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Investigating the Biomarker Potential of Exosomes in Preeclampsia

Preenan Pillay

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Department of Physiology, College of Health Sciences, University of KwaZulu-Natal, Durban

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Preface

The experimental work described in this thesis was conducted at the University of KwaZulu Natal (Durban, South Africa), University of Witwatersrand (Johannesburg, South Africa) and in conjunction with the University of Oxford (London, United Kingdom) from March 2017 to July 2019, under the supervision of Professor Irene Mackraj.

This work has not been submitted in any form for any degree to any tertiary institution, where use has been made of the work of others, it is duly acknowledged in the text.

21/06/2019

P. Pillay **Date**

As the candidate's supervisor, I agree to the submission of this thesis.

21/06/2019

Professor I Mackraj **Date**

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DECLARATION

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Declaration

I Preenan Pillay declare that:

(i) The research reported in this thesis, except where otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

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

(iv) That my contribution to the project was as follows:

Identification of research topics, experimental design, execution, data analysis and interpretation, manuscript & thesis write-up.

(v) That the contributions of others to the project were as follows:

Professor Irene Mackraj (supervisor) has reviewed the entire project and the write-up of the manuscript and thesis. This project was funded by the NRF (Grant no.: 91544).

Professor Jack Moodley and Dr Manu Vatish has reviewed the clinical design of the project and manuscripts.

Professor Raquel Duarte (University of Witwatersrand) assisted with the experimentation and the review of Chapter 3.

21/06/2019

P. Pillay **Date**

Publications and Presentations

Peer-reviewed Publications contributing to this thesis

Preenan Pillay, Kogie Moodley, Jagidesa Moodley, Irene Mackraj. Placental-Derived Exosomes: Potential Biomarkers of Preeclampsia, International Journal of Nanomedicine. 2017:12. Pages 8009-8023. **(Chapter Two of this thesis)**

Preenan Pillay, Manu Vatish, Raquel Duarte, Jack Moodley, Irene Mackraj. Exosomal microRNA profiling in early and late onset pre-eclamptic pregnant women reflects pathophysiology. Accepted: International Journal of Nanomedicine. **(Chapter Three of this thesis)**

Peer-reviewed Publications not part of the thesis but contributed to the prior knowledge

Preenan Pillay, Niren Maharaj, Jagidesa Moodley, Irene Mackraj. Placental exosomes and pre-eclampsia: Maternal circulating levels in normal pregnancies and, early and late onset pre-eclamptic pregnancies. Placenta. 2016: 45, Pages 18-25. **(Appendix 7.4 – not part of this thesis but was a significant study which lead to the subsequent studies presented in this thesis)**

Sonal Verma, Preenan Pillay, T Naicker, Jagidesa Moodley, Irene Mackraj. Placental Hypoxia Inducible factor −1α & CHOP Immuno-histochemical expression Relative to Maternal Circulatory Syncytiotrophoblast Micro-vesicles in Preeclamptic and Normotensive Pregnancies, European Journal of Obstetrics and Gynecology, 2017:220, Pages 18-24. **(Appendix 7.5 – Not part of the thesis but the significant findings contribute to the prior knowledge)**

Nomfundo Mchunu, Preenan Pillay, Jagidesa Moodley, Irene Mackraj. Circulatory Levels of Tumor Necrosis Factor-α, Interleukin-6 and Syncytiotrophoblast Microvesicles in the First Trimester of Pregnancy. Clinical and Experimental Obstetrics and Gynecology. 2018: 45(4), Pages 575-581. **(Appendix 7.6- Not part of the thesis but the significant findings contribute to the prior knowledge)**

Presentations

P Pillay & I Mackraj. Placental-derived exosomes: Future biomarkers of preeclampsia. Journal of Nanomedicine and Nanotechnology. 2017. 8:6, page 40.

Kaminee Maduray, Preenan Pillay, Jagidesa Moodley, Irene Mackraj. Immunological effects of plasma-derived exosomes on BeWo cells under in vitro hypoxic conditions. Placenta 2018 Apr;64 Suppl 1: S2-S3.

W Phoswa, P Pillay, I Mackraj, K Moodley & K Madurary. Effect of titanium dioxide nanoparticle aggregation on myoblast cytotoxicity and nitric oxide synthesis. Journal of Nanomedicine and Nanotechnology. 2017. 8:6, page 41.

Statement

The following publications have been included as chapters in this thesis:

Chapter Two: Placental-Derived Exosomes: Potential Biomarkers of Preeclampsia. (Published: International Journal of Nanomedicine. 2017:12. Pages 8009-8023).

Chapter Three: Exosomal microRNA profiling in early and late onset pre-eclamptic pregnant women reflects pathophysiology. (Accepted: International Journal of Nanomedicine)

Chapter Four: Exosomal Th1/Th2 Cytokines in Preeclampsia and HIV-positive Preeclamptic Women on Highly Active Anti-Retroviral Therapy (Under Review: Cytokine – CYTO-19-358)

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

21/06/2019

P. Pillay **Date**

21/06/2019

Professor I Mackraj **Date**

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Abstract (English)

Preeclampsia is a hypertensive disorder of pregnancy and one of the leading causes of maternal and perinatal morbidity, affecting up to 7 -10% of pregnancies globally which results in 50 000-76 000 maternal deaths per year worldwide. Preeclampsia remains an enigmatic phenomenon due to its unknown etiology. This is attributed to its multifactorial nature, which complicates clinical diagnosis and management, hence the need for a definitive biomarker. Several candidate biomarkers of preeclampsia have been identified but none have the potential to diagnose preeclampsia in early pregnancy. Exosomes have recently emerged as promising biomarkers of the disease and the present study, therefore, focuses on determining the context of the use of exosomes as biomarkers of preeclampsia. In our approach, we developed a theoretical framework for the evaluation of exosomes as potential biomarkers of preeclampsia in terms of the specified criteria defined by the Food & Drug Administration (US) (FDA) Biomarker Workgroup. This subsequently led to the evaluation of the context of use of exosomes as biomarkers of preeclampsia by determining the role of exosomal microRNA in the pathophysiology of preeclampsia using direct digital detection, computational algorithms and biological databases. We identified distinct exosomal miRNAs signatures involved in the aberrant pathophysiology of preeclampsia which serve as promising biomarkers. Furthermore, in terms of the FDA criteria, it is important to incorporate the intent-to-diagnose preeclampsia in combination with comorbidities associated with the increased susceptibility/risk of preeclampsia. It is clinically evident that HIV-positive pregnant women on (Highly Active Antiretroviral Therapy) HAART are at a greater risk of developing preeclampsia due to an unknown immune-related pathology caused by HAART. We, therefore, determined the potential diagnostic application of exosomal cytokines in preeclamptic and HIV-positive preeclamptic women on HAART. In doing so we have identified altered exosomal cytokine levels in both preeclampsia and HIV-positive pregnant women, which suggests an aberrant mechanism of exosomal cytokine encapsulation, which modulates immune responses in both disease states. Importantly, we show that exosomal Tumor necrosis factor alpha (TNF-α) may have clinical validity in diagnosing preeclampsia and preeclampsia in HIV-infected pregnant women.

Abstract (isiZulu)

I-preeclampsia iyinkinga ephezulu yokukhulelwa kanye neyodwa yezimbangela ezibangela ukugula komama nokubeletha, okuphazamisa ama-7 -10% wezokukhulelwa emhlabeni wonke okuholela ekufeni kwabantu abangu-50 000-76 000 emhlabeni wonke. I-preeclampsia iyisici esiyinkimbinkimbi ngenxa ye-etiology engaziwayo. Lokhu kubangelwa uhlobo olunezinhlobonhlobo zezinto ezihlukahlukene, okubandakanya ukuxilongwa nokuphathwa kwemitholampilo, ngakho-ke isidingo se-biomarker ecacile. Kunezimboni eziningana ze-preeclampsia ezikhethwe yi-candidate kodwa azikho ongakwazi ukuhlolisisa i-preeclampsia ekukhulelwe kokuqala. Ama-Exosome asanda kuvela njengezicikumezi ezithembisayo zesifo futhi isifundo samanje, sigxile ekunqumeni umongo wokusetshenziswa kwama-exosomes njengama-biomarkers we-preeclampsia. Endleleni yethu, sathuthukisa uhlaka lwezinhlelo zokuhlola ama-exosomes njengama-biomarkers angaba yi-preeclampsia ngokwemigomo ebekiwe echazwe yi-Food & Drug Administration (US) (FDA) Biomarker Workgroup. Lokhu kwaholela ekuhlolweni kokusetshenziswa kwama-exosomes njengama-biomarkers of preeclampsia ngokunquma indima ye-exosomal microRNA ekuziphatheni kwe-preeclampsia esebenzisa ngokuqondile ukutholakala kwedatha, ukuhlelwa kwezinto zokusebenzisa izibalo kanye nolwazi lwezinto eziphilayo. Sithole amasignesha ama-miRNA ahlukile asebenzayo ku-perosis of preeclampsia esebenza njengama-biomarkers athembisayo. Ngaphezu kwalokho, ngokwemigomo ye-FDA kubalulekile ukufaka i-preeclampsia ye-inten-to-diagnostic ngokubambisana nama-comorbidities ehambisana nokwehluleka / ukulimala kwe-preeclampsia. Kuyabonakala emitholampilo ukuthi abesifazane abane-HIV abakhulelwe (i-Highly Active Antiretroviral Therapy) i-HAART ingengozini enkulu yokuthuthukisa i-preeclampsia ngenxa yokugula okungaziwa komzimba okubangelwa i-HAART. Ngakho-ke, sinquma ukuthi kungenzeka yini ukuhlolwa kwesifo se-cytokines exosomal ku-preeclamptic nakwabesifazane abane-HIV ngaphambili kwe-HAART. Ngokwenza kanjalo sesiye sabona amazinga e-cytokine ashintshiwe eguquguqukayo kokubili preeclampsia nabesifazane abakhulelwe abane-HIV, okusikisela indlela engavamile yokukhipha i-exosomal cytokine encapsulation, eyenza ukuthi izimpendulo zamasosha omzimba zisetshenziswe. Okubaluleke kakhulu, sibonisa ukuthi i-tumor necrosis factor alpha (TNF-α) ingaba nokusebenza komtholampilo ekuhloleni i-preeclampsia kanye ne-preeclampsia kwabesifazane abakhulelwe abane-HIV.

1. Chapter One: Introduction and Literature Review

Preeclampsia (PE), a pregnancy-specific systemic inflammatory syndrome which affects 7-10% of pregnant women globally, is commonly characterised by new-onset hypertension and proteinuria after 20 weeks of gestation [1, 2]. Reports indicate that the incidence of pre-eclampsia, perinatal death and preterm delivery in women is higher in South Africa in comparison to other low- and middle-income countries, despite access to tertiary care [3]. Preeclampsia remains one of the leading causes of maternal and foetal morbidity and mortality due to its unknown etiopathophysiology [4] and to its lack of biomarkers for early detection. Although the exact etiology of PE is unknown, the pathophysiology [4, 5], is believed to originate in the placenta, due to improper and shallow trophoblast invasion in early pregnancy [1, 6] leading to the maternal clinical symptoms observed.

Reports indicate that the cause of placental maladaptation is due to several factors [7], however, the specific and collective role of these factors in the etiology and pathogenesis of PE remains elusive, which makes it difficult to ascribe biomarker status for early detection of preeclampsia. This further complicates the clinical management and contributes to maternal and foetal morbidity and mortality. Placental 'particles' viz. syncytiotrophoblast microvesicles (STBMS) have been reported to be the central link between the placental maladaptation and the systemic manifestation of PE. However, more recently exosomes, a sub-constituent of STBMS, have been identified as a possible factor involved in the etiology and pathogenesis of PE [8, 9]. Exosomes contain cellular derived 'molecular cargo', including membrane-bound proteins, cytosolic RNA and lipids, which are reported to be involved in transferring specific biological codes in mediating physiological as well as disease processes [10].

In a preliminary study, undertaken in 2016, we identified increased exosomes in PE [11]. It is these characteristics and findings, which make exosomes promising biomarkers of PE; however, there is still limited evidence supporting their role in the pathogenesis of PE. This, therefore, necessitates research into unravelling the nature and characteristics of exosomes in the pathogenesis of preeclampsia, based on an internationally regulated framework to facilitate their clinical application as biomarkers.

1.1 Preeclampsia: A Hypertensive Disorder of Pregnancy

Hypertensive disorders of pregnancy (HDP) account for approximately 10% of pregnancy-related complications and are a major cause of maternal/perinatal morbidity and mortality [12]. Hypertensive disorders in pregnancy can be classified into four groups: (1) Preeclampsia and eclampsia (2) Chronic hypertension (of any cause) (3) Chronic hypertension with superimposed preeclampsia and (4) Gestational hypertension. Preeclampsia (PE), the predominant HDP, is associated with a greater risk of foetal and maternal morbidity [13]. Preeclampsia is defined as new onset hypertension with proteinuria [13, 14] and in the absence of proteinuria, it can be identified as hypertension in conjunction with thrombocytopenia, impaired liver function, new onset renal insufficiency, pulmonary oedema, or new onset cerebral or visual disturbances [12].

Preeclampsia can be sub-categorised into early- and late-onset [14-17], due to the differences in the timing and severity of the clinical manifestation [16]. Early-onset pre-eclampsia (EOPE) is defined by new onset hypertension at < 33 weeks plus 6 days of gestation and, late-onset pre-eclampsia (LOPE) defined by new onset hypertension > 34 weeks of gestation [15-17]. Although the clinical features of EOPE and LOPE overlap, they are associated with different maternal and foetal outcomes.

Early-onset PE occurs in approximately 5-20% of PE cases and is closely associated with the placental maladaptation due to the restriction of blood flow as a result of inadequate trophoblast invasion and transformation of spiral arteries [15, 17]. This results in poor foetal development thereby causing the more severe clinical outcomes associated with EOPE. In contrast, LOPE occurs in 8-75% of PE [18] cases and is associated with normal placental volume and weight of newborns [19]. In addition, LOPE is associated with more favourable maternal and neonatal outcomes and the possibility of pregnancy prolongation. One of the major differences between the PE subtypes is that EOPE is usually complicated by intrauterine growth restriction (IUGR), which may be a consequence of the severe placental maladaptation associated with EOPE [24] in comparison to LOPE. Additionally, there are distinct differences in placental pathological features between the PE subtypes [20]. Early onset PE is more closely associated with uteroplacental lesions and placental malperfusion in comparison to LOPE [21] due to the

uteroplacental spiral artery maladaptation in early pregnancy, resulting in oxidative stress and infarction which damages placental tissue [22]. Since EOPE and LOPE both result from malperfusion, with different causes, it is critical that the subtypes are studied as separate entities due to the differences in their etiopathophysioloy.

While the pathogenesis of PE and its subtypes are partially understood, the etiology of preeclampsia remains unclear. The difficulties in unravelling this disorder are attributed to the multiple maternal and foetal factors which contribute to the placental maladaptation [4, 5]. Extensive research has focused on various biological factors involved in the preeclampsia disease pathway including identification of maternal and foetal factors, which may serve as potential biomarkers of preeclampsia. Identification of key biological factors in preeclampsia would enable definitive clinical diagnosis/prognosis in early pregnancy and serve as a useful screening tool. This could lead to the development of strategies that would enable early therapeutic intervention in this disorder.

1.2 Etiopathogenesis of Preeclampsia

1.2.1 Unifying Theory

Although there have been recent advances in understanding the etiopathogenesis of PE, the pathophysiology triggering the disease is not fully understood. Nevertheless, preeclampsia is believed to originate in the placenta due to abnormal placentation [11]. Based on current literature, the etiopathogenesis of PE can be segmented into two parts (i.e. pre-clinical and clinical phase) consisting of six stages (Fig. 1). The pre-clinical phase (Stages 1-3) occurs in the first half of pregnancy and is characterised by poor placentation whereas the clinical phase (Stages 4-6) occurs in the second half of pregnancy and is characterised by the hypoxic placenta which results in the clinical manifestations of PE.

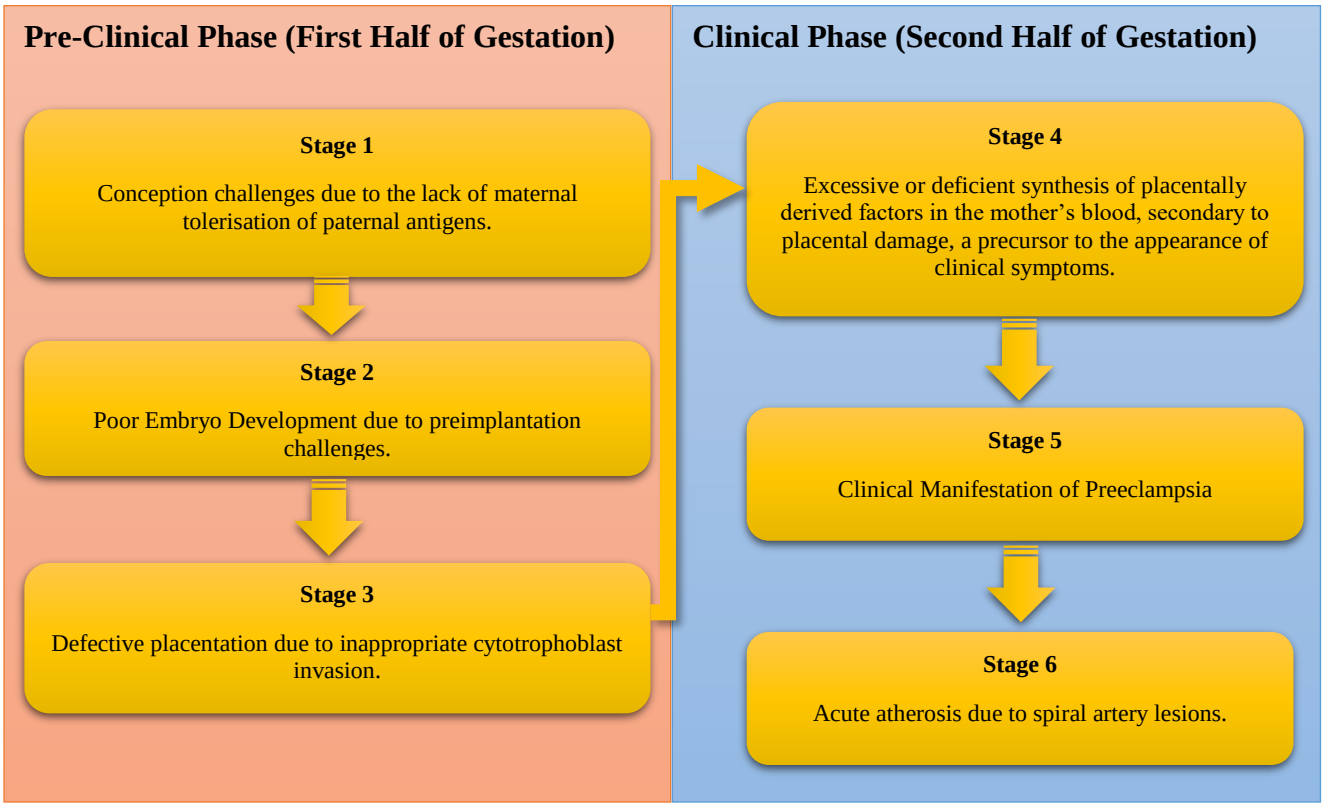

Figure 1: Current Understanding of the Etiopathogenesis of Preeclampsia. The figure represents the etiopathogenesis of PE as a disorder occurring in two phases with six stages which contribute to the disease progression. This model is based on theoretical principles presented by (Redman, 2014: [23]).

In the preclinical stage of PE, it is hypothesised that the maternal immune response to paternal antigens contributes to abnormal embryogenesis and implantation which subsequently leads to abnormal placentation caused by inappropriate extravillous trophoblast invasion (Fig 2). This ultimately leads to the clinical phase whereby the stressed placenta releases excessive or insufficient quantities of biological factors (i.e antiangiogenic protiens, signaling molecules and nucleic acids) which contribute to the maternal endothelial dysfunction, resulting in the clinical manifestation of PE [24]. On the contrary, in normal pregnancy, placentation results in structural alterations which are associated with physiological processes whereby the placental spiral arteries are modified to become low-resistance vessels, and thus they are less sensitive to vasoconstrictive substances [5]. This is a carefully controlled process, involving tightly regulated molecular mechanisms, thereby ensuring appropriate cytotrophoblast invasion which is a requirement for proper spiral artery remodelling during pregnancy (Fig 2).

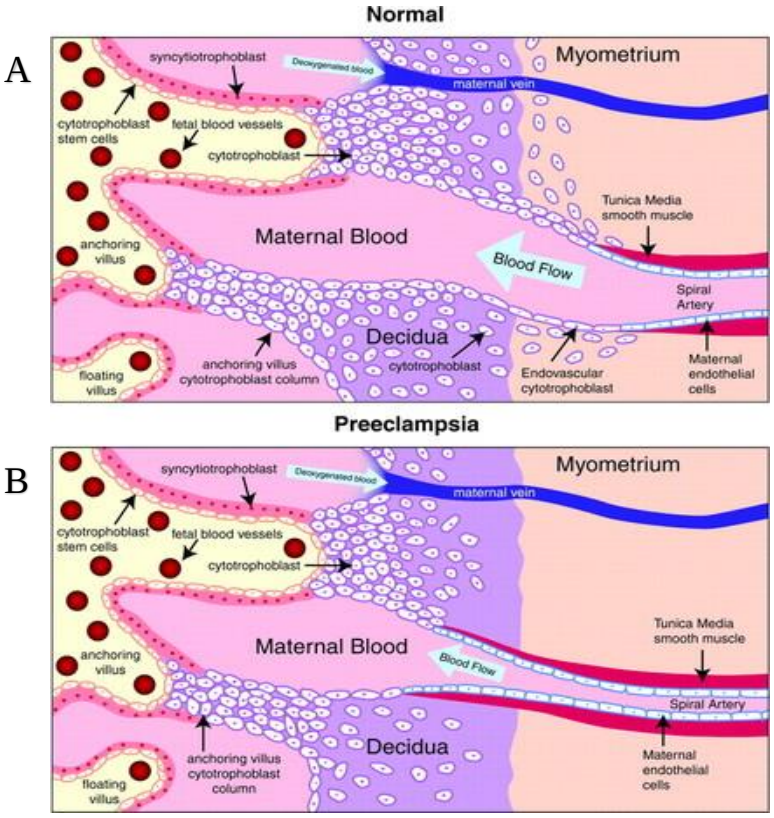

Figure 2: Abnormal Placentation and Preeclampsia. **(A)** During normal placental development, the foetal cytotrophoblasts invade the maternal spiral arteries, converting them from high to low resistance vessels capable of providing adequate placental perfusion to sustain the growing foetus. **(B)** In preeclampsia, cytotrophoblasts fail to invade the maternal spiral arteries. Instead, invasion of the spiral arteries is shallow and they remain high resistance vessels (Powe *et al.*, 2011:[25]).

Although the cause of PE remains unknown, evidence supports the immunological origin of PE due to the inability of the mother to accommodate the genetic foreignness of her unborn child [26]. Reports suggest that the trophoblast signaling to decidual immune cells in PE is weak and fails to stimulate collaboration, which is essential for placentation [1]. The current theoretical understanding of this phenomenon suggests that it may be due to the unknown link between the primary placental causes and the secondary maternal systemic inflammatory response in the third trimester of pregnancy, which may be triggered by a single or a cohort of defined and undefined biological factors (i.e antiangiogenic protiens, signaling molecules and nucleic acids) [27-32].

Although the immunological basis for PE has been assessed in terms of the maternal response to pro- and anti-inflammatory cytokines [7, 9, 32], the role of dNK (decidual Natural Killer)

cells have recently emerged as central factors involved in the etiopathogenesis of PE. The uterus contains immune cells consisting of approximately 70% of dNK cells, which have unique properties involved in regulating placental immunity and development during pregnancy [33, 34]. Previous studies have shown the beneficial function of dNK cells in the remodelling of placental vasculature and the recruitment of trophoblasts at the maternal-foetal interface in normal pregnancy [33, 35-37] which occurs through carefully regulated receptor interactions between dNK and trophoblast cells [34]. Therefore, defective communication between trophoblasts and dNK cells may provide insight into the etiopathogenesis of preeclampsia. The exact mechanisms involved in this process remain unknown.

1.2.2 Angiogenic factors associated with the pathophysiology of preeclampsia

Angiogenic factors, antiangiogenic factors, and cytokines have been identified as modulators of PE pathogenesis and serve as potential biomarkers of the disease [4-10]. To date, combined biomarkers, Soluble Flt-1(sFlt) and Placental Growth Factor (PIGF) are of clinical significance in the diagnosis of overt preeclampsia in the late second trimester of pregnancy [8-11]. Even though these biomarkers may be effective, there is still a clinical need for the diagnosis of PE in early pregnancy.

1.2.2.1 Soluble fms-like kinase-1, Placental Growth Factor & Vascular Endothelial Growth Factor

The widespread endothelial cell dysfunction associated with preeclampsia occurs in response to ischemic or hypoxic conditions resulting in the release of circulating placental pro- and anti-angiogenic factors which modulate the pathogenesis of PE [38]. Soluble fms-like kinase-1 (sFlt-1) has emerged as one of the key anti-angiogenic proteins involved in the pathophysiology of PE by inhibiting the activity of pro-angiogenic vascular endothelial growth factor (VEGF) and Placental Growth Factor (PlGF) produced by invasive cytotrophoblasts [39].

PlGF has the ability to enhance the actions of VEGF by binding to the Flt-1 receptor, subsequently sequestering those binding sites and forcing VEGF to bind to Flt-1 instead [40], which is important for VEGF's proliferative activity [41]. Therefore, the co-expression of VEGF or PlGF has the ability to modulate blood pressure [42]. Furthermore, since clinical

studies have shown that a decrease in circulating levels of PlGF correlated with the increased sFlt-1 and decreased VEGF, in PE women [43] these factors, therefore, have a key role to play in the pathophysiology of preeclampsia. Moreover, since circulating levels of sFlt-1 and PlGF are dysregulated in PE women, the ratio between these factors has been shown to reflect the pathogenesis of PE [44]. Therefore, the sFlt-1/ PlGF ratio is a promising biomarker of PE but at very high cut-off values, which mostly has application in the late second trimester of pregnancy.

1.2.2.2 Soluble Endoglin

Soluble endoglin (sEng) is a glycosylated transmembrane protein, which acts as an auxiliary receptor of several members of the TGF-β superfamily of proteins [45]. Additionally, it has a role in regulating angiogenesis and has been shown to be highly expressed in endothelial and syncytiotrophoblast cells [46]. Notably soluble endoglin (sEng) is elevated in the sera of preeclamptic individuals, and correlates with disease severity and falls after delivery, supporting its role in the pathophysiology of PE. The increased expression of soluble endoglin contributes to the pathophysiology of PE by inhibiting TGF-β1 binding, which results in the dysregulation of endothelial cell proliferation and tubule formation in PE [47].

Several *in vivo* studies have shown that sEng, led to a severe preeclamptic phenotype in mice due to the manifestation of foetal growth restriction, proteinuria and hypertension [25, 47, 48]. In addition, low platelet levels and elevated lactate dehydrogenase was observed, both of which are hallmarks of the manifestation of haemolytic anaemia, elevated liver enzymes, low platelet count (HELLP) in severe preeclampsia [49]. These findings directly implicate sEng as a factor involved in the pathogenesis of PE.

1.2.3 Cytokines in Preeclampsia

Recent research has shown that cytokines are involved in several pregnancy-related events such as ovulation, implantation, placentation, and parturition, which are critical requirements for successful pregnancy [51]. Granulocyte-macrophage-colony-stimulating factor (GM-CSF), colony-stimulating factor-1 [53], IL-4 [54], IL-10 [55], IL-2, TNF-α and IFN-γ [56] are key cytokines involved in ensuring successful pregnancy, however, their dysregulation is

associated with the pathophysiology of PE [52]. Numerous studies have supported the role of cytokines in the pathogenesis of PE in early placentation and in the clinical manifestation of the disorder [50-52]. These studies have shown that increased levels of pro-inflammatory Th1 cytokines (IL-2, TNF-α, and IFN-γ) induce several cell-mediated cytotoxic and inflammatory reactions in PE pathogenesis. In addition, Th2 anti-inflammatory cytokines (IL-4, IL-5 and IL-10) are commonly decreased in PE which promotes the proinflammatory state [52]. Moreover, cytokine levels in maternal circulation remain controversial as the exact source and mechanism of release remains unknown. What is known however is that these cytokines are released in a controlled ratio which is shifted towards Th2-type immunity in normal pregnancy, whereas an intensified shift towards Th1 immunity is associated with PE (Fig 3) [59].

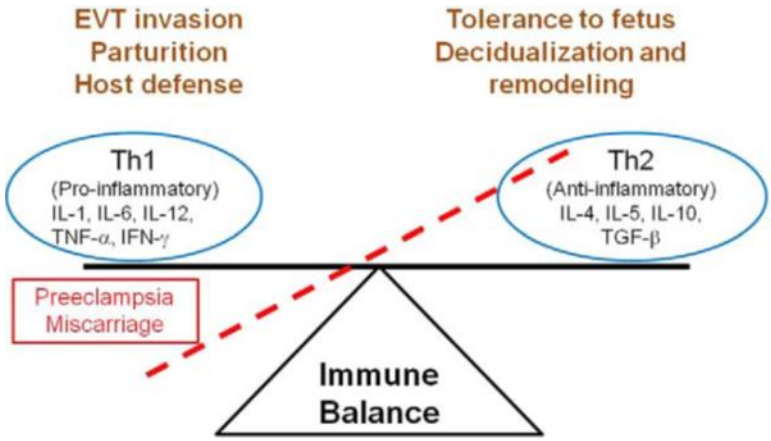

Figure 3. The Th1/Th2 cytokine-mediated maternal immune balance in normal pregnancy and Preeclampsia. The proinflammatory Th1 immune response is responsible for extravillous trophoblast invasion, parturition, and host defence. The anti-inflammatory Th2 reaction is crucial for tolerance to the foetus, decidualisation, and remodelling. Imbalance of immune response will lead to adverse pregnancy outcomes such as preeclampsia and miscarriage. Th1 indicates type 1 $CD4^+$ helper T (Yeh *et al.*, 2013: [59]).

1.2.4 microRNAs in the pathophysiology of Preeclampsia

Over recent years, miRNAs have emerged as central role players in the pathophysiology of preeclampsia since numerous studies have linked the aberrant expression of several miRNAs with PE and other pregnancy-related complications [60, 61]. MicroRNAs are small (~22–25 nt), non-coding, single-stranded RNAs which preferentially regulate gene expression. They are regulatory molecules, which play an important role in the post-transcriptional regulation of gene expression by instigating translational inhibition or cleavage of mRNA that silences gene

expression [62]. MicroRNAs are involved in critical cellular processes such as cell differentiation and migration. Therefore, they contribute to the biological aspects of pregnancy and related complications such as PE by regulating key processes such as immune tolerance, inflammation, apoptosis and angiogenesis [63]. Recently a number of miRNA have been isolated from placental tissue (Table 1). These have been found to be linked to the pathophysiology of PE and pregnancy, however, the exact mechanism of miRNA cellular discharge and the manner in which they contribute to endothelial dysfunction and hypertension in pregnancy remains unknown. However, it is proposed in this study that the cytokines and miRNA involved in the pathogenesis of PE may be encapsulated in microvesicles which serve as a mechanism of delivery to distant or target cells.

Table 1: Placenta-associated microRNAs involved in preeclampsia.

Function	miRNAs involved	Known targets/pathways
Angiogenesis	miR-16, miR-29	Vascular endothelial growth factor (VEGF)-A
	miR-494	CDK6/CYCD1
	miR-17 miR-20a, miR-20b	Ephrin B2, B4
	miR-125b-1-3p	S1PR
	miR-155	CYR 6, VEGF-A
	miR-21	PTEN, positive regulator of VEGF-A and HIF-1α
	miR-210	EFNA3, HOXA9, HSD17
Trophoblast proliferation	miR-16, miR-29b	Inhibits trophoblast proliferation
	miR-34a	SERPINA3
	miR-210	KCMF-1
	miR-155	CYCD1
	miR-378a-5p, miR-376c, miR-21	Promotes trophoblast proliferation by nodal signaling pathway
	miR-17-92 cluster	Differentiation of primary trophoblasts

Bounds *et al.*, (2017:[63])

1.2.5 Extracellular vesicles in the pathophysiology of Preeclampsia

Syncytiotrophoblast microvesicles (STBMs) are directly released from the placenta into maternal circulation and consist of a variety of microvesicles of different sizes and functional properties (Fig. 4) [64]. These include exosomes (30–100 nm), apoptotic bodies (1–5 μm) and syncytial nuclear aggregates (20–500 μm) which are released by syncytiotrophoblast cells of the placenta [7, 64]. Previous studies have identified STBMs as key factors involved in immune modulation, angiogenesis and endothelial dysfunction in pregnancy and PE [9, 65]. According to Redman & Sargent (2005), oxidative stress, as a result of abnormal placentation, induces the release of 'placental debris' which stimulates apoptosis and necrosis, resulting in an exaggerated maternal systemic inflammatory response (MSIR) [1, 9]. In addition, these authors claim that the normal process of regeneration of syncytiotrophoblast cells of the placenta is dependent on cellular apoptosis, which is modulated by placental microvesicles which activate the maternal endothelium [66].

Syncytiotrophoblast microvesicles have been reported to provoke a balanced MSIR in normal pregnancies, but, in PE, the increase in placental debris contributes to the pathogenesis by enhancing the MSIR [9, 32, 67]. Moreover, recent scientific evidence suggests that exosomes, a key constituent of STBM's, may play a significant role in the etiology and pathogenesis of PE due to their role in feto-maternal immune tolerance and endothelial cell migration [68-70]. Scientific evidence to support this hypothesis is currently emerging [1]. Research within our group in collaboration with others is underway to validate STBMs and its constituent (i.e. exosomes) as an early biomarker of PE.

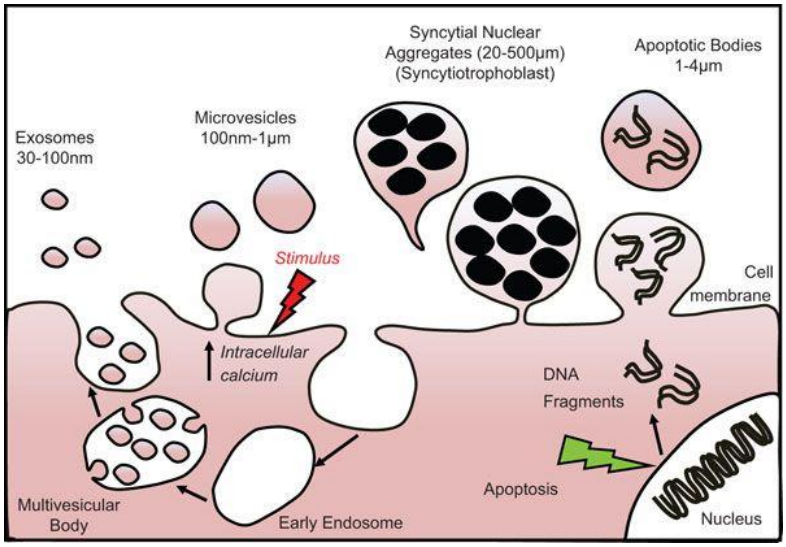

Figure 4: Subtypes of Syncytiotrophoblast Microvesicles. Exosomes (30–100nm) are produced from reverse budding of the endosome membrane, resulting in a multivesicular body, which fuses with the plasma membrane of the cell and releases exosomes by exocytosis. Microvesicles (100nm–1µm) are produced by direct budding of the plasma membrane in response to stimuli that trigger an increase in intracellular calcium levels. Apoptotic bodies (1–5µm) are released from cells undergoing apoptosis, while syncytial nuclear aggregates (20–500µm) are released from the syncytiotrophoblast on the placental surface. (Tannetta *et al.*, 2013:[7]).

1.3 Exosome Morphology and Biogenesis

1.3.1 Morphology

Exosomes are a constituent of STBMs, which fall within the smaller size spectrum of about 20 – 130nm [17-19]. These nanovesicles serve as vehicles for the transfer of 'exosomal cargo' which consist of nucleic acids, cytosolic and membrane-bound proteins, and lipids (Fig 5C). These vesicles function mainly by communicating with cells to re-programme their phenotype and regulate cell function [71, 72]. Exosomes have a defined morphology (Fig 5A and B) and are synthesised by several cell types such as hematopoietic cells, epithelial cells, neural cells, adipocytes, fibroblasts, stem cells, tumour cells, as well as the syncytial cells of the placenta [65]. These vesicles are found in biological fluids such as blood, urine, saliva, breast milk, mucus and cerebral spinal fluid [73]. The key physiological function of exosomal cargo is to stimulate cell-to-cell communication, and hence serve as mediators in biological processes [74]. Cellular communication is complex and is an essential requirement for normal cellular functioning. If the normal cellular function is disrupted it may result in pathological states such as cancer, autoimmunity and diabetes [75-77].

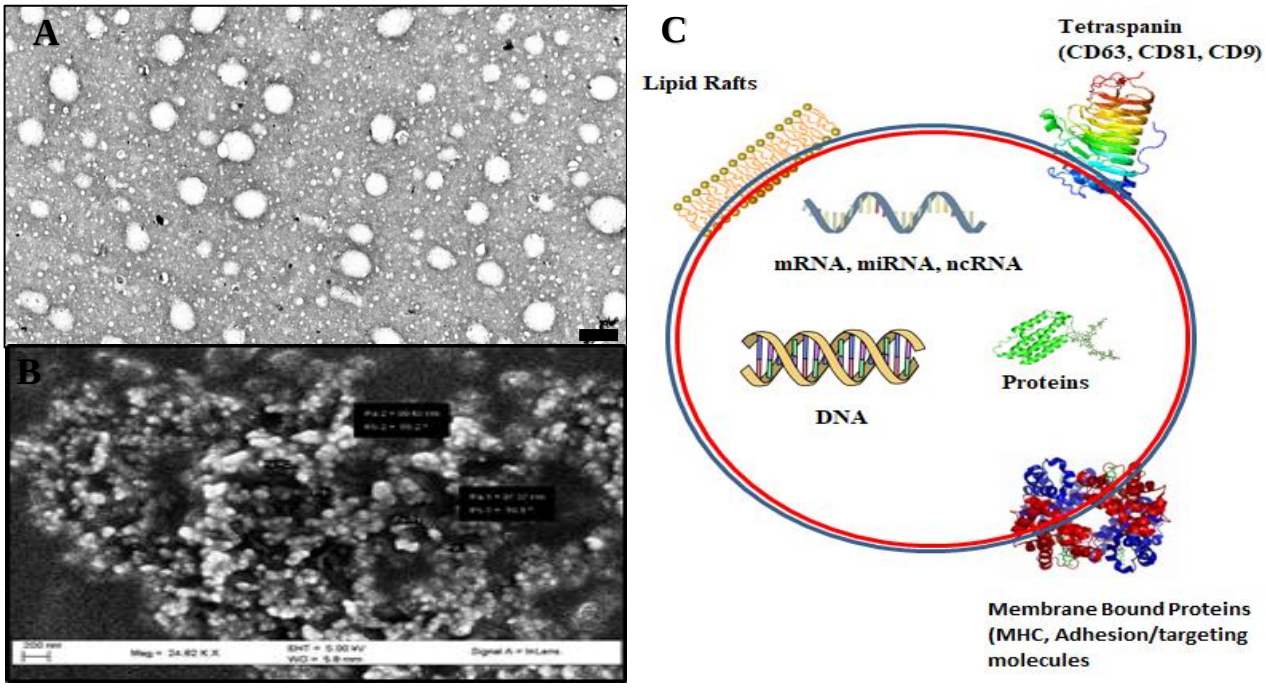

Figure 5. Exosome Morphology. Morphological characterisation of exosomes isolated from maternal circulation by (A) Transmission Electron Microscopy (TEM) and (B) Scanning Electron Microscopy (SEM). Electron microscopy reveals the structure of exosome-like vesicles from the plasma of pregnant women. For TEM the purified exosomes were fixed, stained with 2% uranyl acetate and analysed using electron microscopy. SEM samples were dropped cast and air dried before analysis. Scale bar, 100 nm (original magnification X45 K). (C) Illustration of the types of molecular cargo associated with exosomes [78].

1.3.2 Biogenesis of Exosomes

The exact cellular mechanisms involved in the biogenesis of exosomes is unknown. Nevertheless, it has been shown that the Endosomal Sorting Complex Required for Transport (ESCRT) machinery and associated proteins (MHC class II) are involved in protein sorting and the formation of the intraluminal vesicles (ILVs) destined to be secreted as exosomes [79]. The ESCRTs consist of twenty proteins that agglomerate into four soluble complexes (i.e. ESCRT 0, I, II & III). These complexes have associated proteins such as VPS4 (Vacuolar protein sorting-associated protein 4), VTA1 (Vesicle tracking associated protein 1) and ALIX (ALG-2-interacting protein X) [80, 81].

Figure 6 illustrates the pathway of exosome biogenesis. Endosomal sorting complex required for the transport-0 complex is involved in endocytosis and the recruitment of ubiquitinated proteins on the cell surface [82]. Endosomal sorting complex required for transport I & II, work

in homeostasis in late endosomal formation by co-ordinating membrane budding & scission and ESCRT III is responsible for the fusion of MVB's with the cell wall to release exosomes *via* exocytosis [83]. Exosomes usually contain a variety of molecules that are native to the originating cell and are enriched with endosome-associated protein markers such as RAB proteins (Ras-related proteins), ALIX, TSG101 (Tumor susceptibility gene 101) and endocytic proteins which provide definitive information about their origin [73]. Further understanding of the molecular mechanisms of exosome biogenesis will lead to resolving the complexities of many diseases such as PE.

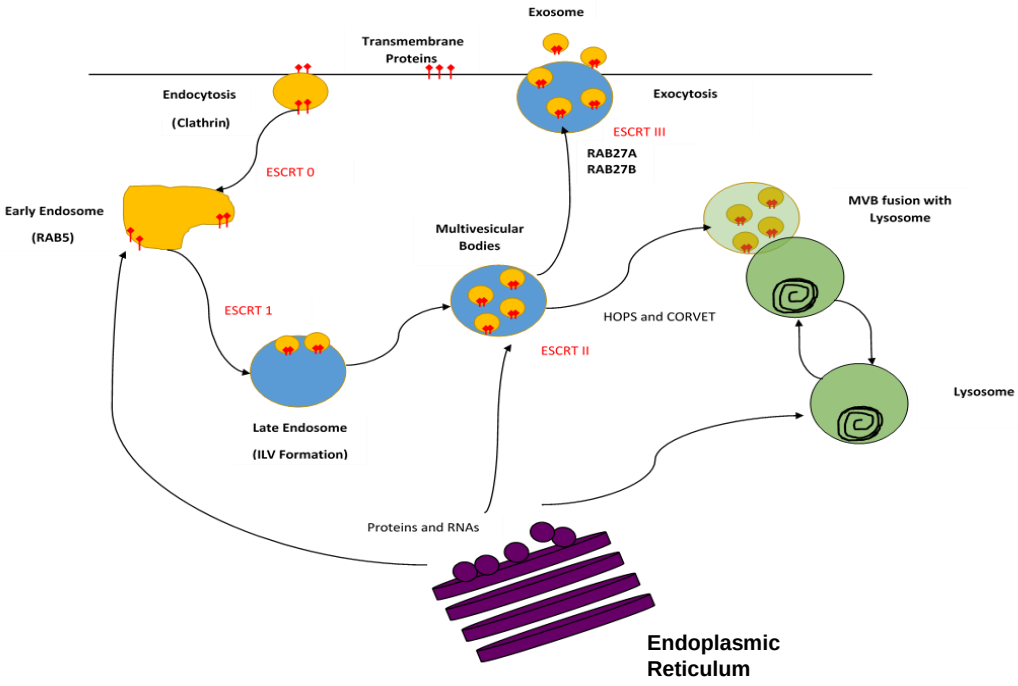

Figure 6. Intracellular mechanisms involved in exosome biogenesis. Schematic representation of the origin and release of exosomes by eukaryotic cells. Exosomes are formed as intraluminal vesicles (ILVs) by budding into early endosomes and MVBs. Several molecules are involved in the biogenesis of ILVs, such as the ESCRT machinery, lipids (such as ceramide) and the tetraspanins. It is still unknown whether these mechanisms act simultaneously on the same MVB or on different MVBs. The fate of MVBs can be either fusion with lysosomes or fusion with the plasma membrane, which allows the release of their content to the extracellular milieu. Several RAB proteins (RAB11, RAB27 and RAB35) have been shown to be involved in the transport of MVBs to the plasma membrane and in exosome secretion (Alenquer & Amorim, 2014: [84]).

1.4 Exosomes in normal and preeclamptic pregnancies

1.4.1 Exosomes in Pregnancy

Pregnancy has often been described as an immunological challenge to the maternal immune system, as it entails a foeto-maternal co-existence which has been described as a 'paradox of nature' [70]. Whilst, there is no single unifying theory for maternal tolerance to the foetus, this adaptation requires a balance of maternal and foetal factors, essential for successful pregnancy [85]. Factors such as cytokines, angiogenic proteins and STBM's, have been shown to have a role in the foeto-maternal immune balance as well as the maintenance of placental vasculature [25, 85, 86].

