleic acid

SCFA | Short-chain fatty acids

SPE | Severe Preeclampsia

Th | T-helper

TLR | Toll-like receptor

TMAO | Trimethylamine-N-oxide

TNF- α | Tumor necrosis factor- α

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1 Introduction

Preeclampsia (PE) is a multi-systemic syndrome that affects approximately 2 to 8 percent of all pregnancies¹. This condition is part of the spectrum of hypertensive disorders of pregnancy (HDP), responsible for 16,1% of maternal deaths in developed countries^{2,3}. PE accounts for over 70,000 maternal and 500,000 foetal deaths and so stands out among the hypertensive disorders as one of the main causes of perinatal and maternal mortality and morbidity^{4,5}. The sphere of complications developing from PE includes, on the maternal side acute kidney failure, hepatic failure, disseminated intravascular coagulation, and cardiovascular collapse, but also regarding obstetric outcomes, placental abruption, preterm birth, intrauterine growth restriction, and low birth weight^{1,6,7}. This HDP can also evolve into eclampsia, characterised as a new onset of seizures that cannot be attributed to another cause, or HELLP syndrome, which encompasses haemolysis, elevated liver enzymes, and thrombocytopenia⁸.

PE was classically defined as hypertension after 20 weeks of gestation and new-onset proteinuria, in a previously normotensive woman⁵. Although in most cases proteinuria is present, a significant proportion of women with hypertension develop only systemic signs of PE such as thrombocytopenia, liver or renal dysfunction, in the absence of proteinuria^{9,10}. The presence of proteinuria in acknowledgement of the syndromic nature of PE is no longer necessary for the diagnosis, relying otherwise on the presence of analytical or clinical signs of maternal organ dysfunction⁵. The disorder is in that case further classified as PE with severe features (**table I**). The International Society for the Study of Hypertension in Pregnancy also determined that hypertension with uteroplacental dysfunction (such as foetal growth restriction, abnormal umbilical artery doppler waveform or stillbirth) could pose as a sufficient criterion for the diagnosis¹¹.

In addition to the designation of preeclampsia with or without severe features, as shown in **table 1**, this condition can also be subdivided into early or late-onset, if diagnosed before or after 34 weeks of gestation, respectively^{12,13}. Early-onset PE is primarily associated with impaired placental development and late-onset PE with dysfunction in the maternal endothelial surface¹⁴.

Although PE can only clinically be defined after 20 weeks, the pathophysiology related to its development is believed to begin earlier. Described as a two phase model, firstly there is abnormal placentation occurring in early pregnancy (first and second trimester) which progresses to the maternal syndrome with systemic vascular dysregulation in the late second and third trimesters⁵. The protagonist in PE appears to be the placenta due to inappropriate angiogenesis, inadequate spiral uterine artery remodelling, and trophoblast invasion¹⁵⁻¹⁷. Several theories have been proposed for the development of an abnormal placentation which include oxidative stress, abnormal uterine natural killer cells in the decidua, and genetic and environmental factors^{5,17}.

Although no conclusive evidence has been identified regarding the pathogenesis of this first phase, the same is not true in the second phase with an association between uteroplacental ischemia and the development of a maternal syndrome^{5,14}. In response to the ischemic placenta, an excessive release of anti-angiogenic factors is triggered which ultimately results in inflammation, endothelial dysfunction, a cytokine storm and ultimately a systemic disease leading to the characteristic hypertensive multi-organ involvement of PE⁵ (**figure 1**).

Nonetheless, the precise etiology of PE remains unknown and there are no approved therapies that specifically target this disease, with the few available applied for the management of symptoms and with delivery being the ultimate option for resolution^{5,18–20}. Although currently there are no accurate measures for the prevention or screening tools that can predict PE before pregnancy⁵, some risk factors are known and include a history of PE, chronic hypertension, antiphospholipid syndrome, pre-gestational diabetes mellitus, pre-gestational obesity (BMI>30), as well as periodontal diseases^{21,22}. In comparison, some of these risk factors have also been associated with the capacity to alter the microbiota composition^{23–26}.

The microbiome encompasses the microbiota, living microorganisms such as bacteria, archaea, fungi, algae and small protists, and their products, such as structural elements and metabolites, as well as molecules produced by co-existing hosts and genetic material including relic deoxyribonucleic acid (DNA)²⁷. This ecological niche continuously adapts and responds to external stressor events to guarantee the maintenance of eubiosis, which is characterized as the balanced host–microbiome interaction²⁸. Moreover as the female body adapts to pregnancy it suffers multiple changes in order to accommodate and maintain the healthy development of a foetus²⁹. Accompanying the physiological changes that occur in the cardiovascular, endocrine, respiratory, gastrointestinal and reproductive tract during pregnancy, a similar plasticity is seen in the oral, gastrointestinal and reproductive tract microbiota^{30,31}.

The inability to establish an aetiology for PE leads to suboptimal treatment options and diagnostic strategies. Since to date there is no way of knowing who will develop PE and given the association between risk factors of PE and microbial variation, the present review will explore the role of the placental microbiome in PE, possibly furnishing a cause for this hypertensive disorder. The establishment of a relationship between the placental microbiome, could also pose significant implications regarding preventions and therapeutic management of this disorder.

2 Methods

This bibliographic review was based on scientific articles published in English found in the PUBMED database between January 2000 and December 2021. The literature search analysed articles based on the following keywords: *preeclampsia, microbiota, pregnancy complications, gastrointestinal microbiome, placenta diseases, placenta microbiome, preeclampsia AND placental microbiome OR gut OR vaginal OR oral microbiome*. From this initial search, the selection of articles was made by the evaluation of titles and abstracts. Articles were selected based on relevance and scientific quality from high impact factor journals and preference was given to original articles and research population studies, in addition to review papers and guidelines for context information.

When necessary or relevant, a manual search was conducted using the bibliographic references of the included publications.

3 Objectives

Previous studies have demonstrated the relationship between gut, reproductive tract microbiome and preeclampsia. The objective is to review the literature for the possibility of detecting placental microbiome involved in the development of PE in hope to discover preventive measures and decrease maternal and foetal morbidity.

4 Microbiome and its implications in pregnancy

The microbiome can be defined based on ecological, host dependent or method dependent theories²⁷. The combination of these theories describes it as a dynamic and interactive ecosystem consisting of a community of micro-organisms, the microbiota, in a defined environment, encompassing both biotic and abiotic factors, the genome of the host and of the microorganisms as well as the environmental features of the habitat²⁷. Research efforts to establish commensal microbial communities, in the Human Microbiome Project ^{32,33} discovered the presence of a microbiome in many human anatomical niches, including the gastrointestinal, mouth cavity, skin and vagina. Aside from variations in the living commensal bacteria at each location within each individual (α -diversity), there is also diversity in the microbial composition at each site that differs between individuals (β -diversity)³³ meaning the variability of the microbiome is both interindividual and intraindividual, with each body compartment having its own microbiota³²(**table II**). Diet, host genetics, body weight, early microbial exposure, and antibiotics all have an impact on these different environments³⁴.

This micro-ecosystem is crucial to the homeostasis as it influences the metabolic and immunological balance^{35,36}. Currently, microbial gut colonization established during early life has been linked with the development of immune response and protection against pathogenic invasion ³⁷. Moreover disruption of the gut microbiota, termed dysbiosis, has shown to be linked with the development of noncommunicable diseases later in life such as diabetes mellitus³⁸, allergies³⁹, inflammatory bowel disease⁴⁰, obesity²⁵ and autism ⁴¹. As a dynamic ecosystem, it has been shown to modify in response to physiological changes like pregnancy, and so during the gestational period alterations occur to the gut and reproductive tract microbiomes^{30,31}.

