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Extracellular vesicles during the three trimesters of pregnancy

Danilo Buca [a], Alessandro Lucidi [a], Davide Vincenzo Buca [b,c], Francesca Di Sebastiano [a], Emanuela D'Angelo [d], Simone Vespa [b,c], Marco Liberati [a], Paola Lanuti [b,c], Francesco D'Antonio [a,*]

[a] Center for Fetal Care and High-Risk Pregnancy, Department of Obstetrics and Gynecology, University of Chieti, Italy
[b] Department of Medicine and Aging Sciences, University "G. d'Annunzio" of Chieti-Pescara, 66100 Chieti, Italy
[c] Center for Advanced Studies and Technology (C.A.S.T.), University "G. D'Annunzio" of Chieti-Pescara, Italy
[d] Department of Medical, Oral and Biotechnological Sciences, University of Chieti-Pescara, Italy

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ABSTRACT

Objectives: Extracellular vesicles (EVs) are cell-derived particles released during different pathophysiological processes and emerging as relevant players in inter-cellular crosstalk. Previous studies have highlighted the role of EVs as potential biomarkers for several pregnancy complications, including miscarriage, pre-eclampsia and gestational diabetes. Despite that, the actual distribution of EVs through gestation has not been reported yet. The aim of this study was to report the concentration of different sub-types of EVs in the first, second and third trimester of pregnancy and to correlate them with different pregnancy and ultrasound characteristics.

Study designs: Prospective observational study including uncomplicated pregnancies in the first, second and third trimester of pregnancy. The first aim of the study was to report the concentration of the EVs derived from endothelial, epithelial, platelet and leukocyte cells of maternal peripheral blood samples in the first, second and third trimester pregnancy using polychromatic flow cytometry. The secondary aim was to correlate EVs with neonatal birthweight and fetal Dopplers, including uterine and umbilical arteries. Un and multivariate analyses were used to compute the data.

Results: 64 women (20 in the first, 22 in the second and 22 in the third trimester of pregnancies) were included in the analysis. There was no difference in the median concentration of either platelet, leukocyte and endothelial EVs between the first, second and third trimester of pregnancy. The concentration of epithelial derived EVs was higher in the third compared to first and second trimester of pregnancy. When analyzing the percentage of EV vesicles through gestation, there was no difference in the percentage of either leukocyte or endothelial EVs through gestation. Conversely, the median percentage of platelet derived vesicles was higher in the first (48.7 %, IQR 34.1–58.5) compared to second (34.0 %, IQR 22.7–44.9) and third (9.13 %, IQR 5.01–12.1) trimester of pregnancy, while the median percentage of third trimester (6.01, IQR 2.42–7.34) epithelial derived vesicles was higher than that of the second (1.53 %, IQR 0.65–2.98), but not of the first (4.45 %, IQR 1.44–6.07) trimester. Finally, we found no association between the median concentration or percentage of endothelial, epithelial, leukocyte vesicles, neonatal birthweight and fetal or maternal Dopplers.

Conclusions: Distribution of EVs examined does not change during the three trimesters of pregnancy and is not influenced by neonatal birthweight or maternal and fetal Dopplers. The findings from this study allows a more objective interpretation of studies comparing EVs in pregnancies with compared to those without obstetric complication. EVs in future can be used for "liquid biopsy" for the early diagnosis of pathological pregnancies up to the development of possible screening protocols.

Abbreviations: EVs, Extracellular vesicles; IQR, interquartile ranges; NO, Nitric Oxide; PB, Peripheral Blood; PBS, Phosphate-buffered saline; PE, pre-eclampsia; sFlt-1, soluble fms-like tyrosine kinase 1; sEng, soluble endoglin.

* Corresponding author.
E-mail address: francesco.dantonio@unich.it (F. D'Antonio).

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1. Introduction

Extracellular vesicles (EVs) are cell-derived particles released during different pathophysiological processes, such as cell growth, activation, proliferation, apoptosis, or senescence (Simeone et al., 2020a, 2020b).

EVs circulate in many body fluids and mediate the inter-cellular crosstalk (Buca et al., 2020) playing a crucial role in a multitude of medical conditions, including malignancies, cardiovascular, inflammatory, metabolic, and autoimmune diseases (Lucidi et al., 2020; Brocco et al., 2022; Buca et al., 2022).

More recently, EVs have been proposed to be involved in several processed related to embryo implantation and placentation and their absence has been related to pregnancy loss (Jørgensen et al., 2020).