More recently, exosomes have emerged as key biological factors in pregnancy, as they are involved in intracellular signalling which may be responsible for maternal immune adaptation [87]. In normal pregnancies, it has been reported that placental-derived exosomes with no associated pathologies are biologically active molecules with a regulatory role in endothelial cell migration [88, 89]. Additionally, placental-derived exosomes in maternal circulation are known to increase with gestational age and has been shown to be directly correlated with endothelial cell migration in the first trimester. However, in the second and third trimester of pregnancy, the lower ratio of placental-derived exosomes to total exosomes in maternal circulation results in the inhibition of endothelial cell migration [88, 89].

1.4.2 Exosomes in Preeclampsia

Under pathological conditions, the dysregulation of the biogenesis of exosomes results in target cell dysfunction, which directly influences cellular activities such as angiogenesis, translational activity, proliferation, metabolism and apoptosis [90]. Since exosomes are central factors involved in cellular signalling in normal pregnancies, it is possible that their dysregulation is involved in the etiopathology of preeclampsia. Importantly, exosomes released from the hypoxic placenta could increase circulating levels of exosomes in maternal plasma. Placental- or maternal-derived exosomes in maternal circulation could result in endothelial dysfunction, resulting in the clinical manifestations of preeclampsia. The identification of new proteins and biomolecules in placental-derived exosomes are of considerable interest for early diagnostic, drug delivery and therapeutic applications.

It is known that preeclampsia is a disorder that originates in the placenta, as a result of poor placentation due to inadequate trophoblast invasion, with the exact cause of this maladaptation being unknown [24]. Exosomes could be involved in this maladaptation, due to their role in cell signalling. Therefore, determining its role in preeclampsia could provide valuable insight into its pathogenesis and serve as an early definitive biomarker. Importantly, the exosomes released from the hypoxic placenta could increase the levels of circulating exosomes in maternal plasma. These circulating exosomes could affect the endothelial cells and therefore result in the manifestation of the clinical symptoms of the disease.

In our preliminary experiments, we have identified a dysregulation in the relative concentration of placental-derived exosomes in early and late onset preeclampsia [11]. Our data indicate that; (i) the concentration of total exosomes are significantly increased in early and LOPE ($p<0.05$) (ii) The concentration of Placental Alkaline Phosphatase (PLAP+) exosomes significantly increases in early onset pre-eclampsia ($p<0.05$) but decreases in LOPE ($p<0.05$) (iii) The ratio of PLAP+ exosomes to the total number of exosomes significantly decreases in EOPE ($p<0.01$) and LOPE ($p<0.05$) (iv) A positive correlation between the CD63+ and PLAP+ exosomes was obtained in normotensive pregnancies ($r = 0.60$, $p<0.05$) and EOPE ($r = 0.51$, $p<0.05$) with the inverse in LOPE ($r = -0.62$, $p<0.001$). These findings obtained may relate to the dissimilarities in the etiology of the two subtypes, suggesting that placental-derived exosomes could play a pathological role in PE. Our findings, therefore, form the rationale for the determination of the role of placental-derived exosomes in PE and suggests that exosomes may be a useful tool/biomarker for the prognosis/diagnosis of PE.

1.5 Association of Highly Active Antiretroviral Therapy and Preeclampsia

It is well known that untreated pregnant women with HIV have a decreased risk of developing PE due to the maternal immune compromised state which facilitates foeto-maternal immune tolerance [48]. However, several studies have implicated Highly Active Anti-Retroviral Therapy (HAART) with the pathophysiology of PE in HIV-positive pregnant women [48-50] since 10.2% of these women develop preeclampsia [48]. The increased risk seems to be as a result of the maternal immune reconstitution induced by HAART whereby the maternal immune reconstitution (restoration of CD4+ T cells) may contribute to an inflammatory state which dysregulates the foeto-maternal immune tolerance and subsequently leads to PE [48]. Even though the immunological events leading to these alterations remain unclear, it is

evidently associated with the abnormal activation of the immune system, which plays a pivotal role in the etiology and pathogenesis of preeclampsia.

Immune-related studies in PE and HIV-positive PE pregnant women on HAART have suggested that the changes in cytokine levels may occur due to the alteration in the Th1/Th2 dichotomy (Fig. 6), which subsequently contributes to PE [49]. This may provide novel perspectives in determining the pathological mechanisms in PE and identifying definitive biomarkers of PE in HIV-positive pregnant women on HAART since clinically, antiretroviral therapy could overlap and complicate diagnosis and management in these patients. We therefore, incorporated the isolation and characterisation of exosomes in PE, and PE with HIV/HAART into this study, as we will be able to gain insight into the possible role of exosomal cytokines in immune function. To the best of our knowledge, this has not been previously investigated. Therefore the observations made from this study will contribute to understanding the possible associations between Highly Active Antiretroviral Therapy and PE.

1.6 Purpose of the study

Current medical biotechnological applications have revolutionised the way we perceive, diagnose and treat diseases leading to innovative medical breakthroughs. Exosome diagnostics, an emerging medical biotechnological approach, provides a powerful platform to identify promising biomarkers and therapeutic agents for diseases, due to their specific molecular content/cargo (i.e. lipids, proteins, nucleic acids), which carry precise biological codes relating to their cellular origin, capable of changing the functioning of target cells in normal and pathological states.

In recent years, studies conducted have identified exosomes and their cargo in normal pregnancies, however, there has been no conclusive and robust studies pertaining to specific exosomal molecular cargo in preeclampsia *per se*, and their role in the etiopathogenesis. This is a critical requirement for evaluating the validity of exosomes as biomarkers, for preeclampsia, as defined by the FDA Biomarker Workgroup [91, 92]. Therefore, our preliminary studies have focused on the assessment of exosomes as potential biomarkers of preeclampsia. The presented studies contribute to the foundational framework in support of the

clinical application of exosomes as biomarkers of preeclampsia, with a focus on its context of use. The preliminary scientific evidence in support of this was categorised into three studies as follows:

i) **AIM 1: To determine the theoretical framework for the evaluation of exosomes as biomarkers of preeclampsia (Chapter two).**

Objectives

- To evaluate the current state of biomarkers of preeclampsia and unmet medical needs, which the proposed biomarker must address; and

- To evaluate the application of exosomes in diagnosing preeclampsia in terms of the FDA biomarker criterion and unmet medical needs.

ii) **AIM 2: To determine the context of use of exosomes with respect to the specific role of exosomal miRNA in the pathophysiology of preeclampsia (Chapter 3):**

Objectives

- To isolate and characterise exosomal microRNA objectively in maternal circulation using rapid and reliable technology in support of its application in the clinical setting.

- To validate results statistically using analytical systems (nSolver -R based platform) which support the validity of the diagnosis.

- To use Computational Merging and Meta-analytical Pathway Analysis (Diana miRPATH) to determine the role of exosomal miRNA in the pathophysiology of preeclampsia.

- To identify significant exosomal miRNA signatures as candidate biomarkers of preeclampsia and its subtypes.

iii) **AIM 3: To evaluate the use of exosomal cytokines in diagnosing preeclampsia in HIV-positive pregnant women on HAART due to the increased prevalence of the comorbid state (HIV and PE).**

Objectives

- To isolate and characterise exosomes objectively in HIV-positive pregnant woman on HAART.

- To determine the expression levels of total and placental-derived exosomes in maternal circulation of HIV-positive preeclamptic pregnant women on HAART.

- To determine the levels of exosomal Th1 and Th2 cytokines in HIV-positive preeclamptic pregnant women on HAART.

- To evaluate the biomarker potential of exosomal cytokines in diagnosing PE in HIV-positive pregnant women on HAART.

The outcomes from this study are novel and will hopefully contribute to the global understanding of the pathophysiology of preeclampsia and preeclampsia/HAART, which may lead to the subsequent development of biomarkers for the diagnosis of preeclampsia, within the FDA approved guidelines.

2. Chapter Two: Manuscript One

Placenta-Derived Exosomes: Potential Biomarkers of Preeclampsia

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This publication critically reviews the current state of PE biomarkers within the context of their specific sensitivity, specificity and shortcomings in diagnostic/prognostic potential, that subsequently formed the 'needs assessment' of the current biomarker status. This formed the 'constitutional matrix' to evaluate exosomes as potential biomarkers of PE in terms not only of the specific FDA biomarker criterion but also of any unmet clinical considerations.

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REVIEW

Placenta-derived exosomes: potential biomarkers of preeclampsia

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Preenan Pillay[1,2]
Kogi Moodley[1]
Jagidesa Moodley[3]
Irene Mackraj[3]

[1]Discipline of Human Physiology, Nelson R Mandela School of Medicine, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa; [2]Pearson Institute of Higher Education, Midrand, South Africa; [3]Women's Health and HIV Research Group, Nelson R Mandela School of Medicine, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa

Abstract: Preeclampsia remains a leading cause of maternal and fetal mortality, due to ineffective treatment and diagnostic strategies, compounded by the lack of clarity on the etiology of the disorder. Although several clinical and biological markers of preeclampsia have been evaluated, they have proven to be ineffective in providing a definitive diagnosis during the various stages of the disorder. Exosomes have emerged as ideal biomarkers of pathological states, such as cancer, and have more recently gained interest in pregnancy-related complications, due to their role in cellular communication in normal and complicated pregnancies. This occurs as a result of the specific placenta-derived exosomal molecular cargo, which may be involved in normal pregnancy-associated immunological events, such as the maintenance of maternal–fetal tolerance. This review provides perspectives on placenta-derived exosomes as possible biomarkers for the diagnosis/prognosis of preeclampsia. Using keywords, online databases were searched to identify relevant publications to review the potential use of placenta-derived exosomes as biomarkers of preeclampsia.

Keywords: placenta-derived exosomes, preeclampsia, biomarkers

Introduction

Preeclampsia (PE) is a multiorgan systemic hypertensive disorder of pregnancy (HDP) that is a leading cause of maternal and fetal mortality.[1–3] The exact etiology of PE is difficult to establish, due to the multifactorial nature of the disorder; however, the general theoretical framework underlying the etiology of PE centers on abnormal placentation due to inadequate spiral-artery remodeling caused by poor trophoblast invasion.[4,5] Moreover, it is now understood that increased placental oxidative stress is triggered by a predisposing condition, which stimulates the release of microvesicles from the syncytial layer of the placenta.[6,7] These extracellular vesicles (EVs), termed syncytiotrophoblast microvesicles (STBMs), may bind to monocytes and stimulate the production of proinflammatory cytokines, which results in altered maternal systemic inflammatory response (MSIR) in normal and preeclamptic pregnancies.[8–11] In addition, ex vivo studies have shown that STBMs have a key role in maternal immunomodulation that results in regulation of T-helper 1 (T_h1)/T_h2 immunoresponses in normal pregnancy,[8] and may thus be responsible for the T_h1 skewness in PE.[12]

It is now recognized that the exact nature and composition of STBMs has yet to be defined with regard to proportion of exosomes, apoptotic bodies, and other vesicles. In light of this, recent literature has indicated that the constituents of STBMs should be classified as EVs and thus be termed STBEVs.[13] Though prior knowledge of STBEVs indicates that these vesicles in their entirety can be used as circulating STB biopsies,[14] they cannot be clearly defined as biomarkers of PE, as studies have

Correspondence: Irene Mackraj
Women's Health and HIV Research Group, Nelson R Mandela School of Medicine, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal 1 University Road, Westville, Durban 4001, South Africa
Tel +27 729 085 646
Fax +27 260 7071
Email mackraji@ukzn.ac.za

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not demonstrated the objective isolation and detection of PE-specific biomarkers. In addition, studies conducted by the Obstetrics and Gynaecology Unit at the University of Oxford have shown that exosomes may be a key constituent of STBMs involved in the pathogenesis of the disorder.[7,8,10,12–19] These fundamental observations support the potential of placenta-derived exosomes as a biomarker of PE. Therefore, this review aims to evaluate the potential application of exosomes as a biomarker of PE.

Understanding the spectrum of pregnancy-related hypertensive disorders

HDPs are classified into four groups: 1) PE and eclampsia, 2) chronic hypertension (of any cause), 3) chronic hypertension with superimposed PE, and 4) gestational hypertension. Clinical classification of the types of HDP is based primarily on gestational age, blood pressure, and compounding complications (Figure 1). PE is defined as an HDP that has been characterized extensively in clinical practice, as it is associated with an increased risk of fetal and maternal morbidity and mortality.[20] Without early-detection strategies, PE is clinically diagnosed as new-onset hypertension with proteinuria,[20,21] and can be further subcategorized into early- and late-onset PE.[21–24] Based on gestational age, early-onset PE is defined by new-onset hypertension at <33 weeks plus 6 days of gestation and late-onset PE defined by new-onset hypertension >34 weeks of gestation.[22–24] However, the severity of PE pregnancies can be differentiated by their clinical manifestations, as outlined by clinical practice guidelines that present the adverse conditions and severe complications of PE according to the end organs affected.[25]

Adverse conditions in PE are associated with risks that increase the odds of severe maternal and fetal complications, and when exaggerated complications lead to severe (that warrant delivery).[25] Diagnosis of these complications is primarily based on clinical maternal symptoms and laboratory tests. In addition, gestational hypertension, superimposed PE, and PE can progress to severe PE if one or more severe complications manifest, which thus necessitates improved clinical monitoring-management systems using defined prognostic and diagnostic methods. Thus far, the only known cure of PE is the early delivery of the fetus and placenta to prevent worsening of disease progression and associated maternal complications, which leads to prematurity and its subsequent complications.[25,26] All these complications emphasize the need for a biomarker that would enable early diagnosis and development of a treatment method for PE to prevent the inherent distress induced by it.

Biomarkers of PE: the current state

Biomarkers are universally defined as biochemical alterations that can be objectively detected and quantified in

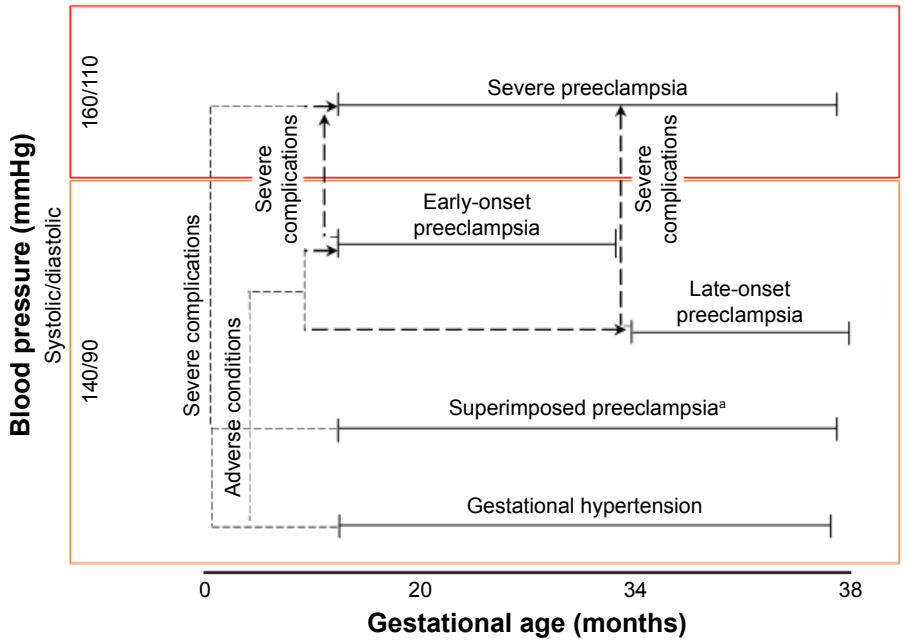

Figure 1 Classification of hypertensive disorders in pregnancy.
Notes: Hypertensive disorders with preeclampsia subclassified into early- and late-onset preeclampsia. Adverse conditions are defined as conditions that increase the risk of severe complications. Adverse conditions and severe complications are categorized into the organ system affected (ie, central nervous system, cardiorespiratory, hematological, renal, hepatic, and fetoplacental), as described by Magee et al.[25] [a]Hypertension present either at prepregnancy or that developed at 20 weeks prior to gestation.

human tissue, cells, or fluids and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention.[27] It is thus imperative that these criteria are met when identifying potential biomarkers of PE to develop an efficient clinical test for early diagnosis and differentiation of the severity of PE. However, this is a challenge, due to the complex etiology and pathology of PE.

Clinical markers, such as maternal factors, blood pressure, proteinuria, and uterine-artery Doppler velocimetry remain the most reliable methods in PE monitoring and supportive care of the mother and fetus. However, they cannot be used in the early diagnosis of PE,[21,28] but may still serve as useful markers when used in combination with other PE biomarkers. Previous studies (Table 1) have shown that combined clinical and biological markers have improved detection rates in overt PE, which could be incorporated into clinical care for early screening and assessment of patient-specific risks of PE.[29–31] However, the limitation of combined clinical markers and biological markers is the inability of these markers to provide definitive differentiation of the severity of PE and assessment of the PE-disease pathway. Currently, combined biomarkers of PE include cytokines, proteins, angiogenic and antiangiogenic factors that have a fundamental role in the pathogenesis and etiology of PE, as well as other related HDPs.[32–34]

Angiogenic and antiangiogenic factors

Thus far, antiangiogenic sFlt1 and angiogenic PGF in combination serve as the most promising biomarkers for the diagnosis of PE.[35–38] This is mainly attributed to the synergistic role of these molecules in the pathogenesis of PE, whereby an altered angiogenic balance induced by sFlt1, inhibits PGF signaling, resulting in vascular endothelial dysfunction, a key feature of PE.[32,37–39] Although the precise physiologic role of these factors is not fully understood, the elevated circulating levels of sFlt1 and decreased PGF observed in overt PE highlight their combined biomarker potential,[33,37,38,40] Furthermore, a recent study presented the differential diagnostic potential of the sFlt1:PGF ratio in PE.[37,38,41,42] This ratio proved to be effective in the diagnosis of overt PE in the third trimester of pregnancy, together with differential diagnosis of severe gestational and chronic hypertension at ≥34 weeks' gestation.[35,31] Regardless of the favorable sensitivity and specificity of the sFlt1:PGF ratio, its clinical usage is restricted as a late-pregnancy diagnostic and management tool for severe or overt HDP.[42] A case–control multicenter study showed that use of differential cutoffs for the various

Table 1 Overview of potential, combined and candidate biomarkers in preeclampsia

Biomarker	Biomarker type	Detection stage	Role	Sensitivity	Specificity	Advantages	Disadvantages	Reference(s)
Potential biomarkers/combined biomarkers								
Cystatin C	Protein	Preclinical (first trimester: ≥11 weeks)	Protease inhibitor involved in impaired renal function and hypothesized to disrupt placental development in PE	91.7	85.7	May be a useful first-trimester biomarker due to its elevation in overt PE, with a 93% detection rate when used in combination with maternal BMI	Indirect measure of glomerular filtration impairment and may be unrelated to placental dysfunction; may have application in severe preeclampsia, though the possibility that this occurs as a result of renal impairment due to increase protein load in maternal circulation should not be excluded The study was inconclusive, as data regarding clinical markers, such as arterial pressure and Doppler PI were not been measured; inclusion of other HDPs need to be taken into consideration in future studies	114

(*Continued*)

Table 1 (*Continued*)

Biomarker	Biomarker type	Detection stage	Role	Sensitivity	Specificity	Advantages	Disadvantages	Reference(s)
HTRA3	Enzyme	Clinical (second trimester: 13–14 weeks)	Serine protease found to be associated with alterations in placental oxygen transfer, and thus may be involved in blood-vessel remodeling	ND	ND	Elevation of HTRA3 in PE indicates biomarker potential; additionally, the ratio of HTRA3-L to total HTRA3 was lower in early-onset PE	Involvement of the enzyme in the pathogenesis of PE is unknown; enzyme not a marker of placental function and thus cannot be used as a specific marker of placental function in PE	62
NGAL	Glycoprotein	Clinical (late second trimester, early third trimester: 24–26 weeks)	Involved in the modulation of oxidative stress in normal cellular physiology; its dysregulation has been associated with inflammation and cancer	75	94.5	Elevated levels of NGAL obtained in PE that were positively correlated with blood pressure and proteinuria	Involvement of NGAL in pathophysiology of PE unknown; findings need to evaluated in larger population	66
PP13	Protein	Preclinical (first trimester: 6–10 weeks)	Immunoregulatory protein involved in placental development	80	20	Significantly reduced levels of PP13 found in PE in early pregnancy and thus may have application as potential early predictor of PE	Marker has poor detection sensitivities, and detection has to be based on predetermined cutoff values	72
Serum lipids	Oxidized cholesterol and glycerophosphocholine	Preclinical (first trimester: 6–10 weeks)	Lipids like oxidized cholesterol contribute to vascular atherosclerosis	91	82	Combined lipid biomarkers show good ability to detect PE in early pregnancy	Role of these lipids still unknown in pathogenesis of PE; in addition, further studies required to determine if these markers are specific for PE or associated with all types of pregnancy-related complications	76
IL10, TNFα, and IFNγ	Cytokines	Clinical (second trimester: 14–18 weeks)	IL10 is an important anti-inflammatory cytokine in pregnancy that inhibits upregulation of MMP2 and MMP9 and promotes the termination of T_h1 inflammatory rejection reactions against the fetal placental unit TNFα involved in systemic endothelial cell activation IFNγ associated with idiopathic recurrent spontaneous abortion	ND	ND	Reduced levels of these cytokines may be involved in the modulation of etiopathogenesis of PE and thus show promise as early clinical stage biomarker	Larger population size required to validate biomarker potential	55, 56

RASSF1A	cfDNA/cffDNA	Clinical (second trimester: 17–30 weeks)	It is hypothesized that the reason cffDNA increases in maternal circulation before onset of the clinical symptoms is increased placental perfusion caused by inadequate spiral-artery remodeling; oxidative stress, apoptosis, and necrosis the main physiological processes that disrupt placental development, leading to increase levels of cfDNA and cffDNA in maternal circulation	87.5/100	75/50	Reliable predictor of PE with ROC (AUC) values of 0.94 and 0.83 for cfDNA and cffDNA, respectively; predictive capability of woman with risk of PE who developed PE and woman at risk who did not develop PE had an ROC (AUC) value of 0.78 and 0.81 for cfDNA and cffDNA, respectively; combined cfDNA and cffDNA quantification of RASSF1A may be used as a late preclinical stage biomarker in women with high risk of PE	Cannot be used as a very early biomarker (0–16 weeks of gestation) cffDNA has low specificity and thus needs to be used in combination with cfDNA The exact physiological role of these molecules in PE has not been elucidated	50, 51
Combined clinical markers/potential biomarkers								
MFs, MAP, UtA PI, PGF, PAPPA	Clinical measurements, angiogenic factor, and protein	Preclinical (first trimester: 11–13 weeks)	Clinical markers (MFs, MAP, and UtA PI) direct measures of cardiac malfunction PGF an angiogenic factor required for normal placental development PAPPA involved in cellular proliferation	ND	ND	Combined markers have potential to detect severe EOPE at detection rate of 93% with false-positive rate of 5% Algorithm for the detection of severe EOPE may serve as a useful screening tool Method has a greater detection rate of PE than previous traditional methods, which relied entirely on maternal history	Poor detection rates for LOPE (35%) and GH (18.3%) obtained; this proves that the combined marker cannot differentiate between PE subtypes	31
MFs, MAP, UtA PI, PAPPA, free βhCG	Clinical measurements, protein, and hormone	Preclinical (first trimester: 8–12 weeks)	βhCG involved in implantation and blastocyst development and replaced PGF in Poon et al[31]	ND	ND	At a false-positive rate of 5%, 10% detection rates for EOPE were 69.2% and 80.8%; however, LOPE-detection rates were lower at 29.4% and 39.6% May be a useful tool in early screening of EOPE	Cannot differentiate between PE subtypes Cannot be used as screening test, as larger studies with inclusion of angiogenic factors are required to validate potential to be used in differential diagnosis of PE	29
Combined candidate biomarkers								
sFlt1 and PGF	Angiogenic and antiangiogenic factors	Clinical (third trimester: 20–33 weeks)	Directly involved in the pathogenesis of PE; PGF and angiogenic factor required for normal placental development and sFlt1 an antiangiogenic factor	88–95	94–99.5	Ability to detect PE with high sensitivity and specificity. In addition, differential phase cutoffs improve the diagnostic accuracy of the ratio Favorable sensitivity and specificity are attributed to their direct involvement in normal pregnancy	Larger studies required to validate differential diagnosis, due to high constructed incidence of PE in this study and multifactorial nature of the disorder Cannot be used as an early biomarker of PE	38

Abbreviations: BMI, body-mass index; EOPE, early-onset PE; GH, gestational hypertension; HDPs, hypertensive disorders of pregnancy; LOPE, late-onset PE; MAP, mean arterial pressure; MFs, maternal factors; ND, not determined; PE, preeclampsia; UtA PI, uterine-artery pulsatility index; βhCG, β-human chorionic gonadotropin.

gestational phases enhanced the diagnostic potential of the sFlt1:PGF ratio.[38] This study, however, did not show false-positive or false-negative values, due to the high constructed incidence of PE and variability of the disorder. Therefore, validation of the sFlt1:PGF ratio in a clinical setting with a larger, homogeneous population is required. sEng, a key antiangiogenic factor implicated in PE, has been found to be elevated in maternal circulation in the second trimester in PE-predisposed patients.[43–45] Although sEng values alone or in combination with other pro- and antiangiogenic factors are significantly altered in PE serum, it has proven to be a poor biomarker of the disorder.[46]

Cell-free fetal DNA

cfDNA and cffDNA are also probable combined biomarkers of PE, mainly due to their sensitivity and specificity in biological fluids.[47,48] The identification of the methylated *RASSF1A* promoter gene, which is elevated in early-PE pregnancies,[49–51] offers a promising alternative to the quantification of cffDNA, using Y-chromosome-specific sequences.[52] Notably, high doses of human cffDNA did not induce PE-like symptoms in a murine in vivo model,[53] reinforcing the notion that the increase in *RASSF1A* methylation is a consequence rather than a cause of PE. Nevertheless, when used in combination with other markers, cffDNA *RASSF1A* methylation may have potential in monitoring PE development in the second trimester.[49–51]

Cytokines

Cytokines are significantly involved in the pathophysiology of PE, as they are instrumental in the maintenance of the T_h1–T_h2 balance in normal pregnancy.[9] During normal pregnancy, there is a shift toward T_h2 immunity that results in the synthesis of IL4, IL5, IL6, and IL10, whereas in PE there is a distinct shift toward T_h1 immunity, resulting in the production of IL2, TNFα, and IFNγ.[54] It is this shift toward T_h1-associated immunity in PE that results in the exaggerated MSIR. Therefore, T_h1- and T_h2-associated cytokines have gained interest as potential PE biomarkers. Research conducted in patients in the early second trimester of pregnancy has indicated that IL10-, TNFα-, and IFNγ-expression levels were significantly altered in PE during weeks 14–18 of gestation.[55–58] Therefore, these cytokines in combination may serve as potential early biomarkers of PE if validated in a larger sample population. In addition, GDF15, a macrophage-inhibiting cytokine possessing cardioprotective and biomarker potential in cardiovascular disease, has been found to be significantly reduced in PE women.[59] However, GDF15 cannot be used as a biomarker of PE in the first trimester of pregnancy, as no marked differences between normotensive and PE pregnancies have been observed. Although cytokines may be involved in the pathophysiology of PE, their application as possible biomarkers is questionable, since key cell-mediated factors are involved in their regulation during pregnancy.

High-temperature-requirement A3 enzyme

HTRA3, a pregnancy-related serine protease expressed by placental extravillous trophoblast cells, may have biomarker potential in the early detection of PE.[60] It has been found to be associated with altered placental oxygen transfer and blood-vessel remodeling in the first trimester.[61] Elevated levels of HTRA3 in weeks 13–14 of gestation have been observed in PE-predisposed patients.[62] Although the potential application of HTRA3 as a biomarker is promising, it has limited application, since factors responsible for altered expression of this enzyme in PE are still unclear. Additionally, this enzyme is not a specific marker of placental function, and thus cannot be used an indicator of placental maladaptation in PE.

Pregnancy-associated plasma protein A

PAPPA, a more relevant, pregnancy-related protease involved in early placental development, has been found to be dysregulated in intrauterine growth restriction, PE, placental abruption, and premature birth.[63] PAPPA is responsible for the cleavage of IGFBP4, hence regulating the synthesis of IGF, a key growth factor in the regulation of placental and fetal growth.[64] Dysregulation of this enzyme may be linked to a variety of pregnancy-related complications associated with generalized placental function. Due to the aspecificity of this enzyme, it cannot be used as a marker of PE, but may have diagnostic application in combination with other clinical measurements and biomarkers.[65]

Neutrophil gelatinase-associated lipocalin

NGAL, a lipocalin-type glycoprotein involved in iron sequestration normally associated with inflammation, neoplastic transformation, and renal damage, has been found to be linked to PE.[66] Increased serum NGAL is proportional to the severity of PE in the late second trimester of pregnancy, with sensitivity and specificity of 75% and 94.5%, respectvely.[67] However, NGAL is not linked exclusively to placental maladaptation, and its role in PE is unclear. Additionally, maternal serum AFP, in combination with maternal factors, the uterine-artery pulsatility index, and mean arterial pressure,

has shown potential in the diagnosis of PE in weeks 19–24 of gestation in combination with other biomarkers.[68]

Placental protein

PP13 has been reported extensively in early placental development,[69,70] and may be involved in T-cell and macrophage apoptosis, regulation of maternal immunoresponses, and maternal–fetal immunomodulation.[70,71] Due to its association with early placental development, it may be useful in detecting PE during early pregnancy.[72] A significantly lower concentration of PP13 in weeks 6–10 of gestation has been found in PE-predisposed patients, which was attributed to impaired trophoblastic regulation and spiral-artery remodeling in PE.[72] Studies involving PP13 as a biomarker are limited, due to the lack of sample-population homogeneity and poor detection sensitivity; in addition, sensitivity and specificity for detection has to be determined using predefined cutoff values. Interestingly, STBEVs have been shown to be immunopositive for PP13,[70] suggestive of an immunomodulatory role. Therefore, future studies must evaluate PP13-biomarker potential in association with STBEVs and exosomes.

Syncytiotrophoblast extracellular vesicles

STBEVs may play a role in maternal–fetal immunoadaptation.[73] In addition, in vitro research has indicated that PE-derived STBEVs differentially affect immune cells, endothelial cells, and platelet activation, suggesting a key role in maintenance of the MSIR.[74] STBEVs have been identified in maternal circulation in the first trimester of normal pregnancy and also found to be significantly elevated in early-onset PE.[75] Moreover, it is known that STBEVs contain subpopulations of EVs.[16] As such, it is difficult to utilize the entire STBEV population as a biomarker of PE solely, as there are a multitude of biological factors associated with each EV subtype.

Lipids

New developments in lipidomic research have led to the identification of multimarker lipid biomarkers of PE. Lipid biomarkers with a mass:charge ratio (m/z) of 383, 784, 796, 798, and 920 were able to identify PE with sensitivity of 91% and specificity of 82%. Lipids with an m/z of 383 have been identified as oxidized cholesterol, which has been shown to contribute to vascular atherosclerosis in PE.[76] However, lipids with an m/z of 784, 796, 798, and 920 belong to the glycerophosphocholine-lipid class, which may be elevated in PE pregnancies due to enhanced cellular apoptosis.[76] Although lipidomics provide a new avenue for the identification of PE biomarkers, further studies are needed to determine if these markers are specific for PE.

In summary, biomarkers of PE can be described as angiogenic and antiangiogenic factors involved in placental maladaptation, improper spiral-artery remodeling, and immune factors that are synthesized as a result of the predominant T_h1 immunoshift in PE (Figure 2). The dysregulation of these factors in PE indicates that they may be involved in the etiopathogenesis of the disorder. However, the upregulation of proangiogenic factors, AFP, and HTRA3 indicates that these factors are consequences of the disorder, rather than the cause. Consolidation of the current state of biomarkers in PE indicates that there is no single biomarker of the disorder that can be objectively utilized as a definitive early biomarker of PE. However, placenta-derived exosomes, a key constituent of STBEVs, may best fit the required biomarker criterion of PE and provide key evidence supporting the etiology and pathogenesis of the disorder. This review thus aims to evaluate the potential of placenta-derived exosomes as biomarkers of PE within the approved US Food and Drug Administration (FDA) biomarker-criterion framework.

Placenta-derived exosomes as potential biomarkers of PE

Placenta-derived exosomes are synthesized by STB cells via the lysosomal pathway, whereby multivesicular bodies fuse with the cell membrane via exocytosis, resulting in the release of placental exosomes into maternal circulation. During the process of exosome biogenesis, selective packaging of molecules into exosomes occurs within the originating cell. These native molecules contained within the exosome thus consist of genetic and proteomic information that could serve as direct markers of pregnancy-related complications/placental function. However, the rationale for presenting exosomes as a candidate biomarker of PE is yet to be evaluated in terms of the FDA-approved biomarker criterion.[27] In this review, we thus incorporate the FDA-biomarker criterion in evaluating placenta-derived exosomes as biomarkers of PE, whereby placenta-derived exosomes were evaluated in view of the following criteria. Can they be objectively isolated, detected, and quantified from biological fluids? Have they been shown to increase with an increase in gestational age in normal pregnancy? Have they been shown to be significantly altered in PE? Do they contain key molecular markers that can improve biomarker sensitivity and specificity? Can they be used as a biotherapeutic agent to reprogram the dysfunctional placenta?

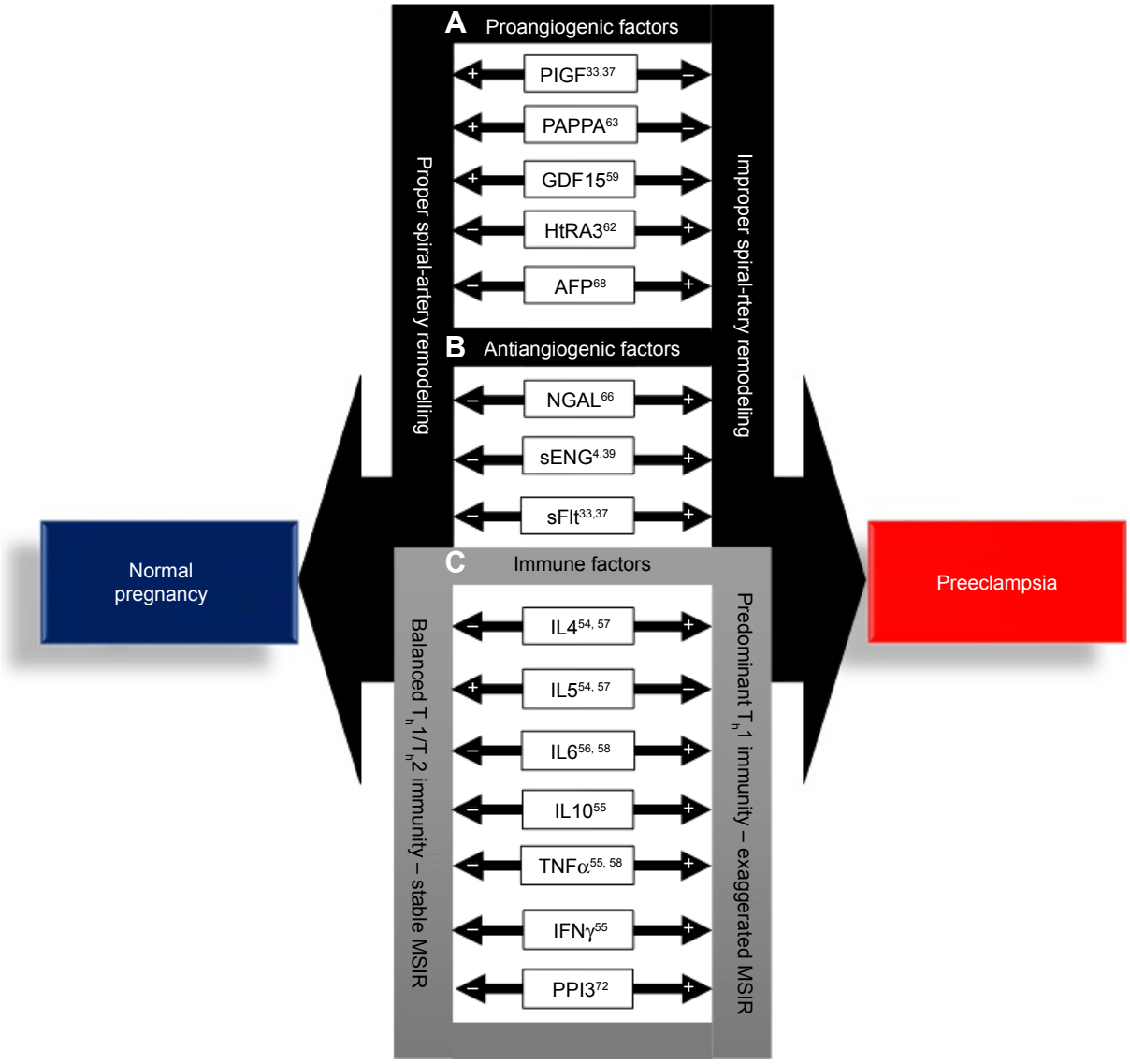

Figure 2 Potential biomarkers involved in the pathogenesis of preeclampsia (PE).
Notes: (**A**) Proangiogenic and (**B**) anti-angiogenic factors associated with improper spiral-artery remodeling in PE. (**C**) Immune factors associated with the pathogenesis of PE as a result of the predominant T_h1 immunity of the disorder. $^{+}$Significantly increased expression in comparison to normal pregnancy; $^{-}$significantly lowered expression in comparison to PE pregnancies.
Abbreviations: MSIR, maternal systemic inflammatory response; T_h, T-helper.

Isolation, detection, and quantification of placenta-derived exosomes in maternal circulation

Blood is the biofluid of choice when isolating exosomes for research and diagnostic purposes; however, various factors, such as the choice of anticoagulant, venipuncture, blood handling, circadian systems, time of draw, fasting status, platelet activation, and hemolysis, affect the composition and characteristics of exosomes in downstream sample processing and may result in inaccurate results.[77] It is thus a requirement that when investigating the use of placenta-derived exosomes as biomarkers of PE that standardized blood-collection protocols as recommended by the International Society for Extracellular Vesicles be considered to prevent the artificial release of exosomes, which would result in false positives and negatives.[77] Exosomes from maternal circulation are generally isolated based on their buoyant density of 1.13–1.19 g/mL using density-gradient or static-gradient ultracentrifugation and characterized by microscopic morphology and proteomic identification of exosome-associated tetraspanins, such as CD63, CD9, CD81, and HSP70.[78] Furthermore, PLAP is used as a placenta-specific marker for the isolation and

quantification of placenta-derived exosomes.[79–81] Alternative technologies, such as analytical size-exclusion chromatography and immunoaffinity capture,[82,83] for the isolation of PLAP+ exosomes from maternal circulation have been shown elsewhere; however, these methods, especially the latter, result in analytical challenges with regard to the reproducibility and precision of the quantification of placenta-derived exosomes in maternal circulation. Alternatively, the direct quantification of placenta-derived exosomes from total purified exosomes using PLAP as a placenta-specific marker has been achieved.[79–85] In contrast to immunocapture methods, this method eliminates inaccuracies attributed to the lower exosomal yield obtained using immunocapture techniques. However, the direct quantification of placenta-derived exosomes in maternal circulation is largely dependent on the purity of the exosomal fraction. It is thus advisable that density-gradient ultracentrifugation and analytical size-exclusion exosome-isolation methods be utilized in combination to improve the purity and yield of maternal exosomes, thus enhancing the objective identification of placenta-derived exosomes.

The objective isolation of exosomes in maternal circulation coupled with recent advancements in nanotechnology, such as nanoparticle-tracking analysis, enables the accurate characterization and quantification of exosomal populations based on particle-size distribution using dynamic light scattering and Brownian motion.[80,85,86] Additionally, this technology enables the use of placenta-specific fluorescent probes, such as PLAP and other exosome-specific protein markers, for the quantification of placenta-derived vesicles.[86,87] With advances in exosome-isolation and -detection technologies, the expected challenges would not only include the discovery of specific PE exosomal markers but also how to incorporate these markers into regulatory decision-making and clinical practice.

Placenta-derived exosomes in normal and complicated pregnancies

Altered levels of circulating placenta-derived exosomes in PE

Previously, studies have focused on supraphysiological levels of enriched STBEVs in ex vivo systems.[12,19,88] These studies have demonstrated an increase in STBEVs in ex vivo placental perfusates in PE in comparison to normal pregnancies. Furthermore, it has been established that STBEVs have a key role in immunomodulation during normal and complicated pregnancies,[12,83,89] and not as purified populations of constituent vesicles, such as exosomes. Exosomes are regarded as a key constituent of STBEVs, due to their ability to reprogram cells and consequently alter normal cellular physiology, which thereby contributes to various pathological states. Although STBEVs in normal pregnancies have been shown to play a role in immunoregulation[12] and placenta-derived exosomes have been shown to increase with gestational age and alter in vitro endothelial cell migration,[80,90] there is a lack of knowledge in understanding the role of placenta-derived exosomes in complicated pregnancies, such as those with PE, as well as the relative proportion of these vesicles in relation to other STBEVs.

Role of placenta-derived exosomes in maternal immunomodulation

Placenta-derived exosomes were initially identified from placental tissue by Frängsmyr et al, and have been shown to contain the transmembrane protein FasL, which suggests that placenta-derived exosomes are involved in immunoregulation during pregnancy by initiating the local deletion of activated maternal lymphocytes that identify placental paternal antigens.[91] In addition, a similar study by Abrahams et al indicated that exosome-mediated secretion of FasL promotes immunoprivilege of semi-allograph invading trophoblastic cells by inducing apoptosis in Fas-bearing immune cells during implantation and pregnancy.[92] However, a more recent study showed that B7 immunomodulatory molecules and HLAG5 are secreted via exosomes from early and term placentas.[93] This study offered a novel perspective in which human placenta-derived exosomes may have served directly as key mediators of maternal immunotolerance to the semiallogeneic fetus. The identification of these key immunoregulatory proteins bound to placenta-derived exosomes supports the fact that exosomes have an immunomodulatory role in preventing the degradation of invading trophoblastic cells by inducing maternal T-cell apoptosis, which could play a key role in maternal complications, such as PE, fetal rejection, and intrauterine growth restriction.

MSIR and placenta-derived exosomes in maternal circulation

According to Redman and Sargent, in PE, placental oxidative stress as a result of abnormal placentation induces the release of STBEVs that may control cellular apoptotic and necrotic events in circulation, thus resulting in an exaggerated MSIR.[7,15] In addition, these authors postulated that in normal pregnancy, the process of regeneration of placental

STB cells is dependent on cellular apoptosis, which is modulated by STBEVs.[7,15] In PE, the increase in STBEVs results in an exaggerated MSIR,[7,10,86] which is induced when the maternal immune system reacts adversely in an exaggerated manner upon exposure to STBEVs.[17] Since placenta-derived exosomes are a key constituent of STBEVs, we and others[16,94] thus hypothesize that the marked differences in magnitude of placenta-derived and total exosomes in PE in comparison to normal pregnancies may control T_h1/T_h2 immunity during pregnancy, which in turn has a direct relationship with the size of the MSIR (Figure 3). In particular, this model proposes that a possible alteration in magnitude of placenta-derived and total exosomes during the pathological progression of early- or late-onset PE may cause adverse conditions or severe complications, which manifest in either severe PE or eclampsia, respectively. Additionally, this hypothesis can be authenticated by the incorporation of a mathematical algorithm whereby the rate of change of placenta-derived exosomes, in relation to total exosomes, within a specified gestational age would enable accurate and reliable diagnosis of PE throughout gestation. This is based on the theory that placenta-derived and total exosomes increase throughout gestation in normal pregnancy,[80,81] which have been found to be altered in early- and late-onset PE.[85] This model represents the classification of early- and late-onset PE based on the ratio of the rate of change of total exosomes to placenta-derived exosomes within a specified gestational age range represented by the following elementary formulae:

$$\frac{\Delta PLAP^+ \text{ exosomes}}{\Delta CD63^+ \text{ total exosomes}} = \frac{PLAP^+_2 - PLAP^+_1}{G_2 - G_1} = \frac{CD63^+_2 - CD63^+_1}{G_2 - G_1} = \frac{PLAP^+_2 - PLAP^+_1}{CD63^+_2 - CD63^+_1}$$

where $PLAP^+$ exosomes are placenta-derived exosomes, $CD63^+$ total exosomes, and G gestational age.

With this hypothetical model, at any specified time period during pregnancy, the calculation of the rate of change in total exosomes to placenta-derived exosomes can be used to assess placental function, impending PE, and the severity of the complication. The model presents the rate of change of $\Delta PLAP^+$ exosomes/$\Delta CD63^+$ exosomes as a relative measure of impending PE during the preclinical and clinical phases of PE. However, this model is still to be evaluated in a clinical setting, in order to determine the appropriate rate of change for diagnosis/prognosis.