4.1 Pregnancy microbiome

4.1.1 The gut microbiome

The gut microbiome during pregnancy has a predominantly anti-inflammatory profile during most of the gestation with low-grade inflammation present during implantation, placentation and towards birth^{42,43}. In the first trimester, the composition of the maternal gut microbiota is similar to that of a non-pregnant woman, characterized by *Firmicutes*, mainly *Clostridiales*, over *Bacteroidetes*⁴⁴ (**figure 2**). Subsequently, from the first to the third trimester, significant changes are observed characterized by an increase in diversity (β -diversity) and decrease in richness (α -diversity)⁴⁴. Complementing the shift toward a pro-inflammatory state, Le Chatelier et al. revealed that low gut microbiome α -diversity is associated with a higher occurrence of low-grade inflammation⁴⁵.

Regarding gut microbial constitution, increased levels of *Blautia*, *Ruminococcus*, *Bilophila* and *Fusobacterium* have been associated with high maternal blood pressure values and liver enzyme levels^{46–48}. Women with PE also showed a reduction in *Faecalibacterium*, *Methanobrevibacter*, both producers of short-chain fatty acids (SCFAs), and *Akkermansia*⁴⁶. SCFAs act to reduce systemic blood pressure and inflammation, therefore, reduced levels of these species are associated with an increased risk of high blood pressure^{49,50}. Moreover, lipopolysaccharides (LPS), known inflammatory factors, are identified at a higher concentration in the faeces and plasma of PE patients in comparison to healthy controls, while *Akkermansia* levels were negatively associated with LPS levels⁴⁶.

In relation to gut dysbiosis and PE, other microorganisms were detected, with the major operational taxonomic units (OTUs) at phylum level belonging to *Firmicutes* (most abundant), followed by *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. The remaining bacterial population were *Fusobacteria*, *Verrucomicrobia*, *Tenericutes*, *Cyanobacteria*, and *Melainabacteria*⁵¹ (**figure 2**). Interestingly this investigation identified two major gut-microbial products at higher levels in preeclamptic women, LPS and trimethylamine-N-oxide (TMAO)⁵¹. In the functional analysis of faecal samples, the microbiota of PE patients revealed a striking representation towards the LPS biosynthesis and, coincidentally the *Bacteroidetes* species are a primary contributor for LPS formation⁵¹. The relationship between this gut metabolite and the pathogenesis of preeclampsia is suggested through the activation of Toll-like receptor (TLR)⁴⁵². Concurrently TMAO has revealed its relationship with the pathogenesis PE through increased expression of inflammatory markers which promote nuclear-factor- κ B (NF- κ B) activation and release of pro-inflammatory cytokines such as interleukin (IL)-1 β and IL-18 as well as the ability to inhibit the secretion of nitric oxide (NO) in human umbilical vein endothelial cells. This metabolite can also modulate cholesterol and sterol metabolism⁵³ possibly contributing towards the formation of atherosclerotic lesions found in placental spiral arteries of PE patient's⁵⁴.

Further highlighting the possible effect of these inflammatory molecules, and variation of microbial community with gestational period, a longitudinal study of PE women between the second and third trimester noted a reduction in *Firmicutes* phylum and increase in *Bacteroidetes* phylum exclusive to the third trimester⁵⁵. These bacterium are respectively producers of SCFAs and LPS⁵⁵. Wang et al. revealed that in comparison to control groups, subjects with preeclampsia have an increase in *Proteobacteria* at phylum level and *Enterobacteriaceae* at family level, bacterium which has been proven to enhance bacterial intestinal translocation, and are associated with epithelial dysfunction and activation of inflammatory pathways⁵⁵.

4.1.2 The vaginal microbiota

Longitudinal studies of the vaginal microbiome have described it as being dynamic, as it suffers several transformations with ageing, sexual activity and development, contraceptive use, gestation, menstruation, hygiene practice and hormonal levels^{56,57}. In asymptomatic women, five microbial community state types (CSTs) can be identified and divided according to the prevalence of the several species (**figure 3**). The norm for a healthy vaginal microbiome was typically described as *Lactobacillus*-dominated. However in several asymptomatic women a diverse microbiome was discovered⁵⁸, with the later finding that a significant disparity exists depending on racial and ethnic group^{59–61}. The variations between CST also occur at an individual level with some women experiencing a stable CST pattern while others frequently transitioning between CSTs⁵⁶. Pregnancy is characterized by an overall decrease in microbial diversity and increase in stability^{58,62}. There is a shift towards the predominance of *Lactobacillus* spp. followed by *Clostridiales*, *Bacteroidales* and *Actinomycetales* and rarely is there a transformation to CST IV-A or CST IV-B, reflecting the importance of *Lactobacillus* spp. in the vaginal ecosystem^{58,62} (**figure 2**). The benefit of a stable microbiome, with low α -diversity emphasizes the colonization effect. This effect describes that the predominance of one microorganism competes with potential invaders for nutrients and given it's highly adapted nature to that environment prevents the foreign microbial replication^{63,64}.

Associations between altered vaginal microbial community structure, bacterial vaginosis and preterm birth (PTB) emphasize the importance of the microbiota for maternal and foetal health⁶⁵. A transition from healthy *lactobacilli*-dominated vaginal microbiota to a relative abundance of facultative anaerobic bacteria has been linked to a variety of negative obstetric and gynaecologic outcomes^{66–69}. Intrauterine infection is associated with approximately 25% of all PTB's⁷⁰ and can be acquired through the ascension of pathogens from the vagina^{71–73}. About 25-30% of PTB cases are preceded by preterm premature rupture of membranes (PPROM)⁷², and multiple studies have revealed that women who present with PPRM have a characteristically intermediate or low *Lactobacillus* sp. dominance with a highly diverse vaginal microbiota^{66,74,75}.

In the vaginal mucosa of women with PE, researchers discovered a significant drop in *Lactobacillus delbrueckii* and *Lactobacillus* spp., as well as an increase in *S.haemolyticus*, *S.aureus*, and β -*haemolytic* streptococcus populations⁷⁶. Additionally in relation to PE in 2020, Chia-Ying-Lin et al., in a study of the vaginal microbiome profiles of Taiwanese women with severe preeclampsia (SPE) exhibited a higher relative abundance of *Prevotella bivia* (OR 6.01). In the confirmation cohort, which also revealed higher levels of *Prevotella bivia*, plasma levels of tumour necrosis factor- α (TNF- α) were also greater in comparison to controls⁷⁷(**figure 2**). PE as previously mentioned is

characterized by a systemic inflammatory response following placental ischemia, so there might be a correlation with the increase in TNF- α detected in the maternal bloodstream⁷⁸. Noticeably other *Prevotella* species, *Prevotella amnii* and *timonensis* were detected in the placental tissues. Within these species, *Prevotella oral* species was also associated with hypertension in older women⁷⁹. Consequently, the authors propose that the *Prevotella* genus could not only play a general role in the aetiology of hypertension in general but also in hypertensive diseases of pregnancy⁷⁹.

5 The placental microbiota

The fetoplacental unit was traditionally considered germ-free, acting as a barrier between the foetus and colonized maternal urogenital tract with the first foetal exposure assumed to occur after or at birth, by passage through the vaginal canal or by environmental microbes in new-borns delivered by caesarean section³⁷. The identification of pathogens in the placenta or embryonic fluids was correspondingly associated with a state of disease and potential danger to the gestation, correlating with the association between infections and PTB^{71,80}. Being the main organ responsible for foetal nutrition and gas exchange, as well as immune tolerance, and since placental infection and inflammation are strongly associated with adverse pregnancy outcomes, placental sterility was considered a pre-requisite for an uncomplicated pregnancy⁷². However bacterial colonization has also been proven to be crucial in the physiological and immunological development of new-borns raising curiosity towards the importance and role of the microbiome⁸¹.

The first discovery of bacterial colonization of the placenta was made through histological analysis of 195 patients that detected in 27% of subjects, Gram-positive and negative intracellular bacteria of varied morphologies in the basal plate of placentas, within extra-villous trophoblasts, regardless of clinical or histological evidence of chorioamnionitis⁸². The presence of bacteria in term placentas regardless of apparent infection, as well as their absence in a significant proportion of preterm placentas, led to the hypothesis of commensal bacteria in the basal plates, with no pathologic effect⁸². Subsequent studies with advanced technological methods such as, polymerase chain reaction (PCR) amplification and sequencing, metagenomics sequencing and whole-genome shotgun sequencing, report the detection of bacterial DNA in the placenta, amniotic fluid, meconium and even in the developing foetus itself, challenging the idea of the sterile womb, and supporting the existence of a rich microbial community in utero and the hypothesis of intrauterine colonization of the foetus^{24,83–86}.

By 2014, Aagard and colleagues proceeded to identify the presence of bacterial DNA sequences in placental specimens of 320 subjects⁸³. The posterior metagenomic analysis described a unique placental microbiome constituted at phylum level by *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes* and *Fusobacteria*, with *Proteobacteria* being the most abundant⁸³. The *Proteobacteria* dominance, in particular *Enterobacteriaceae*, at phylum level in the placental tissue, was verified in a later culture based 16S ribosomal ribonucleic acid (rRNA) gene profiling study⁸⁵. The most prevalent species identified were *Escherichia coli*, as was noticed in a subsequent study^{83,84}. This microorganism is also commonly present in large quantity in the meconium of new-borns, and thus the detection of commensal E.coli in new-borns led to a theory of intrauterine seeding, being the placenta the likely source⁸³. Similarly in a comparison study between foetal and maternal tissue samples, the meconium samples revealed that more than 50% of bacterial phylotypes were found in the placenta and amniotic fluid reinforcing the intrauterine acquisition from a placental source⁸⁵. A surprising discovery by Aagard et al. also revealed that placental microbiome profiles were most similar to the human oral microbiota (with a Bray-Curtis dissimilarity of 0.3), with this resemblance reproduced in later studies⁸³. By comparison with oral samples, the oral microbiome species that were identical to those found in the placenta were *Prevotella tanneri* and non-pathogenic *Neisseria* species, typically encountered in the gingival fissures and mucosal surfaces respectively⁸³.

The microbial community profile in term placentas has also shown to exhibit a spatial variation. Parnell et al. through genomic DNA sequencing of the bacterial 16S rRNA gene, has exhibited that according to sampling location the abundance and diversity may differ⁸⁷. Regarding the microbial community abundance, the basal plate (BP) was most abundant, like the placental villi (PV), whilst foetal membranes (FM) showed a lower copy number. Considering the diversity of the microbial communities, α - and β -diversity diverged amongst the different locations. Both BP and PV denoted a greater richness in comparison to FM whilst β -diversity was significantly lower in BP, followed by FM samples and finally PV. Specific distinct taxa in each sampling location were also identified. The BP sampling found a predominance of *Proteobacteria* followed by five other bacterial phyla which include *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Spirochaetes* and at species level highest prevalence included *Ralstonia insidiosa* and *Mesorhizobium* spp. The PV harboured five different types of phyla of *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Proteobacteria* and *Spirochaetes* with no consistent dominating phyla identified. At last, the FM samples were dominated by *Firmicutes*, alongside the other five phyla identified which included *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Spirochaetes* and *Tenericutes*. The most prevalent species in the FM were *Lactobacillus crispatus*, *iners* and *Ureaplasma nucleatum* with a special prevalence of the

Lactobacillus species, which are frequent inhabitants of the intestinal and vaginal flora, suggesting the ascendant pathway of foetal membrane colonization through the vagina^{62,67}.

Conversely, in a study aiming to investigate the impact of existing human papillomavirus infection on the placental microbiota, a placental microbiome was revealed in the negative control group. The composition of this microbiome described as the most prevalent phylum *Firmicutes* (58.3%) followed by *Proteobacteria* (21.1%), *Actinobacteria* (13.8%) and *Bacteroidetes* (6.5%). At the family level, *Staphylococcaceae* (22.5%), *Enterococcaceae* (15.6%), *Veillonellaceae* (8.8%), *Corynebacteriaceae* (6.3%) and *Moraxellaceae* (6.1%) were the main groups encountered. The predominant microorganisms at the genus level were *Staphylococcus* (22.9%), unclassified *Enterococcaceae* genus (15.6%), *Corynebacterium* (6.3%), and *Acinetobacter* (6.0%)⁸⁸.

Subsequent studies have indicated that placental microbiome profile can differ, not only in location but also depending on the characteristics of the population of study, as well as in the presence of obstetric complications. Therefore microbial variations were observed in women with spontaneous PTB, with excess maternal weight gain and with gestational diabetes mellitus^{23,24,84}. By the identification of a placental microbiome with metabolic and pathological changes, the concept of the colonization of microbes in the maternal-foetal unit has been further strengthened.

5.1 Placental microbiome variations depending on metabolic and obstetric outcomes

Concerning the relationship between higher body index and microbial changes a study revealed that whilst microbial placental composition did not change in term pregnancies with excess gestational weight gain (GWG) or in obese women, the same did not apply to preterm subjects in which a change was observed in women with GWG above the recommended threshold according to the Institution of Medicine⁸⁹. When examined at phyla level relative to taxonomic abundance, in preterm women with excessive maternal weight gain there was a significant increase in abundance of *Firmicutes*, *Actinobacteria* and *Cyanobacteria* as opposed to the decrease in abundance of *Proteobacteria*²⁴. Whilst β -diversity in obese women suffered no significant difference, the same did not apply in women with GWG in excess who delivered preterm²⁴. Analysis of the α -diversity among preterm deliveries revealed that excess GWG was associated with a decrease in overall species richness and variant abundance²⁴.

Regarding the effect of chorioamnionitis in placental microbiome, it was negatively associated with α -diversity, in particular the species diversity⁸⁴. Similarly in previous microbial gut studies, a decrease in α -diversity was associated with clinical infection and significant histological

inflammation⁸⁴. The same study revealed that preterm subjects with chorioamnionitis had a greater similarity in taxa with the vaginal microbiome (by the presence of *Ureaplasma* species and *Streptococcus agalactiae*) and with the oral microbiome (*Fusobacterium* species and *Streptococcus thermophilus*), highlighting the possible acquisition modes for placental microbiome in pathological settings⁸⁴.

Doyle et al. compared bacteria DNA from the superficial layer placental membranes in several modes of delivery as well as in the presence of chorioamnionitis and PTB. Pre-term placentas had higher levels of *Fusobacterium*, *Streptococcus*, *Mycoplasma*, *Aerococcus*, *Gardnerella*, *Ureaplasma*, and *Enterobacteriaceae*⁹⁰. The same author unveiled in 2017 a similar discovery however with distinct phyla in severe chorioamnionitis characterized by a higher mean load of *Fusobacteria* and mean *Tenericutes* load in comparison to the control group⁹¹. At the family level, a higher mean bacterial load of *Mycoplasmataceae*, *Leptotrichiaceae* and *Veillonaceae* was found in the severe chorioamnionitis subjects whilst revealing that *Fusobacterium nucleatum*, *Ureaplasma sp.* and *Gemella asaccharolytica* were associated with shorter duration of pregnancy⁹¹. In resemblance to the studies previously mentioned, this investigation revealed similar bacteria in placental and foetal tissues in comparison to vaginal and oral samples⁹¹. A greater number of similar OTUs between the vaginal and placental microbiome were identified⁹¹.

In women with gestational diabetes mellitus (GDM), another shift in the microbial community was discovered, with an increase in *Proteobacteria* and a decrease in *Bacteroidetes* and *Firmicutes* in comparison to non-GDM women²³. *Proteobacteria*, the most prevalent phylum in GDM women, demonstrated a negative correlation with insulin levels. The possible association between bacterial composition and glucose metabolism is further suggested in this pilot study as different bacterial species are correlated with certain metabolic pathways²³. Insulin concentration had lower levels in the presence of *Pseudomonas* while leptin and IGF-1 increased in the presence of *Staphylococcus* and *Allobaculum*²³. Furthermore, decreased relative abundance of *Pseudomonadales* was linked to lower blood neutrophil and lymphocyte counts in the second and third trimesters of pregnancy, as well as reduced placental expression of IL10 (an anti-inflammatory cytokine)⁸⁹. However conflicting evidence regarding the placental microbiota changes in GDM emerged with Bassols and colleagues study⁸⁹. They found a greater proportion of *Firmicutes* (7%) and lower of *Proteobacteria* and *Bacteroides* (5% and 2% respectively) compared to levels in non-GDM women. *Pseudomonadales* order and *Acinetobacter* genus relative abundance were also found to be of a considerably lower count in women with GDM compared to controls⁸⁹.