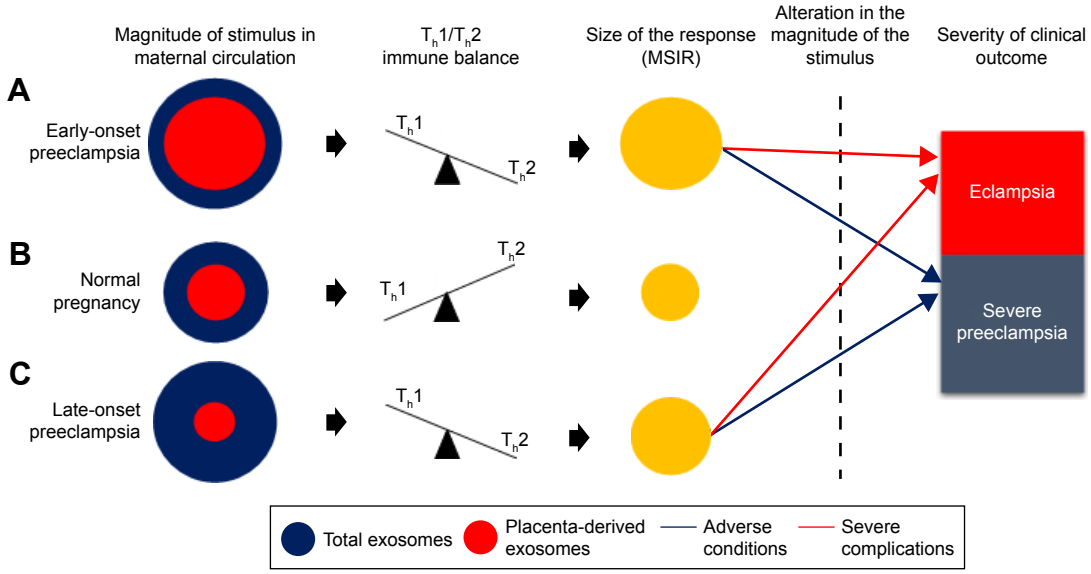

Figure 3 Hypothetical model of exosomes in relation to immunological and clinical outcomes in EOPE, LOPE, and normal pregnancy.
Notes: (**A**) In EOPE, an increase in placenta-derived and total exosomes in comparison to normal pregnancy mediates a shift toward T_h1 immunity and thus results in an exaggerated MSIR. (**B**) In normal pregnancy, there is a balance between placenta-derived and total exosomes that mediates a shift toward T_h2 immunity, thereby maintaining a balanced MSIR, a requirement for successful pregnancy. (**C**) In LOPE, the increase in total and decrease in placenta-derived exosomes in comparison to normal pregnancy mediates a shift toward T_h1 immunity, which exaggerates the MSIR to a degree that does not exceed the MSIR in EOPE. (**A**, **C**) Alteration in the magnitude of placenta-derived and total-exosomes during the manifestation of LOPE or EOPE results in adverse conditions or severe complications that lead to the clinical manifestation of severe PE or eclampsia.
Abbreviations: EOPE, early-onset preeclampsia; LOPE, late-onset PE; MSIR, maternal systemic inflammatory response; T_h, T-helper.

Molecular markers of placenta-derived exosomes in normal and preeclamptic pregnancies

Exosomes contain cargo, such as mRNA, miRNA, cytosolic proteins, membrane-bound proteins, and lipids, which are selectively packaged during exosomal biogenesis. Exosomes usually contain a variety of molecules that are native to the originating cell and enriched with endosome-associated protein markers, such as tetraspanins (CD63, CD81, CD82, CD9, CD37), HSP70, RAB proteins, ALIX, TSG101, endocytic proteins, and cell-specific proteins, which provide information regarding their cellular origin.[95] Pregnancy-associated exosomes have been found to contain a number of membrane-bound protein markers, such as NKGD2 ligands,[96] FasL,[82,83] TRAIL,[82] and syncytin 1,[97] which suggests that placenta-derived exosomes play a key role in maintaining maternal–fetal tolerance (Figure 4). Other specific pregnancy-associated exosomal proteins, such as TGFβ, indicate that placenta-derived exosomes are involved in the control of STB growth, proliferation and differentiation.[98] Although these exosomal membrane-bound proteins are associated with pregnancy, they cannot be used as definitive markers of placenta-derived exosomes, due to their lack of placental specificity; therefore, placental ALP remains the marker of choice when isolating and identifying placenta-derived exosomes.[79–81,85,90,99,100] Even though it is known that exosomes contain mRNA and miRNA, which are key molecular mediators in translational and posttranscriptional modification, the exact composition of these exosomal constituents has not been elucidated in placenta-derived exosomes. Therefore, future studies should incorporate identification of novel nucleic acid sequences in placenta-derived exosomes in normal and PE pregnancies to enhance the specificity and sensitivity of these vesicles as biomarkers of PE.

Perspectives on engineered exosomes in PE therapeutics

Stem cell (SC)-derived exosome therapy is of emerging clinical importance as an alternative to other gene therapies, since exosomes have no risk of aneuploidy and a lower rate of immunorejection following allogeneic administration.[101] In addition, they serve as ideal vectors for the reprogramming of diseased cells in various pathological states, such as PE, since they have the ability to change the phenotype of the target cell though various signaling cascades and transfer of RNA.[102,103] Mesenchymal SCs (MSCs) are rich in their application as regenerative agents of damaged or diseased

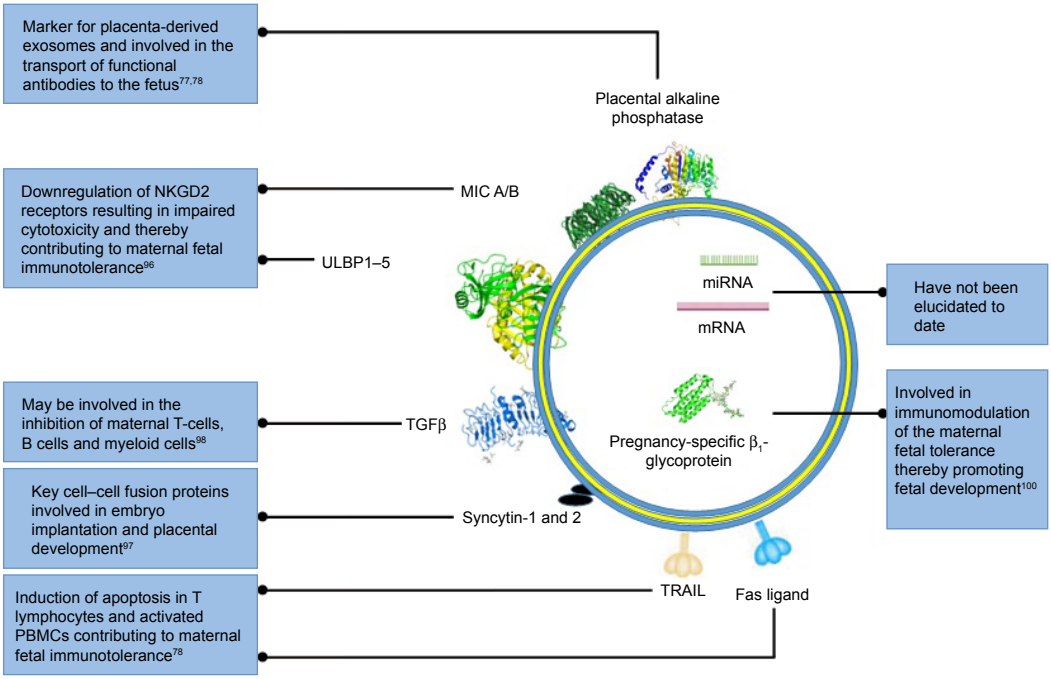

Figure 4 Schematic representation of key pregnancy-associated exosomal molecular cargo and their function.
Notes: This figure highlights key transmembrane and cytosolic proteins involved in immunomodulation during normal pregnancy. Key pregnancy-associated exosomal nucleic acids have not been identified to date. Common exosomal markers are not shown.
Abbreviation: PBMCs, peripheral blood mononuclear cells.

cells, due to their unique complement of proteins, which promote biological processes and pre-miRNA that facilitate intercellular communication.[104–106] MSC-derived exosomes have been shown to act as promising therapeutic agents in cardiovascular disease,[107,108] kidney injury,[109] immune disease,[110] tumor growth,[111] and neurological diseases.[112,113] However the use of MSC-derived exosomes to treat PE has its challenges, due to the complexity of the pathogenesis of the disease. It is thus a prerequisite to determine the role of placenta-derived exosomes in the pathogenesis of PE through isolation and characterization of exosomal cargo and functional activity, and to understand the mechanism of biogenesis of placenta-derived exosomes in normal and PE pregnancies.

Engineering MSC-derived exosomes for the specific treatment of PE has potential, based on the understanding that exosomes may be primary-role players in the etiopathogenesis of PE. This process would involve the in vitro genetic manipulation of isolated MSCs using plasmid-based vectors to package dsRNA selectively during in vitro MSC-exosome biogenesis to enable the silencing of specific genes involved in the pathogenesis of PE and to engineer the surface proteins of MSC exosomes to result in their specific delivery to STB cells, allowing for more targeted exosome-cargo delivery. This would enable the effective targeted delivery of RNAi for reprogramming of the diseased placenta.

Conclusion

The future of the diagnosis of PE is dependent on the identification of novel biomarkers, which in turn may lead to personalized medicine during pregnancy in the form of liquid biopsies. Exosomes are the future of liquid biopsies, as they contain molecular cargo that can be used to diagnose such diseases as cancer without the need for a conventional biopsy. Although there is much controversy regarding the collection, isolation, and characterization of exosomes, the concept of these vesicles as novel biomarkers of the disease remains promising. We project that these shortcomings will be resolved with the implementation of carefully regulated exosomal standardization and validation methods. Additionally, rapid advances in such technologies as nanoparticle-tracking analysis, immunocapture, and next-generation sequencing would improve the efficiency of exosomal isolation and characterization and lead to the identification of novel placenta-derived exosomal protein and nucleic acid markers. The development of algorithms based on the model presented would add value in creating a personalized approach in managing PE and other pregnancy-related complications. These perspectives form the fundamental basis for the use of placenta-derived exosomes in liquid biopsies of the diseased placenta in PE.

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Disclosure

The authors report no conflicts of interests in this work.

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3. Chapter Three: Manuscript Two

Exosomal microRNA profiling in early and late onset pre-eclamptic pregnant women reflects pathophysiology. (Accepted: International Journal of Nanomedicine. 2019)

The theoretical framework outlined in manuscript one **(chapter 2)** supports the scientific role of exosomes in the pathogenesis of PE and consequently its future biomarker potential and 'context of use' in the clinical setting. Therefore, this publication focused on the purpose and nature of exosomal miRNA in PE, enabling a clearer understanding of their specific role in the pathophysiology of PE. This was determined within the contextual framework of the FDA biomarker criterion [92] by:

(1) Using a technology platform that directly detects miRNAs, and offers fast and reliable detection of small amounts of miRNA;
(2) Using validated computational merging and meta-analytical algorithms in identifying specific exosomal miRNA biological pathway interactions related to the pathology of preeclampsia; and
(3) Identifying specific exosomal miRNA signatures, which may serve as candidate biomarkers.

The findings from this study are significant as they highlight the context of use of exosomes as biomarkers of PE.

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ORIGINAL RESEARCH

Exosomal microRNA profiling in early and late onset preeclamptic pregnant women reflects pathophysiology

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Preenan Pillay[1]
Manu Vatish[2]
Raquel Duarte[3]
Jack Moodley[4]
Irene Mackraj[5]

[1]Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of Kwazulu-Natal, Durban, South Africa; [2]Nuffield Department of Women's and Reproductive Health, Women's Centre, John Radcliffe Hospital, University of Oxford, Oxford, UK; [3]Department of Internal Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa; [4]Women's Health and HIV Research Group, Department of Obstetrics and Gynaecology, University of Kwazulu-Natal, Durban, South Africa; [5]School of Laboratory Medicine and Medical Sciences, University of Kwazulu-Natal, Durban, South Africa

Correspondence: Preenan Pillay; Irene Mackraj
University of KwaZulu-Natal, 1 University Road, Westville, Durban 4001, South Africa
Tel +27 260 4603; +27 72 908 5646
Email ppillay01@gmail.com; mackraji@ukzn.ac.za

Background: Preeclampsia is the leading cause of maternal and fetal mortality due to the inability to diagnose and treat the disorder early in pregnancy. This is attributed to the complex pathophysiology and unknown etiology of the disorder, which is modulated by several known and unknown factors. Exosomes have recently been implicated as possible mediators of the pathogenesis of preeclampsia, with, however, no evidence linking these nanovesicles to the pathophysiology of preeclampsia and its subtypes.

Methods: To better understand the pathophysiological role of exosomes in preeclampsia, we have analyzed the exosomal microRNA in early and late onset preeclamptic women in comparison to their gestationally matched normotensive controls using Digital Direct Detection (NanoString Technologies).

Results: For the first time, distinct exosomal microRNA signatures in early and late onset preeclampsia have been identified. Moreover, these signatures indicate that exosomes are involved in key pathological features associated with preeclampsia and differentiate between the subtypes.

Conclusion: This study forms the basis for the diagnostic and functional validation of the identified signatures as biomarkers of preeclampsia and its subtypes.

Keywords: biomarkers, preeclampsia, exosomes, microRNA

Introduction

Preeclampsia (PE), a hypertensive disorder of pregnancy (HDP), remains one of the leading causes of maternal and neonatal mortality and morbidity, affecting 5–8% of pregnancies globally.[1–3] The clinical management of this disorder is complicated, partly due to the heterogeneity of the disease which is attributed to its complex etiopathophysiology, caused by the dysregulation of a host of unknown and known factors. Even though the exact etiology of PE remains unknown, the placenta is central in the pathogenesis of PE. The functional unit of the placenta, the chorionic villous, is a vascular projection of fetal tissue surrounded by the chorion. The embryonic-derived chorion consists of an inner cytotrophoblast layer and outer syncytiotrophoblast layer which, importantly, forms the interface between maternal blood from the fetal vasculature and may be a potential source of circulating factors.

Preeclampsia is commonly categorized into early onset-PE (EOPE) and late onset-PE (LOPE), which can manifest with or without clinical features, such as epigastric pain, visual disturbances, persistent headache, nausea and vomiting,

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culminating in eclampsia if untreated.[4] Notably, early onset-PE occurs at ≤33 weeks of gestation and is associated with greater risk of maternal and fetal complications in comparison to late onset-PE,[5–7] which is reflective of the differences in the pathogenesis between the subtypes. The pathophysiology of early onset-PE is characterised as a two-stage disorder, namely, (1) improper placentation due to shallow trophoblast invasion, impacting upon spiral artery remodelling leading to decreased uteroplacental blood flow (due to the under-perfused placenta) and oxygenation,[5] which results in (2) enhanced placental oxidative stress which is a precursor to the maternal systemic inflammatory response observable later in pregnancy[6] with lasting effects. Late onset PE occurs at ≥34 weeks of gestation and manifests as a result of villous overcrowding at term, leading to competitive inhibition of cellular growth due to limited gaseous exchange and nutrient supply which culminates in placental oxidative stress,[7] leading to the clinical disease state. Late onset-PE is often not associated with significant fetal involvement, but commonly, in both early and late onset-PE, there are two principle features: (1) syncytiotrophoblast stress occurring in the placenta and (2) systemic endothelial dysfunction affecting the mother.

Although the key events and factors initiating the pathophysiology of PE remain largely unclear, biologically-derived nanovesicles, termed exosomes, have emerged as potential mediators of this process[8-11] partly due to their increased levels in maternal circulation.[8] Furthermore, in a recent study, we identified differences between early and late onset-PE in the levels of total and placenta-derived exosomes in maternal circulation,[12] alluding to the possible role of exosomes in the pathophysiology of early and late onset-PE and their subsequent application as biomarkers.[8]

Exosomes (20–130 nm) derived from multivesicular bodies contain nucleic acids, proteins and lipid rafts, which have been reported to promote physiological function through autocrine, paracrine and endocrine signaling mechanisms.[8,13] Exosomes can bind to target cells or organs, reprogramming them, either by direct signaling via surface proteins or by depositing their vesicular cargo intracellularly.[14] Hence, in disease states, it is this property which confers the ability for these vesicles to directly mediate end-organ damage; forming ideal candidate biomarkers for early disease diagnosis/prognosis. More recently, exosomal microRNAs (exomiRNA), in particular, have emerged as key biomarkers of several other disease states, as they are selectively packaged into exosomes[15] and are subsequently released by the cell, destined to participate in cellular reprogramming.[16,17] A few studies have described the key physiological role of miRNAs in regulating normal placental development, as well as the pathogenesis of PE,[18–22] leading to our proposal that a comprehensive understanding of exosomal miRNA will provide key functional insights into the role of these nanovesicles in the pathophysiology of PE.

Evidence of miRNA as a constituent of exosomal RNA cargo has been reported in PE using next-generation sequencing (NGS).[23] However, there is still a lack of information regarding the pathophysiological role of exomiRNA in early and late onset-PE, especially given its powerful role in the modification of gene expression. Importantly, the analysis of exomiRNA is complicated by the limitations of this technology given the small amounts of exomiRNA present. Therefore, studies involving the detection and quantification of exomiRNA would require more specific, rapid analytical strategies for easy clinical application. NanoString technology fulfils this criterion because of its digital precision, enhanced sensitivity, reproducibility and technical robustness using small amounts of starting RNA, without the need for further validation.[24,25] Importantly, standard assessment of small amounts of RNA involves amplification which can skew the relative abundance of short RNA sequences.[24] NanoString technology does not utilise amplification processes thus enabling a more accurate representation of resident miRNA expression levels. Therefore, in this study, we aimed to identify exomiRNA signatures associated with the pathophysiology of early and late onset-PE using the NanoString nCounter platform (Seattle, WA, USA). We highlight the contrasting exomiRNA profiles associated with the pathophysiology of early and late onset-PE as well as the possible overlapping miRNA target-related pathways linked to preeclampsia pathology.

Methods

Ethics statement

Regulatory ethical and institutional approval were obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE310/15), South Africa. Patients were recruited from the Prince Mshiyeni Hospital, Kwa-Zulu Natal, South Africa. All participants were recruited via written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

Study group

Controls [Gestationally matched normotensive pregnant woman (N)] and experimental groups [Early onset-PE (EOPE) and late onset-PE patients (LOPE)], were enrolled in this study (n=15 per group). Normotensive patient controls were gestationally matched to the EOPE and LOPE groups (Table 1), and, identified as blood pressure of 120±10/80±5 (systolic/diastolic mm Hg) with absent proteinuria as detected by a rapid urine dipstick test (Markomed®, South Africa). Early onset preeclampsia was classified as new-onset hypertension (diastolic blood pressure of ≥90 mm Hg and systolic blood pressure of ≥140 mm Hg) and proteinuria (≥300 mg) at ≤33 weeks. Late onset-preeclampsia was defined by new-onset hypertension (diastolic blood pressure of >90 mm Hg and systolic blood pressure of >140 mm Hg) and proteinuria (≥300 mg) at ≥34 weeks gestational age. All recruited women had singleton pregnancies with no evidence of any infections or medical, surgical or other obstetric complications. Blood from maternal circulation was collected at the time of clinical diagnosis of PE as specified above together with gestationally matched normotensive [BD Vacutainer Tubes (EDTA), Becton Dickinson and Company, South Africa] and the plasma samples were stored at −80 °C for analyses.[27,28] Samples were processed according to the accepted guidelines pertaining to exosomes.[24]

Exosome isolation

Exosomes were isolated using the exosome miRCURY isolation kit (Qiagen, USA) according to the manufacturer's instruction with modifications. Briefly, plasma was centrifuged at 300 g for 10 min the supernatant was then centrifuged at 2000 g for 30 min to remove dead cells. Thereafter the recovered supernatant was centrifuged at 12,000 g for 45 min to remove cell debris. To ensure removal of all unwanted cells and debris the supernatant was further purified using a 0.22 µm spin column (Corning, USA). Thrombin was then added to the filtrate for de-fibrination. This was followed by centrifugation at 10 000 g for 5 min. Precipitation buffer was thereafter added to the recovered supernatant and incubated at 4 ºC for 4hrs. Post incubation the exosomes were recovered by centrifugation at 500 g for 5min at 20 ºC. The exosomal pellet was resuspended in exosome resuspension buffer as prescribed by the total exosomal Protein and RNA isolation kit (Life Technologies).

Exosomal protein and RNA isolation

Exosomal RNA was isolated using the total exosome RNA and protein isolation kit (Life Technologies) as per the manufacturer's instructions. The total RNA concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Isolated exosomes were re-suspended in exosome resuspension buffer as per the total exosomal protein and RNA isolation kit (Life Technologies). The total protein concentration was determined using the RC DC Protein Assay as per manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Exosome characterization

Nanoparticle tracking analysis

Quantification and size distribution of exosomes were determined using Nanoparticle Tracking Analysis as described in our previous publication (NanoSight500

Table 1 Clinical characteristics of participants

Variables	Normotensives		Preeclampsia	
	≤33 weeks (n=15)	≥34 weeks (n=15)	Early onset (n=15)	Late onset (n=15)
Age (years)	28.43±2.23	26.12±3.62	25.25±5.13	27.11±5.23
Weight (kg)	59.88±3.25	67.57±5.22	71.12±7.85	87.21±9.21
Height (cm)	151.91±4.89	158.33±5.61	159.12 3±3.11	155.82±5.23
BMI	26.14±3.24	27.91±3.66	28.24±2.11	35.14±6.12†††
Gestational Age (weeks)	31.21±1.56 (26–33)	37±1.9 (34–38)	32.42±2.19 (28–33)	38.14±3.41 (34–39)
Systolic/diastolic blood pressure (mm Hg)	121/79±3.47/2.54 (90–120/50–80)	118/81±8/5 (90–120/50–80)	162/98±6/8 (>140/90)***	157/95±7/5 (>140/90)†††
Urine Protein (mg/dl)	ND	ND	491±71.54	381±68.99

Notes: All values are represented by mean ± SEM. All pregnancies were singleton without intrauterine infection or any other medical condition. The patients recruited had a parity of 1±1. In systolic/diastolic blood pressure early onset-PE versus N (≤33 weeks), ***p<0.001 and late onset-PE versus N (≥34 Weeks), p<0.001. In BMI late onset-PE versus early onset-PE, $^{\dagger\dagger\dagger}p$<0.05.

Abbreviations: ND, not detected; N, normotensive pregnancies; PE, preeclampsia.

NTA 3.0 Nanoparticle Tracking Analysis Release, Version Build 0069).[12] In Brief, samples were diluted with PBS in order to obtain particle distribution of between 10 and 100 particles per image (optimal, 50 particles per image). Samples were introduced into the instrument using the following script: PUMPLOAD, REPEATSTART, PRIME, DELAY 10, CAPTURE 60, REPEAT 5. Videos were recorded at a camera level of 10, a camera shutter speed of 20 ms and camera gain of 600, these settings were kept constant between samples. Each video was then analyzed to give the mean particle size together with the concentration of particles. The size of the exosomes was represented as the mean particle size ± SEM.

Western blotting

Exosomes isolated from plasma samples were lysed with RIPA buffer (1X) at room temperature and the protein content determined using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Laemmli buffer (2X) was added to the samples and incubated at 95 °C for 5 min. Thirty micrograms of exosomal protein per well were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. Polyvinylidene fluoride membranes were probed with the primary antibody, CD63 (ExoAB, System Biosciences, Mountain View, CA) and Placental Alkaline Phosphatase (PLAP) monoclonal antibody (5B1) (Thermo Fisher Scientific). Post incubation at room temperature for 1 h, membranes were washed in Tris buffer saline (pH 7.6) and incubated for 1 h at room temperature with the exosome validated secondary conjugated horseradish peroxidase antibody (System Biosciences, Mountain View, CA). Membranes were incubated with the chemiluminescent substrate and visualised with the ChemiDoc® Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

Transmission electron microscopy

Exosomes were placed onto a continuous carbon grid and negatively stained with 2% uranyl acetate. The morphology of the particles was examined using a JEOL 1010 transmission electron microscope (JEOL, Peabody, MA, USA).

Quantification of total and placenta-derived exosomes

Total and placenta-derived exosomes were determined as per our previous publication.[12] Total exosomes in maternal circulation were determined by the quantification of total immunoreactive exosomal CD63 via an enzyme-linked immune absorbency assay (ExoELISA™, System Biosciences, Mountain View, CA), as per manufacturer's instructions. In brief, 30 µg of isolated exosomal protein was immobilised onto a microtiter plate overnight at 37 °C using exosome binding buffer supplied the manufacturer (System Biosciences). Plates were washed and incubated at room temperature for 1 h with exosome specific primary antibody (CD63), followed by a wash step and incubation with secondary antibody (1:5000) at room temperature for 1 h with agitation. Plates were thereafter washed and incubated with Tetramethylbenzidine ELISA substrate at room temperature for 45 min with agitation. The reaction was thereafter terminated using stop buffer and the absorbance was measured at 450 nm. The number of exosomes/ml, (ExoELISA™ kit) was obtained using an exosomal CD63 standard curve that was generated using the calibrated exosome standard that was supplied.

The relative concentration of placenta-derived exosomes was determined by the quantification of human placental alkaline phosphatase in the exosomal fraction using a commercial ELISA kit (Elabscience, E-EL-H1976, WuHan, P.R.C), as per manufacturers instruction. In brief, 30 µg of exosomal protein was allowed to bind to the primary PLAP specific antibody coated plates by incubation at 37 °C for 90 min. Plates were washed with buffer and 50 µl of HRP-conjugate was added to each well and incubated at 37 °C for 20 min. Plates were washed and incubated with 50 µl of substrate A and 50 µl of substrate B at 37 °C for 15 min. The incubation was terminated using 50 µl of stop solution at room temperature for 2 min under agitation. Absorbance was measured at 450 nm. Exosomal PLAP was expressed as pg/ml plasma. The quantification of PLAP in the exosomal fraction indicates the relative concentration of placenta-derived exosomes ($PLAP^+$ exosomes) in maternal circulation.

Nanostring ncounter system miRNA assay

One hundred nanograms of exosomal RNA from each sample group (n=15 per group, pooled into 3 technical replicates) was analyzed using the NanoString nCounter SPRINT Profiler (NanoString Technologies®, Inc., Seattle, WA, USA). Briefly, miRNAs were ligated to a species-specific tag sequence (nCounter miRtag) through a thermally controlled splinted ligation. Un-ligated miRtags was removed by enzymatic purification, and the miRtagged mature miRNAs were

then hybridized with the nCounter Human (V3) miRNA Expression Assay Code Set overnight at 65 °C. Samples were then injected into the nCounter SPRINT™ cartridge and read using the nCounter® SPRINT Profiler (NanoString Technologies®, Inc., Seattle, WA, USA). The instrument performed the necessary magnetic bead separation, liquid transfers and immobilization of molecular labels on the sample surface before being finally analyzed by the system. The counts of the reporter probes were documented for each sample by the nCounter Digital Analyzer, which was subsequently analyzed using the nSolver Software (V 4.0). Before data normalization was performed, the nCounter data imaging QC metrics were assessed using the nSolver program, using the following parameters; imaging, binding density, positive control linearity, and positive control limit of detection. No significant discrepancy between the fields of view attempted and the fields of view counted were obtained. The binding density for the samples ranged between 0.20 and 0.74 within the recommended range. The normalisation factor by the nSolver program accounts for technical noise such as; variations in hybridization, purification, binding efficiency. The geometric mean of the positive controls was utilised for code count normalization, and the background was estimated using the mean of the negative controls. Input amounts of RNA were normalized to the geometric mean of five housekeeping miRNA controls (RPLP0, RPL19, B2M, GAPDH, and ACTB) and spike-in miRNA (osa-miR422, osa-miR414, osa-miR254, osa-miR248 and osa-miR159a) included in the assay, which was finally normalised to total miRNA count.

Statistical data analysis

All data analyses and graphical representations were performed and generated in GraphPad Prism 6.0 (CA, La Jolla) and nSolver Analysis Software 4.0.7 (NanoString Technologies Inc, USA). Exosomal miRNA ratio analysis between groups was selected based on an arbitrary |fold change|≥1 and False Discovery Rate (FDR) ≤0.05 with all expression data represented in Log2 scale using the nSolver analysis software. Subsequently, advanced analysis of the data was done using average-linked hierarchical clustering of a Spearman's Rank correlation similarity matrix of the PE and normotensive samples using the nSolver analysis software. All functional Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis was performed using Descriptive Intermediate Attributed Notation for Ada (DIANA) miRPATH (V 3.0) together with DIANA-microT-CDS 5.0 algorithms (interactions with a p-value<0.00001 was considered).

Results

Participant clinical characteristics

Early and late onset-PE groups were gestationally matched as shown in Table 1 below. Proteinuria was elevated in EOPE (491±71.54 mg/dl) and LOPE (381±68.99mg/dl). As expected, a significant increase in blood pressure was observed in EOPE (162/98±6/8 mm Hg) and LOPE (157/95±7/5 mm Hg) in comparison to the N groups (≤33 weeks: 121/79±3.47/2.54, ≥34 weeks: 118/81±8/5, p<0.001, Table 1) respectively. However, there was no significant difference in blood pressure and proteinuria between the N (≤33 weeks) and N (≥34 weeks) groups. It is notable that the BMI of the early onset-PE group was significantly greater than the late onset-PE group (35.14 ±6.12 vs 28.24±2.11 p<0.001).

Isolation and characterization of exosomes in maternal circulation

Isolated exosomes from each group were analyzed for size distribution using Nanoparticle Tracking Analysis and transmission electron microscopy. Isolated exosomes were within the accepted exosomal size range (20–130 nm, Figure 1E–G). The total number of exosomes in maternal circulation was quantified using an exosome validated ELISA kit which detected the exosome CD63 marker. Total exosomes in EOPE ($8.9\pm0.5\times10^{10}$ total exosomes/ml) and LOPE ($5.3\pm0.5\times10^{10}$ total exosomes/ml) were significantly higher than total exosomes in N (≤33 weeks: $1.06\pm0.2\times10^{10}$ total exosomes/ml, p<0.0001) and N (≥34 weeks: $2.54\pm0.32\times10^{10}$ total exosomes/ml, p<0.0001) respectively. Additionally, the exosome concentration in EOPE was significantly higher in comparison to LOPE (Figure 1A, p<0.0001). In conjunction with the ELISA, the Western blot analysis was positive for the exosomal CD63 marker (Figure 1B).

In order to determine the contribution of placenta-derived exosomes present in maternal plasma, the PLAP content per exosome was determined using ELISA (Figure 1C). Placental alkaline phosphatase is a marker of the syncytiotrophoblast and therefore its presence confirms placental origin.[26] A significant increase in placenta-derived exosomes was observed in early onset-PE in comparison to N (≤33 weeks) (427.1±8.9 vs 231±10.92 pg/ml, p<0.0001).

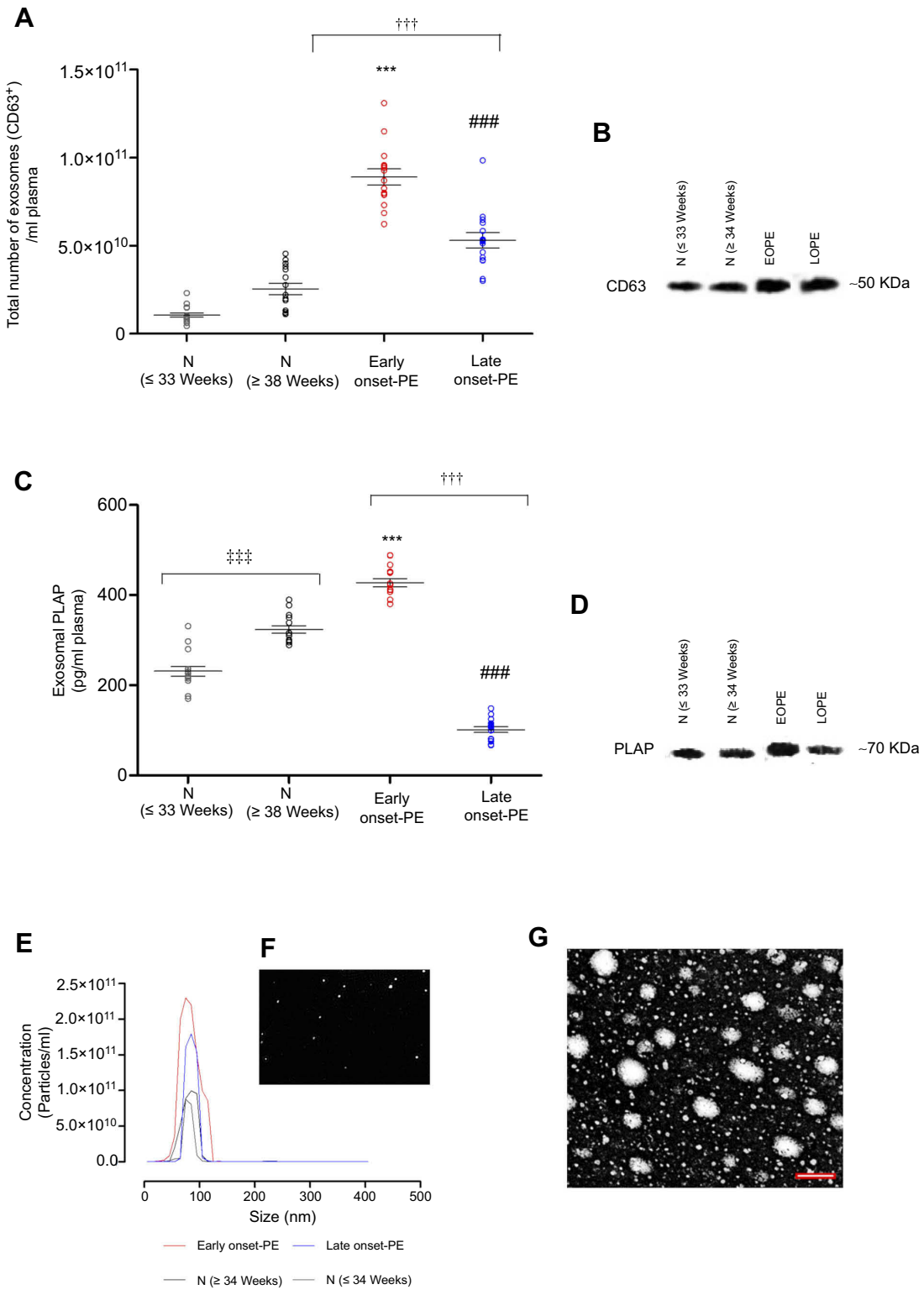

Figure 1 Identification and Characterization of Exosomes from Maternal Circulation. Exosomes from early onset-PE, late onset-PE and normotensive pregnant women were isolated and characterized. (**A**) Total exosome concentration was determined by the quantification of exosomal CD63 marker per ml of plasma using ELISA. (**B**) Western blot analysis for the CD63 exosome enriched marker. (**C**) Placenta-derived exosomes (ie exosomal placental alkaline phosphatase (PLAP) was quantified per ml of plasma by ELISA. (**D**) Western blot analysis for the exosomal PLAP marker. (**E**) Nanoparticle Tracking Analysis (NTA) illustrating the representative vesicle size distribution (nm). (**F**) Representative NTA video frame of the isolated exosomes. (**G**) Electron micrograph of isolated exosomes, scale bar 100 nm. In A and C the data is expressed as aligned dot plot and values are mean ± SEM. ***p<0.0001 early onset-PE vs N (≤33 weeks), p<0.0001 late onset PE vs N (≥34 weeks), $^{\dagger\dagger\dagger}p$<0.001 early onset-PE vs late onset-PE, $^{\ddagger\ddagger\ddagger}p$<0.0001 N (≤33 weeks) vs N (≥34 weeks).

Abbreviations: N - Normotensive Pregnant Women, PE - Preeclampsia, ELISA - Enzyme-Linked Immunosorbent Assay.

Additionally, a significant decrease in placenta-derived exosomes in LOPE compared to N (≥34 weeks) was observed (323.6±8.01 vs 101.4±6.22 pg/ml, $p<0.0001$). Moreover, a significant decrease in placenta-derived exosomes in LOPE in comparison to early onset-PE was observed (427.1±8.9 vs 101.4±6.22 pg/ml, $p<0.0001$). Furthermore, isolated exosomes were positive for membrane-bound PLAP marker by Western blot analysis (Figure 1D).

Exosomal miRNA expression profiles in early and late onset preeclampsia

In order to determine the profile of exomiRNA detected by the NanoString Human miRNA reference panel, we performed hierarchical clustering using all detected miRNAs within the specified range (FDR ≤0.05) in the EOPE vs N (33≤weeks, Figure 2) and LOPE vs N (≥34 weeks, Figure 3) groups. Average-linked hierarchical clustering of a

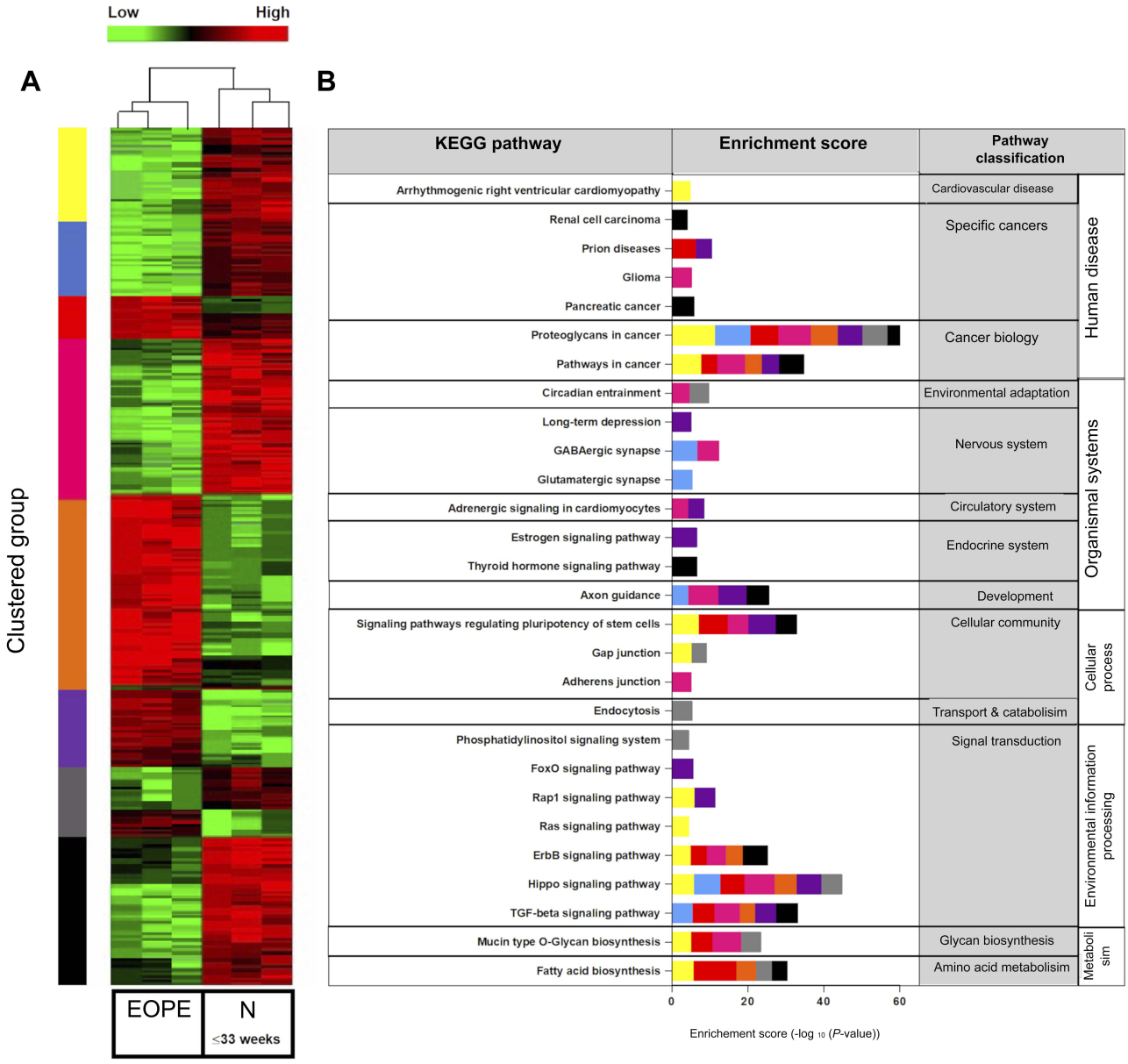

Figure 2 Comparison of exomiRNA expression profiles of Early onset Preeclamptic (EOPE) vs Normotensive derived exosomes. (**A**) Heatmap representing hierarchical clustering of differentially expressed miRNAs in EOPE (n=15, pooled into three technical replicates) and normotensive (n=15, pooled into three technical replicates) derived exosomes using average linkage clustering and Spearman Rank as distance metrics. miRNA profiles are clustered in 7 different subgroups (as indicated by the color bar on the right of the heatmap) defined by the miRNA expression patterns. Samples are shown in columns, miRNA in rows. Heat map from green to red represents relative miRNA expression as indicated in the key bar above the dendrogram. (**B**) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway mapping for top canonical pathways represented by the differentially expressed miRNA within each cluster, as determined by Descriptive Intermediate Attributed Notation for Ada (DIANA) miRPATH V.3.0 software.

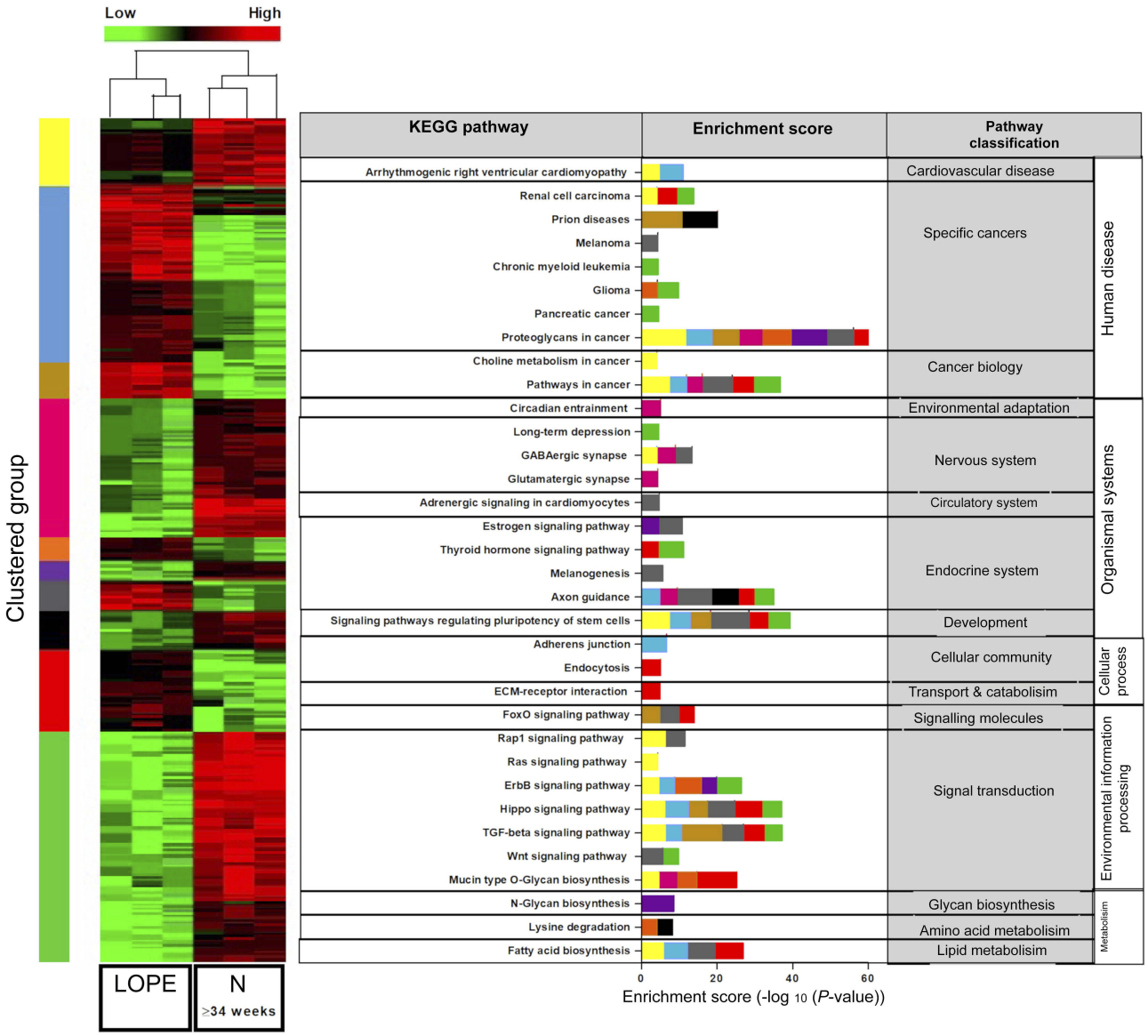

Figure 3 Comparison of exomiRNA expression profiles of Late onset Preeclamptic (LOPE) vs Normotensive derived exosomes. (**A**) Heatmap representing hierarchical clustering of differentially expressed miRNAs in LOPE (n=15, pooled with three technical replicates) and normotensive (n=15, pooled with three technical replicates) derived exosomes using average linkage clustering and Spearman Rank as distance metrics. miRNA profiles are clustered in 8 different subgroups (as indicated by the color bar on the right of the heatmap) defined by the miRNA expression patterns. Samples are shown in columns, miRNA in rows. Heat map from green to red represents relative miRNA expression as indicated in the key bar above the dendrogram. (**B**) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway mapping for top canonical pathways represented by the differentially expressed miRNA within each cluster determined using microT-CDs target predicting with (Descriptive Intermediate Attributed Notation for Ada) DIANA miRPATH V.3.0 software.