5.2 Where do placental microorganisms originate?

The striking resemblance between the microbial communities discovered in the intrauterine environment with the ones from oral, gut, and vaginal microbiome poses the question of where the intrauterine microbiota derives. Direct ascension from the vaginal canal or haematogenous spread from distal sites such as the oral cavity and the gastrointestinal system have been proposed as primary routes of transmission ⁹². In support of the hypothesis of a vaginal origin of placental microbiota, human and rodent uteri have shown the ability to uptake bioluminescent bacteria and particles radioactively labelled from the vaginal canal through the cervix into the intrauterine environment⁹³. The possible origin of microbiota in the placental foetal compartment from the gastrointestinal environment emerges as there is the identification of bacterial DNA from the intestinal lumen in peripheral blood mononuclear cells, particularly dendritic cells, bearing great resemblance to the bacterial DNA found in breast milk, and thus supporting the bacterial gut translocation theory ⁹⁴.

Interestingly an identical rRNA sequence of *Bergeyella* strain, a bacteria specific to the oral cavity, was detected in the amniotic fluid, in the absence of identification of this organism in the vaginal samples of the study⁹⁵. Given the distance between the intrauterine environment and the oral cavity, the only possible transmission route would be via bloodstream, as postulated by the respective authors⁹⁵. Complementing this hypothesis of haematogenous dissemination as an alternative acquisition pathway, similar bacteria are found between the oral and placental tissue microbiota in experimental studies of model pregnant mice. Through the injection of human salivary, subgingival samples and specific microorganisms such as *Fusobacterium nucleatum*, into the tail veins of mice, the bacterial DNA or the microorganism resembling these mouth cavity bacterial communities were retrieved in the placental samples. ^{96,97}. Considering the experimental study with *F. nucleatum*, this microorganism was unable to colonize other highly vascularized organs such as the spleen or liver leading to the author's conclusion that the bacteria found in the placenta were not exclusively due to low-grade bacteremia⁹⁷. The mechanism by which *F. nucleatum* can reach the intravascular environment involves the adhesin FadA binding to cadherin on the vascular endothelium⁹⁸. The interaction between these receptors enhances the vascular permeability and allows other microbial species to disseminate through endothelial barriers justifying the abundance of *E. coli* species, as well as other *Fusobacterium* species in the placental microbiome described by Aagard et al. ^{83,98} (figure 4).

6 The placental microbiome of women with preeclampsia

Given that the placental microbial community varies with GDM, GWG, PTB and chorioamnionitis, discussion ensues whether PE could cause such a change and how alterations in the microbiota could be linked to the disease's pathogenesis. Amarasekara et al. found that the communities of pathogenic commensals in PE are distinct from the microbiome previously described by Aagard et al., the perceived commensals to the placenta in normal pregnancies⁹⁹. In this study of fifty-five primiparous women with PE, and 55 primiparous healthy pregnant women, the 16S rRNA gene was found in seven (12.7 %) of the placental tissue samples from women with PE⁹⁹. *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella*, *Escherichia*, *Klebsiella pneumonia*, *Anoxybacillus*, and trace amounts of *Variovorax*, *Prevotella shahii*, *Porphyromonas*, and *Dialister* were detected in the seven placental tissue samples from women with PE, and all controls were negative⁹⁹. These women had a microbiome composed of several agents and the existence of a single-agent/sparse colonization was absent⁹⁹. According to the author, the microorganisms detected involve microbial species commonly found in respiratory, gastrointestinal infections and periodontitis⁹⁹. Furthermore, the lack of these infectious agents in women's venous blood, as well as urine samples retrieved at the same time, shows that they are restricted to the foetal-maternal surface of the placenta⁹⁹.

Solidifying the relationship found between the oral and placental microbiome, several studies and meta-analyses have identified a correlation between placental microbial constitution, preeclampsia and periodontal disease^{100–102}. In a study of 16 placenta samples retrieved from caesarean sections of preeclamptic women *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum* spp., *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* and *Treponema denticola* were all detected using PCR and their counts were statistically significantly higher in women with preeclampsia in comparison to control groups²⁶. The amniotic membranes, in all of the cases, were intact, making the bacterial entrance into the uterus exceedingly unlikely, further reinforcing the proposed haematogenous transmission of placental microbiota²⁶.

Periodontal infections and related virulence factors enter the bloodstream via transitory bacteraemia during chronic periodontal inflammation with these factors having the ability to diffuse across numerous organ systems and create a negative effect through the induction of an inflammatory response or by directly colonizing these tissues^{103,104}. *Porphyromonas gingivalis*, a common Gram-negative anaerobic bacterium of the oral cavity, is found in samples of the decidua/placental basal plate¹⁰⁵. *Porphyromonas gingivalis* can disseminate into the placental mesenchyme via uterine-trophoblast by invasion into extra-villous trophoblast cells, bypassing host cells¹⁰⁶. Infected rodents with this strain developed pathological changes consistent with PE which

included uterine vasculitis, uteroplacental thrombosis/haemorrhage and structural changes in the placental vascular layer^{105,107–109}.

7 Pathophysiology involved in preeclampsia and microbial variations

The activation of antiangiogenic and inflammatory pathways, which degrades endothelial and trophoblastic activities and raises blood pressure, is a viable explanation for the link between the bacterial community and PE¹¹⁰ (**figure 5**). Microbial products particularly those present in the placenta, like LPS, lipoproteins, peptidoglycans and lipoteichoic acid have shown to play a significant role in PE through toll-like receptors^{111–113}. TLRs are transmembrane proteins which act as pattern recognition receptors (PRRs) and have the ability to identify pathogen-associated molecular patterns in different microorganisms, from bacteria to fungi and parasites as well as damage-associated molecular patterns¹¹⁴. These PRRs are present in placental immune cells and trophoblast cells in the chorion, and during pregnancy, are essential contributors in maintaining the homeostasis through immunological tolerance³⁵. There are 10 different TLRs which are all constitutively expressed in the placentas of healthy pregnancy subjects¹¹⁵. However in PE, TLR-3, TLR-4, TLR-7, TLR-8 and TLR-9 are found to be upregulated in the placental tissue^{116,117}. The upregulation of TLR-2 in PE samples hasn't been consistently revealed¹¹⁸. The persistent activation of placental TLRs in PE samples has been associated with the imbalance between immune cells and trophoblasts, abnormal vascular remodelling, and endothelial dysfunction, with their upregulation in animal studies associated with the onset of preeclamptic signs and symptoms^{117,119(p2)}. Depending on the activating receptor a Myd88-dependent or independent signalling pathway follows¹²⁰ with both leading to NF-kB activation and the production of inflammatory cytokines such as TNF- α , IL-1, IL-2, IL-6, IL-8, IL-10, IL-12 and the promotion of T-helper (Th)1 differentiation which in turn secretes interferon- γ (IFN- γ)¹²¹. Through the excessive activation of NF-kB the pathogenesis of PE is initiated, including excessive inflammatory cytokine release, abnormal trophoblastic invasion and consequently defective spiral arterial remodelling, endothelial dysfunction leading to the pathological maternal syndrome¹²².

LPS, a molecule found in excess in PE studies, arises from Gram-negative bacteria and activates an inflammatory response primarily through TLR-4 but also by the upregulation of TLR-3, TLR-7 and TLR-8^{123–125}. The study published by Holmlund et al. described the TLR function in the placenta and discovered that stimulating TLR-2 and TLR-4 in trophoblast surface with zymosan and LPS resulted in the generation of cytokines IL-6 and IL-8, which have pro-inflammatory functions⁵². In experimental animal studies with the infusion of a low dose of LPS, these subjects developed a form of pre-eclamptic syndrome with high blood pressure, proteinuria, elevated transaminases,

low platelets, increased anti-angiogenic factors and endothelial dysfunction^{112,119,126,127}. LPS-induced PE-like circumstances in rats resulted in defective spiral artery remodelling of the placenta as well as local and systemic inflammatory responses¹¹². This implicates the lipopolysaccharide molecule as one of the primary PE mediators.