Spearman's Rank correlation similarity matrix of the data was performed using the NanoString nSolver analysis software (Version 4.0). As expected, two distinct patient clusters were identified (early and late onset-PE vs gestationally matched controls) and distinct gene clusters indicated by the colour bar represented in the heat map (Figures 2 and 3). In its entirety, there were 578 and 611 differentially expressed exomiRNA in EOPE vs N (33≤weeks) and LOPE vs N (≥34 weeks) respectively. Moreover, the KEGG pathway enrichment analysis of each cluster was performed using DIANA miRPath (V 3.0) together with DIANA-microT 5.0 algorithms. Significantly enriched KEGG pathways (p-value <0.00001), indicated that differentially expressed exomiRNA are involved in key signaling pathways associated with PE pathophysiology (Rap1, ErbB, TGF-β, Ras, Hippo, FOX, adrenergic cardiomyocyte and Thyroid hormone signaling, Figures 2 and 3). Interestingly, the differential expression of exomiRNA

mirrors the molecular pathways associated with cancer biology (glioma, renal cell carcinoma, pancreatic cancer, proteoglycans and pathways in cancer, Figures 2 and 3) which links placental development with cancer biology.[27] Further analysis of the significantly dysregulated exomiRNA in each comparison explores these relationships in more detail.

Dysregulated exomiRNA in early and late onset preeclampsia in comparison to gestationally matched normotensive pregnant women

Differential expression of exomiRNA was identified using the nSolver Analysis Software (V 4.0). All fold changes (FC) are represented in log 2 scale (log FC). Statistically significant exomiRNAs were selected based on an arbitrary $|FC| \geq 1$ and FDR ≤ 0.05 (Figure 4B–D). To evaluate the function of the significantly dysregulated exomiRNA within the complementary and intersecting group comparisons (Figure 4A), KEGG pathway and GO: Biological process target enrichment analysis was done using DIANA mirPATH together with DIANA-microT-CD 5.0 algorithms (Figure 5A and B respectively). These findings directly linked the identified exomiRNAs to validated pathways and biological functions involved in the pathophysiology of preeclampsia.

Complement analysis

In [EOPE vs N (≤33 weeks)] and [LOPE vs N (≥34 weeks)], 59 and 30 exomiRNAs in total were differentially expressed respectively, of which 39 were unique to EOPE and 19 LOPE with 3 common miRNAs between the groups (Figure 4, Supplementary file 1). In EOPE vs N (≤33 weeks), of the 39 uniquely expressed exomiRNA, KEGG pathway enrichment of the 14 upregulated exomiRNA (Figure 4, Supplementary file 1) represented involvement in lipid metabolism (Fatty acid biosynthesis), endocrine system functioning (Thyroid signaling pathway) and biological processes associated with tumor progression (Proteoglycans in cancer and Glioma) (Figure 5A, $p<0.00001$). Furthermore, GO: biological process target enrichment indicated that the upregulated EOPE exomiRNA is involved in several key biological processes associated with the pathophysiology of EOPE, these include; platelet activation, blood coagulation, cell death and protein modification, Figure 5B, $p<00001$). In contrast, KEGG enrichment of the downregulated exosomal miRNAs in EOPE in comparison to N (≤33 weeks) indicate dysregulation in key signaling pathways (Hippo, TGF-beta, Wnt, ErbB, FoxQ signaling pathways), cellular processes (Endocytosis, Adherens junction, Regulation of the pluripotency of stem cells) and organismal systems (Axon guidance, Glutamatergic synapse) (Figure 5A, $p<00001$).

Of the 19 exclusively dysregulated exomiRNA in [LOPE vs N (≥34 weeks)], KEGG enrichment of the 7 upregulated exomiRNAs (Figures 4 and 5, Supplementary file 1) represented involvement in lipid metabolism (Fatty acid metabolism and fatty acid biosynthesis), signaling pathways (Hippo signaling pathway and signaling pathways regulating pluripotency of stem cells) and cancer biology (Glioma) (Figure 5A, $p<0.00001$). GO target enrichment of the upregulated exomiRNA in LOPE indicates involvement in several signaling pathways (phosphatidylinositol-mediated, fibroblast growth factor receptor, epidermal growth factor, Fc-epsilon receptor and Neurotrophin TRK signaling pathways) and cellular processes (Cellular lipid metabolism, biological process, synaptic transmission, cellular protein metabolism, cellular component assembly, catabolism, blood coagulation, small molecule metabolism, gene expression, biosynthesis and cellular protein modification) associated with EOPE with however no involvement in cell death, platelet activation, response to stress and Toll-like receptor signaling pathways as displayed in EOPE which distinguishes the pathology of EOPE vs LOPE (Figure 5B, $p<0.00001$). KEGG pathway enrichment of the downregulated exomiRNA in LOPE vs N (≥34 weeks) indicates dysregulation in cell survival, migration and invasion associated with tumor progression (proteoglycans in cancer) and signaling pathways (Hippo and regulation of pluripotency of stem cells) (Figure 5A, $p<0.00001$).

Intersect analysis

In the intersect between [EOPE vs N (≤33 weeks)] and [LOPE vs N (≥34 weeks)] exosomal miR-2113 (logFC −1.69 vs −1.12 respectively) and miR-374c-5p (logFC −1.7 vs −1.21) were downregulated. KEGG enrichment indicated that exosomal miR-2113 regulates fatty acid biosynthesis which is a key biological process in lipid metabolism and miR-374c regulates inflammation (KEGG pathways: TGF-beta signaling, Hippo and Wnt signaling pathway) which are key biological processes commonly linked to the pathophysiology of EOPE and LOPE (Figure 5A, $p<00001$).

In [EOPE vs LOPE] vs [LOPE vs N (≥34 weeks)] and [EOPE vs LOPE] vs [EOPE vs N (≤33 weeks)] 6 and 15 commonly dysregulated exomiRNA (Figure 4, Supplementary file 1) were identified between the groups respectively, suggesting the pathophysiological role of

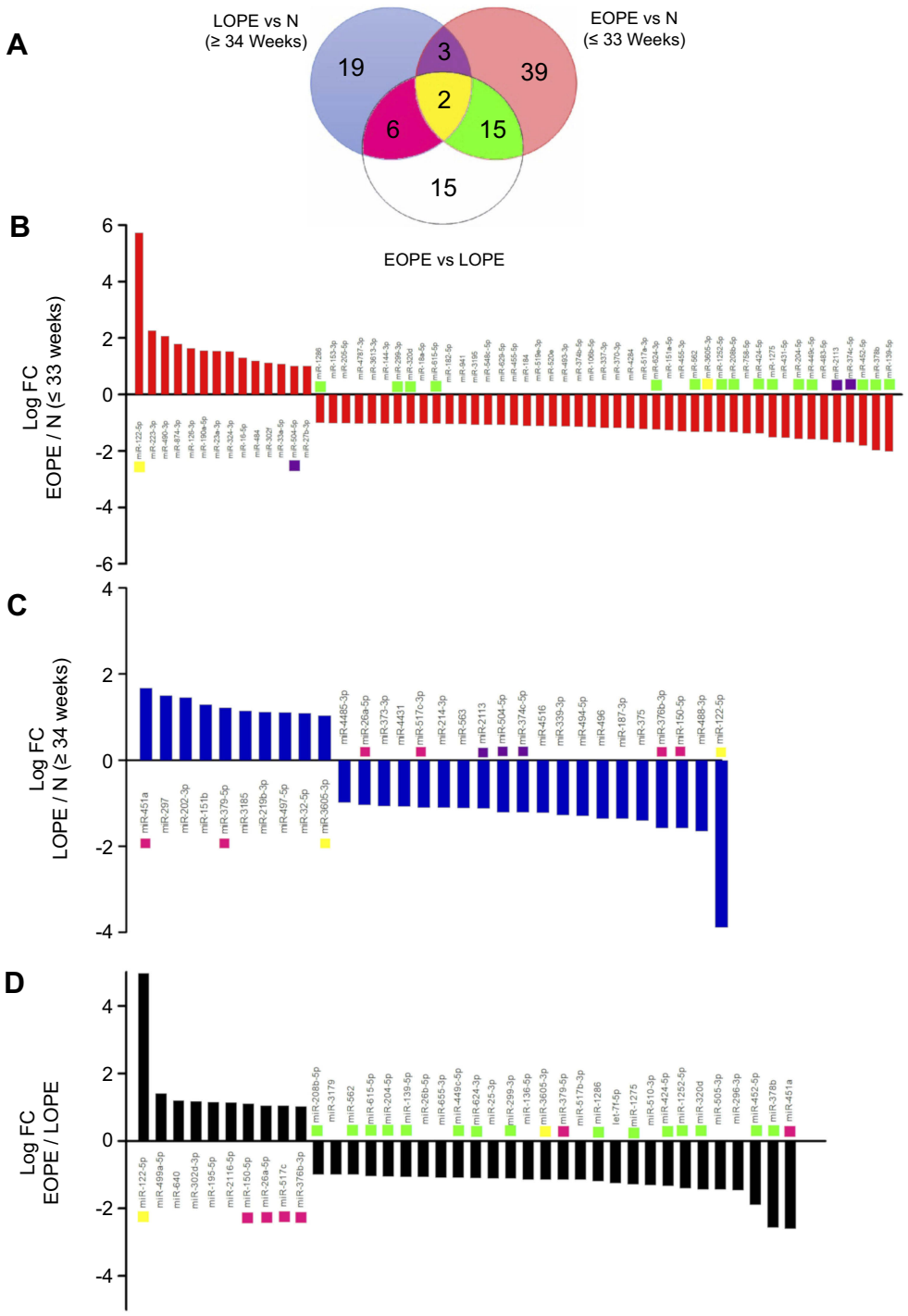

Figure 4 Differential expression analysis of exosomal miRNA in early and late onset preeclampsia. (**A**) Venn diagram representing differentially expressed exomiRNA observed in the comparisons amongst Early-onset Preeclampsia (EOPE), Late-onset Preeclampsia (LOPE) and the respective gestationally matched controls (|fold-change| ≥ 1.0, FDR ≤ 0.05). (**B**) Log FC values of differentially expressed miRNA in EOPE in comparison to N (≤33 Weeks). (**C**) Log FC values of differentially expressed miRNA in LOPE in comparison to N (≥34 Weeks). (**D**) Log FC values of differentially expressed miRNA in EOPE in comparison to LOPE. In B, C and D the x-axis represents the LogFC of differentially expressed miRNA. In B, C and D, respective colors indicate the common miRNA between intersecting groups: ■ commonly deregulated exosomal miRNAs in the LOPE vs N (≥34 Weeks) and EOPE vs N (≤33 Weeks) comparisons, ■ commonly deregulated exosomal miRNAs in the LOPE vs N (≥34 Weeks) and EOPE vs LOPE comparisons, ■ commonly deregulated exosomal miRNAs in the EOPE vs LOPE and EOPE vs N (≤33 Weeks) comparisons, ■ commonly deregulated exosomal miRNAs in all comparisons.

Abbreviations: FC - Fold Change, N - Normotensive pregnant women.

exosomes in EOPE and LOPE in comparison to their respective normotensive controls. In EOPE vs LOPE and EOPE vs N (≤33 weeks), KEGG pathway enrichment (Figure 5A) of the 15 dysregulated exomiRNAs (Figure 4, Supplementary file 1)

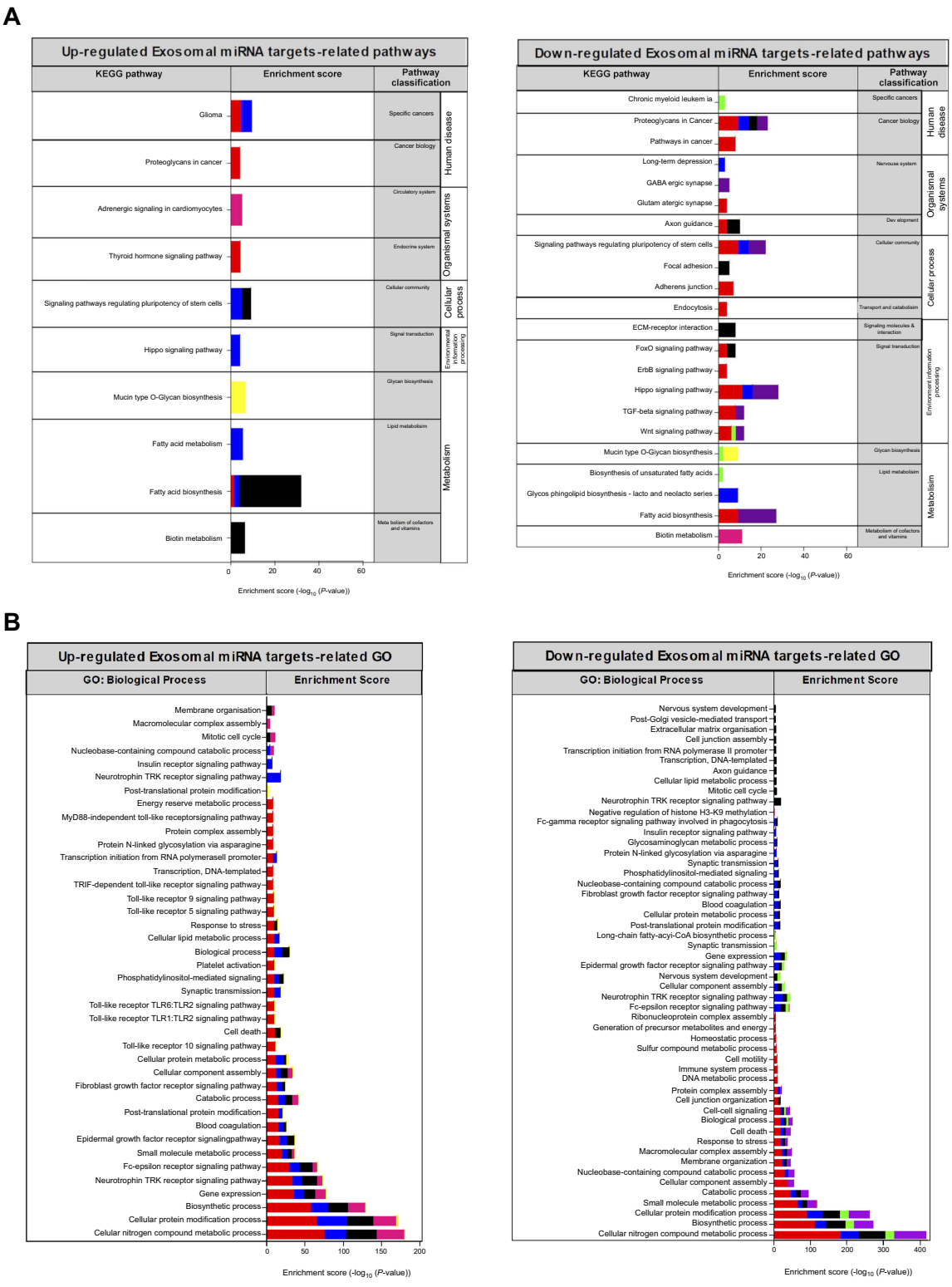

Figure 5 Pathway and Gene Ontology enrichment analysis of the exosomal miRNA targets differentially expressed in early and late onset preeclampsia. (**A**) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway mapping for top canonical pathways and (**B**) Biological process of Gene Ontology (GO) enrichment analysis represented by the differentially up and downregulated exosomal miRNAs within each group determined using microT-CDs target predicting with Descriptive Intermediate Attributed Notation for Ada (DIANA) miRPATH V.3.0 software. Each group is represented by the following respective colors: ■ uniquely dysregulated exosomal miRNA in the EOPE vs N (≤33 Weeks) comparisons, ■ uniquely dysregulated exosomal miRNA in the LOPE vs N (≥34 Weeks) comparisons, ■ commonly deregulated exosomal miRNAs in the LOPE vs N (≥34 Weeks) and EOPE vs to N (≤33 Weeks) comparisons, ■ commonly dysregulated exosomal miRNAs in the LOPE vs N (≥34 Weeks) and EOPE vs LOPE comparisons, ■ commonly deregulated exosomal miRNAs in the EOPE vs LOPE comparison, ■ commonly deregulated exosomal miRNAs in the EOPE vs LOPE and EOPE vs N (≤33 Weeks) comparisons, ■ commonly deregulated exosomal miRNAs in all comparisons.

Abbreviations: N - Normotensive Pregnant Women, EOPE - Early -onset Preeclampsia, LOPE - Late -onset Preeclampsia

specifies participation of exosomes in the dysregulation of; protein glycosylation (Mucin type O-Glycan biosynthesis pathway), lipid metabolism (Biosynthesis of unsaturated fatty acids pathway) and signal transduction during cell proliferation and migration (Wnt signaling pathway). This is related to GO biological process targets (Figure 5B) which regulate key cellular (cellular nitrogen compound metabolism, cellular protein modification) and signaling processes (Fc-epsilon receptor signaling pathway, Neurotrophin TRK receptor signaling pathway, Epidermal growth factor receptor signaling pathway, cell-cell signaling) (Figure 5, $p<0.00001$). Intersecting exomiRNAs (hsa-miR-451a and hsa-379-5p) in the EOPE vs LOPE and LOPE vs N (≥34 weeks) comparisons indicate exosomal involvement in cardiomyocyte adrenergic signaling; a direct association with impaired cardiac function which is a key feature of PE.

Importantly in all comparisons 2 distinct exomiRNAs were identified, both of which indicated involvement in mucin-type O-Glycan biosynthesis pathway (KEGG enrichment $p<0.00001$, Figure 5A). Coupled to this finding these exomiRNAs were linked to posttranslational protein modification and cellular protein metabolic processes (GO: Biological process target enrichment $p<0.00001$).

Exosomal miRNA signatures associated with the pathophysiology of early and late onset preeclampsia

In addition to the KEGG and GO enrichment analysis we further analyzed the differentially expressed exomiRNA in each comparison (Figure 4A), for their biological function and gene targets using NCBI gene databases (Tables 2 and 3). This linked the identified exomiRNAs to the validated pathophysiological functions involved in preeclampsia.

Complement analysis

In the complement analysis of EOPE vs N (≤33 weeks) and LOPE vs N (≥34 weeks) and EOPE vs LOPE, exomiRNA with an arbitrary |fold change|≥1.0 and FDR ≤0.05 were selected for further evaluation. It was confirmed that the significantly dysregulated exomiRNA unique to the EOPE vs N (≤33 weeks) comparison is associated with key pathophysiological features related to the more severe clinical outcomes of EOPE, such as angiogenesis, cell proliferation, inflammation, vasoconstriction, cell invasion, myogenesis and lipid metabolism (Table 2). Furthermore, in LOPE vs N (≤33 weeks) the dysregulated exomiRNA indicates the role of exosomes in the misregulation of angiogenesis and glucose homeostasis (Table 2), which is associated with the decreased probability of adverse maternal and fetal complications when compared to EOPE. Exosomal miRNA in the EOPE vs LOPE comparison indicated a physiological role in inflammation, angiogenesis, cell proliferation, blood pressure regulation and electrolytic homeostasis, which are features associated with the pathology of PE (Table 2).

Intersect analysis

In the intersect analysis of comparisons, exomiRNA with an arbitrary |fold change|≥1.0 and FDR ≤0.05 were selected for further evaluation of their physiological function and targets (Table 3). Intersecting exomiRNA in the EOPE vs N (≤33 weeks) and LOPE vs N (≥34 weeks) comparisons indicated three statistically significantly dysregulated exomiRNA which have confirmed biological roles in cell proliferation, invasion and inflammation (Table 3). Intersecting exomiRNA in the EOPE vs N (≤33 weeks) and EOPE vs LOPE comparisons indicated a downregulation of the identified exomiRNA in EOPE in comparison to LOPE and N (≤33 weeks) (Table 3). These exomiRNA play a role in the dysregulation of cell migration and invasion, cell proliferation apoptosis, mesenchymal transition, multiciliogenesis and angiogenesis. Furthermore, the enhanced downregulation of exomiRNAs in EOPE in comparison to LOPE and N (≤33 weeks) suggests that exosomes contribute to the more severe maternal and fetal complications associated with EOPE in comparison to LOPE.

In contrast, intersecting exomiRNA between the LOPE vs N (≥34 weeks) and EOPE vs LOPE comparisons indicate that the pathological association between LOPE in comparison to EOPE and N (≥34 weeks) (Table 3) is linked to cell survival, glucose secretion, angiogenesis, autophagy, cell proliferation and migration and trophoblast invasion. Moreover, the dysregulation of hsa-miR-379-5p in LOPE exosomes in comparison to EOPE and N (≥34 weeks) (Log2: 3.93 vs 2.79 and 2.71 respectively) signifies a possible association with the maternal metabolic syndrome in LOPE through the suppression of glucose-induced insulin secretion. In all intersecting comparisons [EOPE vs N (≤33 weeks) and LOPE vs N (≥34 weeks) and EOPE vs LOPE] two exomiRNA; hsa-miR-122-5p and has-miR-3605-3p were common (Table 3). hsa-miR-122-5p has been confirmed to have a function in cholesterol metabolism by targeting CAT1 which directly links to dyslipidemia in PE.

Table 2 Functional roles and validated targets of unique exomiRNA identified between group comparisons

Group Comparison	Identified Exosomal miRNAs				Function	Validated Targets/Pathways	References
	miRNA	Mean Expression values (Log2)		log FC			
EOPE vs N (≤33 weeks)		**EOPE**	**N (≤33 weeks)**	**EOPE vs N (≤33 weeks)**			
	Upregulated						
	hsa-miR-223-3p	7,21	4,95	2,26	Angiogenesis	Inhibits angiogenesis by targeting the RPS6KB1/hif-1a signaling pathway	28
	hsa-miR-490-3p	3,66	1,58	2,08	Apoptosis and Cell Cycle	Targets HMGA2 in cancer	29
	hsa-miR-874-3p	4,59	2,8	1,79	Apoptosis and Cell Proliferation	Controls cell fate by targeting PIN1 expression in cancer	30
	hsa-miR-126-3p	4,96	3,32	1,64	Inflammation	Inhibits VCAM1 expression and limits leukocyte adherence to endothelial cells	31
	hsa-miR-190a-5p	5,01	3,46	1,55	Vasoconstriction	Enhances artery constriction by targeting the Kþ–channel KCNQ5	32
	hsa-miR-23a-3p	5,45	3,9	1,54	Inflammation	Targets ATG12-mediated autophagy	33
	hsa-miR-324-3p	3,52	2	1,53	Cell proliferation and Invasion	Targets the Hedgehog pathway transcription gene GLI3 in cancer	34
	Downregulated						
	hsa-miR-431-5p	3,37	4,9	−1,53	Myogenesis	Promotes myogenesis through the regulation of Smad4 mRNA, which encodes one of the downstream effectors of TGF-β signaling	35
	hsa-miR-758-5p	1,2	2,8	−1,6	Lipid Metabolism	Regulates cholesterol efflux by repressing ABCA1	36

(*Continued*)

Table 2 (Continued).

Group Comparison	Identified Exosomal miRNAs				Function	Validated Targets/Pathways	References
	miRNA	Mean Expression values (Log2)		log FC			
LOPE vs N (≥34 weeks)		**LOPE**	**N (≥34 weeks)**	**LOPE vs N (≥34 weeks)**			
	Upregulated						
	hsa-miR-297	4,62	3,13	1,5	Angiogenesis	Negatively regulate VEGFA expression	37
	hsa-miR-202-3p	4,16	2,71	1,45	ND	ND	38
	Downregulated						
	hsa-miR-375	3,76	5,17	−1,41	Glucose homeostasis	Regulates glucose metabolism by targeting 3′-Phosphoinositide–Dependent Protein Kinase-1	39
	hsa-miR-488-3p	2,93	4,58	−1,65	ND	ND	
EOPE vs LOPE		**LOPE**	**EOPE**	**EOPE vs LOPE**			
	Upregulated						
	hsa-miR-499a-5p	5,55	6,96	1,41	Cell Proliferation and Migration	Promotes cell proliferation and migration in atherosclerosis by targeting *MEF2C*	40
	hsa-miR-640	4,09	5,29	1,2	Angiogenesis	Inhibits H2S mediated angiogenesis	41
	Downregulated						
	hsa-miR-505-3p	3,22	1,79	−1,44	Inflammation	Regulates chemokine receptor upregulation in macrophages	42
	hsa-miR-296-3p	4,46	3,01	−1,46	Blood pressure and Electrolyte Homeostasis	Regulates blood pressure by suppressing hWNK4	43

Note: All fold changes associated with the complement analyses are represented in Log2 scale (LogFC), statistically, significant exomiRNAs were selected based on an arbitrary |FC| ≥1.4 and FDR ≤0.05. Mean expression values are presented in Log2 scale.

Abbreviations: ND, not detected; N, normotensive pregnancies; EOPE, early onset preeclampsia; LOPE, late onset preeclampsia; FC, fold change.

Table 3 Functional roles and validated targets of interesting exomiRNA identified between group comparisons

Group Comparison	Identified Exosomal miRNAs							Function	Validated Targets/Pathways	References
	miRNA	Mean (Log2)				log FC				
		LOPE	EOPE	N (≤33 weeks)	N (≥34 weeks)	EOPE vs N (≤33 weeks)	LOPE vs N (≥34 weeks)			
EOPE/N (≤33 weeks) vs LOPE/N (≥34 weeks)	hsa-miR-504-5p	2,09	3,01	2	3,3	1,01	-1,21	Cell Proliferation and Invasion	Targets LOXL2 in cancer	44
	hsa-miR-2113	3,76	3,01	4,7	4,88	-1,69	-1,12	ND	ND	
	hsa-miR-374c-5p	2,09	2,2	3,9	3,3	-1,7	-1,21	Inflammation	Regulates MID1 protein expression	45
		LOPE	EOPE	N (≤33 weeks)		EOPE vs N (≤33 weeks)	EOPE vs LOPE			
EOPE/N (≤33 weeks) vs EOPE/ LOPE	hsa-miR-204-5p	3,57	2,52	4,08		-1,56	-1,05	Epithelial-to-Mesenchymal Transition	Repression of TGF-β2–induced Epithelial-to-Mesenchymal Transition in the presence of SMAD4 small interfering RNA.	46
	hsa-miR-139-5p	3,85	2,79	4,8		-2,02	-1,07	Cell proliferation and apoptosis	Regulates cell proliferation an apoptosis by targeting the Brg1 gene	47
	hsa-miR-449c-5p	4,46	3,37	4,95		-1,58	-1,09	Multiciliogenesis	Targets the cell cycle and notch pathway via DLL1 and NOTCH1	48
	hsa-miR-1275	4,29	3,01	4,52		-1,51	-1,28	cell migration, invasion and proliferation	Targets IGF-1R and CCR7 in tumor progression	49
	hsa-miR-320d	4,81	3,37	4,39		-1,02	-1,44	Apoptosis	Regulates apoptosis by targeting TRIAP1 and NET1	50
	hsa-miR-452-5p	3,67	1,79	3,58		-1,8	-1,89	Cell proliferation and Migration	Targets the CPEB3/EGFR axis in tumor progression	51
	hsa-miR-378b	3,76	1,2	3,17		-1,97	-2,56	Angiogenesis	Promotes angiogenesis by targeting Suppressor of fused (SuFu) and Fus-1 expression	52

(*Continued*)

Table 3 (Continued).

Group Comparison	Identified Exosomal miRNAs							Function	Validated Targets/Pathways	References
	miRNA	Mean (Log2)				log FC				
		LOPE	EOPE	N (≥34 weeks)		LOPE vs N (≥34 weeks)	EOPE vs LOPE			
LOPE/N (≥34 weeks) vs EOPE/ LOPE	hsa-miR-451a	12,75	10,15	11,1		1,68	-2,6	Cell Survival	Inhibition of cell survival by targeting calcium-binding protein 39 (CAB39, also known as MO25a) to inhibit the key AMPK kinase LKB1 (liver kinase B1), causing AMPK inactivation	53
	hsa-miR-379-5p	3,93	2,79	2,71		1,22	-1,15	Glucose Secretion	Suppresses glucose induced insulin secretion by targeting Myotrophin (Mtpn)	54
	hsa-miR-26a-5p	3,67	4,72	4,71		-1,04	1,05	Angiogenesis	targets VEGF-A expression through PIK3C2α	55
	hsa-miR-376b-3p	2,35	3,37	3,93		-1,58	1,02	Autophagy	Regulates autophagy by targeting ATG4C and BECN1 (Beclin 1)	56
	hsa-miR-150-5p	3,35	4,45	4,93		-1,58	1,1	Cell Proliferation and Migration	HIF-1α expression	57
	hsa-miR-517c-3p	3,67	4,72	4,77		-1,1	1,05	Trophoblast invasion	Overexpression decreases invasion through the dysregulation of TNFSF15 expression and sFLT1	58
EOPE/N (≤33 weeks) vs LOPE/N (≥34 weeks) vs EOPE/ LOPE		LOPE	EOPE	N (≤33 weeks)	N (≥34 weeks)	EOPE vs N (≤33 weeks)	LOPE vs N (≥34 weeks)	EOPE vs LOPE		
	hsa-miR-122-5p hsa-miR-3605-3p	0,76 4,16	5,72 3,01	0,2 4,32	4,65 3,13	5,72 -1,31	-3,88 1,03	4,96 -1,15	Cholesterol Metabolism ND	Targets CAT1 ND	59

Note: All fold changes associated with the complement analyses are represented in Log2 scale (LogFC), statistically, significant exomiRNAs were selected based on an arbitrary |FC| ≥1.4 and FDR ≤0.05. Mean expression values are presented in Log2 scale.

Abbreviations: ND, not detected; N, normotensive pregnancies; EOPE, early onset preeclampsia; LOPE, late onset preeclampsia; FC, fold change.

Discussion

Whilst studies have supported the role of exomiRNA as potential biomarkers of PE, their role in the etiopathophysiology of PE and its subtypes remains unclear. Therefore, for the first time, this study has profiled exomiRNA and determined the pathophysiological role of dysregulated exomiRNA in early and late onset-PE using Direct Digital Detection and computational algorithms.

The data observed from the characterisation and quantification of total and placenta-derived exosomes are consistent with our previous study[12] reinforcing that differential levels of placenta-derived exosomes in maternal circulation are attributed to the differences in the pathophysiology of the disease subtypes. Moreover, the significant increase of placenta-derived exosomes in EOPE vs N (≤33 Weeks) and decrease in LOPE vs N (≥34 Weeks), which may reflect the role of the placenta in the pathogenesis of PE.

Exosomal miRNA expression profiles in EOPE vs N (≤33 weeks) and LOPE vs N (≥34 weeks) indicate a clear dysregulation and difference in exomiRNA expression between the PE subtypes and the matched normotensive controls. KEGG pathway enrichment of clusters of dysregulated exomiRNA (Figures 2 and 3) reveal top canonical pathways which are associated with cellular signaling, cancer biology, metabolism, cellular processes, cardiovascular disease and organismal systems. Further analysis of highlighted KEGG target genes indicates that exomiRNA in maternal circulation are likely to be involved in modulating key biological processes involved in cell proliferation, invasion, migration and apoptosis, which are known to be dysregulated in PE. Our findings, therefore, support the role of exosomes in reprogramming adjacent or distant cells through autocrine and paracrine signaling mechanisms thereby facilitating the pathogenesis of preeclampsia. Moreover, we identified specific clusters of exomiRNA in both EOPE and LOPE, which are linked to arrhythmogenic right ventricular cardiomyopathy (Figures 2 and 3) indicating that exosomes in maternal circulation may be involved in the dysregulation of cardiac function – a known complication of the disease.[60]

Computational analysis of statistically significant fold changes in exomiRNA (LogFC ≥1, FDR ≤0.05) between the EOPE vs N (≤33 weeks), LOPE vs N (≥34 weeks) and EOPE vs LOPE groups supports the pathophysiological role of exomiRNA in PE. Enrichment of exclusively upregulated exomiRNA in EOPE vs N (≤33 weeks) indicates that the dysregulation of exomiRNA in EOPE in comparison to N (≤33 weeks) is responsible for the irregular expression of proteoglycans (KEGG pathway: Proteoglycans in cancer, p-value ≤0.00001), which concomitantly contribute to placental proteoglycan deficiency, resulting in placental thrombotic lesions: a key morphological feature of PE placentas.[61] Additionally, this pathway interaction indicates that the upregulated exomiRNA in EOPE induces a micro-environment which favours abnormal metastatic-related properties resulting in abnormal cell growth, adhesion, migration and invasion. Moreover, the pathway interactions of significantly upregulated exomiRNA in EOPE are associated with dysregulation of the thyroid hormone signaling pathway by targeting the thyroid hormone receptor-β (THRE), a regulator of calcineurin 1(RCAN1) and Ca2+ transporting ATPase (ATP2A2), which controls cardiac muscle contraction and angiogenesis. Therefore, our findings highlight the potential cardiovascular involvement of exomiRNA in preeclampsia which we are further investigating.

In contrast, the enrichment of exclusively upregulated exomiRNA in LOPE (LogFC ≥1, FDR ≤0.05) indicated a dysregulation in lipid metabolism by targeting fatty acid synthase (FASN) expression which indicates that LOPE exosomes enhance a state of adipogenic trans-differentiation which promotes oxidative stress, chronic inflammation, and, potentially, insulin resistance in LOPE; which are key metabolic features which may be linked to pathophysiology of LOPE or reflective of the increased BMI in LOPE in comparison to EOPE (38.14±3.41 vs 32.42±2.19, p<0.001).[62] Moreover, KEGG enrichment of upregulated exomiRNA in EOPE vs N (≤33 weeks) and LOPE vs N (≥34 weeks) indicates a significant involvement in the glioma cancer pathway (p<0.00001), suggesting that EOPE- and LOPE-derived exosomes have the ability to target glial cells through a network of genes responsible for signaling in glial progenitor cells (KEGG highlighted target genes: TGFA, PDGFA, SOS1, AKT3, PTEN and IGF1R) which may provide the possible links to placental branching and vasculogenic, abnormalities that occur during the development of EOPE and LOPE.[63]

Intersecting exomiRNA hsa-miR-122-5p and hsa-miR-3605-3p were common in [EOPE vs N (≤33 weeks)], [LOPE vs N (≥34 weeks)] and [EOPE vs LOPE]. Exosomal hsa-miR-122-5p expression in all groups may target the polypeptide N-acetylgalactosaminyltransferase

gene which regulates the biosynthesis of mucin-type O-glycosylated antigen (Tn antigen); a tumor-associated carbohydrate antigen which is not normally expressed in peripheral tissues or blood cells.[64] It is possible that exosomal mediated dysregulation of Tn antigen synthesis may be an important recognition element, mediating cell-cell interactions involved in placental organotrophic metastasis.[27,64] Additionally, the upregulation of exosomal hsa-miR-122-5p in EOPE vs LOPE (LogFC 5.72 vs 0.76) could indicate that this exomiRNA in EOPE is associated with the aberrant glycosylation of the Tn antigen which is linked to the enhanced maternal systemic inflammatory response which is commonly observed in PE with severe features.[59] Moreover, recent studies have identified that the aberrant glycosylation of proteins in PE is linked to the synthesis of novel proteins involved in hepatic and renal dysfunction[65] which suggests that total and placenta-derived exosomes may be involved in causing end-organ complications associated with severe forms of PE.

The identification of unique signatures of exomiRNA in [EOPE vs N (≤33 weeks)], [LOPE vs N (≥34 weeks)] and [EOPE vs LOPE]; revealed key pathophysiological differences between the groups. Significantly dysregulated exomiRNA (LogFC ≤1.4) in [EOPE vs N (≤33 weeks)] were confirmed to have a pathophysiological role in cellular maintenance, inflammation, vasoconstriction, myogenesis and lipid metabolism, all of which are associated with the more adverse pathological features of EOPE in comparison to LOPE.[65] In contrast, since significantly dysregulated exosomal hsa-miR-297 and hsa-miR-375 (LogFC ≤1.0) in LOPE vs N (≥34 weeks) has been previously shown to negatively regulate VEGFA expression and glucose homeostasis[36,38] suggests that this could contribute to more favourable maternal and neonatal outcomes in comparison to EOPE.[66] Furthermore, dysregulated (LogFC ≤1.0) exomiRNAs in EOPE vs LOPE but not in comparison to their physiologic controls (Table 3) indicate that differential severity of EOPE compared to LOPE is mediated by exomiRNAs which modulate cell proliferation and migration, angiogenesis, inflammation and blood pressure regulation. These events and factors are central to the pathogenesis of PE. Interestingly even though no confirmed targets are available for hsa-miR-3605-3p, it has been shown to be linked to Multiple System Atrophy (MSA), a sporadic neurodegenerative disorder characterized by a combination of various degrees of Parkinson cerebellar ataxia and autonomic dysfunction, which might provide a link to the autonomic nervous system control of key physiological processes such as blood pressure regulation in EOPE and LOPE.

Moreover, we found that the exomiRNAs in normotensive and preeclamptic maternal circulation is linked to biological pathways associated with cancer biology. This concurs with a current theory that placental trophoblast and cancer cells can develop a microenvironment which supports immunologic privilege and angiogenesis through molecular mechanisms which regulate hyper-proliferation, invasion, angiogenesis and immunoevasion.[27] Therefore, our future studies would seek to compare and integrate the pathophysiological role of exomiRNA in pregnancy, PE and cancer biology to identify possible interrelated mechanisms to better understand the intriguing and natural phenomenon of pregnancy.

This study highlights the possible roles of exomiRNA in the pathophysiology of preeclampsia and its subtypes which warrants further validation of their biomarker potential with a larger and dynamic patient cohort. Furthermore, this study is limited by the unknown cellular origin of the exosomal miRNA identified therefore it becomes challenging to understand the diverse exosome-mediated interactions involved in the pathophysiology. The NanoString Direct Digital Detection technology used in this study has proven to be valuable in a clinical setting due to rapid detection, precision and accuracy. Therefore, future studies will focus on (1) identifying the specific origin of the sub-populations of exosomes for in-depth analysis and (2) validating exosomes and their molecular cargo as biomarkers of preeclampsia and their subtypes.

Conclusion

This novel study identifies exomiRNA signatures in EOPE and LOPE which are implicated in the dysregulation of key physiological processes associated with the etiopathology of PE. Importantly, the interconnectedness of cancer biology and PE is revealed through pathway associations which supports the notion that exomiRNAs in pregnancy and its associated complications, including PE, is related to the "pseudo-malignant like" physiological processes. Furthermore, the discovery of distinct exomiRNA signatures in EOPE and LOPE support the existence of the differential pathophysiology between the subtypes. Therefore, the observations made in this study warrant the diagnostic and functional validation of the signatures as biomarkers of PE and its subtypes.

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Author contributions

All authors contributed towards data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interests in this work.

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4. Chapter Four: Manuscript Three

Exosomal Th1/Th2 Cytokines in Preeclampsia and HIV-positive Preeclamptic Women on Highly Active Anti-Retroviral Therapy. (Under Review: Cytokine CYTO-19-358)

Our previous studies presented in Chapters 2 and 3 have contextualised the basis of exosomes as a biomarker of PE. Defined conditions of use must be characterised when evaluating potential biomarkers. This must incorporate the intent-to-diagnose the disease in combination with other conditions which increase the susceptibility/risk of the disease. We, therefore, evaluated the biomarker potential of exosomes in diagnosing PE in HIV-positive pregnant women on HAART. Highly Active Anti-Retroviral Therapy has been closely associated with the increased risk of PE due to maternal immune reconstitution [93], which alters the Th1/Th2 immune balance as represented by the variations in cytokine levels in circulation [94]. Therefore our findings of altered exosomal cytokines in PE and PE in HIV-positive pregnant women on HAART lends greater significance to the role of exosomes as biomarkers of PE.

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Exosomal Th1/Th2 cytokines in preeclampsia and HIV-positive preeclamptic women on highly active anti-retroviral therapy

Preenan Pillay[a,*], Kogi Moodley[a], Manu Vatish[b], Jagidesa Moodley[c], Raquel Duarte[d], Irene Mackraj[e,*]

[a] Discipline of Human Physiology, School of Laboratory Medicine & Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
[b] Nuffield Department of Women's & Reproductive Health, Women's Centre, John Radcliffe Hospital, University of Oxford, Oxford, United Kingdom
[c] Women's Health and HIV Research Group, Department of Obstetrics and Gynaecology, University of KwaZulu-Natal, Durban, South Africa
[d] Department of Internal Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa
[e] School of Laboratory Medicine & Medical Sciences, University of KwaZulu-Natal, Durban, South Africa

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TNF-α

ABSTRACT

Preeclampsia (PE) is a hypertensive disorder of pregnancy which is a leading cause of maternal and foetal morbidity and mortality. Furthermore, HIV/Highly Active Anti-Retroviral Treatment has been associated with the increased risk of preeclampsia due to maternal immune reconstitution, which complicates the clinical diagnosis of PE in these patients. It is therefore necessary to identify biomarkers involved in the pathology of both disorders with the intent to diagnose. Exosomal cytokines represent ideal biomarkers of PE and inflammatory conditions due to their immunomodulatory role in pregnancy. We therefore quantified exosomal Th1 (IL-2 and TNF-α) and Th2 cytokines (IL-10) in maternal circulation. A significant dysregulation in total exosomes, placental-derived exosomes and exosomal cytokines in PE and HIV-positive PE pregnant woman on Highly Active Antiretroviral Treatment (HAART) was observed ($p < 0.01$). Additionally, we observed a significant shift towards Th1 immunity in PE which becomes amplified in HIV-positive PE pregnant woman on HAART ($p < 0.01$). Moreover, we show the potential application of exosomal Tumor necrosis factor alpha (TNF-α) as a biomarker of PE and PE in HIV-positive pregnant women on HAART (CI: 95%, LHR > 10, sensitivity of 100% and specificity of 90%). These findings are in support of exosome release and exosome cytokine encapsulation as a tightly regulated process in favour of maintaining the immune microenvironment, which can orchestrate either normal pregnancy, or the pathogenesis of preeclampsia and preeclampsia in HIV/HAART pregnancies.

1. Introduction

Preeclampsia (PE) remains a leading cause of maternal and foetal morbidity [1] which is further complicated in HIV-positive pregnant women receiving Highly Active Anti-Retroviral Therapy (HAART) who are at a greater risk of developing PE [2]. Therefore, definitive biomarkers of PE and PE in HIV-positive pregnant women are required within the clinical setting.

Pregnancy is a well-coordinated immune state regulated by a network of cytokines which modulate a balance between both pro-inflammatory (Th1) and anti-inflammatory (Th2) immune responses [3,4]. In order for the mother to tolerate the semi-allogenic foetus a Th2 mediated immune response modulated by cytokines such as IL-10 is essential [3,4], whereas the Th1 mediated pro-inflammatory immune state modulated by cytokines such as TNF-α and IL-2 is essential for trophoblast invasion, parturition and defense against infections [5,6]. Therefore, the balance between Th1/Th2 immune response is a critical requirement for successful pregnancy. Conversely, the dysregulation in Th1/Th2 immune balance during pregnancy results in complications such as PE. Preeclampsia is associated with a shift from Th2 to Th1 dominant immunity [7] due to cell-mediated immune responses activated by known and unknown factors, which induce an enhanced maternal systemic inflammatory response (MSIR). Similar to normal pregnancy, a shift from Th1 to Th2 immunity has been observed during the progression of HIV infection [8]. However, the immune status is reversed with HAART [9,10] which increases the risk of PE. It has been established that circulating cytokines are key immunomodulating factors involved in HIV-associated immune activation [11] and PE [12].

* Corresponding authors at: School of Laboratory Medicine & Medical Sciences, University of KwaZulu-Natal, 1 University Road, Westville, Durban 4001, South Africa.
E-mail address: mackraji@ukzn.ac.za (I. Mackraj).

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Recent reports have established that most circulating cytokines are encapsulated in extracellular vesicles (viz. 70%-IL-2, 50% TNF-α and 100% IL-10) in comparison to the soluble form [13].

Extracellular vesicle (EV) cytokine encapsulation is described as a regulated property of the immune system, whereby, T-cells and monocytes predominantly release IL-2, IL-10 and TNF-α in EVs [13]. This emphasises the significant role of cytokine EV-entrapment: (1) as a mechanism to dispose of over-produced cytokines; (2) as protection from degradation, enhancing their half-lives in circulation and (3) to facilitate delivery to distant targets [13–16]. Therefore, cytokine EV-encapsulation can be viewed as a carefully regulated process, involving cell-cell communication which facilitates and is reflective of adaptation to specific physiological and pathophysiological immune requirements [15–17].

Exosomes (20–130 nm) are important sub-constituents of circulating EVs due to their role in key physiological and pathophysiological processes [14]. However, their immunomodulatory role in PE and PE in HIV/HAART remains unexplored therefore the presented study examined exosomal levels of pro-inflammatory Th1 cytokines (IL-2 and TNF-α) as well as anti-inflammatory Th2 cytokine (IL-10) in maternal circulation [12,18]. Moreover, to identify biomarkers and therapeutic targets in PE and PE in HIV-positive women on HAART, the complex immune interactions in these disease states needed to be understood. Therefore, the present study focused on elucidating: (1) the concentration of exosomes in maternal circulation and (2) exosomal Th1 and Th2 cytokine levels in preeclamptic and HIV-positive preeclamptic women on HAART.