The products of gram-positive bacteria include lipoproteins, peptidoglycans and lipoteichoic acid which in turn activate TLR2¹²⁸. TLR-2 then binds to TLR-6 or TLR-1 and can similarly lead to NF-κB upregulation via a cascade of intermediary steps and in this way associated with PE pathogenesis¹²⁹. Isolated the TLR-2 activation is also associated with trophoblast apoptosis by the activation of Fas-mediated death-domain activation through Myd88-signalling pathway¹²⁹. It's role in PE is associated with excessive apoptosis, diminished trophoblast invasion and spiral artery remodelling altering the first phase of placentation.

As previously mentioned, the placental microbiome is similar to the oral microbiome. Among common microbes resident in the mouth's environment, nitrate and nitrite-reducing bacteria are a source of bioactive NO, which is crucial in the endothelial cell function and in the modulation of blood pressure^{130–132}. Moreover the presence of active maternal periodontal disease during pregnancy is linked to an increased risk of PE²². Periodontal diseases can result in a systemic inflammatory activation through the production and spread of bacterial endotoxins¹³³. An association study by Chaparro et al., while revealing an association between the presence of *P. gingivalis* (OR 7.59) and *T. denticola* (OR 9.39) in the placental tissue of pregnant women with periodontal disease and hypertensive disorders, it also demonstrated an increase of TLR-2 expression¹³⁴. According to current findings, TLR-2 appears to be the primary receptor for *P. gingivalis*^{107,134} further highlighting the possible correlation between innate immunity dysfunction, microbial variations, and PE. *P. gingivalis* has also shown to diminish IL-10 levels in experimental mice studies¹⁰⁷. IL-10 is positively associated with Th2 polarization, and as one of the key cytokine of Th2 response it is beneficial in the control of inflammation and vascular function plus in a successful placentation¹³⁵. Furthermore, the investigative effort of the placental microbiome by Bassols et al. also revealed that lower *Pseudomonadales* order and *Acinetobacter* genus were also correlated to variations in inflammatory cytokines. A lower placental expression of the anti-inflammatory cytokine IL-10 was observed with a reduced relative abundance of both *Acinetobacter* and *Pseudomonadales*⁸⁹

Continuing the relationship between periodontal disease and PE placental variations, in early pregnancy, the onset of preeclampsia in affected women was accompanied by an increase in IL-6 in the gingival cervical fluid¹³⁶. IL-6 in a series of meta-analysis has been shown to be increased in

preeclampsia^{78,137}. This cytokine is involved in placental invasiveness during implantation and in an experimental study with pregnant rats its ability to diminish the blood vessel relaxation was revealed^{138,139}. Oral infections have also been correlated with an immune-inflammatory response by the local increase of proinflammatory cytokines, such as IFN- γ , IL-17, TNF, IL-1 and IL-6¹⁴⁰. Regarding the abnormal immune response and oral microbiome, infected mice with *Porphyromonas gingivalis* exhibited an increase in placental Th1/Th2 cytokine ratio and maternal TNF- α levels¹⁰⁷. An aberrant shift towards the Th1 phenotype is described in preeclampsia, as opposed to the Th2 polarization happening in healthy pregnancies¹⁴¹. The result is insufficient trophoblast invasion and consequently the first phase of PE is initiated. Furthermore, species found in the oral and placental microbiome of PE patients were identical to the ones detected in atherosclerotic plaques in parallel studies^{26,54,104}. Preeclampsia's placental lesion can manifest as acute atherosclerosis, with similar histopathological characteristics as atherosclerotic plaques^{54,103,104,108}. The narrowing of spiral arteries by atherosclerosis could lead to placental ischemia and consequently oxidative stress, the release of inflammatory markers and endothelial dysfunction⁵⁴.

Dormant bacteria, which are generally inconspicuous in traditional microbiology examinations, have also been shown to generate antigens as well as other inflammatory factors¹¹¹. Cross-reactivity of such antigens with host epitopes can result in the formation of autoantibodies, and assault the host via trophoblast termination of placentation inducing the first phase of PE¹¹¹. This is referred to as molecular mimicry. The Gram-negative bacteria LPS has also shown to act in this manner with the capacity of activating corticotrophin-releasing hormone or mitogen-activated protein kinase and thus terminating placentation^{123,142}.

Moreover, the current bacterial community in the placenta is metabolically enriched with genes involved in the metabolism of fatty acids (especially SCFAs), tryptophan, and benzoate. In preeclampsia, the microbial dysbiosis may affect tryptophan and fatty acid metabolism, resulting in disturbed maternal-foetal energy homeostasis, which may trigger or amplify pre-eclamptic episodes, especially during late gestation, resulting in severe PE¹⁴³. SCFAs such as acetate, propionate, and butyrate have also been linked with hypertension via activation of G-protein-coupled receptors⁴⁹.

At last, in the Amarasekara study that revealed “dysbiotic placental microbiome” associated with PE, the authors suggested that given the microbial similarity with infectious states in other locations, these microorganisms could potentially trigger a similar recruitment of inflammatory cells as they do in the systemic diseases in which they are involved, leading to an imbalance

between pro-and anti-angiogenic molecules, reduced trophoblast function culminating in endothelial dysfunction and placental hypoxia/ischaemia, ultimately elevating maternal blood pressure and resulting in PE⁹⁹. Complementing this theory, the presence of an infection has been associated with increased risk of PE¹⁴⁴.

8 Future approaches

Intriguing therapeutic possibilities emerge as the placental microbial community could serve as a potential target for personalized treatment. With the hope of microbiome modulation in mind and seeing as PE is a catastrophic condition with an unknown aetiology and no approved treatments, understanding the pathogenesis of PE and developing treatments would enhance pregnancy outcomes and long-term health in women and their children. The microbiome is undeniably important in regulating proper host physiology. Novel therapeutic techniques can be generated by understanding the host-microbial connection¹⁴⁵.

Probiotics are generally thought to be safe during pregnancy and perhaps beneficial in PE¹⁴⁶. In a trial of primiparous women, the consumption of probiotic milk during pregnancy reduced the likelihood of developing PE, particularly with regular high maternal intake (>200mL/d) of lactobacilli-rich dairy products (OR 0.6 to develop SPE)¹⁴⁷. This effect was not evaluated for early and before pregnancy probiotic use and the study failed to assess changes in the composition of the gut/vaginal microbiome¹⁴⁷. The findings are especially intriguing in light of a study by Yeganegi et al. in which *L. rhamnosus* GR-1 was found to influence the LPS response in placental trophoblast cells, linking modification of cell activation and inflammatory response¹⁴⁸. The administration of this probiotic inhibited the LPS-stimulated TNF- α and increased IL-10¹⁴⁸.

Regarding prebiotics, it is the consensus that all prebiotics are fibres, although the same is not true for all fibres^{149,150}. Fibre ingestion is linked to higher blood SCFA levels and improves gut health by reducing gut inflammation^{151–153}. The effects of circulating SCFA's include decreasing blood pressure, attenuating inflammation and enhancing endothelial function, particularly SCFA's acetate and propionate, usually depleted in PE patients^{49,154,155}. Butyrate reveals its therapeutic potential as levels inversely correlate with pro-inflammatory markers and nitric oxide levels¹⁵⁶.

Oxidative stress is also associated with PE, hence antioxidants such as vitamin C (ascorbic acid) were anticipated to be protective¹⁵⁷. However, this was not transversal to all antioxidants. While polyphenolic antioxidants that chelate iron appeared to be beneficial¹⁵⁸, the same was not the case

for vitamin C ^{157,159}. A possible explanation for the absence of a positive effect was the fact that ascorbic acid is a pro-oxidant in the presence of unliganded iron¹⁶⁰.