2. Materials and methods

2.1. Ethics statement

Regulatory ethical and institutional approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, South Africa (BE310/15). Patients were recruited from the Prince Mshiyeni Hospital, KwaZulu Natal, South Africa. All participants gave written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

2.2. Study groups and samples

HIV-positive and HIV-negative pregnant women with PE (early onset and late onset), in the third trimester, were recruited (n = 30 per group). HIV-positive and HIV-negative normotensive pregnant women recruited and matched to PE groups based on their gestational (n = 30 per group). HIV was detected using a rapid test kit and confirmed using an Enzyme-linked immunosorbent assay (ELISA) performed by the South Africa National Health Laboratory Services (Prince Mshiyeni Hospital, Kwa-Zulu Natal, South Africa). All HIV-infected patients were on HAART (tenofovir, emtricitabine, efavirenz) as per the CDC guidelines. Normotensive patients were classified by a blood pressure of 120 ± 10/80 ± 5 (systolic/diastolic mm Hg) and absent proteinuria as detected by a rapid urine dipstick test (Markomed®, South Africa). Early onset pre-eclampsia was classified as new-onset hypertension (diastolic blood pressure of ≥90 mm Hg and systolic blood pressure of ≥140 mm Hg) and proteinuria (≥300 mg) at ≤33 weeks. Late onset-pre-eclampsia was defined by new-onset hypertension (diastolic blood pressure of > 90 mm Hg and systolic blood pressure of > 140 mm Hg) and proteinuria (≥300 mg) at ≥34 weeks gestational age. Gestational age matching was done on all samples collected. All patients had singleton pregnancies and those with evidence of any infections or medical, surgical or other obstetric complications were excluded. Blood samples were collected [BD Vacutainer Tubes ethylenediamine tetra-acetic acid (EDTA), Becton Dickinson and Company, South Africa] and the plasma samples were stored at −80 °C for analyses. All samples analysed were not stored for more than 1 month.

2.3. Isolation and characterisation of exosomes from the plasma of HIV-positive and HIV-negative pregnant women

2.3.1. Exosome isolation

Total exosomes were isolated according to the method described by Théry et al. [19]. Plasma (1 ml) was diluted with an equal volume of phosphate buffered saline (PBS; pH 7.4). Exosomes were isolated and purified by differential ultracentrifugation using a 30 per cent sucrose cushion. In brief, centrifugation was initially performed at 2000*g* at 4 °C for 30 min, the supernatant was collected and then centrifuged at 12,000× for 45 min. Thereafter the collected supernatant was centrifuged at 110,000*g* at 4 °C for 120 min (Optima™ MAX-XP Ultracentrifuge, fixed angle MLA-55 rotor, Beckman Coulter Inc., Brea, CA, USA). The pellet was suspended in Phosphate Buffered Saline (PBS) and filtered through a 0.22 μm filter (Cellulose acetate, GVS™, Europe). The filtrate was centrifuged at 110,000*g* at 4 °C for 70 min, the pellet re-suspended in PBS (pH 7.4) and centrifuged at 110,000*g* for 70 min, 4 °C. The exosome pellet was suspended in 1 ml of PBS and subsequently purified from contaminating HIV using a 6–18% iodixanol gradient (Opti-prep, Sigma) [20,21]. One millilitre of the solution was then placed on top of the 11 ml gradient and was centrifuged at 200 000*g* for 120 min at 4 °C. Thereafter twelve 1 ml fractions were collected for further characterisation.

2.3.2. Acetylcholinesterase (AChE) assay

Acetylcholinesterase activity was measured according to the method described by Ellman et al. [22]. This assay was used to determine the fractions which segregate the exosomes from the HIV particles [20,21]. In Brief, 100 μl of each exosomal fraction was suspended in 100 μl of substrate solution (1.25 mM acetylthiocholine in PBS, pH 8, mixed with 0.1 mM 5,5-dithio-bis(2-nitrobenzoic acid) in PBS pH 7 in a final volume of 200 μl). The solution was then incubated in pre-warmed (37 °C) substrate solution for 10 min and thereafter absorption was measured at 450 nm. Fractions with high AchE activity were considered to have a high concentration of exosomes which was confirmed using ELISA, immunoblot identification, electron microscopy and nanoparticle tracking analysis.

2.3.3. Nanoparticle tracking analysis

Quantification and size distribution of exosomes was determined using the NS500 equipped with a 405 nm laser and a sCMOS camera as previously described (NanoSight NTA 3.0 Nanoparticle Tracking and Analysis Release, Version Build 0069) [23]. Samples were diluted with PBS prior to analysis in order to obtain particle distribution of 10 and 100 particles per image before analysis with the NTA system. Samples were introduced into the sample chamber using the following script: PUMPLOAD, REPEATSTART, PRIME, DELAY 10, CAPTURE 60, REPEAT 5. Videos were recorded at a camera level of 10, a camera shutter speed of 20 ms and camera gain of 600, these settings were kept constant between samples. Each video was then analysed to give the mean particle size together with the concentration of particles.

2.3.4. Exosomal protein quantification

Isolated exosomes were re-suspended in PBS and the total protein concentration determined using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The bovine γ-globulin protein standard was used.

2.3.5. Western blotting

Isolated exosome pellets were lysed with radioimunoprecipitation assay (RIPA) buffer (1X) at room temperature for 5 min and the protein content determined using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Laemmli buffer (2X) was added to the samples and incubated at 95 °C for 5 min. Twenty micrograms of exosomal protein per well were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto

polyvinylidene fluoride (PVDF) membranes which were probed with CD63 primary antibody (ExoAB, System Biosciences, Mountain View, CA) post-incubation at room temperature for 60 min. Membranes were washed in Tris buffer saline (pH 7.6) and incubated for 60 min at room temperature with the exosome validated secondary conjugated goat rabbit horseradish peroxidase antibody (ExoAB, System Biosciences, Mountain View, CA). Membranes were incubated with chemiluminescent substrate and visualised using the ChemiDoc® Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

2.3.6. Transmission electron microscopy

Exosomes were applied to a continuous carbon grid and negatively stained with 2% uranyl acetate. The size and morphology of the particles were examined using a JEOL 1010 transmission electron microscope (JEOL, Peabody, MA, USA) at the Electron Microscopy Unit, University of KwaZulu-Natal.

2.4. Quantification of total and placental-derived exosomes

2.4.1. Quantification of total exosomes in maternal circulation

The concentration of total exosomes in maternal circulation was determined by the quantification of total immunoreactive exosomal CD63 via an enzyme-linked immune absorbency assay (ExoELISA™, System Biosciences, Mountain View, CA), as per manufacturer's instructions. In brief, 30 μg of isolated exosomal protein was immobilised on microtiter plates overnight at 37 °C using exosome binding buffer supplied by the manufacturer (System Biosciences). Plates were washed and incubated at room temperature for 60 min with exosome specific primary antibody (CD63), followed by a wash step and incubation with secondary antibody (1:5000) at room temperature for 60 min with agitation. Plates were thereafter washed and incubated with tetramethylbenzidine ELISA substrate at RT for 45 min with agitation. The reaction was thereafter terminated using stop buffer and the absorbance was measured at 450 nm. The number of exosomes, (ExoELISA™ kit) was obtained using an exosomal CD63 standard curve that was generated using the calibrated exosome standard that was supplied.

2.4.2. Quantification of placental-derived exosomes in maternal circulation

The relative concentration of placental-derived exosomes was determined by the quantification of human Placental-Like Alkaline Phosphatase (PLAP) in the exosomal fraction using a commercial ELISA kit (Elabscience, E-EL-H1976, WuHan, P.R.C), as per manufacturer's instruction. In brief, 30 μg of exosomal protein was allowed to bind to the primary PLAP specific antibody coated plates by incubation at 37 °C for 90 min. Plates were washed and 50 μl of HRP-conjugate was added to each well and incubated at 37 °C for 20 min. Plates were washed and incubated with 50 μl of substrate A and 50 μl of substrate B at 37 °C for 15 min. The reaction was terminated using 50 μl of stop solution at RT for 2 min under agitation. Absorbance was measured at 450 nm. Exosomal PLAP was expressed as pg/ml plasma. The quantification of PLAP in the exosomal fraction indicates the relative concentration of placental-derived exosomes ($PLAP^+$ exosomes) in maternal circulation.

2.5. Quantification of exosomal cytokines

2.5.1. Multiplex quantification of cytokines IL-2, IL-10 and TNF-α

Exosomal IL-2, IL-10 and TNF-α were analysed using the Milliplex MAP Human Cytokine Panel (Merck, Germany) according to manufacturer's instructions. Exosomes were solubilised by incubating the intact exosomes with Triton X (1% v/v). The protein concentration was then determined using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). In brief, the microtiter plate was pre-wet with 200 μl assay buffer. Fifty micrograms of sample and standards were added to the appropriate wells followed by the addition of the magnetic beads. The plate was incubated for 240 min with agitation at room temperature, the fluid was then removed, and the wells washed twice with wash buffer. Detection antibodies (anti-IL-2, IL-10 and TNF-α) were then added to each well and incubated for 60 min at room temperature. Thereafter the fluorescent Streptavidin-Phycoerythrin conjugate was added to each well and incubated for 30 min at room temperature. The fluid was then removed, and the wells were washed twice with buffer. The analytes were then resuspended in sheath fluid and analysed using the Luminex 100 instrument (Bio-Rad). Data were analysed using the Bio Manager Analytical software (BioRad) with the lower detection limit at 3.2 pg/ml for all analytes with an intra-assay variability of less than 10%.

2.6. Statistical analysis

All data analyses and graphical representations were performed and generated using GraphPad Prism 6.0 (CA, La Jolla). Data distribution was determined using the Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lilliefor P-value, all data were found to be normally distributed. One-way Analysis of Variance (ANOVA) followed by Tukey post-hoc testing was done to determine the level of significance of differences between groups. Statistical significance was defined as $p < 0.05$. Where applicable data are represented as the mean ± SEM. Graphical representation of data is summarised using box and whisker plots including median and interquartile range. Cut-off values for exosomal markers, cytokines and ratios which diagnose PE and its subtypes were conducted using receiver operating characteristics analysis (GraphPad Prism 6.0). Cut-off values of diagnostic relevance were defined as having a sensitivity of 100% and a specificity of 90% with a positive likelihood ratio (LHR) of 10 at a CI of 95%.

3. Results

The focus of the analysis was conducted on PE and PE in HIV-positive pregnant women on HAART vs. their respective controls. The differentiation between EOPE and LOPE is of clinical importance irrespective of HIV status. All significant findings between each of the groups described above was summarised in Table 1.

3.1. Participant clinical characteristics

There was no significant difference in blood pressure and proteinuria between the N (≤33 weeks) and N (≥34 weeks) groups. All CD4 counts were above 400 cells/mm^3 with no significant differences between groups ($p > 0.05$). A significant increase in blood pressure was, however, observed in PE vs. N [HIV-positive (EOPE: 177/99 ± 4.23/6.66 vs. 118/77 ± 4.71/3.66 and LOPE: 166/96 ± 4/3 vs. 128/85 ± 2.77/3.11 mm Hg) and HIV-negative (EOPE: 166/96 ± 4/3 mm Hg vs. 124/75 ± 2.11/1.56 and LOPE: 165/91 ± 3.2/5.42 vs. 123/80 ± 2/4 mm Hg) total exosomes/ml, Table 2, $p < 0.001$].

Proteinuria was significantly elevated in EOPE in comparison to LOPE (HIV-positive 466 ± 64.99 vs. 345 ± 54.21 and HIV-negative 495 ± 66.41 vs. 389 ± 60.90 mg/dl, Table 1). It is notable that the BMI of the early onset-PE group was significantly greater than the late onset-PE group [(HIV-positive: 36.80 ± 3.32 vs. 28.10 ± 2.08) and (HIV-negative: 36.10 ± 4.41 vs. 28.0 ± 1.08 $p < 0.001$)].

3.2. Isolation and characterisation of exosomes from HIV-infected and non-infected pregnant women

Exosomes were isolated using density gradient ultracentrifugation and were distinguished from HIV particles by acetylcholinesterase (AChE) activity. The fractions with a lower-density collected from the top of the iodixanol gradient were enriched for exosomes as confirmed by AChE activity (1–5, Fig. 1). Discarded HIV particles were segregated in the higher-density fractions (at the bottom of the iodixanol gradient) and were confirmed to have no AChE activity (6–12, Fig. 1). These results are in accordance with previous studies [20,21]. The presence of

Table 1
Summary of the statistical significance of all group comparisons in terms of the specific tests and analyses conducted.

Comparisons	Total and Placental-derived Exosomes			Exosomal Cytokines							
	Quantification		Ratio	Quantification(pg/ml plasma)			Ratio:Th1/Th2		Ratio:Exosomal cytokines/placental-derived exosomes		
	Total Exosomes -CD63 + (number/ml plasma)	Placental-derived Exosomes (pg/ml plasma)	Placental-derived/ total exosomes	IL-10	IL-2	TNF-α	TNF-α/IL-10	IL-2/IL-10	IL-10/Placental-derived exosomes	IL-2/Placental-derived exosomes	TNF-α/Placental-derived exosomes
HIV-positive vs. -negative N (≤ 33 weeks)	↑***	↑***	ns	↓**	↑***	ns	ns	↑***	ns	ns	ns
HIV-positive vs. -negative N (≥ 34 weeks)	↑**	↑***	ns	↓***	↑***	ns	ns	↑***	ns	ns	ns
HIV-positive vs. -negative EOPE	↑***	↓***	↓***	↓***	↑***	↑***	↑***	↑***	↑***	↑***	↑***
HIV-positive vs. -negative LOPE	↑***	↑***	↑***	↓***	↑***	ns	ns	↑***	ns	ns	ns
HIV-positive EOPE vs. N (≤ 33 weeks)	↑***	↓***	↓***	↓***	↑***	↑***	↑***	↑***	↑***	↑***	↑***
HIV-negative EOPE vs. N (≤ 33 weeks)	↑***	↑***	↓***	↓**	↑***	↑***	↑**	ns	ns	ns	ns
HIV-positive LOPE vs. N (≥ 34 weeks)	↑***	↑***	↓***	↓**	↑***	↑***	↑***	↑***	ns	ns	ns
HIV-negative LOPE vs. N (≥ 34 weeks)	↑***	↓***	↓***	↓***	↑**	↑***	↑***	ns	↑***	↑***	↑***
HIV-positive EOPE vs. LOPE	↑***	↓***	↓***	ns	↑***	↑***	ns	↓***	↑***	↑***	↑***
HIV-negative EOPE vs. LOPE	↑***	↑***	↑***	ns	↑***	ns	ns	ns	ns	ns	↓***

N = normotensive pregnancies; EOPE = early onset pre-eclampsia; LOPE = late onset pre-eclampsia; NS = no statistical significance ($p > 0.05$); ↑ = Statistically Significant Increase; ↓ = Statistically Significant Decrease.
The level of significance was defined as:
*$p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 2
Clinical Characteristics of Participants.

Variables	Normotensives				Pre-eclampsia			
	HIV-		HIV+		HIV-		HIV+	
	≤ 33 weeks (n = 30)	≥34 weeks (n = 30)	≤ 33 weeks (n = 30)	≥34 weeks (n = 30)	EOPE (n = 30)	LOPE (n = 30)	EOPE (n = 30)	LOPE (n = 30)
Age (years)	27.11 ± 1.35	27.81 ± 2.21	28.1 ± 1,56	26.21 ± 0.81	26.97 ± 1,45	26.61 ± 2.23	28.22 ± 1.88	26.46 ± 1.41
Weight (kg)	59.21 ± 4.18	68.21 ± 4.44	58.54 ± 1.15	69.42 ± 3.47	72.08 ± 5.77	82.89 ± 4.25	70.22 ± 8.54	85.99 ± 8.45
Height (cm)	158.89 ± 6.78	159.12 ± 5,32	155.74 ± 3.25	159.99 ± 6.88	160.45 ± 4.28	151.55 ± 6,25	158.21 ± 7.56	152.76 ± 4,23
BMI	23,5 ± 4.89	26.9 ± 2.22	24,89 ± 1,08	27,1 ± 2,12	28.0 ± 1.08	36.10 ± 4.41†††	28.10 ± 2.08	36.80 ± 3.32†††
Gestational Age (weeks)	32.15 ± 2.13 (26–33)	37,56 ± 2.54 (34–38)	33.12 ± 1.66 (26–33)	36 ± 1.57 (34–38)	31.58 ± 1.94 (28–33)	37.88 ± 1.65 (34–38)	31.84 ± 1.89 (28–33)	37.14 ± 2.86 (34–38)
Systolic/ diastolic blood pressure (mm Hg)	128/85 ± 2.77/3.11(90–120/50–80)	123/80 ± 2/4 (90–120/50–80)	118/77 ± 4.71/3.66(90–120/50–80)	124/75 ± 2,11/1,56 (90–120/50–80)	166/96 ± 4/3 (> 140/90)***	165/91 ± 3,2/5,42 (> 140/90)$^{\#\#\#}$	177/99 ± 4,23/6,66 (> 140/90)***	169/91 ± 5,23/7,19 (> 140/90)$^{\#\#\#}$
Urine Protein (mg/dl)	ND	ND	ND	ND	495 ± 66.41	389 ± 60.90	466 ± 64.99	345 ± 54.21
CD4 Count (cells/ mm^3)	483 ± 150	501 ± 111	489 ± 100	498 ± 121	520 ± 177	477 ± 133	509 ± 144	481 ± 135

ND = not detected; N = normotensive pregnancies; EOPE = early onset pre-eclampsia; LOPE = late onset pre-eclampsia. All values are represented by mean ± SEM. All woman were of black ethnicity. All pregnancies were singleton without intrauterine infection or any other medical condition. The patients recruited had a parity of 1 ± 1. In systolic/diastolic blood pressure early onset-PE (HIV+ & HIV-) versus N (≤33 weeks, HIV+ & HIV−) respectively.

*** $p < 0.001$ and late onset-PE (HIV+ & HIV−) versus N (≥34 Weeks, HIV+ & HIV−) respectively.

$^{\#\#\#}$ $p < 0.001$. In BMI late onset-PE (HIV+ & HIV−) versus early onset-PE (HIV+ & HIV−) respectively.

††† $p < 0.05$.

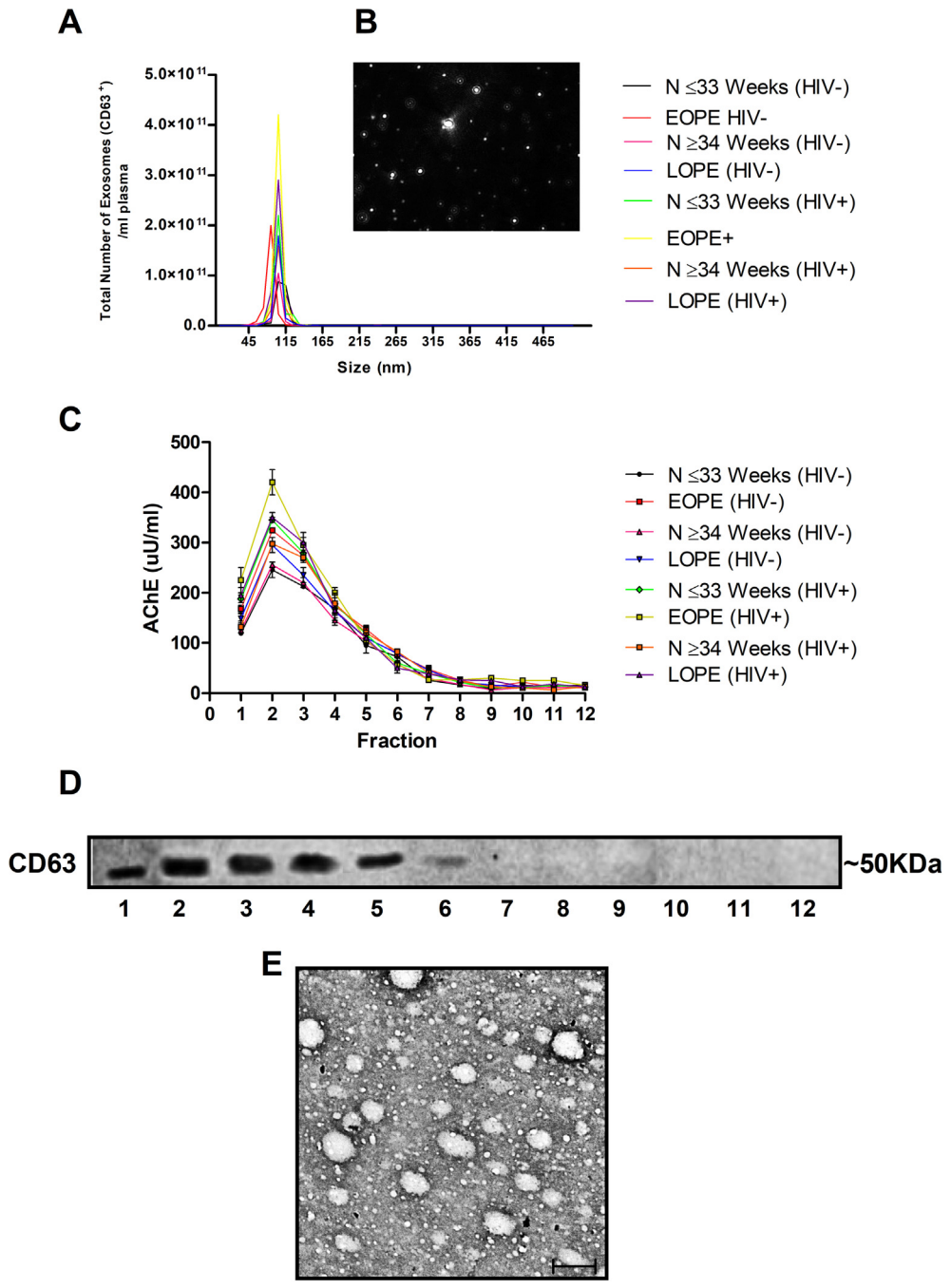


Fig 1. Isolation and Characterisation of Exosomes from HIV-infected and non-infected pregnant woman. Exosomes from preeclamptic and normotensive pregnant women were isolated and characterised. (A) Nanoparticle Tracking Analysis (NTA) illustrating the representative vesicle size distribution (nm). (B) Representative NTA video frame of the isolated exosomes. (C) Enzymatic assay for acetylcholinesterase (AChE) and (D) Western blot analysis for exosomal CD63. (E) Electron micrograph of isolated exosomes, scale bar 100 nm.

exosomes was further confirmed by western blot analysis using the CD63 exosomal marker, transmission electron microscopy and nanoparticle tracking analysis (Fig. 1). Isolated exosomes were within the accepted exosomal size range of 20–130 nm (Fig. 1).

3.3. Quantification of total and placental-derived exosomes in maternal circulation

3.3.1. Quantification of total exosomes

Significantly increased levels of total exosomes were observed in HIV-positive vs. negative normotensive pregnant women [(N ≤ 33 weeks: $9.96 \pm 0.04 \times 10^9$ vs. $6.16 \pm 0.05 \times 10^9$) and (N ≥ 34 weeks: $1.22 \pm 0.04 \times 10^{10}$ vs. $8.45 \pm 0.05 \times 10^9$) total exosomes/ml plasma, $p < 0.001$, Fig. 2A]. Similarly, a significantly higher total exosome concentration in HIV-positive in comparison to HIV-negative PE pregnant women was observed [EOPE: $3.71 \pm 0.06 \times 10^{10}$ vs. $2.73 \pm 0.01 \times 10^{10}$ and LOPE: $2.15 \pm 0.07 \times 10^{10}$ vs. $1.71 \pm 0.06 \times 10^{10}$ total exosomes/ml plasma, $p < 0.001$].

The total exosome concentration in [HIV-positive PE vs. N] and [HIV-negative PE vs. N] pregnant women was significantly higher [HIV-positive: (EOPE: $3.710 \pm 0.6 \times 10^{10}$ vs. $9.96 \pm 0.4 \times 10^9$ and LOPE: $2.15 \pm 0.05 \times 10^{10}$ vs. $1.22 \pm 0.05 \times 10^9$) and HIV-negative (EOPE: $2.273 \pm 0.05 \times 10^{10}$ vs. $6.16 \pm 0.4 \times 10^9$ and LOPE:

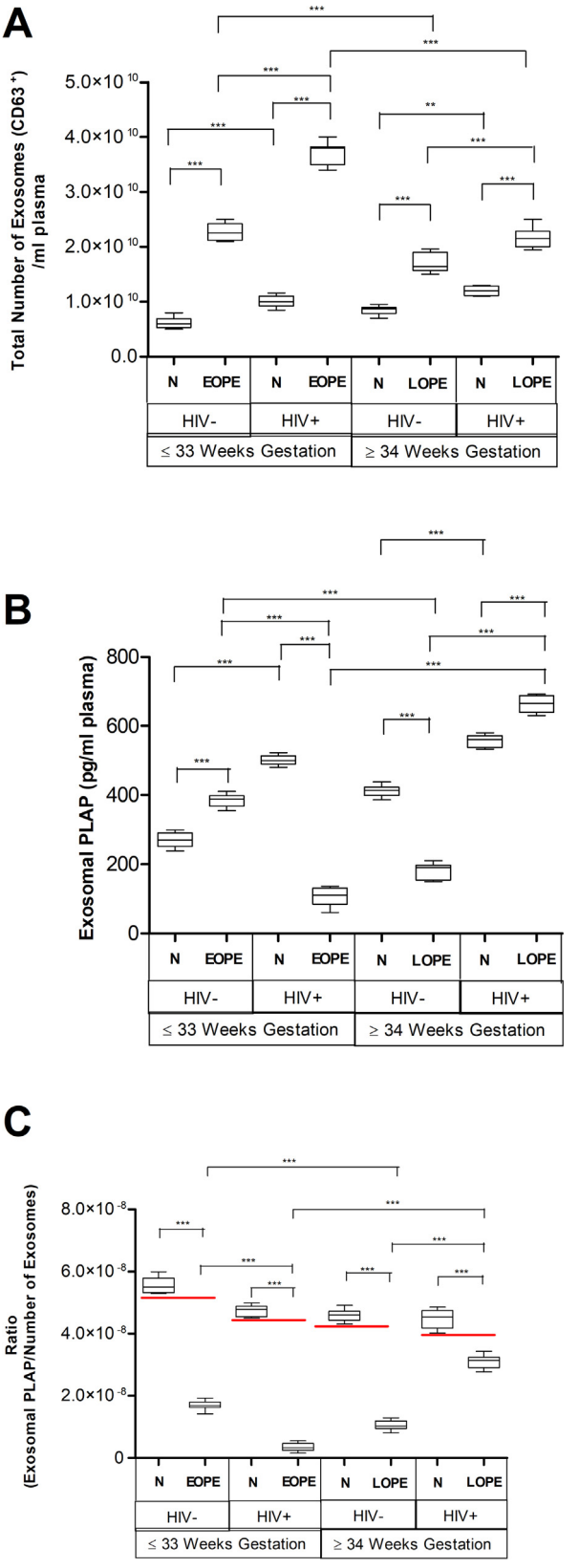


Fig 2. Quantification of total and placental-derived exosomes in maternal circulation. Total and placental-derived exosomes were quantified in HIV-positive and -negative preeclamptic and normotensive pregnant women. (A) Total exosome concentration was determined by the quantification of exosomal CD63 marker per ml of plasma using ELISA. (B) Placental-derived exosomes (i.e. exosomal placenta alkaline phosphatase (PLAP) was quantified per ml of plasma by ELISA. (C) The ratio of placental-derived exosomes ($PLAP^+$) to total exosomes ($CD63^+$). In A, B and C the horizontal bars indicate the level of significance of the difference between two groups at either end of the bar tails, *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$ using one-way ANOVA followed by Tukey post-hoc testing. Red horizontal bars indicate ROC cut-off values at a sensitivity of 100% and specificity of 90% with a positive LHR ≥ 10 (CI: 95%). Data are presented as box and whisker plots including mean and interquartile range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$1.71 \pm 0.05 \times 10^{10}$ vs. $8.45 \pm 0.05 \times 10^{9}$) total exosomes/ml plasma, $p < 0.001$].

The total exosome concentration was significantly higher in HIV-positive EOPE ($3.71 \pm 0.06 \times 10^{10}$/ml plasma) vs. LOPE ($2.15 \pm 0.07 \times 10^{10}$/ml plasma) ($p < 0.001$). Additionally, the total exosome concentration in HIV-negative EOPE ($2.27 \pm 0.08 \times 10^{10}$/ml plasma) vs. LOPE ($1.71 \pm 0.05 \times 10^{10}$/ml plasma) was significantly higher ($p < 0.001$).

3.3.2. Quantification of placental-derived exosomes

The relative concentration of the placental-derived exosomes was determined by quantifying the exosomal PLAP concentration (Fig. 2B). Placental-derived exosome concentration in HIV-positive pregnant women was significantly higher in comparison to HIV-negative normotensive pregnant women [(N ≤ 33 weeks: 557.7 ± 5.63 vs. 412.3 ± 4.91 pg/ml) and (N ≥ 34 weeks: 501.8 ± 4.48 vs. 270.4 ± 6.64) pg/ml, Fig. 2B $p < 0.001$]. Significantly increased placental-derived exosomes were observed in HIV-positive (662.8 ± 7.3 pg/ml) vs. HIV-negative (179.8 ± 7.39 pg/ml), LOPE was observed ($p < 0.001$). On the other hand, the concentration of placental-derived exosomes was significantly decreased in HIV-positive (107.5 ± 8.39 pg/ml) compared to HIV-negative EOPE (384.3 ± 6.01 pg/ml), $p < 0.001$.

Significantly increased placental-derived exosomes was observed in HIV-positive LOPE (662.8 ± 7.3 pg/ml) in comparison to N [≥ 34 weeks, (557.7 ± 5.63 pg/ml)] ($p < 0.001$). However, a significant decrease in the concentration of placental-derived exosomes in HIV-positive EOPE (107.5 ± 8.39 pg/ml) vs. N [≤ 33 weeks, (501.8 ± 4.48 pg/ml)] was observed ($p < 0.001$).Significantly increased placental-derived exosomes was observed in HIV-negative EOPE (384.3 ± 6.01 pg/ml) in comparison to N [≤ 33 weeks, (270.4 ± 6.64 pg/ml)] ($p < 0.001$). On the other hand, a significant decrease in the concentration of placental-derived exosomes in HIV-negative LOPE (179.8 ± 7.39 pg/ml) vs. N (412.3 ± 4.91 pg/ml) was observed ($p < 0.001$).

A significant decrease in placental-derived exosomes in HIV-positive EOPE (107.5 ± 8.39 pg/ml) vs. LOPE (662.8 ± 7.3 pg/ml) was observed ($p < 0.001$). We also observed a significant increase in placental-derived exosomes in HIV-negative EOPE (384.3 ± 6.01 pg/ml) vs. LOPE (179.8 ± 7.39 pg/ml) ($p < 0.001$).

3.3.3. The relative contribution of placental-derived exosomes to total exosomes in maternal circulation

The contribution of placental-derived ($PLAP^+$) exosomes to total exosomes in maternal circulation was determined by calculating the ratio of PLAP content per exosome (Fig. 2C). Significant differences in the ratio of $PLAP^+$ exosomes to total exosomes were observed (Fig. 2C). Significantly decreased ratio of $PLAP^+$ exosomes to the total number of exosomes was identified in HIV-negative EOPE ($1.69 \pm 0.04 \times 10^{-8}$) in comparison to N [≥ 33 weeks, ($5.55 \pm 0.05 \times 10^{-8}$)]. Similarly a decreased ratio of $PLAP^+$ exosomes to the total number of exosomes was identified in HIV-negative LOPE ($1.05 \pm 0.08 \times 10^{-8}$) in comparision to N [≥ 34 weeks, ($4.59 \pm 0.06 \times 10^{-8}$)], Fig. 2C, $p < 0.001$. Additionally, a significant decrease in the ratio of $PLAP^+$

exosomes per number of total exosomes in HIV-positive PE vs.N was observed [EOPE: ($3.49 \pm 0.04 \times 10^{-9}$ vs. $4.73 \pm 0.08 \times 10^{-8}$) and LOPE: ($3.09 \pm 0.08 \times 10^{-8}$ vs.$4.93 \pm 0.06 \times 10^{-8}$), Fig. 2C, $p < 0.001$].

Moreover, a significant decrease in the ratio of $PLAP^{+}$ exosomes to the total number of exosomes was identified in [HIV-positive ($3.49 \pm 0.041 \times 10^{-9}$) vs. HIV-negative ($1.69 \pm 0.04 \times 10^{-8}$) EOPE] whereas a significant increase in [HIV-positive ($3.09 \pm 0.04 \times 10^{-8}$) vs. HIV-negative ($1.05 \pm 0.04 \times 10^{-8}$) LOPE] was observed (Fig. 2C, $p < 0.001$).

A significantly decreased ratio of $PLAP^{+}$ exosomes to total exosomes between HIV-positive EOPE ($3.49 \pm 0.041 \times 10^{-9}$) vs. LOPE ($3.09 \pm 0.06 \times 10^{-8}$) was observed (Fig. 2C, $p < 0.001$). On the other hand, a significant increase in the ratio between HIV-negative EOPE ($6.40 \pm 0.1 \times 10^{-9}$) vs. LOPE ($4.59 \pm 0.6 \times 10^{-8}$) was observed (Fig. 2C, $p < 0.001$).

3.4. Quantification of exosomal cytokines

3.4.1. IL-10 (Th2 anti-inflammatory cytokine)

Significantly decreased exosomal IL-10 was observed in [HIV-positive PE vs. N, (EOPE: 48 ± 0.7 vs. 56.85 ± 1.25 and LOPE: 41.82 ± 1.4 vs. 53.02 ± 1.65) pg/ml] and [HIV-negative PE vs. N, EOPE: 56.23 ± 0.93vs.68.81 ± 1.12 and LOPE: 50.80 ± 1.25 vs. 66.59 ± 1.64) pg/ml], Fig. 3C, $p < 0.001$. Similarly, a significant decrease in exosomal IL-10 was observed in HIV-positive vs. HIV-negative normotensive pregnant women [N: ≤33 weeks (56.83 ± 1.24 vs. 63.8 ± 1.12) and N: ≥34 weeks (53 ± 1.65 vs. 66.6 ± 1.64) pg/ml, $p < 0.01$, Fig. 3A]. Moreover, a significant decrease in exosomal IL-10 in HIV-positive vs. HIV-negative PE was observed [EOPE: 48 ± 0.71 vs. 56.23 ± 0.93 and LOPE: 41.83 ± 1.48 vs 50.66 ± 1.25 pg/ml, $p < 0.001$, Fig. 3A].

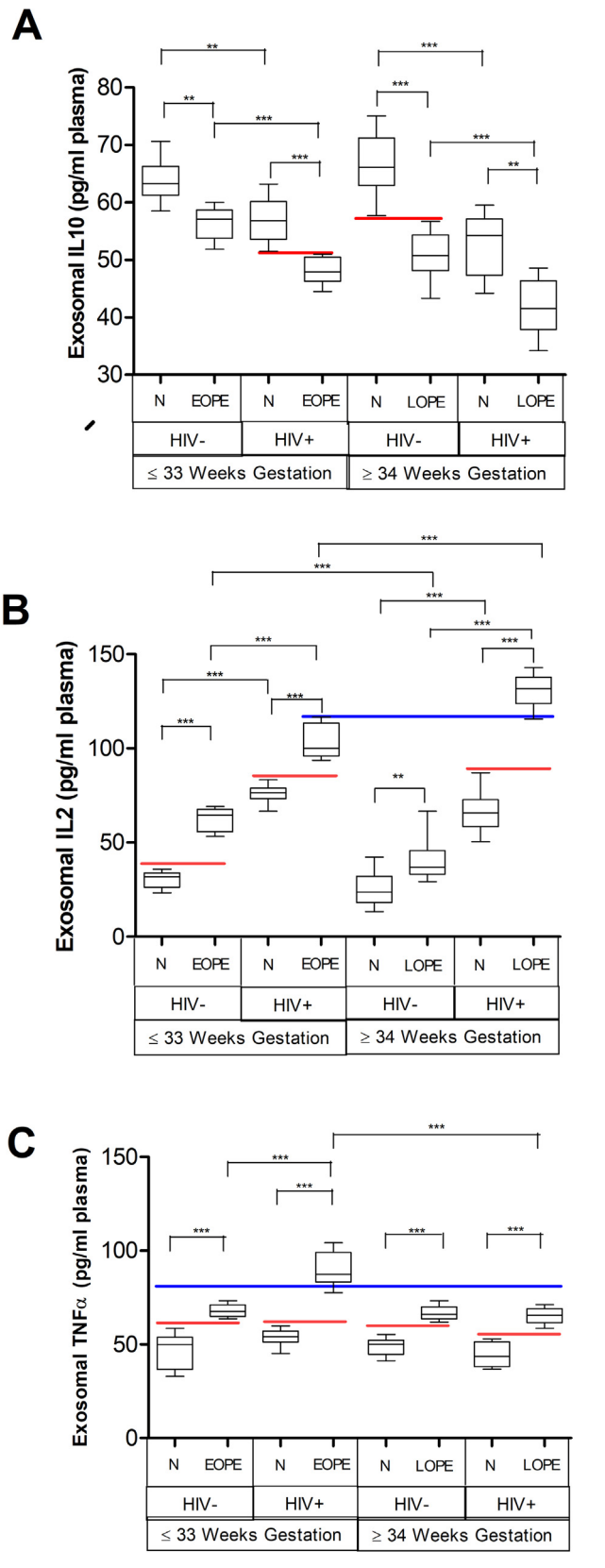
3.4.2. IL-2 and TNF-α (Th1 pro-inflammatory cytokines)

Significantly increased exosomal TNF-α was observed in HIV-positive PE vs. N, (EOPE: 90.26 ± 2.85 vs. 53.86 ± 4 and LOPE: 65.38 ± 1.4 vs. 44.71 ± 2.03 pg/ml) and HIV-negative PE vs. N, (EOPE: 67.85 ± 1.05 vs.46.37 ± 1.41 and LOPE: 66.84 ± 1.15 vs. 49.03 ± 1.41 pg/ml), Fig. 3C, $p < 0.001$. Moreover, a significant increase in exosomal TNF-α was observed in HIV-positive (90.26 ± 2.85 pg/ml) vs. HIV-negative (67.85 ± 1.05 pg/ml) EOPE, Fig. 3C, $p < 0.001$.

Significantly increased exosomal IL-2 was observed in HIV-positive vs. HIV-negative normotensive pregnant women [N ≤ 33 weeks (76.14 ± 1.45 vs. 30.70 ± 1.37) and N ≥ 34 weeks (65.87 ± 3.43 vs. 25.08 ± 2.78) pg/ml, $p < 0.01$, Fig. 3B].

Similarly, a significant increase in exosomal IL-2 in HIV-positive PE vs. N, (EOPE: 103.5 ± 2.86 vs. 76.14 ± 1.45 and LOPE: 130 ± 2.9 vs. 66 ± 3.43 pg/ml) and HIV-negative PE vs. N, (EOPE: 62.73 ± 1.91 vs. 30.70 ± 4.12 and LOPE: 40.80 ± 3.62 vs. 25.08 ± 2.78 pg/ml) was observed, Fig. 3B, $p < 0.01$. Moreover, a significant increase in exosomal IL-2 in HIV-positive vs. HIV-negative PE was observed [EOPE: 103.5 ± 2.86 vs. 62.73 ± 1.91, $p < 0.001$ and LOPE: 130.7 ± 2.9 vs. 40.8 ± 3.62 pg/ml, Fig. 3B, $p < 0.01$].

Significantly increased exosomal IL-2 was observed in HIV-negative EOPE (62.73 ± 1.91 pg/ml) in comparision to LOPE (40.8 ± 3.62 pg/ml), Fig. 3B, $p < 0.001$. Moreover, a significant increase in exosomal TNF-α in HIV-positive EOPE (90.28 ± 2.85 pg/ml) in comparison to LOPE (65.38 ± 1.40 pg/ml) was observed, Fig. 3C, $p < 0.001$. On the other hand, a significant decrease in exosomal IL-2 was observed in HIV-positive EOPE (103.5 ± 2.86 pg/ml) in comparision to LOPE (130.7 ± 2.9 pg/ml), Fig. 3B, $p < 0.001$.



(caption on next page)

3.5. Th1/Th2 exosomal cytokine ratio in maternal circulation

3.5.1. TNF-α (Th1)/IL-10 (Th2)

The ratio of exosomal TNF-α (Th1) to IL-10 (Th2) in maternal

Fig 3. Quantification of Exosomal Cytokines in maternal circulation. Exosomal (A) IL-10, (B) IL-2 and (C) TNF-α was quantified in HIV-positive and -negative preeclamptic and normotensive pregnant women (pg/ml plasma). Horizontal bars indicate the level of significance of the difference between two groups at either end of the bar tails, *p < 0.01, **p < 0.001, ***p < 0.0001 using one-way ANOVA followed by Tukey post-hoc testing. Data are presented as box and whisker plots including mean and interquartile range. In B and C the red horizontal lines indicate the significant ROC cut-off values in distinguishing between experimental and control groups whereas the blue horizontal line distinguishes the significant ROC cut-off values between HIV-positive EOPE and LOPE at a sensitivity of 100% and a specificity of 90% with a positive LHR ≥ 10 (CI: 95%). No significant cut-off values between HIV-positive EOPE and LOPE were observed (p > 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

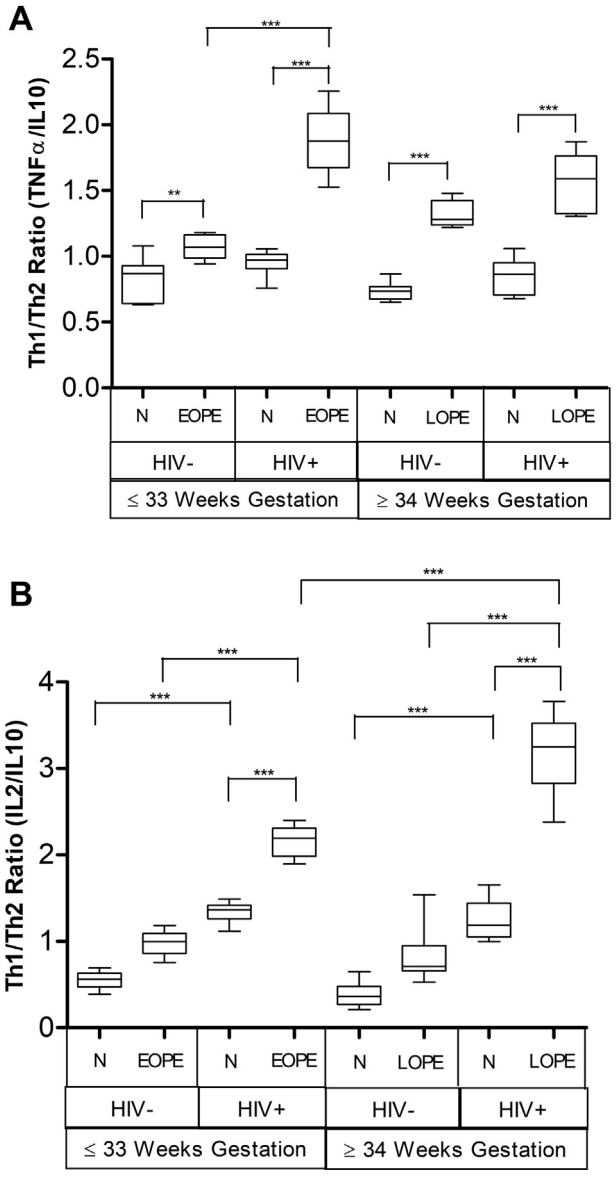


Fig 4. Th1/Th2 ratio analysis of exosomal cytokines. The Th1/Th2 ratio of exosomal (A) TNF-α/IL10 and (B) IL2/IL10 and was determined in HIV -positive and -negative preeclamptic and normotensive pregnant women. Horizontal bars indicate the level of significance of the difference between two groups at either end of the bar tails, *p < 0.01, **p < 0.001, ***p < 0.0001 using one-way ANOVA followed by Tukey post-hoc testing. Data are presented as box and whisker plots including mean and interquartile range.

circulation was calculated (Fig. 4A). A significantly increased exosomal TNF-α/IL-10 ratio was observed in HIV-positive PE vs. N (EOPE: 1.88 ± 0.1 vs. 0.95 ± 0.03, p < 0.01, and LOPE: 1.58 ± 0.07 vs. 0.85 ± 0.04, p < 0.001) and HIV-negative PE vs. N (EOPE: 1.07 ± 0.03 vs. 0.82 ± 0.05 and LOPE: 1.32 ± 0.03 vs. 0.74 ± 0.02, pg/ml, Fig. 4A, p < 0.01). Similarly, a significant increase in exosomal TNF-α/IL-10 ratio was observed in HIV-positive (1.88 ± 0.1, pg/ml) incomparision to HIV-negative PE (1.07 ± 0.03 pg/ml) EOPE, Fig. 4A, p < 0.001. No significant intragroup associations were observed (p > 0.05).

3.5.2. IL-2 (Th1)/IL-10 (Th2)

The ratio of exosomal IL-2 (Th1) to IL-10 (Th2) in maternal circulation was calculated (Fig. 4B). A significantly increased exosomal IL-2/IL-10 ratio was observed in HIV-positive vs. HIV-negative normotensive pregnant women [N ≤ 33 weeks (1.34 ± 0.03 vs. 0.54 ± 0.03) and N ≥ 34 weeks (1.25 ± 0.07 vs. 0.38 ± 0.04) pg/ml, p < 0.01, Fig. 3B].

Significantly increased exosomal IL-2/IL-10 ratio was observed in HIV-positive in comparison to HIV-negative pregnant women [EOPE: (2.16 ± 0.06 vs. 1.0 ± 0.04) and LOPE: (3.16 ± 0.13 vs. 0.82 ± 0.1), Fig. 4B, p < 0.001]. Moreover, a significant increase in the exosomal IL-2/IL-10 ratio in HIV-positive PE in comparision to N was observed [EOPE: 2.16 ± 0.06 vs. 1.34 ± 0.03, and LOPE: 3.16 ± 0.13 vs. 1.25 ± 0.07 pg/ml, Fig. 4B, p < 0.001)]. On the other hand a significant decrease in the exosomal IL-2/IL-10 ratio in HIV-positive EOPE vs. LOPE was observed (2.16 ± 0.06 vs. 3.16 ± 0.13, Fig. 4B, p < 0.001).

3.6. The relative contribution of exosomal cytokines to placental-derived exosomes in maternal circulation

A significant increase in the ratio of exosomal cytokines to PLAP$^+$ exosomes was observed in HIV-positive in comparison to HIV-negative EOPE [IL-10/PLAP: (0.47 ± 0.04 vs. 0.14 ± 0.01), IL-2/PLAP: (1.02 ± 0.08 vs. 0.16 ± 0.01) and TNF-α/PLAP: (0.9 ± 0.09 vs. 0.17 ± 0.01), pg/ml, Fig. 5, p < 0.001]. Similarly, a significant increase in the ratio in HIV-positive EOPE vs. N (≤33 weeks) was observed [IL-10/PLAP: (0.47 ± 0.04 vs. 0.11 ± 0.01), IL-2/PLAP: (1.02 ± 0.08 vs. 0.15 ± 0.01) and TNF-α/PLAP: (0.9 ± 0.09 vs. 0.1 ± 0.01), pg/ml, Fig. 5, p < 0.001]. Moreover, a significant increase in the ratio in HIV-negative LOPE vs. N (≥34 weeks) was observed [IL-10/PLAP: (0.29 ± 0.01 vs. 0.16 ± 0.01), IL-2/PLAP: (0.22 ± 0.02 vs. 0.06 ± 0.005) and TNF-α/PLAP: (0.38 ± 0.02 vs. 0.12 ± 0.01), pg/ml, Fig. 5, p < 0.001].

Significantly increased ratio of exosomal cytokines to PLAP$^+$ exosomes in HIV-positive EOPE incomparision to LOPE was observed [IL-10/PLAP: (0.47 ± 0.05 vs. 0.06 ± 0.001), IL-2/PLAP: (1.02 ± 0.08 vs. 0.2 ± 0.01) and TNF-α/PLAP: (0.9 ± 0.09 vs. 0.09 ± 0.005), Fig. 5, p < 0.001]. On the contrary, a significantly decreased ratio of exosomal TNF-α to PLAP$^+$ exosomes in HIV-positive EOPE in comparison to LOPE was observed [TNF-α/PLAP: (0.18 ± 0.01 vs. 0.37 ± 0.02), Fig. 5, p < 0.001].

3.7. Diagnostic value of exosomes in preeclampsia in HIV-Positive and HIV-Negative pregnant women

3.7.1. Total and placental-derived exosomes

The ratio of placental derived to total exosomes suggests diagnostic potential in determining PE but it cannot differentiate between the subtypes. The ratio predicted PE in HIV-positive and -negative pregnancies with a cut-off value of 4.51×10^8 at a sensitivity of 100% (95% CI: 69.15–100.0%) and specificity of 90% (95% CI: 55.50–99.75%) with a positive LHR of 10.

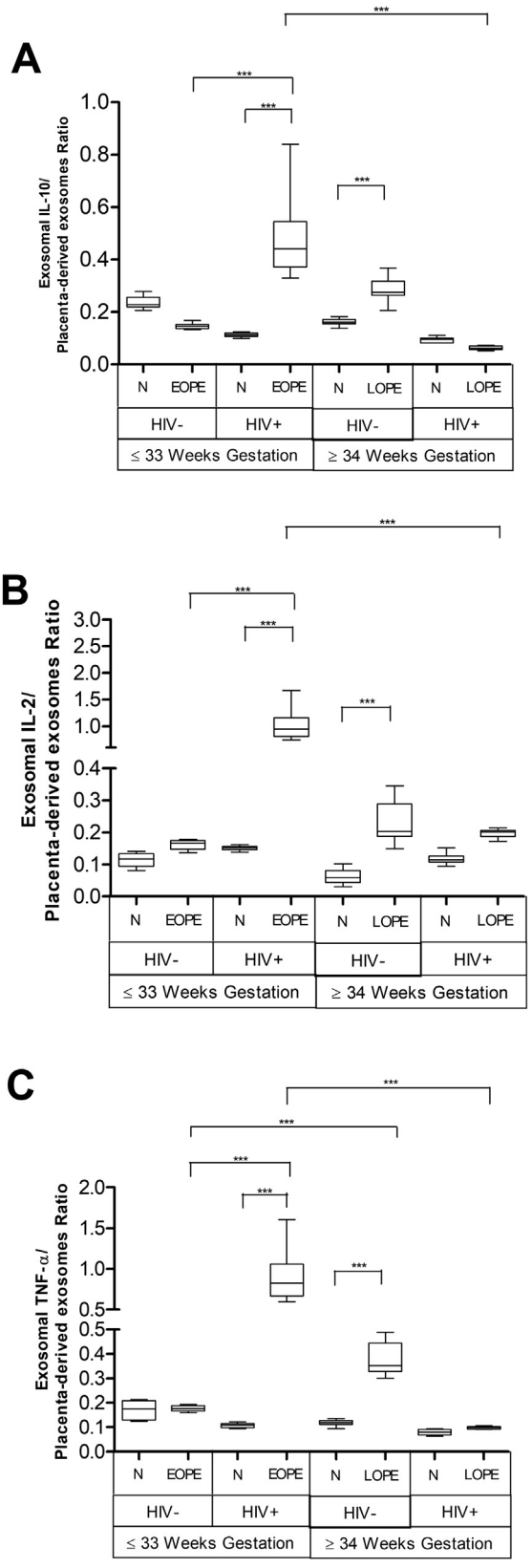


Fig 5. The relative contribution of exosomal cytokines to placental-derived exosomes in maternal circulation. The ratio of (A) IL-10/placental-derived exosomes, (B) IL-2/placental-derived and (C) TNF-α/placental-derived exosomes from maternal circulation was determined in HIV -positive and -negative preeclamptic and normotensive pregnant women. Horizontal bars indicate the level of significance of the difference between two groups at either end of the bar tails, **$p < 0.001$, ***$p < 0.0001$ using one-way ANOVA followed by Tukey post-hoc testing. Data are presented as box and whisker plots including mean and interquartile range.

3.7.2. Exosomal cytokines

It is important to note that exosomal IL10, Th1/Th2 cytokine ratios & exosomal cytokine/placental-derived exosome ratios do not indicate any strong application in diagnosing PE in comparison to controls (LHR < 10). However individual exosomal cytokines IL-2 and TNF-α showed promising potential in diagnosing PE (Table 3).

3.7.2.1. IL-2. Exosomal IL-2 levels predicted PE in the HIV-positive EOPE, HIV-positive LOPE and HIV-negative EOPE in comparison to their controls with cut-off values of 82, 36 and 83 pg/ml respectively at a sensitivity of 100% (95% CI: 69.15–100.0%) and specificity of 90% (95% CI: 55.50–99.75%) with a positive LHR of 10. Additionally, it was only able to differentiate EOPE from LOPE in the HIV-positive patient cohort with a cut-off value of 117 pg/ml at a sensitivity of 100% (95% CI: 69.15–100.0%) and specificity of 90% (95% CI: 55.50–99.75%) with a positive LHR of 10.

3.7.2.2. TNF-α. Exosomal TNF-α levels predicted PE in the HIV-positive EOPE, HIV-positive LOPE, HIV-negative EOPE and HIV-negative LOPE in comparison to their controls with cut-off values of 59, 53, 58 and 52 pg/ml respectively at a sensitivity of 100% (95% CI: 69.15–100.0%) and specificity of 90%(95% CI: 55.50–99.75%) with a positive LHR of 10. Additionally, it was only able to differentiate EOPE from LOPE in the HIV-positive patient cohort with a cut-off value of 79 ~~117~~ pg/ml at a sensitivity of 100% (95% CI: 69.15–100.0%) and specificity of 90% (95% CI: 55.50–99.75%) with a positive LHR of 10.

4. Discussion

The present study demonstrates alterations in levels of total exosomes, placental-derived exosomes and exosomal cytokines (IL-10, IL-2 and TNF-α) in preeclamptic pregnant women as well as HIV-positive preeclamptic pregnant women on HAART. The findings obtained support the probable role of exosomes in the pathogenesis of preeclampsia and preeclampsia in HIV-positive women on HAART. Importantly, these findings support the clinical application of exosomes in diagnosing PE and PE in HIV-positive pregnant woman.

4.1. Total and placental-derived exosomes and their relative contribution

The significant increase in total and placental-derived exosomes observed in PE which is consistent with our previous study where we associated this finding with the pathophysiology of PE and its subtypes [23]. Moreover, the observations made in this study indicated a similar increase in HIV-positive pregnant woman on HAART. This could possibly be attributed to the enhanced maternal immune response induced by the HAART reconstituted maternal immune system.

We also observed a significantly lower ratio of placental derived to total exosomes in PE. Since, exosomes are known to regulate foeto-maternal immune tolerance in normal pregnancy [24], this may be a consequence of the foeto-maternal immune imbalance which negatively affects placental development. Moreover, the significantly lower ratio of placental-derived to total exosomes in HIV-positive EOPE suggests that HAART may be responsible for the augmentation of the more severe clinical outcomes associated with EOPE in HIV-positive pregnant women on HAART. The implication is that HAART immune reconstitution may contribute to the foeto-maternal immune maladaptation, contributing to the improper placental development, which culminates in the clinical manifestation of PE.

Table 3
The sensitivity and specificity of exosomal cytokines in preeclamptic and HIV-positive preeclamptic pregnant woman.

Exosomal Cytokine	Comparison	Cut-off Value	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood Ratio
IL10	**HIV-positive**						
	EOPE vs. N	> 51	100	69.15–100.0%	90	55.50–99.75%	10
	LOPE vs. N	> 49	70	34.75–93.33%	90	55.50–99.75%	7
	EOPE vs. LOPE	< 45	70	34.75–93.33%	90	55.50–99.75%	7
	HIV-negative						
	EOPE vs. N	> 60	90	55.50–99.75%	90	55.50–99.75%	9
	LOPE vs. N	> 56	100	69.15–100.0%	90	55.50–99.75%	10
	EOPE vs. LOPE	< 52	60	26.24–87.84%	90	55.50–99.75%	6
IL2	**HIV-positive**						
	EOPE vs. N	> 82	100	69.15–100.0%	90	55.50–99.75%	10
	LOPE vs. N	> 83	100	69.15–100.0%	90	55.50–99.75%	10
	EOPE vs. LOPE	> 117	100	69.15–100.0%	90	55.50–99.75%	10
	HIV-negative						
	EOPE vs. N	> 36	100	69.15–100.0%	90	55.50–99.75%	10
	LOPE vs. N	< 44	70	34.75–93.33%	90	55.50–99.75%	7
	EOPE vs. LOPE	< 82	90	55.50–99.75%	80	44.39–97.48%	4.5
TNF-α	**HIV +**						
	EOPE vs. N	> 58	100	69.15–100.0%	90	55.50–99.75%	10
	LOPE vs. N	> 52	100	69.15–100.0%	90	55.50–99.75%	10
	EOPE vs. LOPE	< 79	100	69.15–100.0%	90	55.50–99.75%	10
	HIV −						
	EOPE vs. N	> 59	100	69.15–100.0%	90	55.50–99.75%	10
	LOPE vs. N	> 53	100	69.15–100.0%	90	55.50–99.75%	10
	EOPE vs. LOPE	< 64	30	6.674–65.25%	90	55.50–99.75%	3

N = normotensive pregnancies; EOPE = early onset pre-eclampsia; LOPE = late onset pre-eclampsia.

4.2. Quantification of pro- and anti-inflammatory exosomal cytokines

4.2.1. IL-2 and TNF-α (Th1 pro-inflammatory cytokines)

The increase in exosomal IL-2 and TNF-α observed in PE and in PE in HIV-positive pregnant women on HAART may contribute to the systemic pro-inflammatory state commonly associated with PE pathophysiology [25]. Moreover, the significant increase in exosomal IL-2 in HIV-negative EOPE vs. LOPE suggest that exosomal IL-2 may contribute to the more severe clinical symptoms associated with EOPE. These results imply that exosomes may be directly involved in the pathophysiology of PE since increased IL-2 and TNF- α expression has been associated with endothelial cell injury [26,27].

A significant increase in exosomal IL-2 and TNF-α was observed in HIV-positive preeclamptic women on HAART. These proinflammatory cytokines are known to have a role in maintaining $CD4^+$ T cell proliferation and differentiation [28,29] we therefore speculate that the dysregulation of exosomal proinflammatory cytokines occurs due to the HAART reconstituted maternal immune system, whereby the improper restoration of specific T-cell memory phenotypes result in immune activation [30,30–32]. This could further enhance maternal immune activation in response to the allogenic foetus, subsequently leading to the immune pathogenesis associated with PE. Importantly, from these findings we postulate that the link between the pathogenesis of PE and HAART-induced maternal immune reconstitution occurs through an exosomal microenvironment, which modulates immune activity and enhances the potency of pro-inflammatory cytokines in maternal circulation.

4.2.2. IL-10 (Th2 anti-inflammatory cytokine)

The significant decrease in exosomal IL-10 observed in PE may be reflective of the role of exosomes in the immune pathogenesis of PE [33]. IL-10 is considered a master regulator of immunity in pregnancy and sufficient levels are required to suppress active maternal immunity to allow for the acceptance of the foetal allograft [34]. Reports indicate that extracellular vesicle IL-10 encapsulation is representative of approximately 100% of circulating IL-10 [13]. We therefore hypothesise that the decreased exosomal encapsulation of IL-10 in PE promotes a pro-inflammatory phenotype, resulting in an immune and angiogenic imbalance [35], which leads to endothelial dysfunction and subsequent hypertension, organ damage and proteinuria [36].

Similarly, we identified a decrease in exosomal IL-10 in HIV-positive PE women on HAART. This may occur as a result of inappropriate immune reconstitution, whereby, decreased levels of memory $CD4^+$ T cells and increased reconstitution of naïve $CD4^+$ T-cells commonly associated with HAART [32,37], alter the immune microenvironment, which contributes to the pathogenesis of PE.

4.2.3. Th1/Th2 ratio analysis of exosomal cytokines in maternal circulation

In order for the mother to tolerate the semi-allogenic foetus a shift towards Th2 immunity is essential to achieve positive obstetric outcomes [3], whereas Th1 dominant immunity results in subsequent PE and foetal abortion [4]. Therefore, the Th1/Th2 cytokine ratio is an indicator of the normal or compromised immune state of pregnancy. In this study, exosomal Th1/Th2 cytokine ratios (TNF-α/IL-10 and IL-2/IL-10) in all normotensive pregnancies favoured a Th2 type immunity, thereby signifying the role of exosomes in maternal-foetal tolerance in HIV-positive and HIV-negative normotensive pregnancies. On the other hand, the increased exosomal Th1/Th2 ratios (TNF-α/IL-10 and IL-2/IL-10) in PE, indicate a shift towards Th1 immunity which is commonly associated with exaggerated inflammatory responses in PE. These findings support the possible role of exosomes in mediating foeto-maternal immune balance in normal pregnancy, and the immune dysregulation in PE.

Importantly the significantly higher exosomal Th1/Th2 ratios in HIV-positive PE woman on HAART indicate an exosome-mediated Th1 skewness, which may be as result of the overactive maternal immune system to foetal antigens, caused by disproportionate maternal immune reconstitution by HAART [9]. Although the exact mechanisms involved in immune reconstitution remain unknown, we speculate that the HIV proviral latent state may contribute to the irregular activation of the maternal immune system through a network of exosomal cytokines which control viral replication.

4.2.4. Ratio of exosomal cytokines to placental-derived exosomes

The significant increase in the ratio of exosomal cytokines to

placental-derived exosomes observed in HIV-positive vs. HIV-negative EOPE suggests that the more severe outcomes commonly associated with preeclamptic pregnant women on HAART [2,38] may be related to the maternal immune maladaptation caused by HAART and not due to its toxicity. Moreover, the increase in the ratio in HIV-positive EOPE vs. LOPE, suggests that circulating exosomes may reflect the variations in placental and maternal immune-related stress response pathways between the sub-types in HIV-positive pregnant woman on HAART. From these findings we hypothesise that, irrespective of the comorbidity, EOPE and LOPE occur due to differences in their eitopathogensis which clarifies their differences in severity.

4.3. Diagnostic value

4.3.1. The ratio of placental-derived to total exosomes

It is important to note that total or placental-derived exosomes independently do not possess clinical diagnostic utility (LHR < 10), which is possibly due to their diverse roles in modulating immune responses in normal pregnancy and PE. However, the ratio of placental-derived to total exosomes suggests potential diagnostic application in determining PE in HIV-positive and HIV-negative pregnant women.

4.3.2. Ant-inflammatory cytokines

The data analysis showed no clinical significance in the potential of IL-10 (LHR < 10).

4.3.3. Pro-inflammatory cytokines

Pro-inflammatory IL-2 and TNF-α have shown potential clinical significance in diagnosing EOPE and LOPE in HIV-positive women on HAART (CI: 95%, LHR > 10, sensitivity of 100% and specificity of 90%). More importantly, we demonstrate the potential clinical utility of exosomal TNF-α in diagnosing PE in both HIV-positive and -negative pregnant women with a definitive distinction between EOPE and LOPE in only HIV-positive pregnant women on HAART (CI: 95%, LHR > 10, sensitivity of 100% and specificity of 90%).

4.4. Limitations

Due to ethical considerations, untreated HIV-positive pregnant women could not be included in this study. Future *in-vivo* and *in-vitro* functional studies would also aid in elucidating the biological mechanism of action of exosomes in the pathophysiology of PE in HIV-positive pregnant women. A larger prospective observational study together with validation strategies is underway to qualify exosomal cytokines as candidate biomarkers in PE (non-infected) and PE in HIV-positive pregnant women on HAART in early pregnancy.

5. Conclusion

This study investigated the potential use of exosomes and its associated cytokines as biomarkers of PE and PE in HIV/HAART. The findings from this study highlight the immune-related pathophysiological implications of HAART immune reconstitution on pregnancy outcomes, which may be modulated by exosomes. Moreover, the observed alterations in the Th1/Th2 ratio in HIV/HAART pregnancies complicated with EOPE and LOPE implicates exosomes as central facilitators of PE and HIV pathogenesis. Importantly, we have shown that exosomal pro-inflammatory cytokines have promising clinical application in diagnosing preeclampsia and preeclampsia in HIV-positive pregnant women on HAART.

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Author contributions

P.P, K.M, I.M, M.V and J.M conceptualised the study, designed the experiments and performed the data analysis and interpretation. P.P and K.M performed the testing and analysis. P.P, K.M, I.M, M.V and J.M wrote the manuscript. All authors reviewed and edited the manuscript.

Declaration of Competing Interest

The authors report no conflicts of interests in this work

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5. Chapter Five: Synthesis

5.1 Synthesis

The scientific understanding of disease pathogenesis involves the integration of information from numerous, complex physiological systems. Preeclampsia, a debilitating pregnancy-related disorder, is complicated by its unknown etiopathogenesis [95]. Therefore, identification of definitive, early biomarkers of preeclampsia is a challenging area of research. Exosomes have been reported to have numerous roles in normal pregnancy *per se*, especially with regard to immunomodulation [70], endothelial function [96] and placental development [97]. However, exosomes have also been implicated in abnormal pregnancies. In particular, we were the first to have reported altered levels of total and placental-derived exosomes in early and late onset preeclampsia [11], suggesting their involvement in the pathophysiology of preeclampsia which formed the rationale for the evaluation of exosomes as biomarkers of preeclampsia. Therefore the data presented in this study are a progression from our initial findings with the aim of providing evidence further supporting the potential use of exosomes as biomarkers of PE in compliance with FDA criteria.

In publication one **(Chapter Two)**, the theoretical 'context of use' of exosomes as biomarkers of PE was developed, based on the FDA Biomarker Framework [92]. From the perspective of the current state of PE biomarkers, we critically reviewed and evaluated the potential biomarker application of exosomes based on the following criteria: (1) Can exosomes in preeclampsia be isolated and quantified using standardised methods from maternal circulation? (2) Are exosomes distinctly dysregulated in preeclampsia in comparison to normotensive pregnancies? and (3) Do exosomes contain specific and distinct molecular markers, which can improve biomarker sensitivity and specificity for the disease in question? From this review, it was evident that exosomes do have potential in diagnosing preeclampsia which formed the basis for our subsequent experiments.

We have shown that exosomes can be isolated and quantified in maternal circulation, however, this criterion alone does not justify their use as biomarkers of preeclampsia. Therefore, publication two **(Chapter 3)** subsequently focused on the role of exosomal miRNA in the pathophysiology of preeclampsia. We and others have reported altered levels of microRNAs

in the pathophysiology of preeclampsia [63, 98]. However, these miRNAs do not qualify as biomarkers of preeclampsia due to their low diagnostic sensitivity and specificity [63, 99], which may be attributed to the unknown origin of the miRNA in maternal circulation.

Current methods of miRNA quantification apply indirect techniques reliant on the amplification of target genes due to the small amounts of starting miRNA, which lowers the sensitivity and specificity of the assay. Furthermore, we proposed that the exosomal origin of the miRNA would enhance the accuracy and precision of diagnosis, since these nanovesicles are involved in key cellular processes. The analysis of exosomal miRNA is not a straightforward process due to the small amounts of starting nucleic acid, and this is compounded by the available molecular technology. Therefore, the current conventional methods of analysis would not be viable, because in the modern era of precision medicine one requires precise and rapid analytical strategies for easy and accurate clinical application. NanoString technologies have emerged as a promising platform for the detection of exomiRNA due to its digital precision, enhanced sensitivity, reproducibility and technical robustness, using small amounts of starting RNA, hence there is no requirement for further validation by another method [100]. Additionally, the Nanostring technology does not utilise any amplification processes, which therefore enhances the dynamic range of the assay thus enabling a more accurate representation of the miRNA expression levels. Therefore, our current study has provided a sound rationale for the involvement of exosomal miRNA in the pathophysiology of PE using the sensitive Nanostring technology platform for a survey of the entire profile and status of exomiRNA in PE. Using Descriptive Intermediate Attributed Notation for Ada (DIANA) (Web Server 5.0) computational merging and meta-analytical algorithms we uncovered distinct exomiRNA signatures which are involved in key pathophysiological processes associated with preeclampsia; which includes inflammation, cell migration, cell invasion, angiogenesis, apoptosis, vasoconstriction, lipid metabolism and blood pressure homeostasis. Furthermore, the significantly altered expression of specific exomiRNA in preeclampsia indicates their direct involvement in dysregulating key biological processes such as platelet activation, apoptosis, cellular stress responses, blood coagulation, cardiomyocyte signaling, catabolic processes and gene expression; all of which are instrumental in the symptoms observed in preeclampsia. Interestingly, distinct exomiRNA differences were observed between early- and late- onset preeclampsia. This illustrated the clinical differences in severity between the subtypes based on their biological pathway enrichment analysis. These

findings suggest that exosomes may well be part of the basis for the systemic manifestation of preeclampsia, which supports the qualification of exosomes as biomarkers of preeclampsia. Importantly, the distinct exomiRNA signatures identified serve as candidate biomarkers of the disease.

The FDA biomarker definition specifies that biomarkers of disease must be validated with other prevalent clinical disorders associated with the increased susceptibility/risk of the disease (i.e. co-morbid states) [92, 101]. In South Africa, there is a high prevalence of the co-morbid state of HIV and preeclampsia, and importantly there have been reports of an increased risk of preeclampsia in HIV/HAART pregnant woman (all HIV infected pregnant women in this study were on HAART as per the Center for Disease Control and Prevention guidelines, 2016) [3, 93, 102]. Therefore to satisfy the FDA biomarker criterion we have included the treated HIV cohort to validate the biomarker potential of exosomes.

In Chapter four **(manuscript 3)** we explored the biomarker potential of exosomes in the two cohorts namely preeclampsia and HIV positive preeclamptic women on HAART. Notably, both HIV and preeclampsia represent hyperinflammatory states and circulating cytokines are key mediators of these states. Extracellular vesicle cytokine encapsulation represents a powerful system which may explain the dysfunctional immune responses involved in pathological states such as PE. Exosomal cytokines were selected for two reasons (1) both HIV and preeclampsia are immune-related disorders and (2) a large percentage of cytokines were found to be encapsulated in extracellular vesicles (viz. 70%-IL-2, 50% TNF-α and 100% IL-10) [103]. This suggests that exosomal cytokine encapsulation may be a regulated property of the immune system, which is yet to be fully understood. Although there is a substantial amount of literature supporting the general role of cytokines in preeclampsia [104] and HIV [105], there is no scientific evidence linking exosomal cytokine encapsulation in HIV-positive women on HAART and in preeclampsia. The quantification of exosomal IL-2, TNF-α (pro-inflammatory) & IL-10 (anti-inflammatory), was selected for investigation since these cytokines are known to have a significant immuno-modulatory role in the pathophysiological state of both HIV and preeclampsia [106]. Our data indicate that exosomal IL-2 and TNF-α levels are increased, whereas IL-10 levels are decreased in preeclampsia and in HIV-positive preeclamptic women on HAART, suggesting the immuno-modulatory role of exosomes in both

disease states. The ratio between these pro- and anti- inflammatory exosomal cytokines indicates a predominant shift towards Th1 (pro-inflammatory) immunity in preeclampsia which implicates exosomes as factors involved in the exaggerated MSIR commonly associated with the clinical manifestation of preeclampsia. Furthermore, we speculate that the maternal immune reconstitution by HAART may be responsible for the increase in exosomal pro-inflammatory cytokines which contribute to the pathogenesis of preeclampsia. Importantly, we show that exosomal TNF-α and IL-2 have potential in diagnosing preeclampsia and preeclampsia in HIV-positive women on HAART with high sensitivity and specificity (CI: 95%, LHR > 10, sensitivity of 100% and specificity of 90%). Our findings suggest that exosomal cytokine quantification may be a better indication or marker of the pathogenesis of preeclampsia and preeclampsia in HIV-positive women on HAART when compared to non-specific, non-exosomal quantification of cytokines in maternal circulation.

Even though the presented research supports the use of exosomes as biomarkers of preeclampsia further validation in a larger patient cohort is required. Furthermore, these studies are limited because we are unable to pinpoint the specific cellular origin of the exosomal miRNA and cytokines identified. Therefore it becomes challenging to understand the diverse exosome-mediated cellular interactions involved in the pathophysiology of preeclampsia. Additionally, we could not include untreated HIV-positive women in this study due to ethical considerations. Furthermore, intricate *in vivo* and *in vitro* functional studies would also aid in elucidating some of the exact biological mechanisms of exosomes in the pathophysiology of preeclampsia and preeclampsia in HIV-positive pregnant women.

5.2 Conclusion

The findings presented have identified novel exomiRNA signatures and exosomal cytokine levels in PE, which are involved in key biological processes associated with the pathology of the disease. The altered exosomal cytokine levels in preeclampsia and HIV/HAART highlights the pathophysiological implications of HAART immune reconstitution on pregnancy outcomes, which may be modulated by exosomes. Importantly the discovery of distinct exomiRNA signatures and exosomal cytokines in early- and late- onset preeclampsia support the existence of a differential pathophysiology between the subtypes. Therefore, the

observations made in this study support the diagnostic value of exosomal cytokine and miRNA signatures as biomarkers of preeclampsia and its subtypes.

5.3 Recommendations

Our proposed future studies will seek to enhance the scientific understanding of exosomes in the pathogenesis of preeclampsia in order to determine its potential context of use as biomarkers in the clinical environment. Therefore, our future research will focus on developing biomarker technology. This will involve a comprehensive approach in qualifying exosomes as a biomarker of preeclampsia which should subsequently lead to the submission of a qualification package to the relevant regulatory authorities for approval of exosomes for the clinical diagnosis of preeclampsia.

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7. Appendices

7.1 Human Ethics Approval

UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 260-4609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

09 February 2018

Mr P Pillay (210533252)
Discipline of Human Physiology
School of Laboratory and Medical Sciences
210533252@stu.ukzn.ac.za

Protocol: Determine the role of nanoparticles in the pathogenesis of preeclampsia.
Degree: MMedSc
BREC reference number: BE310/15 (*sub-study of BE036/12*).

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	26 October 2017
Expiration of Ethical Approval:	25 October 2018

I wish to advise you that your application for Recertification dated 30 January 2018 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.

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 approval will be ratified by a full Committee at a meeting to be held on 13 March 2018.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc: supervisor: mackie@ukzn.ac.za
cc: postgrads: shalinie.thar@ukzn.ac.za

7.2 Hospital Approval

health

Department:
Health
PROVINCE OF KWAZULU-NATAL

PRINCE MSHIYENI MEMORIAL HOSPITAL
ACTING MEDICAL MANAGER OFFICE
DR. A.Hussain
Private Bag X07, Mobeni 4060
Mangosuthu Highway
Tel.:031-907 6317/ 6304
Fax.:031- 906 1044
Email:: Akdahussain@kznhealth.gov.za
www.kznhealth.gov.za

Enquiry: Dr A.Hussain
Ref No: 27/RESH/2105

TO: Mr Preenan Pillay

RE: LETTER OF SUPPORT TO CONDUCT RESEARCH AT PMMH

Dear Sir

I have pleasure to inform you that PMMH has considered your application to conduct research on **"Determine the Role of Nanoparticles in the Pathogeneses of Preeclampsia"** in our institution.

Please note the following:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Please ensure this office is informed before you commence your research.
4. The institution will not provide any resources for this research.
5. You will be expected to provide feedback on you finding to the institution.

Should the following requirements be fulfilled, a Permission/ Approval letter will follow.

- Full research protocol, including questionnaires and consent forms if applicable.
- Ethical approval from a recognized Ethic committee in South Africa

Thank you.

Dr. A.Hussain
Acting Medical Manager & Special in Family Medicine
MBBS,PGDip in HIV/AIDS (Natal), DCH ADV Health Management
M.Med.Fam.Med (Natal)

7.3 South African Department of Health Approval

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Physical Address: 330 Langalibalele Street, Pietermaritburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3189/ 3123 Fax: 033 394 3782
Email: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

DIRECTORATE:
Health Research & Knowledge Management

Reference: 285/15
NHRD: KZ_2015RP36_802

Dear Mr P. Pillay
Email: ppillay01@gmail.com

Re: Approval of research

1. The research proposal titled **'Determine the Role of Nanoparticles in the Pathogenesis of Preeclampsia'** 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. Make the necessary arrangement with the identified facility before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted 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

Dr E Lutge
Chairperson, Health Research Committee
Date: 19/10/15

Fighting Disease, Fighting Poverty, Giving Hope

7.4 Placental exosomes and pre-eclampsia: Maternal circulating levels in normal pregnancies and, early and late onset pre-eclamptic pregnancies.

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Placental exosomes and pre-eclampsia: Maternal circulating levels in normal pregnancies and, early and late onset pre-eclamptic pregnancies

Preenan Pillay [a], Niren Maharaj [d], Jagidesa Moodley [c], Irene Mackraj [b, *]

[a] Discipline of Human Physiology, School of Basic Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
[b] Nelson R Mandela School of Medicine, School of Laboratory Medicine & Medical Sciences, University of KwaZulu-Natal, Durban, South Africa
[c] Women's Health and HIV Research Group, University of KwaZulu-Natal, Durban, South Africa
[d] Prince Mshiyeni Memorial Hospital, Department of Obstetrics and Gynaecology, Durban, South Africa

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ABSTRACT

Introduction and aim: Exosomes are a subtype of extracellular vesicle (20–130 nm) released by biological cells under normal and pathological conditions. Although there have been reports of circulating exosomes in normal pregnancy, the relevance of placental-derived exosomes in normal and abnormal pregnancies still needs to be elucidated. The aim of this study was to quantify total and placental-derived exosomes in maternal plasma from normal (N), early onset- and late onset-preeclampsia (PE).
Method: Plasma samples were obtained from pregnant women in the third trimester, for the isolation of exosomes by differential ultracentrifugation. Total exosomes were quantified using nanoparticle tracking analysis and immuno-reactive exosomal CD63 quantification. Placental-derived exosomes were quantified using placental alkaline phosphatase (PLAP) as a specific marker. The contribution of placental-derived exosomes to total exosomes in maternal plasma was determined by the ratio of $PLAP^+$ exosomes to $CD63^+$ exosomes.
Results: The concentration of total exosomes significantly increased in early onset-PE and late onset-PE compared to N (≤33 weeks) and N (≥34 weeks). The relative concentration of placental-derived exosomes significantly increased in early onset-PE but decreased in late onset-PE compared to N. The ratio of $PLAP^+$ exosomes to total number of exosomes significantly decreased in early onset-PE and late onset-PE. A positive correlation between total and placental-derived exosomes were obtained in N (≤33 weeks: Pearson's $r = 0.60$, ≥34 weeks: Pearson's $r = 0.67$) and early onset-PE (Pearson's $r = 0.51$, $p < 0.05$) with the inverse in late onset-PE (Pearson's $r = -0.62$, $p < 0.01$).
Conclusion: The differences in the contribution of placental-derived exosomes to total exosomes in maternal circulation suggests a possible pathophysiological role of placental-derived exosomes in pre-eclampsia.

1. Introduction

Pre-eclampsia (PE) is a hypertensive pregnancy disorder unique to human pregnancies. It is defined as new onset hypertension (systolic blood pressure of ≥140 mm Hg and diastolic blood pressure of ≥90 mm Hg), proteinuria (≥300 mg) [1]. Pre-eclampsia is reported to occur in approximately 5–7% of pregnant woman globally [2] and is a major cause of maternal and neonatal morbidity and mortality [3].

The recent classification of hypertensive disorders of pregnancy, recommends that the categorization of PE is categorised into mild to moderate PE, severe preeclampsia, eclampsia and early onset-PE & late onset-PE [1]. Early onset-PE is associated with greater risks of maternal and fetal complications than late onset-PE [4–6]. The distinction in severity between early onset-PE and late onset-PE may reflect the differences in the etiopathogenesis of the subtypes of PE which adds a new dynamic to a disorder with existing complexities. The exact etiology of PE is not known, however, the

* Corresponding author.
E-mail address: mackraji@ukzn.ac.za (I. Mackraj).

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pathophysiology is thought to be a two-stage disorder originating in the placenta [7]. The first stage is theorised to be due to defective trophoblastic invasion of the uterine spiral arterioles, which leads to decreased uteroplacental blood flow and a reduction in oxygenation [7]. Subsequently, the second stage is one in which decreased cellular oxygenation results in the release of proangiogenic factors and an imbalance between pro- and anti-angiogenic factors leading to widespread endothelial damage and the clinical manifestations of PE [7–10]. The placenta, therefore, plays an important role in the pathophysiology of PE. Both early onset-PE and late onset-PE are of placental origin with early onset-PE believed to be superimposed on underlying medical conditions such as diabetes [11,12]. Therefore the severity of placental changes and maladaptation in the uterine spiral arterioles may be greater than that which occurs in late onset-PE.

The aetiology of preeclampsia still remains unknown due to the multifactorial nature of the disorder. Angiogenic, antiangiogenic, *cff*DNA, *cf*DNA, proteins and vasoactive factors have been implicated in the pathogenesis PE [13–15]. More recently, studies have identified syncytiotrophoblast microparticles (STBM) as factors that are immune-stimulatory, antiangiogenic, pro-coagulant and involved in endothelial dysfunction, a key pathological feature of preeclampsia [16–18]. Syncytiotrophoblast microparticles are directly released from the placenta into maternal circulation and consist of vesicles ranging from 20 to 3000 nm in size [19].

Exosomes are a key constituent of STBMs which fall within the smaller size spectrum of 20–130 nm and have a latent role in fetal-maternal immune tolerance and endothelial cell migration [20–22]. Exosomes are formed as products of the lysosomal pathway and secreted by most cell types due to the fusion of intracellular multivesicular bodies with the plasma membrane. These vesicles consist of a bi-lipid membrane containing a variety of signalling molecules including cell adhesion molecules and growth factor receptors [23]. In addition, exosomes contain mRNA and miRNA [24], which are involved in immune function [21,25,26]. Exosomes function primarily by communicating with adjacent or distal cells to re-programme their phenotype and regulate cell function [24]. Reports suggest that placental-derived exosomes play a role in the regulation of immune tolerance during normal and complicated pregnancies [21,23–26]. The variations in the contribution of placental-derived exosomes and bioactivity in normal pregnancies are suggestive of the immune regulatory role of exosomes in a successful pregnancy. The probable role of placental-derived exosomes is to regulate the activity of both proximal and distal target cells, translation, angiogenesis, proliferation, metabolism, and apoptosis [21,27–31].

Exosomes may have a potential role as activating agents in endothelial dysfunction, a hallmark of PE [30]. It has been established that total and placental-derived exosomes with no associated pathologies are biologically active molecules with a regulatory role in endothelial cell migration [27]. Normally, placental exosomes in maternal circulation increase with gestational age which inversely correlates to endothelial cell migration [27]. In the third trimester of pregnancy, however, the lower contribution of placental-derived exosomes in relation to total exosomes contributes to the inhibition of endothelial cell migration [27].

Ex-vivo and *in-vitro* studies have been used to examine enriched preparations (supraphysiological levels) of placental exosomes from placental perfusates [31]. However, the relevance of placental-derived exosomes in pathological pregnancies has not yet been elucidated. Therefore the aim of this study was to determine the levels of placental-derived exosomes from maternal plasma in normal, early onset-PE and late onset-PE pregnancies.

2. Materials and methods

2.1. Ethics statement

Regulatory ethical and institutional approval were obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE310/15), South Africa.

2.2. Study group and samples

Normotensive (N), early onset-PE and late onset-PE patients, in the third trimester were recruited (n = 15 per group). Normotensive patients were classified by a blood pressure of 120 ± 10/80 ± 5 (systolic/diastolic mm Hg) and absent proteinuria as detected by a rapid urine dipstick test (Markomed®, South Africa). Normotensive patients were matched for gestational age according to early onset-PE and late onset-PE patients. Early onset pre-eclampsia was defined by new onset hypertension (diastolic blood pressure of ≥90 mm Hg and systolic blood pressure of ≥140 mm Hg) and proteinuria (≥300 mg) at < 33 weeks plus 6 days of gestation. Late onset-pre-eclampsia was defined by new onset hypertension (diastolic blood pressure of >90 mm Hg and systolic blood pressure of >140 mm Hg) and proteinuria (≥300 mg) at > 34 weeks gestational age. All patients had singleton pregnancies and those with evidence of any infections or medical, surgical or other obstetric complications were excluded. Blood samples were collected [BD Vacutainer Tubes (EDTA), Becton Dickinson and Company, South Africa] and the plasma samples were stored at −80 °C for analyses [27,28].

2.3. Isolation and purification of exosomes from maternal circulation

Exosomes were isolated according to the method described by Théry et al., 2006 (Fig. 1) [32]. Plasma (1 ml) was diluted with an equal volume of phosphate buffered saline (PBS; pH 7.4). Exosomes were isolated and purified by differential ultracentrifugation using a 30% sucrose cushion. In brief, centrifugation was initially performed at 2000 × g at 4 °C for 30 min, followed by 12 000 × g at 4 °C for 45 min. The supernatant was centrifuged at 110 000 × g at 4 °C for 120 min (Optima™ MAX-XP Ultracentrifuge, fixed angle MLA-55 rotor, Beckman Coulter Inc., Brea, CA, USA). The pellet was suspended in PBS and filtered through a 0.22 μm filter (Cellulose acetate, GVS™, Europe). The filtrate was centrifuged at 110 000 × g at 4 °C for 70 min, the pellet re-suspended in PBS (pH 7.4) and centrifuged at 110 000 g for 70 min, 4 °C. The exosome pellet was suspended in 1 ml of PBS and subsequently purified using a 30% sucrose cushion as described by Théry et al., 2006 [32]. The final pellet was resuspended in 100 μl of PBS and stored at −80 °C. The exosomal protein concentration was determined as described by Salomon et al. (2014) using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) [27].

2.4. Nanoparticle tracking analysis

Quantification and size distribution of exosomes were determined using the NS500 equipped with a 405 nm laser and sCMOS camera (NanoSight NTA 3.0 Nanoparticle Tracking and Analysis Release, Version Build 0069). Samples were diluted with PBS prior to analysis in order to obtain particle distribution of 10 and 100 particles per image (optimal, 50 particles per image) before the analysis with NTA system. Samples were introduced into the sample chamber using the following script: PUMPLOAD, REPEAT-START, PRIME, DELAY 10, CAPTURE 60, REPEAT 5. Videos were recorded at a camera level of 10, camera shutter speed of 20 ms and

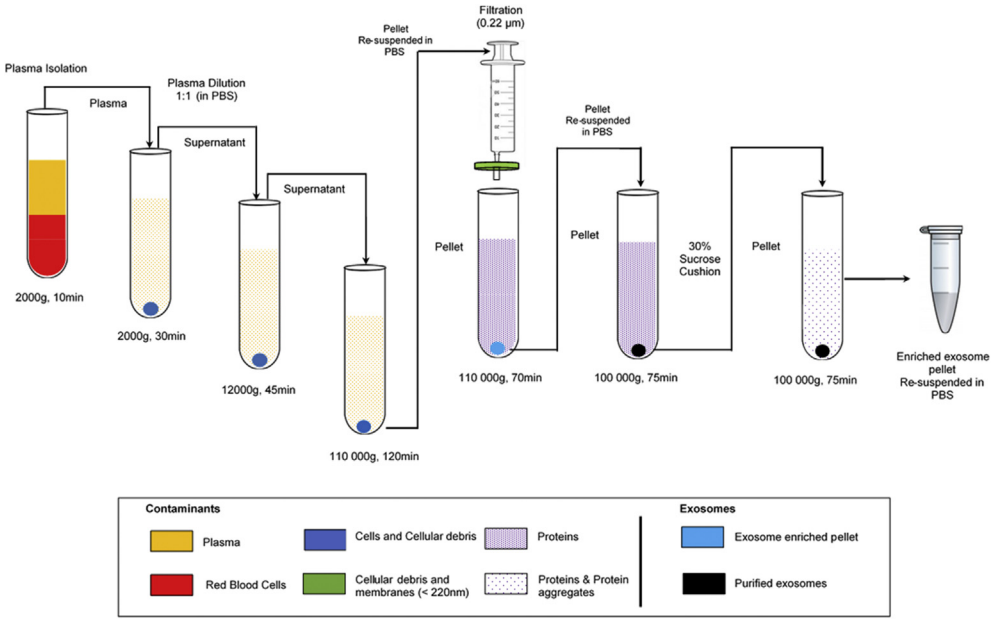

Fig. 1. Flow chart for the exosome purification. Procedure used is based on differential ultracentrifugation methods as described by Théry et al., 2006. The figure illustrates the differential centrifugal steps followed for the removal of contaminants and isolation of purified exosomes.

camera gain of 600, these settings were kept constant between samples. Each video was then analysed to give the mean particle size together with the concentration of particles. The size of the exosomes was represented as the mean particle size ± SD.

2.5. Quantification of placental-derived exosomes

The concentration of total exosomes in the maternal circulation was determined by the quantification of total immunoreactive CD63 enzyme-linked immune absorbency assay (ExoELISA™, System Biosciences, Mountain View, CA), as described by Salomon et al. (2014) [27]. CD63 is not an exosome specific marker but is commonly bound to the exosomal membrane, and hence the method employed uses isolated and purified exosomes which contain the CD63 marker. The kit used consists of an exosome specific primary CD63 antibody developed by the manufacturer. Briefly, exosomes were immobilised on microtiter plates overnight at 37 °C using exosome binding buffer supplied the manufacturer (System Biosciences). Plates were washed and incubated at room temperature for 1 h with exosome specific primary antibody (CD63), followed by a wash step and incubation with secondary antibody (1:5000) at RT for 1 h with agitation. Plates were thereafter washed and incubated with Super-sensitive TMB ELISA substrate at RT for 45 min with agitation. The reaction was terminated using Stop Buffer solution. Absorbance was measured at 450 nm. The number of exosomes/ml, (ExoELISA™ kit) was obtained using an exosomal CD63 standard curve that was generated using the calibrated exosome standard that was supplied.

The relative concentration of placental-derived exosomes was determined by the quantification of human placental alkaline phosphatase in the exosomal fraction using a commercial ELISA kit (Elabscience, E-EL-H1976, WuHan, P.R.C), as described by Salomon et al. (2014) [27]. Briefly, exosomes were allowed to bind to the primary PLAP specific antibody coated plates by incubation at 37 °C for 90 min. Plates were washed and 50 µl of HRP-conjugate was added to each well and incubated at 37 °C for 20 min. Plates were washed and incubated with 50 µl of substrate A and 50 µl of substrate B at 37 °C for 15 min. The incubation was terminated using 50 µl of stop solution at RT for 2 min under agitation. Absorbance was measured at 450 nm. Exosomal PLAP was expressed as pg/ml plasma. The quantification of PLAP in the exosomal fraction indicates the relative concentration of placental-derived exosomes ($PLAP^+$ exosomes) in maternal circulation.

2.6. Western blotting

Exosome pellets isolated from the normal pregnant and pre-eclamptic plasma samples were lysed with RIPA buffer (1X) at room temperature for 5 min and the protein content determined using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Laemmli buffer (2X) was added to the samples and incubated at 95 °C for 5 min 20 µg of exosomal protein per well was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Polyvinylidene fluoride membranes were probed with primary antibody, CD63 (ExoAB, System Biosciences, Mountain View, CA). Post incubation at room temperature for 1 h membranes was washed in Tris buffer saline (pH 7.6) and incubated for 1 h at room temperature with the exosome validated secondary conjugated goat rabbit horseradish peroxidase antibody (ExoAB, System Biosciences, Mountain View, CA). Membranes were incubated with chemiluminescent substrate and visualised using the ChemiDoc® Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Transmission electron microscopy

Exosomes were applied to a continuous carbon grid and negatively stained with 2% uranyl acetate. The size and morphology of the particles were examined using a JEOL 1010 transmission electron microscope (JEOL, Peabody, MA, USA) at the Electron Microscopy Unit, University of KwaZulu-Natal.