Other possible therapeutic approaches include magnesium sulphate which has been used to treat eclampsia, as it prevents LPS-induced cell death and TNF- α expression in an in vitro model of the human placenta ^{161,162} and inorganic nitrate supplementation which was related to lowering blood pressure, due to its ability to alter the oral microbiota, increase levels of plasma nitrite and lead to the vasodilative action of NO ¹³².

9 Controversies in low-density microbial habitats like the placenta

Several studies from 2016 to 2021 have found no evidence to support the existence of a placental microbiome and the limited bacterial presence detected in the placenta was attributed to contamination of used laboratory reagents and extraction kits with bacterial DNA or acquisition during labour and delivery^{80,163–168}. Low microbial biomass samples, as in the case of placental samples, are particularly sensitive to sampling methods, DNA extraction techniques and contamination¹⁶⁹. Salter and colleagues have shown that the lower the amount of bacterial DNA in a sample, the greater the proportion of sequences that can be attributed to contamination ¹⁷⁰. The authors provided a list of bacterial taxa found in negative controls that are commonly found in reagents. Surprisingly, approximately 36% of the total species reported as "the placenta microbiome" by Aagaard and colleagues⁸³ overlap with taxa on this list¹⁷⁰. In fact, despite identifying a unique microbial population within the human placenta, Aagaard did not provide sufficient controls⁸³. Additionally, molecular approaches to the characterization of the placental microbiome have been criticized, namely next-generation sequencing and PCR as critics argue that the detection limit in these low-density bacterial communities is insufficient and is particularly susceptible to contamination ¹⁷¹.

To establish the existence of resident bacterial communities in placental or foetal tissues, bacterial DNA must be identified as distinct from bacterial DNA found in technical controls (e.g., DNA extraction kits, PCR reagents, laboratory environments), detection of quantitative real-time PCR bacterial load should be greater than that of technical controls, microscopic detection of bacteria in placenta must be feasible, and bacteria viability should be guaranteed by the ability to grow in culture as well as the ecological rational possibility must be proven, meaning the bacteria could theoretically survive in these tissues^{165,172}. Lauder and colleagues made a systematic comparison with sequencing data obtained from placental samples with those obtained from different contamination controls such as sterile swabs, swabs exposed to clinical laboratory air, and

extraction blanks from two different DNA isolation kits¹⁶³. This study found that placenta samples contained the same trace levels of bacterial DNA as the extraction blanks and that the bacterial communities found shared a close cluster with the contaminant community of the respective DNA isolation kit. Most notably, no bacterial lineages were found as being distinct to or more abundant in placental samples than in contaminated controls¹⁶³. Bacteria identified in PCR have also failed to be detected upon culture methods¹⁷³.

With PE in mind, Goffau et al. questioned the presence of bacterial DNA in the human placenta and consequently its role in the development of this HDP⁸⁰. Their data showed no evidence for the presence of bacteria in most placental samples and pointed to either bacteria being acquired during labour or being contaminated by laboratory reagents with bacterial DNA⁸⁰. This leads to another aspect regarding contamination dependent on mode of delivery as vaginally delivered placentas are at risk of exposure to vaginal microorganisms during expulsion. A study of 74 preterm and full-term caesarean section and vaginal delivered placental samples evaluated through quantitative PCR discovered that 30 to 43 % of these tissues were positive for bacterial DNA, however when the tissue samples were separated by type of delivery, none of the full-term caesarean section placentas tested positive¹⁷⁴. Similar conclusions were obtained in 2009 as *Bifidobacteria* and *Lactobacillus* retrieved through 16S rDNA sequencing were not found in culture¹⁷³ and in other sterile methodological approaches that involved exclusively retrieved caesarean section placentas no placental microbiome was demonstrated¹⁶⁵. Kuperman et al. further supported the absence of bacteria in human placental samples through immuno-histochemistry and provided a brief conclusion that if a placental microbiome exists, it is of extreme low biomass and most likely of negligible clinical significance¹⁶⁷.

Evidence towards the concept of a sterile womb is backed by the ability to form successful generations of germ-free animals obtained by aseptic transfer of the uterus, via caesarean sections and that a subsequent growth of an offspring occurs in a sterile setting¹⁷⁵. The fact that axenic animals may be produced and maintained microbe-free under sterile circumstances provides compelling evidence that in utero transfer of the microbiome does not occur and by extension supports the sterile placental theory¹⁷¹. Similarly in human pregnancies involving infants at risk of severe congenital immunodeficiencies, by sterile delivery through caesarean section and immediate transfer and maintenance in sterile incubators, the infants were able to maintain sterile¹⁷⁶.

10 Conclusion

Although some studies have found microbial residents in the placenta of PE patients, they have only suggested a link, not a causal relationship. Concomitantly the distinction between commensal placental microbiome and pathogenic microbiota involved in PE is indistinguishable, as similar periodontopathogenic microorganisms were identified in healthy pregnancies and in PE patients and in Amarasekara study⁹⁹ no microbes were found in controls. The microorganism(s) responsible have yet to be elucidated, some of which are controversial. However, there is compelling evidence bridging preeclampsia and distant intrauterine seeding. Through similarities in microbial composition between common gut microbial metabolites and oral microbiome, their production of inflammogenic molecules, a possible pathophysiology between PE and preeclampsia is suggested. The microorganisms linked to a dysbiotic state in other obstetric complications and body niches, could potentially trigger an abnormal immune response in the placenta, an imbalance towards excess of anti-angiogenic molecules, reduced trophoblast invasion and function culminating in placental hypoxia, vascular damage, ultimately leading to hypertension and the systemic complications of PE.

Nonetheless the clinical significance of placental microbiome and PE appears questionable, following the several limitations and controversies in the actual existence of this low biomass microbial community, as most positive studies have exclusively found bacterial DNA, which does not guarantee the viability of the microbial community and therefore its ability to interact and produce a placental immune and inflammatory response. Moreover, possible conflicting conclusions over the presence of placental microbiome may be present as investigations used variable procedures, inclusion criteria, study methodologies and protocols to establish controls.

Although the studies aiming to detect intrauterine microbiome have failed, some have excluded the presence of pregnancy complications like preeclampsia and therefore we propose further investigation with well-controlled protocols to identify the specific bacteria that may be involved in PE. A combination of whole-genome sequencing, metagenomics, and metabolomics is necessary to properly comprehend and interpret the data regarding microbial variations and their effect on maternal metabolism and immune response. The selection of possible therapeutic measures can only be improved by elucidating disease-specific microbial aberration, with expectation still remaining towards the use of microbiome as a targeted disease managing tool.

11 Appendix

Table I- Diagnostic criteria for Preeclampsia according to American College of Obstetricians and Gynaecologists (ACOG) 2020²⁰ including preeclampsia with severe features

(* = systemic symptoms/signs) (^ = sufficient criteria if other quantitative measures are not available or if therapeutic measures are emergent)

DIAGNOSTIC CRITERIA OF PREECLAMPSIA (ACOG) Elevated blood pressure i. Systolic blood pressure ≥ 140 mmHg/ Diastolic blood pressure ≥ 90 mmHg on 2 occasions with a minimum 4-hour interval, after 20 weeks of gestation in a previously normotensive woman OR ii. Systolic blood pressure ≥ 160 mmHg/Diastolic blood pressure ≥ 110 mmHg on 2 occasions at least 4 hours apart AND Proteinuria OR Systemic symptoms/signs*
PREECLAMPSIA WITH SEVERE FEATURES • Systolic blood pressure ≥ 160 mmHg/Diastolic blood pressure ≥ 110 mmHg on 2 occasions at least 4 hours apart on a previously diagnosed women with gestational hypertension • Thrombocytopenia $< 100,000 \times 10^9/L^*$ • Liver function impairment – serum liver transaminases elevated to twice the normal concentration * • Renal insufficiency (serum creatinine concentration more than 1.1 mg/dL or doubling of the serum creatinine concentration in the absence of other renal disease) * • Pulmonary oedema* • Visual disturbances * • New onset of headache unresponsive to medication and not accounted for by alternative diagnosis*