2.8. Statistical analysis

The data is presented as mean ± SEM, with n = 15 per patient

group. Differences in total exosomes, total exosomal protein and PLAP$^+$ exosome concentration between N (≤33 weeks), N (≥34 weeks), early onset-PE and late onset-PE groups were determined using ANOVA with posthoc analyses (Tukey-Kramer). Pearson's correlation was used to assess the relationship between placental-derived exosomes and total exosomes in N (≤33 weeks), N (≥34 weeks), early onset-PE and late onset-PE groups. Statistical analysis was performed using Prism 6 (GraphPad Inc, CA, USA). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Patient clinical data

Table 1 shows the clinical characteristics of the study groups. The N (≤33 weeks), N (≥34 weeks), early onset-PE and late onset-PE groups had a gestational age of 30.8 ± 2.63, 38 ± 1.7, 30.55 ± 2.68 and 36.73 ± 2.18 weeks respectively (Table 1). Proteinuria was evident in early onset-PE (460 ± 82.80 mg/dl) and late onset-PE (300 ± 75.59 mg/dl). As expected, a significant increase in blood pressure was observed when early onset-PE (152/96 ± 12/6 mm Hg) and late onset-PE (155/98 ± 13/9 mm Hg) were compared to the N groups (≤33 weeks: 110/67 ± 6.32/9.29, ≥34 weeks: 111/68 ± 10/7, $p < 0.05$, Table 1) respectively. However, there was no significance between the early onset-PE and late onset-PE groups. It is notable that the BMI of the early onset-PE group was significantly greater than the late onset-PE group (31.14 ± 5.66, $p < 0.05$).

3.2. Exosome isolation and characterisation

Fig. 2A and B shows the size distribution of the exosome-enriched fraction viz. N ≤ 33 weeks (102.9 ± 12.16 nm), N ≥ 34 weeks (100.3 ± 7.78 nm), early onset-PE (101.8 ± 7.68 nm) and late onset-PE (104.1 ± 7.65 nm). A particle diameter of ~100 nm was obtained from electron microscopic analysis (Fig. 2C). The exosome-enriched fraction was analysed for the presence of CD63 exosomal marker by western blot (Fig. 2D). Nanoparticle tracking analysis (NTA) of exosomes demonstrated a significant increase in total exosome concentration in early onset-PE and late onset-PE (Fig. 3A). Total exosomes in early onset-PE ($8.23 \pm 1.59 \times 10^9$ total exosomes/ml) and late onset-PE ($6.14 \pm 1.45 \times 10^9$ total exosomes/ml) were significantly higher than total exosomes in N (≤33 weeks: $5.75 \pm 2.27 \times 10^8$ total exosomes/ml, $p < 0.05$) and N (≥34weeks: $3.88 \pm 0.23 \times 10^{10}$ total exosomes/ml, $p < 0.05$) respectively. In addition, the total exosome concentration in early onset-PE was significantly higher in comparison to late onset-PE ($8.23 \pm 1.59 \times 10^9$ *vs* $6.14 \pm 1.45 \times 10^9$ total exosomes/ml, Fig. 3A $p < 0.05$). Plasma exosomal protein concentration was expressed as mg of exosomal protein per ml of plasma (Fig. 3B). Plasma exosomal protein concentration in the early onset-PE (2.39 ± 0.11 mg exosomal protein/ml plasma) and late onset-PE (2.03 ± 0.14 mg exosomal protein/ml plasma) group was significantly higher in comparison to N (≤33 weeks 0.82 ± 0.04 mg exosomal protein/ml plasma, $p < 0.05$) and N (≥34 weeks:1.83 ± 0.11 mg exosomal protein/ml plasma, $p < 0.05$) respectively. However protein concentration in late onset-PE was not significantly different from early onset-PE (2.03 ± 0.14 *vs* 2.39 ± 0.11 mg exosomal protein/ml plasma, Fig. 3B $p > 0.05$).

3.3. Quantification and contribution of placental-derived exosomes to total exosomes from maternal plasma

In order to determine the relative contribution of placental exosomes to total exosomes present in maternal plasma, the PLAP content per exosome was determined. The concentration of PLAP$^+$ exosomes in maternal plasma is represented in Fig. 4. A significant increase in PLAP$^+$ exosome concentration in comparison to N (≤33 weeks) was observed in early onset-PE (84 ± 10.61 *vs* 300 ± 14.00 pg/ml, $p < 0.05$). However, we found significantly decreased PLAP$^+$ exosome concentration in late onset-PE compared to N (≥34 weeks: 112 ± 7.52 *vs* 261 ± 10.28, $p < 0.05$). In addition, a significant decrease in late onset-PE PLAP$^+$ exosomes was obtained in comparison to early onset-PE (112 ± 7.52 pg/ml *vs* 300 ± 14, $p < 0.05$).

The contribution of PLAP$^+$ exosomes to total exosomes was determined by calculating the ratio of PLAP content per exosome (Fig. 5B). The total number of exosomes was quantified using an ELISA kit for the quantification of the exosomal marker CD63 (Fig. 5A). The total number of exosomes increased in early onset-PE ($1.31 \pm 0.007 \times 10^{10}$ total exosomes/ml) and late onset-PE ($1.09 \pm 0.004 \times 10^{10}$ total exosomes/ml) in comparison to N (≤33 weeks: $5.76 \pm 0.006 \times 10^9$ total exosomes/ml, $p < 0.05$) and N (≥34 weeks: $9.18 \pm 0.09 \times 10^9$ total exosomes/ml, $p < 0.05$) respectively. A significant decrease in the ratio of PLAP$^+$ exosomes per number of total exosomes's were identified (Fig. 5B) in early onset-PE ($2.25 \pm 0.13 \times 10^{-8}$) and late onset-PE ($1.04 \pm 0.12 \times 10^{-8}$) in comparison with and N (≤33weeks: $3.54 \pm 0.31 \times 10^{-8}$, $p < 0.05$) and N (≥34 weeks: $2.93 \pm 0.13 \times 10^{-8}$, $p < 0.05$) respectively.

3.4. Relationship between total exosomes and placental-derived exosomes

Fig. 6 represents the relationship between the concentration of total exosomes and exosomal PLAP concentration (PLAP$^+$ exosomes) in the study groups. The relationship between the concentration of total exosomes and PLAP$^+$ exosomes in early onset-PE and late onset-PE was examined by the determination of the correlation of PLAP$^+$ exosomes (exosomal PLAP pg/ml plasma) per

Table 1
Clinical characteristics of patients.

Variables	Normotensives		Pre-eclampsia	
	≤33 weeks (n = 15)	≥34 weeks (n = 15)	Early onset (n = 15)	Late-onset (n = 15)
Age (years)	27.13 ± 4.63	25.46 ± 5.85	26.93 ± 6.02	26.42 ± 6.66
Weight (kg)	68.51 ± 9.51	69.93 ± 11.56	75.47 ± 15.98	81.85 ± 19.21
Height (cm)	157.26 ± 9.46	157.66 ± 8.69	161.9 3 ± 6.9	161.4 ± 7.16
BMI	27.4 ± 3.24	28.14 ± 3.66	26.8 ± 4.21	31.14 ± 5.66†
Gestational Age (weeks)	30.8 ± 2.63 (26–33)	38 ± 1.7 (34–38)	30.55 ± 2.68 (28–33)	36.73 ± 2.18 (34–39)
Systolic/diastolic blood pressure (mm Hg)	110/67 ± 6.32/9.29 (90-120/50-80)	111/68 ± 10/7 (90-120/50-80)	152/96 ± 12/6 (>140/90)***	155/98 ± 13/9 (>140/90)$^{\#\#\#}$
Urine Protein (mg/dl)	ND	ND	460 ± 82.80	300 ± 75.59

ND = not detected; N = normotensive pregnancies; early onset-PE = early onset pre-eclampsia; late onset-PE = late onset pre-eclampsia. All values are represented by mean ± SEM. All pregnancies were singleton without intrauterine infection or any other medical condition. The patients recruited had a parity of 1 ± 1. In systolic/diastolic blood pressure early onset-PE versus N (≤ 33 weeks), $^{***}p<0.001$ and late onset-PE versus N (≥ 34 Weeks), $^{\#\#\#}p<0.001$. In BMI late onset-PE versus early onset-PE, $^{\dagger}p < 0.05$.

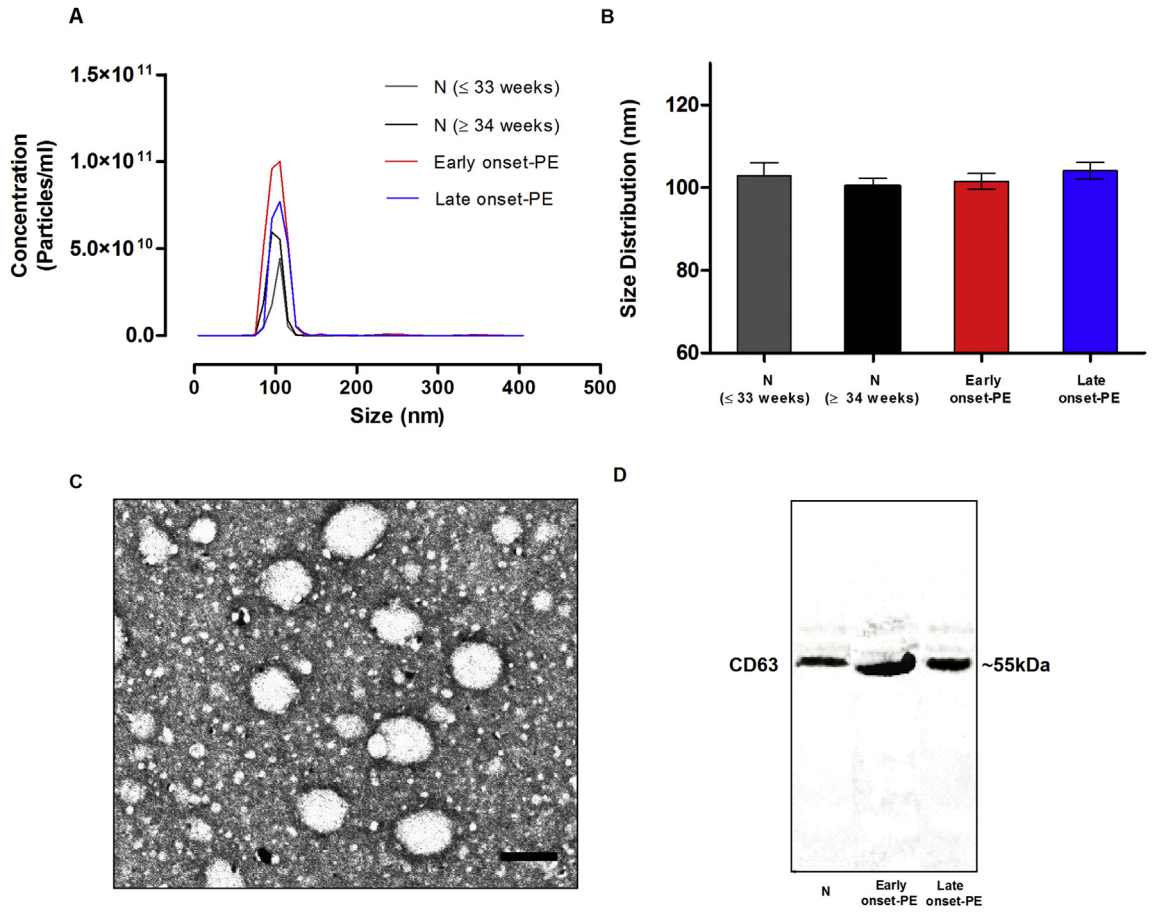

Fig. 2. Characterisation of Exosomes from Maternal Circulation. Exosomes from normotensive pregnant woman and from those with early onset-PE and late onset-PE was characterised using Nanoparticle Tracking Analysis. (A) Representative vesicle size distribution (nm). (B) Vesicle size distribution (C) Electron micrograph of isolated exosomes, scale bar 100 nm. (D) Western blot analysis for the CD63 exosome enriched marker; lane 1: early onset-PE, lane 2: late onset-PE and lane 3: N. Values are presented as mean ± SEM.

total number of exosome vesicles. In the N groups a positive correlation was obtained (≤33 weeks: Pearsons r = 0.60, ≥34 weeks: Pearsons r = 0.67, $p < 0.05$; Fig. 6A and B respectively) which corresponds to the positive correlation in early onset-PE but not in the late onset-PE group. A positive correlation was obtained between PLAP$^+$ exosomes (pg/ml plasma) and total number of exosomes/ml plasma (total exosomes/ml plasma) in early onset-PE (Pearson's r = 0.51, $p < 0.05$; Fig. 6C) with a negative correlation in late onset-PE (Pearson's r = −0.62, $p < 0.05$; Fig. 6D). The correlation analysis indicates that PLAP$^+$ exosomes increase with the increasing total number exosomes in N (≤33 weeks & ≥ 34 weeks) and early onset-PE but decreases in late onset-PE.

4. Discussion

Studies have shown that exosomes increase in maternal circulation during normal pregnancy. However, there is a lack of information on exosomes in early onset-PE and late onset-PE [27,28]. Exosomes, with a mean particle size distribution of 104.5 ± 11.68 nm, were measured across sample groups using NTA. Furthermore, western blot analysis of isolated vesicles was shown to be positive for the CD63 exosomal marker. Characterisation data obtained in the present study was positive for exosomes and is consistent with previously published data [21,27–30,32]. In the present study, we found increased total exosomes in early onset-PE (~55–60%) together with increased PLAP$^+$ exosomes (~25–30%, early onset-PE) compared to N (≤33 weeks). However, whilst the total exosomes in late onset-PE increased (~45% *vs* N ≥ 34 weeks), we found a decrease in PLAP$^+$ exosomes compared to N (≥34 weeks) and early onset-PE groups (~50% *vs* N ≥ 34 weeks and ~61% *vs* early onset-PE). Notably, these findings may account for the differences in the severity between the two sub-types of PE. This study, therefore, provides preliminary data in support for the potential use of exosomes as a biomarker in PE.

In our study, we found that the concentration of PLAP$^+$ exosomes increased in early onset-PE but decreased in the late onset-PE group in comparison to N (≤33 weeks) and N (≥34 weeks) respectively. The decrease in PLAP$^+$ exosomes in late onset-PE could be due to the differences in the etiology of late onset-PE in comparison to early onset-PE. In addition, correlation analysis between PLAP$^+$ and total exosomes indicates a significant positive correlation in early onset-PE (Pearson's r = 0.51, $p < 0.05$) and N (≤33 weeks: Pearson's r = 0.60, $p < 0.05$) and N (≥34 weeks: Pearson's r = 0.67, $p < 0.05$), with a negative correlation in late onset-PE (Pearson's r = −0.62, $p < 0.05$). These differences could be attributed to the differences in the pathophysiology between early onset-PE and late onset-PE. This may provide a useful tool to distinguish the two subtypes of PE and may assist in clinical decision making in the timing of childbirth in PE.

It has been reported that placental-derived exosomes promote vasculogenesis and angiogenesis, which are essential for placental vasculature development, proper fetal circulation [23,27,28] as well as immune modulation [21]. It is, therefore, possible that the increase in PLAP$^+$ exosomes in early onset-PE in comparison to N

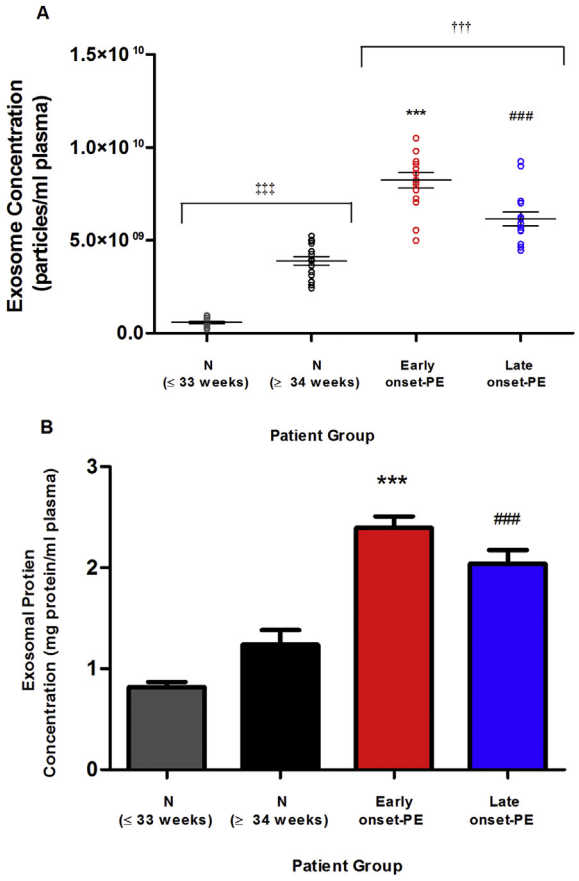

Fig. 3. Exosome concentration in early and late-onset pre-eclampsia. Isolated exosomes were quantified in normotensive pregnant woman and those with early onset-PE and late onset-PE pregnancies using NTA. (A) Number of exosomes per patient group. Data is presented as aligned dot plot and values are mean ± SD. (B) Plasma exosomal protein concentration is expressed as mg exosomal protein per ml of plasma. Data is presented as aligned dot plot and values are mean ± SEM. In A and B $^{***}p < 0.0001$ early onset-PE vs N (≤33 weeks), $^{\#\#\#}p < 0.0001$ late onset-PE vs N (≥34 weeks); In A $^{\dagger\dagger\dagger}p < 0.001$ early onset-PE vs late onset-PE, $^{\ddagger\ddagger\ddagger}p < 0.0001$ N (≤33 weeks) vs N (≥34 weeks).

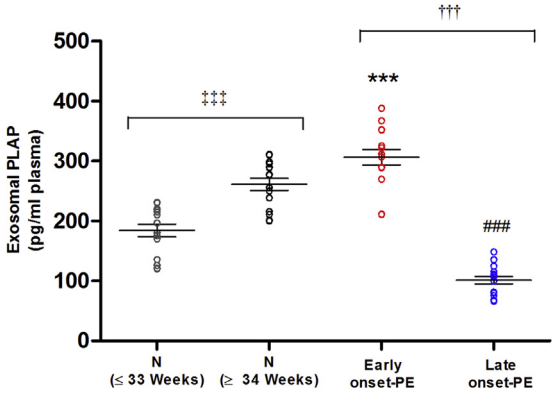

Fig. 4. Placental-derived exosomes in Pre-eclampsia and normotensive pregnancies. Placental-derived exosomes (i.e. exosomal PLAP) was quantified in maternal peripheral plasma by ELISA. The data is expressed as aligned dot plot and values are mean ± SEM. $^{***}p < 0.0001$ early onset-PE vs N (≤33 weeks), $^{\#\#\#}p < 0.0001$ late onset-PE vs N (≥34 weeks), $^{\dagger\dagger\dagger}p < 0.001$ early onset-PE vs late onset-PE, $^{\ddagger\ddagger\ddagger}p < 0.0001$ N (≤33 weeks) vs N (≥34 weeks).

(≤33 weeks) and late onset-PE could be an adaptive response to the hypoxic placenta, which is a pathological manifestation of PE. On

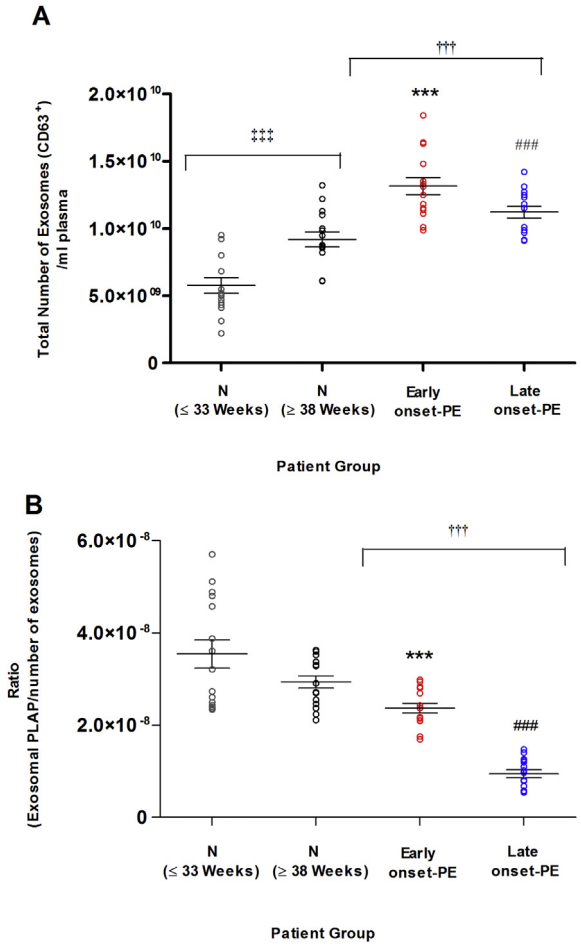

Fig. 5. Contribution of placental-derived exosomes to total exosomes. $PLAP^+$ exosomes (i.e. exosomal PLAP) and total exosomes in N (≤33 weeks), N (≥34 Weeks), early onset-PE and late onset-PE groups were determined by ELISA. (A) Total exosomes were determined by the quantification of exosomal CD63 marker. (B) Ratio of $PLAP^+$ exosomes to total exosomes ($CD63^+$). Values are represented as mean ± SEM. In A and B $^{***}p < 0.0001$ early onset-PE vs N (≤33 weeks), A $^{\dagger\dagger\dagger}p < 0.001$ early onset-PE vs late onset-PE, $^{\#\#\#}p < 0.0001$ late onset-PE vs N (≥34 Weeks); In A, $^{\ddagger\ddagger\ddagger}p < 0.0001$ N (≤33 weeks) vs N (≥34 Weeks).

the other hand, the decreased $PLAP^+$ exosomes in late onset-PE in comparison to N (≥34 weeks) and early onset-PE, may suggest that the progression of late onset-PE is not based on a hypoperfused placenta. This may account for the differences in the clinical expression between the PE groups. These findings are consistent with placental morphological dissimilarities observed in early onset-PE and late onset-PE [33]. In addition, we have identified a positive correlation between the number of exosomes in maternal circulation and BMI in early onset-PE (Pearson's $r = 0.57$, $p < 0.05$) and late onset-PE (Pearson's $r = 0.58$, $p < 0.05$) with no significant correlation in N (≤33 weeks) and N (≥34 weeks). These findings indicate that the increase in exosomes in preeclampsia is associated with an increase in BMI which could be related to the metabolic variations associated with PE [34,35].

Placental-derived exosomes have been reported to be involved in immune tolerance in normal pregnancies by the suppression of T-cell signalling [21] which is mediated by exosomal FasL and PD-L1 transmembrane proteins. These placental-derived exosomal transmembrane proteins have been shown to suppress CD3-zeta expression and induce apoptosis in normal pregnancies, indicating that placental-derived exomes play a role in fetal-maternal immune tolerance [21]. These findings support our study,

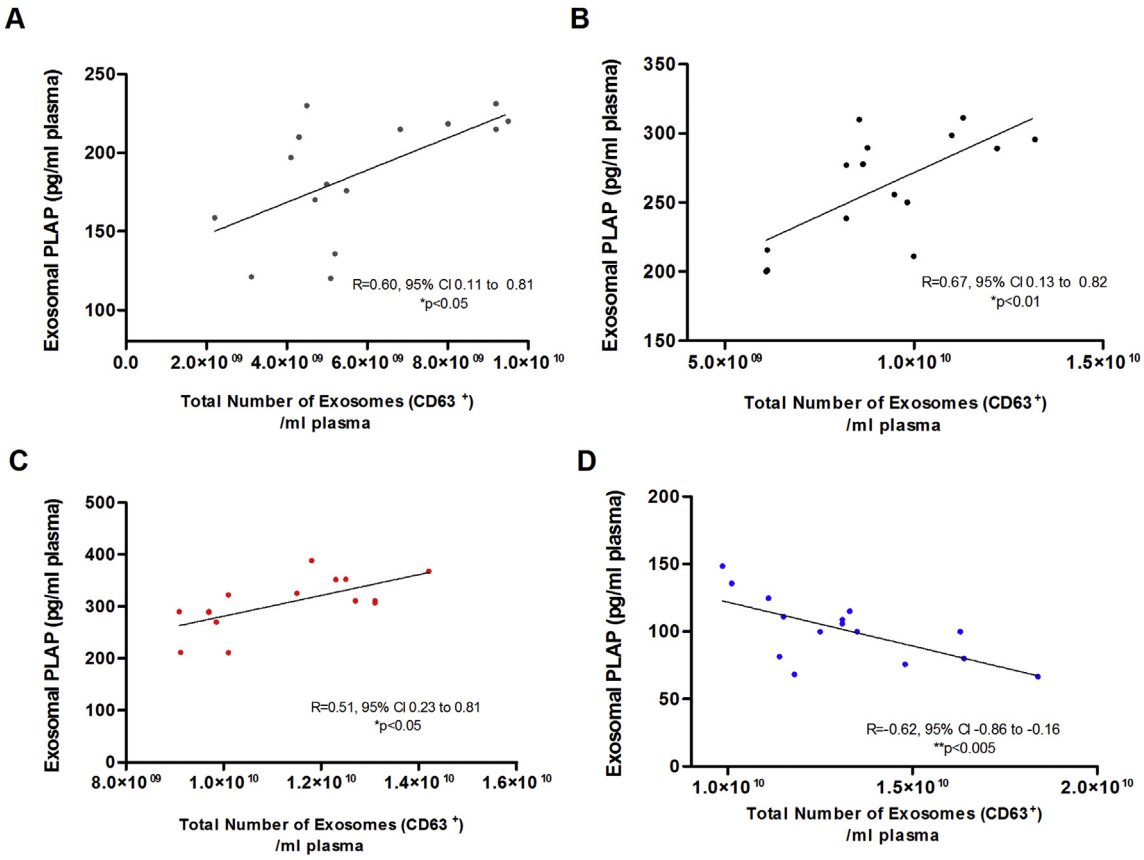

Fig. 6. Relationship between placental-derived exosomes and total exosomes in maternal circulation. The correlation between PLAP$^+$ exosomes (i.e. exosomal PLAP) and total exosomes (i.e. CD63$^+$ exosomes) in (A) N (≤33 weeks), (B) N (≥34 Weeks), (C) early onset-PE and (D) late onset-PE. In A and B a (+) Pearson correlation. In D a (−) Pearson's correlation.

whereby the increase in relative placental-derived exosomes in early onset-PE is probably responsible for the exaggerated maternal immune alterations and cellular apoptosis, thus, resulting in the clinical expression of PE.

The presented study demonstrates the contribution of PLAP$^+$ exosomes in relation to total exosomes by the quantification of exosome membrane bound placental alkaline phosphatase (PLAP). Placental alkaline phosphatase was used as a marker for the quantification of placental-derived exosomes which has been previously utilised [21,27,28]. This marker has been detected in pregnant women in second and third trimesters of pregnancy at a steadily increasing rate [36,37], which subsequently declines after delivery [38]. In addition, sources of circulating exosomes may originate from other tissues such as the endothelium; however, we focussed on the possible role of placental-derived exosomes. Moreover, standardisation of exosomal isolation and purification methods are limited and have not been validated [23], therefore the methods used in this study, based on published protocols requires further validation. Additionally, it cannot be ruled out completely that the PLAP signal could be partly attributed to other contaminating micro-vesicles. Notwithstanding this fact, variations in PLAP signal would be in keeping with variations in PLAP levels from PLAP$^+$ exosomes in maternal circulation.

5. Conclusion

In the presented study, we have shown that the total number of exosomes increased in early onset-PE and late onset-PE compared to N. Notably, we also found significant differences in the relative concentration of placental-derived exosomes between the subtypes. A positive correlation was found between PLAP$^+$ exosomes and total exosomes in early onset-PE, with a negative correlation in late onset-PE. These findings may relate to the dissimilarities in the etiology of the two subtypes, suggesting that placental-derived exosomes could play a pathological role in PE. Our findings, therefore, form the rationale for the determination of the role of placental-derived exosomes in PE and suggests that exosomes may be a useful tool for the prognosis/diagnosis of PE.

Conflict of interest statement

The authors have no conflicts of interests to declare.

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7.5 Placental Hypoxia Inducible factor −1α & CHOP Immuno-histochemical expression Relative to Maternal Circulatory Syncytiotrophoblast Micro-vesicles in Preeclamptic and Normotensive Pregnancies.

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Full Length Article

Placental hypoxia inducible factor -1α & CHOP immuno-histochemical expression relative to maternal circulatory syncytiotrophoblast micro-vesicles in preeclamptic and normotensive pregnancies

S. Verma[a], P. Pillay[a], T. Naicker[b], J. Moodley[c], I. Mackraj[a,*]

[a] Department of Physiology, University of KwaZulu-Natal, South Africa
[b] Department of Optics and Imaging, University of KwaZulu-Natal, South Africa
[c] Women's Health and HIV Research Group, University of KwaZulu-Natal, South Africa

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ABSTRACT

Objectives: Preeclampsia (PE) occurs as a result placental hypoxia-induced oxidative and endoplasmic reticulum stress, and is associated with the activation of hypoxia inducible factor-1α (HIF-1α) and apoptotic CHOP pathways with the consequential shedding of syncytiotrophoblast microvesicles which may be central in mediating the maternal systemic immune response. The aim of this study was to immune-localise and morphometrically analyse CHOP and HIF-1α within the placenta of normotensive and pre-eclamptic pregnancies and concomitantly quantify syncytiotrophoblast released microvesicles in maternal circulation.

Study design: Placental tissue and plasma were obtained from normotensive and pre-eclamptic pregnant women. The expression of CHOP and HIF-1α was analysed using immunohistochemistry. Isolation and size distribution of the circulating maternal microvesicles was determined using nanoparticle tracking analysis. The concentration of syncytiotrophoblast microvesicles was determined using the placental alkaline phosphatase ELISA.

Results: This study demonstrates a significant increase in immunohistochemical expression of HIF-1 α and CHOP in preeclampsia compared to the normotensive women ($p < 0.05$). In keeping with this, a significant increase in the mean syncytiotrophoblast microvesicles concentration was observed in PE, compared to normotensives ($p < 0.05$). A positive correlation between placental expression of CHOP and HIF-1α and STBMs was obtained.

Conclusion: This study demonstrates increased placental expression of HIF-1α and CHOP in preeclampsia compared to normotensive pregnancies which correlate to their increased syncytiotrophoblast microvesicles concentration in maternal circulation. These findings indicate that placental hypoxia and ER stress are interrelated contributory factors to the pathogenesis of PE and the consequential release of placental derived debris into the maternal circulation.

Introduction

Hypertensive disorders of pregnancy constitute the main direct cause of maternal mortality, globally and within South Africa [1]. Preeclampsia (PE) accounts for the majority of maternal and foetal deaths emanating from hypertensive disorders, with the global incidence of PE ranging from 2 to 8% [2].

Preeclampsia is clinically characterised by new-onset hypertension and proteinuria which manifests after the 20th week of gestation [3]. The exact aetiology of PE still remains elusive, although the role of the placenta in the pathogenesis has been well described [4,5]. Preeclampsia is believed to occur as a result of inadequate trophoblastic invasion during pregnancy, which results in improper remodelling of the myometrial spiral arteries, with consequential placental hypoperfusion and an ischemic microenvironment [6]. This results in a hypoxic microenvironment, which induces the expression of hypoxia-inducible factor 1 alpha (HIF-1α) [7]. HIF-1α plays a crucial role in the transcription of numerous oxygen-dependent genes, which encode proteins involved in angiogenesis and cell metabolism in normal pregnancy, promoting the establishment of the placenta. However, overexpression is implicated in the development of several inflammatory conditions, such as PE [7,8].

* Corresponding author.
E-mail address: mackraji@ukzn.ac.za (I. Mackraj).

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This hypoxic/ischemic microenvironment causes oxidative and endoplasmic reticulum stress and subsequent death of placental cells, which may lead to the exaggerated release of trophoblast-derived factors, such as microvesicles and other debris, into the maternal circulation, in comparison to normal pregnancies [9]. Microvesicles originate from the blebbing of membranes or cellular apoptosis during the physiological turnover of cells [10]. The syncytiotrophoblast layer of the placenta constitutes the maternal-fetal interface, which releases microvesicles into maternal circulation, termed as syncytiotrophoblast microvesicles (STBMs). These STBMs have been shown to be involved in maternal and fetal cross-talk, and excessive release stimulates the maternal systemic inflammatory response (MSIR) and endothelial dysfunction, thereby contributing to the development of PE [11].

The syncytiotrophoblast layer of the placenta has a high endoplasmic reticulum (ER) content as it performs many important functions [12]. In PE, ischaemic reperfusion and hypoxia caused by diminished blood flow into the intervillous space, causes oxidative and ER stress, thus generating and accumulating reactive oxygen species through the mitochondrial pathway [13]. This activates a cascade of signalling networks called the Unfolded Protein Response (UPR). The UPR restores ER homoeostasis, promoting cell survival and adaptation [14]. However, this initiation of UPR also leads to a homeostatic imbalance and activation of pro-apoptotic pathways such as CHOP (GADD153) [15]. The apoptotic events occur as a result of the overexpression of CHOP *via* the Bcl-2–inhibitor mechanism [16].

While ER stress and the release of microvesicles have been measured in separate studies, in this study we measured both events in a single patient (both placenta and blood from the same women) in an effort to investigate the relationship between these factors. To the best of our knowledge, this is the first study to try and establish a link between the etiopathology and clinical symptoms of PE triggered by microvesicular release.

Therefore, the aim of this study is to quantify STBMs in Black South African PE and normotensive pregnant women, and to assess whether ER stress in placental tissue is linked to the increased production of these microvesicles.

Materials and methods

Ethics statement

Regulatory ethical and institutional approvals were obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE364/15), South Africa.

Study group and samples

Healthy normotensive (N; n = 15) pregnant and preeclamptic (PE; n = 7) women in the third trimester of pregnancy scheduled to have elective caesarian deliveries for obstetric indications were recruited. The inclusion criteria was designed to minimise any kind of hypoxic and endoplasmic reticulum stress. Normotensive women were classified by a blood pressure of $<$140/90 mmHg (systolic/diastolic mmHg) and absent proteinuria as detected by a rapid urine dipstick test (Markomed®, South Africa). Preeclampsia was defined as new-onset hypertension, characterized by a blood pressure of $\geq$140/90 mmHg, with the presence of protein in the urine ($\geq$300 mg in a 24-h urine sample or a value of $\geq$2+ on dipstick) after the 20th week of gestation [17,18]. All women had singleton pregnancies and those with evidence of any infection or medical, surgical or other obstetric complications were excluded.

Blood samples were collected [BD Vacutainer Tubes (EDTA), Becton Dickinson and Company, South Africa] and the plasma samples were stored at −80 °C for analyses (N; n = 15, PE; n = 7). Placental tissue was dissected both from central and peripheral regions of the placenta. The placental tissue was cut, rinsed with PBS (1X, pH 7.5) and preserved in 10% phosphate buffered formalin within 10 min of delivery.

Immuno-histochemistry

Post fixation, the placental tissue samples were dehydrated and paraffin embedded using the Leica automated tissue processor. Sections of 3–4 μm were cut with a Leica RM 2135 microtome and mounted onto coated slides (X-tra Adhesive, Leica Microsystems, Germany). Specimens were de-waxed in xylene and rehydrated in decreasing order of ethanol concentration. Epitope retrieval was performed with target antigen retrieval (Envision High pH Flex Mini Kit, DAKO) for 20 min. The monoclonal-mouse-anti-HIF-1α [1A3] antibody (Abcam, USA; ab113642, 1:1000) and monoclonal-mouse-anti-GADD153/CHOP [9C8] antibody (Novus Biologicals; NB600-1335, 1:75) served as primary antibodies. Gastric adenocarcinoma and glioblastoma multiform tissue were used as the positive control for HIF-1 α and CHOP, respectively. Primary antibody substituted with buffer served as a negative control. Goat Anti-Mouse IgG [HRP] (Abcam, USA; ab205719) served as the secondary antibody.

Morphometric image analysis of HIF-1 α and CHOP

All specimens were viewed with an Axioscope A1 microscope (Carl Zeiss, Germany). Image capturing, processing and analysis were performed using the AxoVision software (Carl Zeiss, Germany 4.8.3). Auto analysis was done using four fields of view per slide which were randomly selected and captured at an initial objective magnification of 20X.

HIF-1 α and CHOP expression were determined as a percentage of immunostaining (colour: brown) within the villi. Within the placental villi (both exchange and conducting), HIF-1α immunostaining occurred within the cytoplasm of the syncytiotrophoblast and cytotrophoblast cell population (Fig. 1B). Additionally, the immunoexpression was also noted in endothelial cells lining arteries, veins and capillaries across all villi types. The CHOP immunostaining was nuclear and also occurred in the trophoblast cell population in both exchange and conducting villi (Fig. 1A). Four random areas per slide were selected and immunostaining was expressed as a percentage trophoblast area.

Isolation of microvesicles from maternal circulation

Microvesicles were isolated according to a modified method as described by Dragovic et al. [19] on seven normotensive and seven preeclamptic pregnancies to match the equal number of pre-eclamptic and normal samples. Plasma (1 ml) was diluted with an equal volume of phosphate buffered saline (PBS; pH 7.4) and centrifuged at 1500*g* at 4 °C for 20 min. The supernatant was collected and centrifuged at 10 000*g* at 4 °C for 30 min. The pellet was re-suspended in 2 ml PBS (pH 7.4) and thereafter centrifuged at 10 000*g* at 4 °C for 30 min. This step was repeated to reduce contamination by soluble proteins. Microvesicle protein concentration was determined using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

Size distribution of microvesicles using nanoparticle tracking analysis

Quantification and size distribution of microvesicles were determined using the NS500 as previously described by our group (Nano Sight NTA 3.0 Nanoparticle Tracking and Analysis Release, Version Build 0069) [20]. Briefly, samples were diluted with PBS prior to analysis in order to obtain particle distribution of 10 and

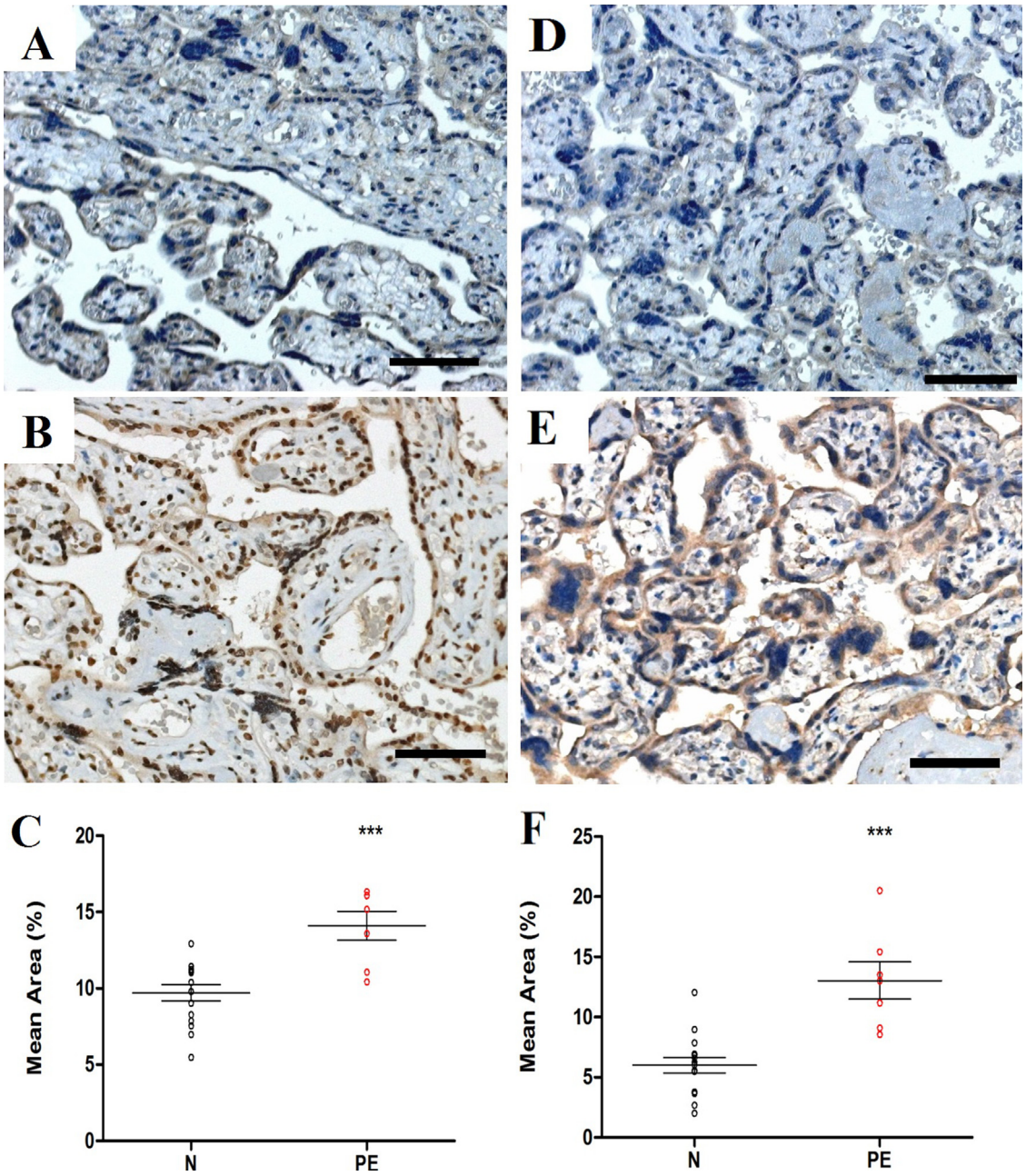

Fig. 1. Placental Immune Histochemical Expression of CHOP and HIF-1α in Preeclamptic and Normal Pregnancies (n = 15). (A) CHOP expression in PE and (B) normotensive pregnancies. (D) HIF-1α expression in PE and (E) Normotensive pregnancies. Mean area (%) of (C) CHOP and (F) HIF-1α in PE and N. In A and B arrows indicate the increased nuclear expression of CHOP in the terminal villi. In D and E arrows indicate the increased cytoplasmic expression of HIF-1α in the terminal villi. In C indicates CHOP expression and F HIF-1α expression, ***$p < 0.0001$.

100 particles per image (optimal, 50 particles per image) before the analysis with NTA system. Samples were introduced into the sample chamber using the following script: PUMPLOAD, REPEAT-START, PRIME, DELAY 10, CAPTURE 60, REPEAT 5. Videos were recorded at a camera level of 10, camera shutter speed of 20 ms and camera gain of 600, these settings were kept constant between samples. Each video was then analysed to give the mean particle size together with the concentration of particles. The size of the exosomes was represented as the mean particle size ± SD.

Quantification of syncytiotrophoblast microvesicles

The relative concentration of syncytiotrophoblast microvesicles was determined by the quantification of human placental alkaline phosphatase in the isolated microvesicle fraction using a commercial ELISA kit (Elabscience, E-EL-H1976, WuHan, P.R.C). Briefly, microvesicles were allowed to bind to the primary PLAP specific antibody coated plates by incubation at 37 °C for 90 min. Plates were washed and 50 μl of HRP-conjugate was added to each well and incubated at 37 °C for 20 min. Plates were washed and incubated with 50 μl of substrate A and 50 μl of substrate B at 37 °C for 15 min. The incubation was terminated using 50 μl of stop solution at RT for 2 min under agitation. Absorbance was measured at 450 nm. Exosomal PLAP was expressed as pg/ml plasma. The quantification of PLAP in the microvesicle fraction indicates the relative concentration of syncytiotrophoblast microvesicles in maternal circulation.

Table 1
The Clinical Characteristics of both study groups.

Variables	Normotensive (N) (n = 15)	Preeclampsia (PE) (n = 7)
Age (years)	30.0 ± 5.1	25.1 ± 7.1
BMI (kg/m^2)	31.6 ± 6.4	26.7 ± 5.9
Nulliparous (number)	1	2
Multiparous (number and percentage)	14	5
Gestational age at sampling/delivery (weeks)	36.2 ± 1.3	35.8 ± 1.5
Systolic blood pressure (mmHg)	108.8 ± 6.7	148.1 ± 11.2*
Diastolic blood pressure (mmHg)	71.8 ± 6.4	96.4 ± 8.8*
Baby weight (kg)	3.4 ± 0.3*	2.7 ± 0.5
Baby gender (Male)	7	3
Baby gender (Female)	8	4

All values are expressed as mean ± SEM. All pregnancies were singleton without any intrauterine infection or any other medical condition. In systolic/diastolic blood pressure PE versus N, * $p < 0.05$. In BMI N vs PE, * $p < 0.05$.

Statistical analysis

Statistical significance was defined as $p < 0.05$. Statistical significance between the two groups was assessed using unpaired *t*-test. Data is presented as the mean ± SEM. Pearson's correlation analysis was done to determine the relationship between syncytiotrophoblast microvesicles in maternal circulation and placental protein expression. Data distribution was determined using the combined D'Agostino and Pearson normality test, all data was found to be normally distributed. Statistical analyses were performed using commercially available packages (Prism 6, GraphPad Inc, La Jolla, CA 92037 USA).

Results

Patient clinical data

Patient demographics are presented in Table 1. The table shows that there were no significant differences in clinical parameters between the N and PE groups ($p > 0.05$) except for increased blood pressure levels in the PE group ($p < 0.05$). The mean birth weight in the N group was significantly higher than that in the PE group (3.4 ± 0.3 *vs* 2.7 ± 0.5 kg, $p < 0.05$).

Expression of CHOP and HIF-1α in preeclamptic and normotensive placenta

Immunohistochemistry showed strong reactivity for HIF-1 α and CHOP in preeclamptic placentas compared to normotensive controls (Fig. 1). The majority of the positive staining was found in the syncytiotrophoblasts. In addition, the analysis of the mean percentage area of CHOP and HIF-1α immunostaining indicated that there was a significant increase in CHOP and HIF-1α immune-expression in PE in comparison to N (13.1 ± 4.1% vs 6.5 ± 4.3% and 14.1 ± 6.8% vs 9.24 ± 2.4%, respectively, $p < 0.0001$; Fig. 1C & F).

Quantification and characterisation of microvesicles in maternal circulation

The size distribution profiles (Fig. 2A) indicate that the microvesicles ranged in size from 50 to 450 nm. A statistically significant difference in the mean microvesicle particle size between preeclamptic and normotensive groups was obtained (127.6 ± 7.93 nm vs 137.4 ± 5.08 nm, $p < 0.005$; Fig. 2B). A significant increase in microvesicle concentration was obtained in PE in comparison to N pregnancies ($1.38 \pm 0.34 \times 10^{11}$ vs $5.06 \pm 0.61 \times 10^{10}$ particles/ml plasma, $p < 0.05$; Fig. 3). The relative concentration of syncytiotrophoblast (STB) microvesicles obtained in PE plasma compared to normotensive pregnancies was greater [574.3 ± 55.99 pg/ml vs 218.8 ± 29.35 pg/ml, respectively, ($p < 0.005$; Fig. 4)].

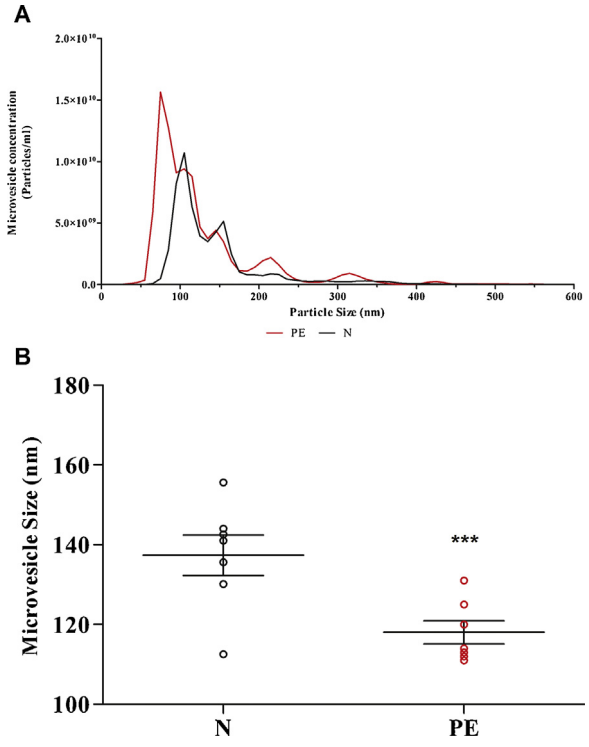

Fig. 2. Characterisation of Micro-vesicles in Preeclamptic and Normotensive (n = 15) Maternal Circulation. (A) Micro-vesicles size distribution profile (nm). (B) Mean particle size (nm), ***$p < 0.0001$.

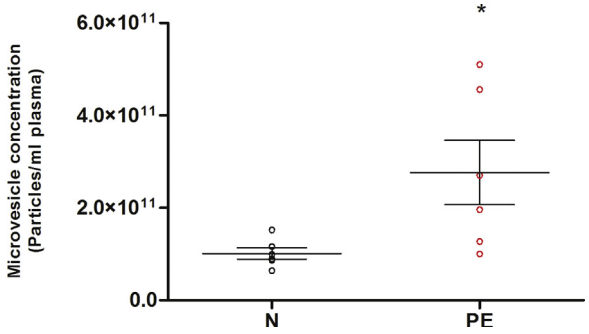

Fig. 3. Micro-vesicle concentration in maternal circulation of normotensive and preeclamptic pregnant woman (n = 15). The data is expressed as aligned dot plot and values are mean ± SEM.***$p < 0.0001$ PE vs N.

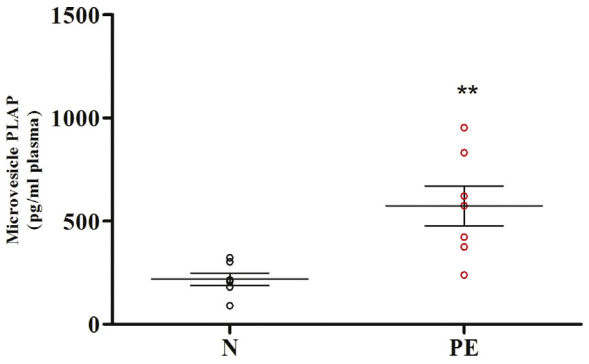

Fig. 4. Syncytiotrophoblast micro-vesicles in preeclampsia and normotensive pregnancies (n = 15). The data is expressed as aligned dot plot and values are mean ± SEM. $^{**}p < 0.005$ PE vs N.

Relationship between syncytiotrophoblast microvesicles in maternal circulation and HIF-1α & CHOP expression

Fig. 5 shows the relationship between the relative concentration of syncytiotrophoblast microvesicles and placental protein (HIF-1α & CHOP) expression. This relationship was determined by the correlation between the $PLAP^{+}$ microvesicles (pg/ml plasma) and protein expression (HIF-1α & CHOP, % area). A positive correlation between the relative concentration of STBMs and placental HIF-1α & CHOP protein expression in PE and N groups were obtained (Pearson's $r > 0.5$, $p < 0.05$; Fig. 5).

Discussion

While increased endoplasmic reticulum stress and circulating microvesicular concentration have been reported separately in the pathology of preeclampsia by Burton et al. [21,22] and Sargent et al. respectively, no study has investigated the combined relationship between these two significant events. The rising interest in microvesicles as biomarkers necessitates this study, whereby we hypothesise that these two important key events may be linked. Our findings demonstrate a significant increase in the immunoexpression of CHOP and HIF-1α in pre-eclamptic placental tissue in comparison to the normotensive group, in keeping with previous studies [23,24]. Concomitantly nanoparticle tracking analysis indicated that microvesicles in PE maternal circulation was significantly increased in comparison to normotensives. The size distribution profile ranged from 50 to 450 nm which is characteristic of microvesicles. Further quantification of the relative concentration of STBMs from the isolated microvesicles by PLAP ELISA indicated that there was a significant increase in STBM concentration in PE in comparison to a normotensive woman. In addition, we report a positive correlation between placental CHOP & HIF-1α expression and STBMs in maternal circulation. These findings support our assertion that the oxidatively stressed placenta results in the expression of ER stress proteins, which may enhance the production of placental derived STBMs.

Hypoxia is essential in the early stages of gestation for a successful pregnancy, however, prolonged hypoxia is observed in PE and other inflammatory conditions [7]. Hence increased expression of placental HIF-1 α in PE could be due to an imbalance

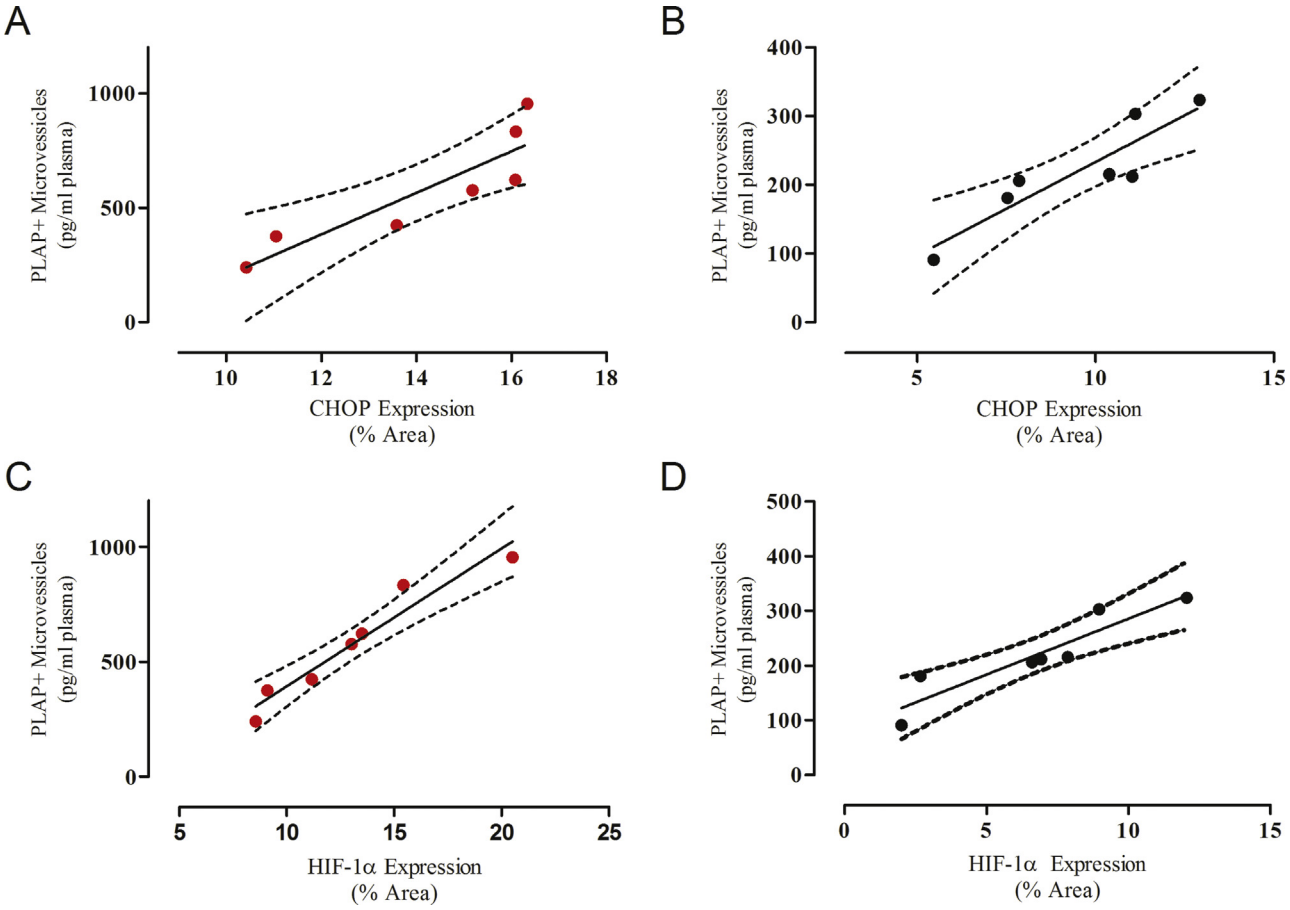

Fig. 5. Relationship between the relative concentration of STBMs in maternal circulation and HIF-1α & CHOP & expression (n = 15). The correlation between; PLAP+ micro-vesicles and CHOP expression (% Area) in (A) PE & (B) N. PLAP+ micro-vesicles and HIF-1α expression (% Area) in (C) PE & (D) N. In A–D (+) Pearsons correlation.

in oxygen homoeostasis due to diminished placental perfusion, caused by improper spiral artery remodelling in early pregnancy, a key feature of PE [23]. Increased HIF-1-α mRNA expression in preeclamptic placentae have also been observed by others [15]. In addition, severe and prolonged imbalances in oxygen homoeostasis could result in antiangiogenic/angiogenic imbalance induced by HIF-1α, which acts in combination with many other factors and potentiates the clinical manifestation of PE [24].

The syncytiotrophoblast performs many key functions such as regulation of oxygen delivery and synthesis of a variety of proteins and possesses abundant free ribosomes and endoplasmic reticulum (ER) [25]. Oxidative stress in syncytiotrophoblast predisposes it to ER stress. CHOP is a known marker of ER stress and is up-regulated in PE [15,26]. Endoplasmic reticulum stress-induced apoptosis is primarily mediated through ATF4-mediated transcriptional induction of the C/EBP homologous protein CHOP [27]. The increased expression of syncytiotrophoblast nuclear CHOP observed in PE in comparison to normotensive placentae suggests that ER stress may be a major contributing factor to the increased apoptosis in PE syncytiotrophoblast. This is supported by the findings of our study whereby the increased concentration of circulating STBMs in PE, positively correlates to placental CHOP and HIF-1α expression. In addition, this relationship suggests that both the ER stress and hypoxic response pathways could potentiate the release of STBMs in maternal circulation.

STBMs are key factors involved in mediating the maternal systemic inflammatory response (MSIR) [28]. It is, therefore, plausible that the increased release of STBMs into maternal circulation, leads to the exaggeration of the MSIR, resulting in PE. In this study, we observed that the isolated vesicles in PE were elevated with a particle size of 60–120 nm, in comparison to the normotensives, and notably this size profile is a key characteristic of microvesicles. This observation is in keeping with our previous study which showed that placental-derived microvesicles and exosomes were increased in early and late onset preeclampsia [20]. This suggests that the cascade of cellular events induced by the enhanced expression of placental CHOP and HIF-1α not only results in the increased release of STBMs, but possibly also the specific biogenesis of nanovesicles such as exosomes. These exosomes serve as signalling vesicles which may contain key DNA, RNA and protein factors which potentiate the MSIR [20]. It is therefore necessary that future studies incorporate the isolation and characterisation of the sub-classes of STBMs, in conjunction with determining their biological relationship with ER stress and hypoxia. Such studies are currently ongoing within our research group.

Limitations

Oxidative stress and ER stress occurs early in complicated pregnancies, however, it was not possible to obtain placental tissue samples during early gestation due to ethical considerations. In addition, the sample size was a limitation as the occurrence of HIV-negative pregnant woman with preeclampsia delivering babies by caesarian section were low.

Conclusion

Our findings show that the placental immunoexpression of CHOP and HIF-1α are elevated in preeclampsia and could be a key placental factor involved in the pathogenesis of PE. Additionally, the correlation between placental protein expression and STBMs indicate that ER stress and hypoxia may potentiate the release of STBMs into the maternal circulation. In combination, these findings form the basis for future research involving the isolation and characterisation of STBM constituents for their elucidation of their function in ER stress and hypoxia.

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7.6 Circulatory Levels of Tumor Necrosis Factor-α, Interleukin-6 and Syncytiotrophoblast Microvesicles in the First Trimester of Pregnancy.
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Circulatory levels of tumor necrosis factor-α, interleukin-6, and syncytiotrophoblast microvesicles in the first trimester of pregnancy

N. Mchunu[1], P. Pillay[1], J. Moodley[3], I. Mackraj[2]

[1]Discipline of Human Physiology, School of Laboratory Medicine & Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban
[2]Nelson R. Mandela School of Medicine, School of Laboratory Medicine & Medical Sciences, Durban
[3]Women's Health and HIV Research Group, University of KwaZulu-Natal, Durban
[4]Prince Mshiyeni Memorial Hospital, Department of Obstetrics and Gynaecology, Durban (South Africa)

Summary

Background: The first trimester of pregnancy remains an unknown immunological adaptation. Cytokines and syncytiotrophoblast microvesicles (STBM) have been identified as key factors involved in the maternal immune adaptation. This study therefore aims to determine the levels of T helper cell 1 (Th1) and 2 (Th2) associated cytokines TNF-α and IL-6 in relation to the relative concentration of STBM's in maternal circulation. *Materials and Methods:* Plasma samples from normotensive pregnant women in the first trimester of pregnancy were obtained. The concentrations of TNF-α and IL-6 were determined using ELISA. STBMs were determined by isolating microvesicles in maternal circulation and quantifying the concentration of PLAP using ELISA. *Results:* TNF-α, IL-6 and STBMs remained at constant levels in weeks 5-10 of gestation. In weeks 11-12 of gestation, TNF-α increased with a decrease in IL-6 and STBMs. Ratio of TNF-α/IL-6 remained constant in weeks 5-6 and significantly increased in weeks 11-12 of gestation. A positive correlation between TNF-α and IL-6 was obtained in weeks 6-10 and a negative correlation in weeks 11-12 of gestation. In addition, a positive correlation between STBMs & TNF-α was obtained and negative correlation between STBMs and IL-6. was observed *Conclusion:* The relationship between TNF-α/IL-6 and STBMs suggests that syncytiotrophoblast microvesicles may have a role in cytokine production and in the maintenance of the Th1/Th2 immune adaptation in normal pregnancy.

Key words: IL-6; TNF-α; Syncytiotrophoblast microvesicles; Early pregnancy.

Introduction

Pregnancy is well recognised as a complex immunological adaptation between mother and fetus [1]. There are a multitude of factors such as placental-derived microvesicles, hormones, and cytokines [2] that synergistically coordinate the maternal immune adaptation in the first trimester of pregnancy, a prerequisite for successful pregnancy. The exact mechanism whereby these factors regulate this process is still unknown although more recent evidence suggests that placental-derived microvesicles may be implicated in the maternal immune adaptation, required for a successful pregnancy [3].

There are distinct fetal and maternal physiological developments that occur in the first trimester of pregnancy. These developments include placentation, embryogenesis, organogenesis, and pregnancy-associated cellular differentiation, which together form part of the co-ordinated natural phenomenon. In the first trimester of pregnancy, trophoblast cells invade the maternal decidua to ensure proper spiral artery remodelling, a tightly regulated process whereby any deviations from the normal could result in complicated or failed pregnancies [4]. These trophoblast cells are modulated by placental factors such as cytokines, hormones, and microvesicles. In particular, syncytiotrophoblast microvesicles (STBMS) are understood to play a role in maternal immune adaptation and systemic inflammatory response (MSIR) during normal and complicated pregnancies [5, 6].

Syncytiotrophoblast microvesicles consisting of subclasses of molecules ranging from 10-1000 nm in size include three main types of vesicles namely: (1) vesicles that bud directly from the cell membrane, (2) exosomes that are derived from multi-vesicular bodies within the cell, and (3) apoptotic bodies [5]. These microvesicles have been shown to have an immune regulatory role in pregnancy and are involved in the acceptance of the semi-allogenic fetus [7-9]. Importantly, STBM's have been shown to induce cytokine release in monocytes and B-cells [5] via cellular signalling cascades leading to the synthesis of inflammatory cytokines such as TNF-α and IL-6 [2]. In-vitro and ex-vivo studies

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Table 1. — *Clinical characteristics of patients.*

Variables	Gestational age (weeks)					
	5 (n = 3)	6 (n = 5)	8 (n = 12)	10 (n = 15)	11 (n = 15)	12 (n = 15)
Maternal age (years)	31.33 ± 4.81	26.83 ± 2.04	24.83 ± 2.19	25.16 ± 0.92	25.20 ± 1.27	25.91 ± 1.27
Parity	1 ± 0.33	1 ± 0.37	1 ± 0.29	1 ± 0.20	1 ± 0.15	1 ± 0.36
Systolic/diastolic Blood Pressure (mmHg)	121.3/83.33 ± 1.33/0.8	122.8/80.67 ± 1.64/2.19	123.2/81.92 ± 0.91/0.56	123.5/82.50 ± 0.60/0.34	124.7/82.65 ± 1.22/0.64	123.6/82.82 ± 1.47/0.63

All values are represented by mean ± SEM. All pregnancies were singleton without intrauterine infection or any other medical condition. Proteinuria was not detected in all patient groups. No statistical significance was identified with parity and blood pressure ($p > 0.05$).

have shown that both TNF-α and IL-6 have a synergistic role in regulating placental morphogenesis by co-ordinating trophoblast proliferation, migration, differentiation, and secretory function [10-12]. Additionally, ex-vivo studies have shown that STBMs stimulate the production of cytokines and therefore have an immune modulatory role in pregnancy. Syncytiotrophoblast microvesicles could therefore be responsible for the shift in Th1/Th2 immune adaptation in normal and complicated pregnancies [5]. It has been established that a shift from type 1 (Th1) to type 2 (Th2) immunity is an essential requirement for normal pregnancy and failure to make this shift results in a compromised or failed pregnancy [7]. The classical Th1/Th2 cytokine paradigm in pregnancy is a universally accepted concept even though placental-derived factors in relation to the maternal immune adaptation are not fully understood [13].

Previous studies have focused on maternal systemic levels of TNF-α and IL-6 in the second and third trimesters of pregnancy without determining the relative concentration of STBMs in maternal circulation [14-16]. In this study, the authors therefore aim to identify the possible relationship between maternal circulatory levels of cytokines (TNF-α and IL-6) and STBMs in the first trimester of pregnancy, the "black-box" period of pregnancy.

Materials and Methods

Regulatory ethical and institutional approval were obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, South Africa (BE036/12). All patients were recruited by informed consent.

Primiparous women in the first trimester of pregnancy at 5, 6, 8, 10, 11, and 12 weeks of gestation (n = 3, 5, 12, 15, 15, and 15, respectively). Normotensive pregnant women were classified by a blood pressure of 120±10 / 80±5 (systolic/diastolic mmHg) and absent proteinuria. Women had singleton pregnancies and those with evidence of any infections or medical, surgical or other obstetric complications were excluded. Blood samples were collected and the plasma samples were stored at -80°C for analyses.

Plasma concentrations of TNF-α and IL6 were measured using commercially available sandwich enzyme-linked immunosorbent assays (TNF-α & IL6 quantification kits). Briefly, specific monoclonal antibody (TNF-α/IL-6) was immobilised onto each well overnight at 4°C using the antibody binding buffer supplied. To prevent non-specific binding; wells were washed and 200 µl of assay diluent was added to each well and incubated at room temperature for one hour with agitation. Plates were thereafter washed with buffer and 100 µl of diluted plasma sample or standards were added to each well. Antigens were allowed to bind to primary antibodies at room temperature for two hours with agitation. Plates were washed with buffer followed by addition of 100 µl of HRP detection antibody into each well and incubated at room temperature for one hour with agitation. Plates were thereafter washed with buffer and 100 µl of Avidin-HRP was added to each well and incubated at room temperature for 30 minutes with agitation. Plates were washed and 100 µl of TMB substrate solution was added to each well and incubated for 15 minutes with no exposure to light. The reaction was monitored for colour change and stopped using the stop solution provided. The absorbance was read at 450 nm.

Microvesicles in maternal circulation was isolated according to a modified method as described by Dragovic *et al.* [17]. Microvesicle protein concentration was determined using a protein assay. Placental alkaline phosphatase concentration in maternal circulation was measured using a sandwich ELISA test. Plates were pre-coated with specific PLAP primary antibodies by the manufacturer. Approximately 25 µg of isolated microvesicles were added to each well and incubated at 37°C for 30 minutes. Plates were thereafter washed and 50 µl of HRP-conjugate was added to each well followed by incubation at 37°C for 20 minutes. Plates were thereafter washed and substrate solution A (50 µl) and B (50 µl) was added to each well and incubated at room temperature for ten minutes with no exposure to light. The reaction was terminated by the addition of 50 µl of stop solution and the absorbencies were read at 450 nm.

The data is presented as mean ± SEM. Differences in STBMs, IL6 and TNF-α concentration between pregnant women were determined using ANOVA with post-hoc analyses (Tukey-Kramer, 95% CI). Statistical analysis was performed using Prism 6. Correlation analysis was done using the Pearson's correlation test. Statistical significance was defined as $p < 0.05$.

Results

The clinical characteristics of the study groups are represented in Table 1. There was no statistical significance in the clinical data between the groups ($p > 0.05$).

The concentration of TNF-α and IL-6 in maternal circulation are represented in Figures 1A and B, respectively. TNF-α and IL-6 levels during early pregnancy (5-10 weeks) remained constant with no statistically significant differences ($p > 0.05$). A significant increase in TNF-α in weeks 11 (8.056 ± 0.15 pg/ml) and 12 (8.46 ± 0.19 pg/ml) was observed in comparison to weeks 5 (7.004 ± 0.20 pg/ml), 6 (7.345 ± 0.120), 8 (7.122 ± 0.12 pg/ml), and 10 (7.36 ± 0.05 pg/ml), $p < 0.05$. Conversely, the IL-6 levels significantly decrease in weeks 11 (14.32 ± 0.75 pg/ml) and 12 (13.47 ± 0.45 pg/ml) in comparison to week 10 (15.75

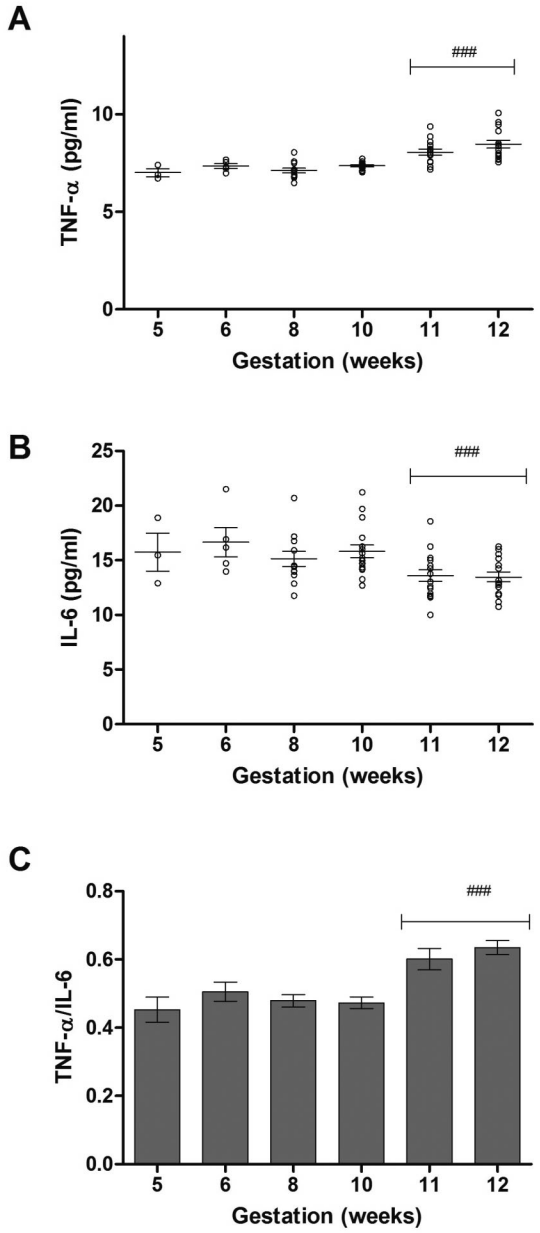

Figure 1. — Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in maternal circulation. Plasma (A) TNF-α (pg/ml) and (B) IL-6 (pg/ml) concentration measured by enzyme linked immunoabsorbency assay and (C) ratio of TNF-α to IL-6 in pregnant women at 5, 6, 8, 10, 11, and 12 weeks of gestation. In A and B data is presented as an aligned dot plot. In C data is presented as a bar graph. Values are represented as mean ± SEM. In A, B, and C $^{###}$ $p < 0.01$ 11 and 12 weeks *vs.* 5 to 10 weeks.

± 1.73 pg/ml) and 6 (16.65 ± 1.32 pg/ml), $p < 0.05$. The ratio of TNF-α to IL-6 in maternal circulation was calculated (Figure 1C). This ratio significantly increased in weeks 11 (0.60 ± 0.03) and 12 (0.64 ± 0.02) in comparison to weeks 5 (0.45 ± 0.04), 6 (0.49 ± 0.03), 8 (0.48 ± 0.02), and 10 (0.47 ± 0.02), $p < 0.05$.

The relationship between TNF-α and IL-6 in pregnant women was further analysed using Pearson's correlation coefficient. The analysis showed a positive correlation in weeks 6 ($r = 0.90$, $p < 0.05$; Figure 2A) and 10 ($r = 0.72$, $p < 0.01$; Figure 2B) of gestation. In contrast, a negative correlation was obtained in 11 ($r = -0.56$, $p < 0.05$; Figure 2C) and 12 ($r = -0.92$, $p < 0.05$; Figure 2D) weeks of gestation.

The differences in the relative concentration of STBMs in maternal circulation are shown in Figure 3. It is noted that the relative STBM concentration remained constant in weeks 5-10 (averages -905 ± 83.74 pg/ml) of pregnancy. However, a decrease in weeks 11 (508.9 ± 35.66 pg/ml) and 12 (556.7 ± 54.91 pg/ml) in comparison to weeks 5-10 was observed ($p < 0.05$).

Pearson's correlation coefficient analysis was used to further analyse the relationship between the relative STBM concentration and inflammatory cytokines (TNF-α and IL-6) during the first trimester of pregnancy. No correlation was obtained in the 8th week of gestation. However, the analysis indicates a positive correlation between relative STBM concentration and TNF-α in weeks 6 ($r = 0.96$, $p < 0.05$; Figure 4A), 10 ($r = 0.55$, $p < 0.05$; Figure 4B), 11 ($r = 0.59$, $p < 0.05$; Figure 4C) and 12 ($r = 76$, $p < 0.01$; Figure 4D) of gestation. In contrast, a negative correlation between relative STBM concentration and IL-6 in weeks 6 ($r = -0.92$, $p < 0.05$; Figure 4E), 10 ($r = -0.79$, $p < 0.01$; Figure 4F), 11 ($r = -0.53$, $p < 0.05$; Figure 4G), and 12 ($r = -0.55$, $p < 0.05$; Figure 4H) of gestation was obtained.

Discussion

In this study, the authors quantified cytokines TNF-α and IL-6 relative to the concentration of STBMs in maternal circulation over the first trimester of pregnancy, an underreported phase of pregnancy due to participant recruitment challenges. The study, therefore, provides data regarding the maternal circulatory levels of cytokines relative to STBMs in the first trimester of pregnancy. The main findings of the study showed constant levels of TNF-α, IL-6, and STBM levels in weeks 5-10 of pregnancy. However, in comparison, at weeks 11 and 12, TNF-α increases while IL-6 and STBM decline. Further analysis of the data showed an increased ratio of TNF-α/IL-6 (Th1/Th2) in weeks 11 and 12 compared to weeks 5-10. Correlation analysis between TNF-α and IL-6 displayed a positive correlation in weeks 6 and 10. Conversely, a negative correlation was observed in weeks 11 and 12, suggesting a slight shift towards Th1 immunity. In addition, the constant positive correlation between STBMs and TNF-α and negative correlation between STBM and IL-6 suggests that STBMs are associated with upregulating Th1 immunity and downregulating Th2 immunity in the first trimester of pregnancy. These findings are indicative of an alteration in immune response during the transitioning from organogenesis (5-10 weeks) to fetal development (≥11 weeks).

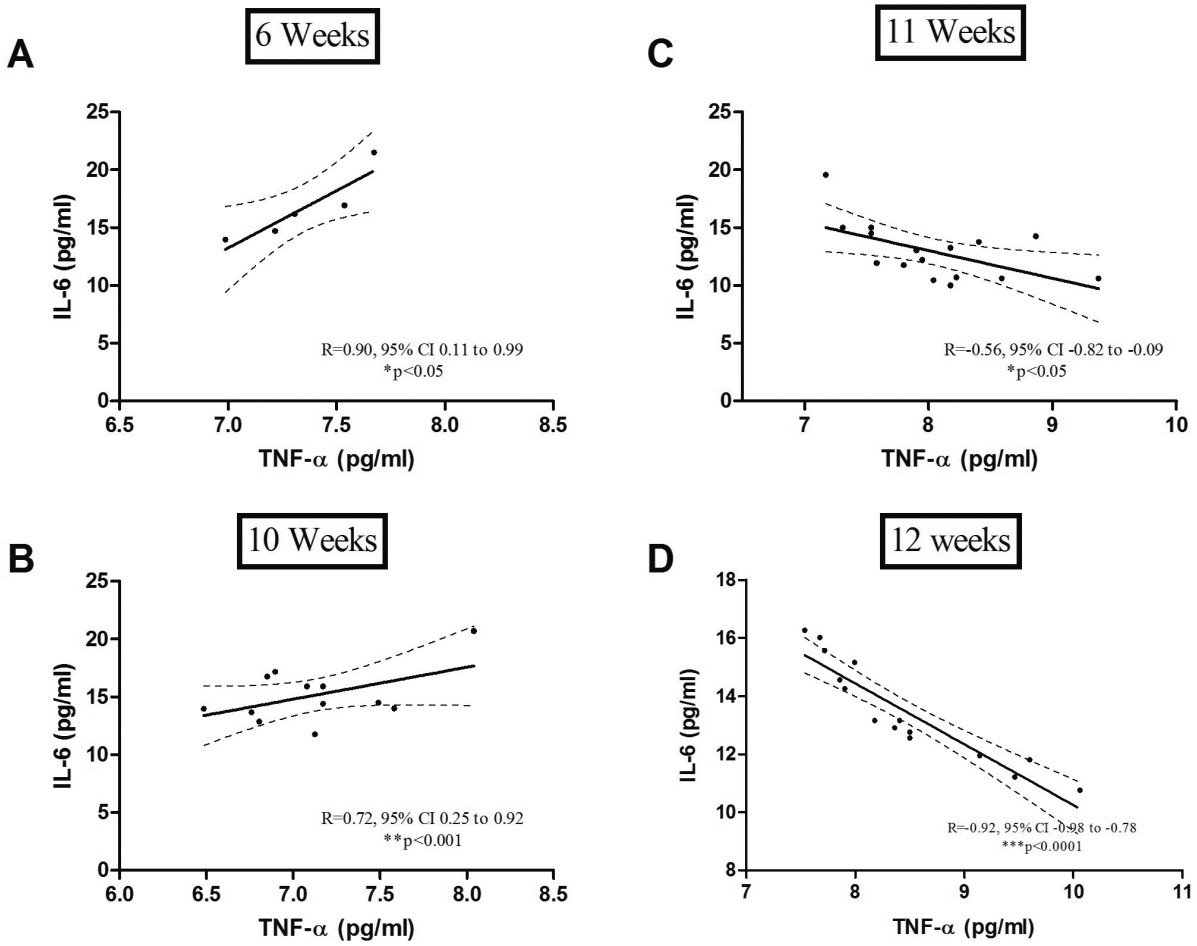

Figure 2. — The relationship between tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels in maternal circulation. The correlation between TNF-α and IL-6 in (A) 6, (B) 10, (C) 11, and (D) 12 weeks of gestation. In A and B a (+) Pearson's correlation. In D and E a (-) Pearson's correlation.

The maintained levels of TNF-α and IL-6 in weeks 5-10 of gestation is in keeping with a previous study which showed that there are no significant alterations in serum TNF-α and IL-6 levels during weeks 8-10 of gestation [18]. This could be representative of the maternal immune adaptation during organogenesis. Previous studies also showed that TNF-α and IL-6 increases in the second and third trimesters of normal pregnancy [14, 16, 18], which is a requirement for the maintenance of Th2 associated immunity. However, in this study, for the first time, the authors have shown that there is an increase in TNF-α and decrease in IL-6 in weeks 11 and 12 of gestation, which represents a slight shift towards Th1 immunity, which could be representative of the transition from organogenesis to fetal development. This finding was further assessed by the analysis of the TNF-α (Th1-pro-inflammatory)/IL-6 (Th2-anti-inflammatory) ratio. The ratio analysis supports the notion that in normal pregnancy there is a shift towards Th2 immunity, which is a common feature of successful pregnancy [19]. However, observations made in this study suggest that there is a greater shift towards Th2 immunity during weeks 5-10 of gestation in comparison to weeks 11 and 12. The slight shift towards Th1 immunity in weeks 11 and 12 of gestation probably occurs due to the oxygen demand for rapid fetal growth, which results in enhanced inflammation to support the increase in trophoblast invasion and vessel transformation; a critical requirement for spiral artery remodelling [4]. These findings support the theory that placental morphogenesis requires both a pro- and anti-inflammatory state during the first trimester of pregnancy [20]. It has been recognised that STBMs, released into maternal circulation could play a regulatory role in Th1/Th2 immunity which may mediate the synthesis of pro- and anti-inflammatory cytokines in maternal circulation [5].

Elevated STBMs in maternal circulation have been associated with pregnancy-related complications, such as preeclampsia and is indicative of placental function [21, 22]. Placental alkaline phosphatase, a syncytiotrophoblast membrane-bound allosteric enzyme, has been used to

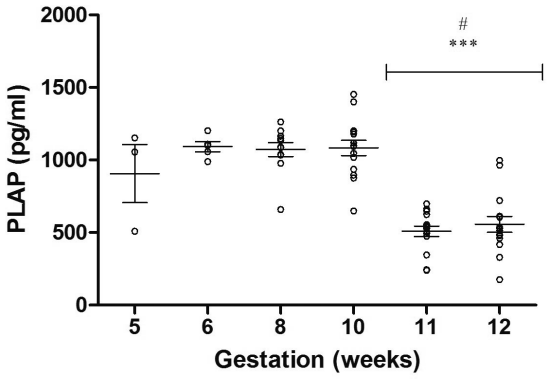

Figure 3. — Syncytiotrophoblast microvesicle concentration in maternal circulation. Syncytiotrophoblast microvesicles were quantified by the measurement of PLAP using enzyme linked immunoabsorbency assay in pregnant women at 5, 6, 8, 10, 11, and 12 weeks of gestation (n = 3, 5, 12, 15, 15, and 15, respectively). Data is presented as an aligned dot plot and values are represented as mean ± SEM. 11 and 12 weeks *vs.* 5 weeks ($^{\#}p < 0.05$), 6-10 weeks ($^{***}p < 0.01$).

measure the relative concentration of STBMs in maternal circulation, mainly in the 2nd and 3rd trimester of pregnancy. There is therefore a lack of information regarding the relative concentration of STBMs in 5-12 weeks of normal pregnancy. In this study, the authors therefore isolated and quantified circulating STBMs by the measurement of PLAP (stimulus) and related it to the concentration of TNF-α and IL-6 (maternal inflammatory response) in maternal circulation.

More recently ex-vivo studies showed that STBMs play a central role in promoting Th2 immunity during normal pregnancy and therefore could possibly play a central role maintenance of MSIR in normal and complicated pregnancies [5]. In this study, maternal circulatory levels of STBMs in weeks 5-10 of gestation remained constant with a significant decrease in weeks 11-12 of gestation ($p < 0.05$), which may occur as a result of the transitioning from organogenesis to fetal growth as discussed above.

The decrease in the ratio between TNF-α/IL-6 in weeks 5-10 is probably as a result of the increase in relative STBM concentration which may have stimulated the Th2 anti-inflammatory response leading to the increased synthesis of IL-6 required for facilitating fetal organ development [11]. In contrast, the rapid decline in STBMs in weeks 11-12 of gestation may have slightly shifted the inflammatory response towards Th1 immunity in comparison to a stronger Th2 response in weeks 5-10 of gestation. This is probably due to the synthesis of pro-inflammatory cytokines (TNF-α) from monocytes which were primed by STBMs earlier in pregnancy [5]. Further correlation analysis between STBMs (magnitude of the stimulus) related to TNF-α and IL-6 (size of the response) in maternal circulation during weeks 5-12 of gestation. The analysis demonstrates that STBMs are positively associated with TNF-α and negatively associated with IL-6 in the first trimester of pregnancy. These findings suggest that STBMs in the first trimester of pregnancy is positively associated with Th1 immunity. This is contrary to previous findings whereby STBMs derived in the third trimester have been shown to stimulate Th2 immunity [5] and could possibly be due to key immunological adaptations that occur throughout the various phases of gestation which ultimately affect the biogenesis of STBMs. In addition, it is important to note that the measurement of PLAP from isolated microvesicles may not be a true representation of STBMs in maternal circulation as STBMs consist of various types of vesicles. However, the measurement of PLAP from isolated microvesicles in maternal circulation would represent the relative concentration of STBMs in maternal circulation and may therefore be a useful tool in monitoring pregnancy when combined with pregnancy associated factors such as TNF-α and IL-6.

Since STBMs consist of sub-classes of vesicles, it is therefore necessary that future studies incorporate the isolation and characterisation of individual sub-classes of STBMs from maternal circulation. Consequently, studies regarding placental-derived exosomes [23] as biomarkers of obstetric complications are ongoing within the present research group. Nonetheless, the findings from this study indicate that combined analysis of TNF-α/IL-6 ratio and STBMs in maternal circulation can be incorporated into a medical algorithm to improve and standardise decisions made regarding normal and complicated pregnancies.

Conclusions

TNF-α (Th1), IL-6 (Th2) and STBM levels in maternal circulation remain constant in weeks 5-10 of gestation; a period of organogenesis. However, in weeks 11-12 of gestation, TNF-α increases with a decline in IL-6 and STBMs, which is suggestive of the physiological transitioning from organogenesis to fetal development as a result of enhanced placental perfusion. Ratio analysis of TNF-α/IL-6 indicates that there is an enhanced shift towards Th1 immunity in weeks 11 and 12 of gestation; this may occur in support of placental spiral artery remodelling, a perquisite for fetal growth. Additionally, the measurement of the relative concentration of STBMs in relation to TNF-α and IL-6 indicates that STBMs may play a key role in the immune adaptation in early pregnancy. This study shows that combined analysis of TNF-α, IL-6, and STBMs may provide a useful medical algorithm in ascertaining successful pregnancy and impeding obstetric complications in the future.

Acknowledgments

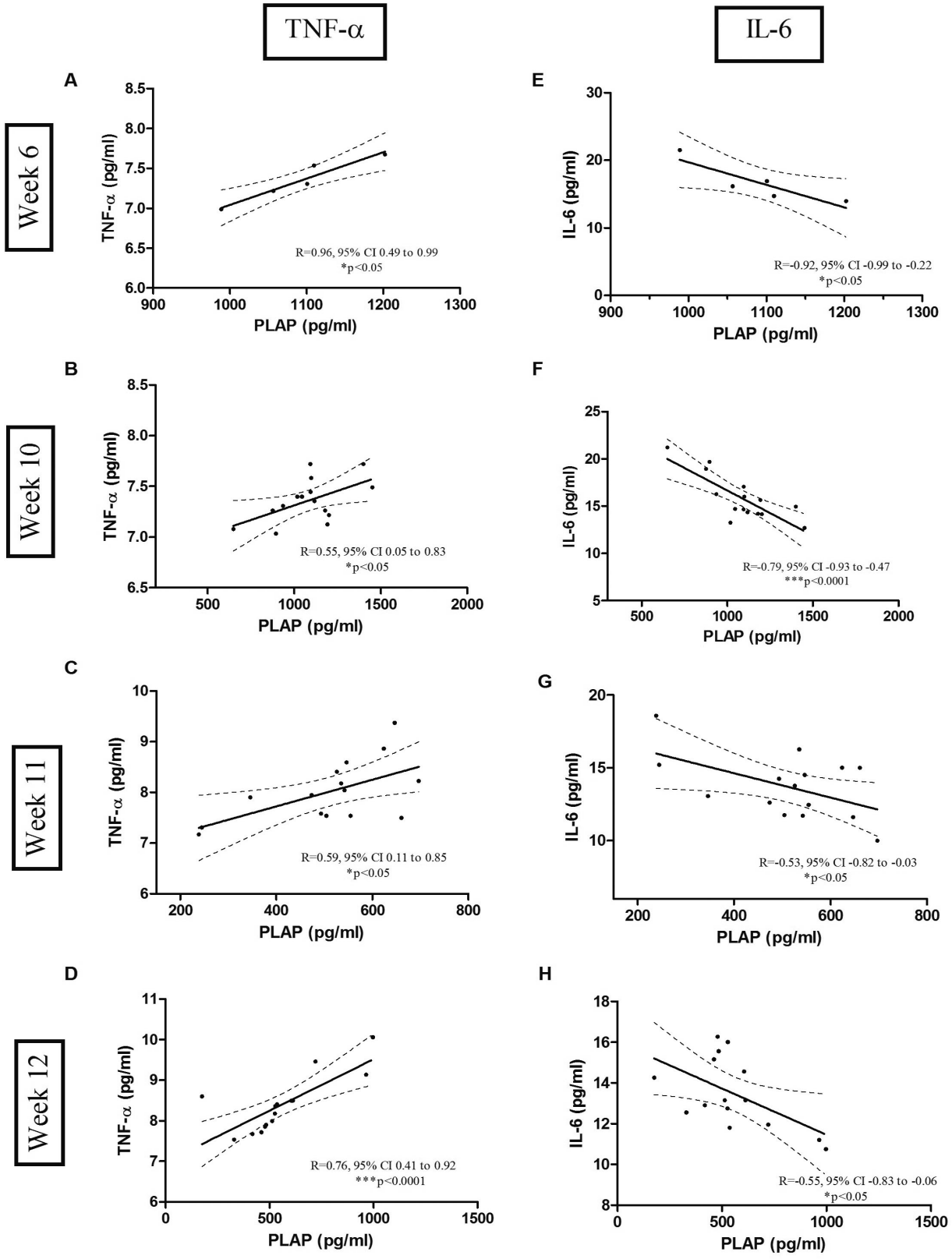

Figure 4. — The relationship between tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and syncytiotrophoblast microvesicles in maternal circulation. The correlation between relative concentration of STBMs by the measurement of PLAP (pg/ml) and TNF-α in weeks (A) 6, (B) 10, (C) 11, and (D) 12 of gestation; IL-6 in weeks (E) 6 and (F) 8, (G) 11, and (H) 12 of gestation. In A, B, C, and D a (+) Pearson's correlation. In E, F, G, and H a (-) Pearson's correlation.

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Corresponding Author:
IRENE MACKRAJ
1 University Road, Westville
Durban, 4001 (South Africa)
e-mail: mackraji@ukzn.ac.za