Table II- Definitions of terms commonly used in microbiome studies

Microbial community: variety of microorganisms living in a body
Diversity: describes the abundance and uniformity of a community by defining the proportion of a bacterial species in relation to the total species present in the microbial community
Richness: number of bacterial species present in a microbial community
Alpha diversity: determines which species and how many different species are present in a microbial community
Beta diversity: considers the difference in the structure of the bacterial community's composition of different environments
Eubiosis: balanced microbial ecosystem. Potentially characterized by a higher abundance of beneficial species whilst pathogenic species may also be present in lower abundance
Dysbiosis: an unbalanced microbial ecosystem. In a dysbiotic ecosystem there is a lack of bacterial symbiotic relationships
Operational taxonomic unit (OTU): basic unit used in numerical taxonomy clustering microorganisms in regard to sequence similarity in the molecular level (typically the threshold is 97% of similarity). The 16s ribosomal RNA gene is commonly used.
Biomass: a measurement of how much living tissue mass for a population is present at one instant in time (or averaged over several periods of time), and its units are mass (or energy) per unit area (e.g., g/m ²)
Bray-Curtis dissimilarity: quantitative measure, used in ecology and biology, that identifies the differences in species populations between two different sites. Numbered between 0 and 1, with convergence towards 0 signifying greater similarity and towards 1, significant difference.
Odds-ratio (OR): measure of association between an exposure and an outcome. The result of 1 indicates no relation, > 1 that exposure is associated with higher odds of outcome and <1 that exposure is associated with lower odds of outcome.

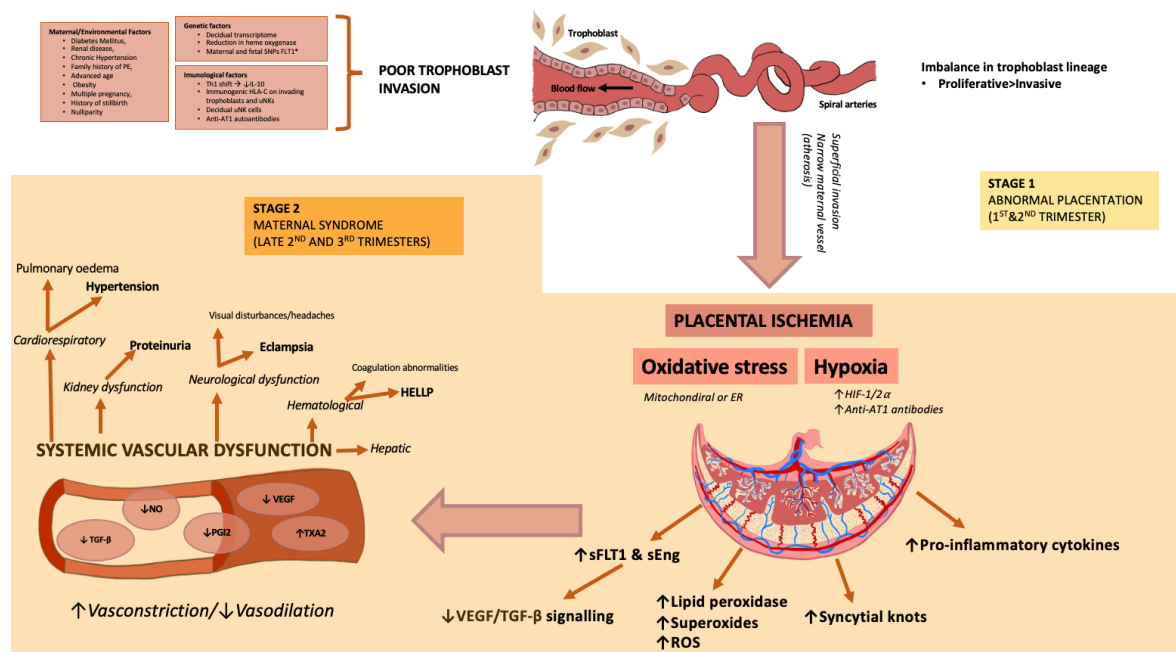


Figure 1- Proposed pathophysiology of preeclampsia

Genetic, environmental, and immunological factors play a role in the development of preeclampsia (PE). In this hypertensive disorder, poor trophoblast invasion in the placenta leads to inadequate spiral artery remodelling and results in placental ischemia and reperfusion injury and inflammation. The abnormal ischemic placentation leads to the release of antiangiogenic factors such as soluble fms-like tyrosine kinase 1 (sFLT1) and soluble endoglin (sENG) binding to vascular endothelial growth-factor (VEGF) and placental growth-factor (PIGF), which are pro-angiogenic factors contributing to superficial placental invasion and narrow maternal blood vessel. These antiangiogenic factors enter the systemic circulation and are key in endothelial dysfunction. The ischemic placenta results in oxidative stress with increase lipid peroxidase, superoxide, and the formation of radical oxygen species (ROS) molecules, which may derive from endoplasmic reticulum (ER). Hypoxia induced factor (HIF)-1/2 α are markers of cellular oxygen deficiency and in response to ischemia, anti-angiotensin-1 (AT1) autoantibodies can stimulate further placental production of sFLT-1 and sENG. Moreover, in preeclamptic women the placenta can produce syncytial debris and pro-inflammatory cytokines which diffuse along the maternal circulation. Angiogenic imbalance along with oxidative stress and inflammations leads to systemic endothelial dysfunction, with increased thromboxane and endothelin-1 expression leading to increased vasoconstriction and decreased prostacyclin and nitric oxide (NO) bioavailability reducing endothelium-dependent relaxation. The clinical manifestations of PE arise from systemic microvascular damage with multi-organ repercussions including the brain, liver, kidney, cardiorespiratory and circulatory system.

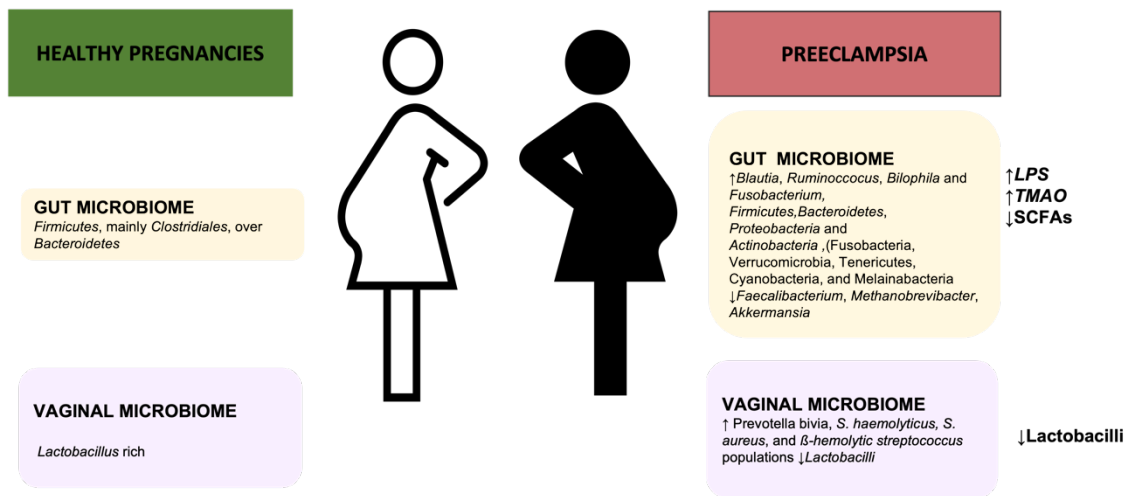


Figure 2- Microbial variations according to location

In preeclamptic women, gut microbiome changes are associated with increased levels of lipopolysaccharide (**LPS**), trimethylamine-N-oxide (**TMAO**) and decreased short chain fatty acids (**SCFAs**), molecules which have been correlated to inflammatory activation and therefore suggested in the development of preeclampsia (**PE**). The vaginal microbiome and PE principal responsibility lies on the levels of Lactobacilli.

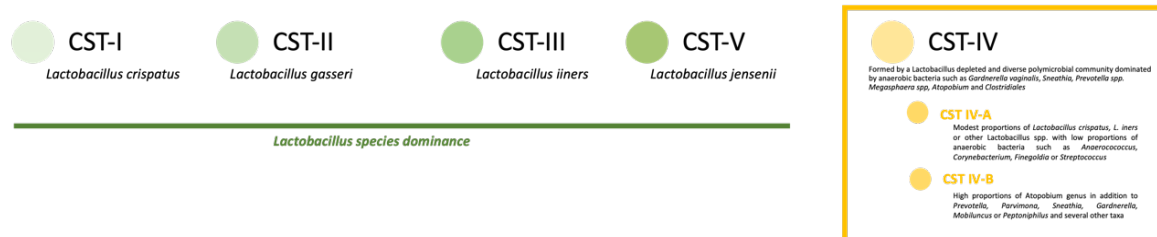


Figure 3- Community State Types (CSTs) of the vaginal microbiome. There are five different types varying in species predominance.

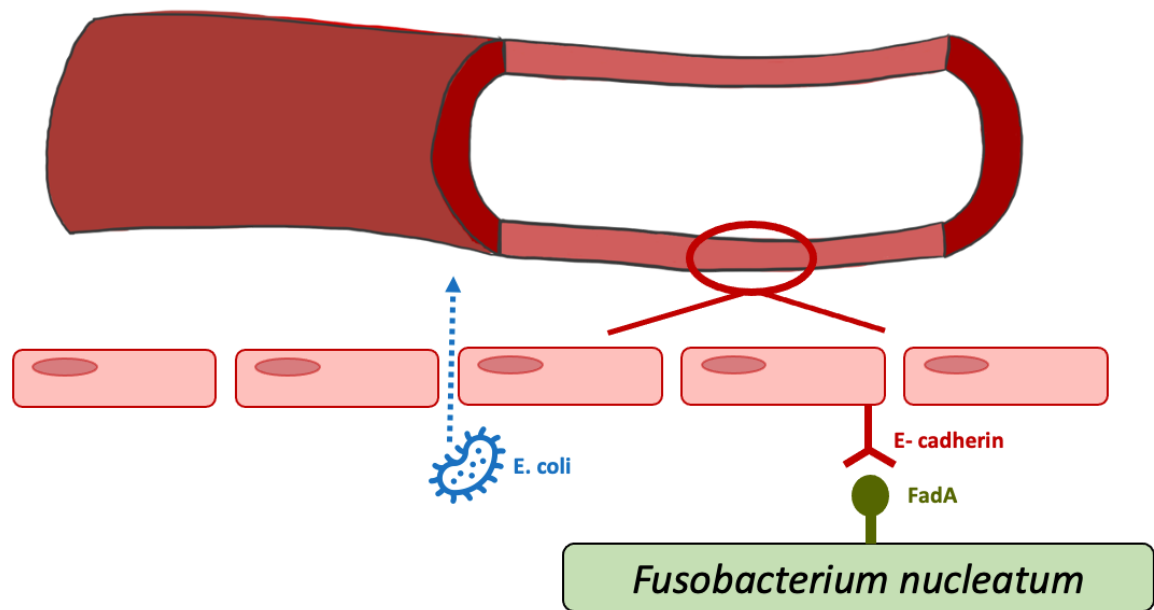


Figure 4- Representative diagram of the mechanism of *Fusobacterium* uptake to the bloodstream.

By binding the FadA adhesin (**FadA**) to the E-cadherin in the endothelial surface, this bacterium obtains access to the circulation. Simultaneously, this interaction increased the endothelial permeability allowing the entrance of other microorganism and possibly explaining how not a single microorganism, but a group are found in placental samples according to Aagard et al. study⁸³.

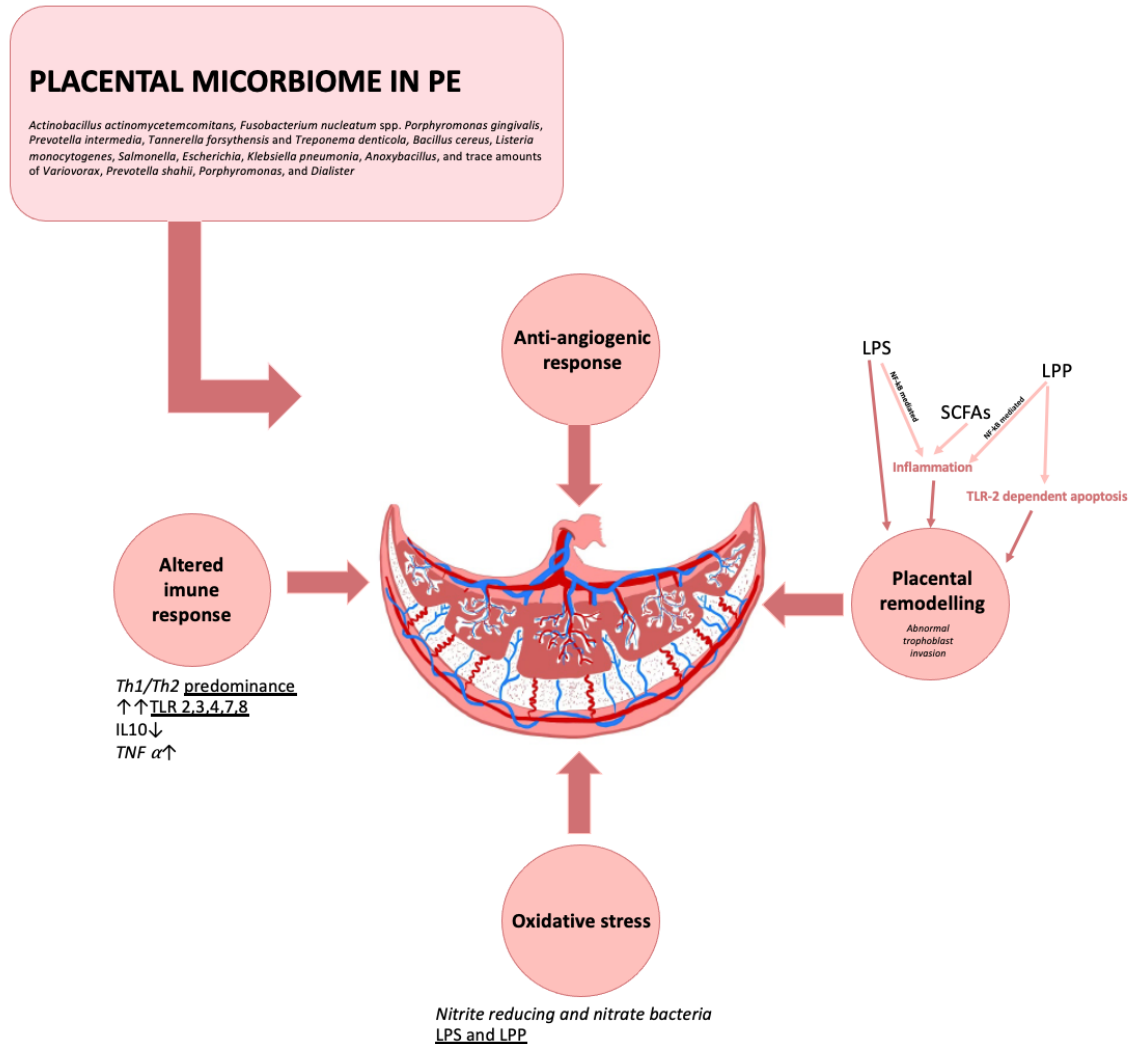


Figure 5- Pathophysiology suggested for preeclampsia (PE) according to microbial variation detected in the placental microbiome and their by-products. An increase in antiangiogenic response, a disruption in placental remodelling through excessive inflammation, and altered immune response and the augmented oxidative stress appear to play a role in the development of PE in response to the resident microbiome in the placenta. (**Th**- T-helper; **TLR**- toll-like receptor; **IL**- interleukin; **TNF**- tumour necrosis factor; **LPS**- lipopolysaccharide; **LPP**- lipoprotein; **SCFAs**- short chain fatty acids)

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