Circulating EVs have been also proposed as reliable biomarkers able to provide relevant information on pathogenic events and responses to treatments in several obstetric conditions, such as preeclampsia, abortion, fetal growth restriction, and gestational diabetes (Hashimoto et al., 2021; Hromadnikova et al., 2019; Tiozzo et al., 2021).

We have previously reported that the median concentration of EVs is different in pregnant compared to non-pregnant women. However, assessment of the actual usefulness of EVs as potential biomarkers for the different obstetric conditions implies the knowledge of their distribution through pregnancy and their potential association with the most recorded maternal and pregnancy parameters, including maternal or fetal Dopplers and neonatal birthweight.

In this context, we designed a prospective study aimed to elucidate the concentration of the different types of EVs in the peripheral blood of uncomplicated pregnant women during the three trimesters of pregnancy and to report their correlation with neonatal birthweight, maternal and fetal Dopplers.

2. Material and methods

This is a prospective observational not longitudinal study, including uncomplicated pregnancies in the first, second and third trimester of gestation, receiving antenatal care at the Division of Maternal Fetal Medicine, University of Chieti, Italy, in collaboration with the Centre for Advanced Studies Technology (CAST), "G. d'Annunzio" University, Chieti, Italy.

This study was approved by the local ethics Committee of Chieti-Pescara and University "G. D'Annunzio". Women were enrolled from March 2022 to June 2022 and written informed consent was signed by all participants.

Inclusion criteria were uncomplicated singleton pregnancies defined as pregnancy with no fetal or maternal complications, including anomalies, growth restriction, medical conditions pre-existing or occurring during pregnancy, gestational age confirmed by the crown-rump length at the 11–14 weeks scan, and delivery in our unit.

Pregnancies complicated by fetal structural or chromosomal anomalies, multiple gestations, maternal smoking or medical complications pre-existing or occurring during pregnancies were excluded from the analysis.

The primary outcome was to report the distribution of the different types of EVs vesicles, including endothelial, epithelial, platelet and leukocyte cells in the first, second and third trimester of pregnancy. The secondary aim was to report the association, if any, with neonatal birthweight and maternal and fetal Doppler indices, including the pulsatility index (PI) in the umbilical and uterine arteries.

The concentration of EVs was calculated using a dedicated flow cytometry instrument (FACSVerse, BD Biosciences) equipped with a volumetric count device, as previously described. (Marchisio et al., 2020; Simeone et al., 2020a, 2020b).

A new protocol of polychromatic flow cytometry was patented by the G. d'Annunzio University, which guarantees the sensitivity and the ability to process multiple parameters in a short period of time (Marchisio et al., 2020).

Briefly, peripheral blood (PB) samples were collected using sodium citrate as an anticoagulant. Samples were stained by adding to each tube 200 µl of PBS. Peripheral blood samples (5 µl/tube) were then added and incubated in the dark at room temperature for 45 min. To avoid immune complex formation and antibody aggregation, each reagent stock solution was centrifuged before its use at 21,000 *g* for 12 min. After incubation, each sample was diluted by adding 500 µl of PBS and 1×10^6 events/sample were acquired by flow cytometry (FACS Verse, BD Biosciences, San Jose, CA, USA). Shapiro-Wilk test was initially used to assess the distribution of the recorded variables, which were summarized using means and standard deviations, or medians and interquartile ranges (IQR).

The concentration of each extracellular vesicle was compared across gestational trimester using standard multivariate analyses. A scatter plot was used to display the concentration of each EV versus:

(1) right uterine artery PI.
(2) left uterine artery PI.
(3) umbilical artery PI.
(4) birthweight.

A locally weighted scatter-plot smoother (LOWESS) curve was built to capture the potential non-linearities of each relationship. The potential association between the collected variables was also evaluated fitting a univariate regression.

Statistical significance was defined as a two-sided p-value < 0.05 for all analyzes, which were performed using Stata, version 13.1 (Stata Corp., College Station, Texas, USA, 2014).

3. Results

64 women (20 in the first, 22 in the second and 22 in the third trimester of pregnancies) were included in the analysis. General characteristics of the included population is reported in Table 1. There is no difference in median maternal age, body mass index, parity and pregnancies conceived via assisted reproductive techniques among the three groups.

Median concentrations of EVs through gestation is reported in Table 1.

There was no difference in the median concentration of either platelet, leukocyte and endothelial EVs between the first, second and third trimester of pregnancy. The concentration of epithelial derived EVs was higher in the third compared to first and second trimester of pregnancy. When analyzing the percentage of EV vesicles through gestation, there was no difference in the percentage of either leukocyte or endothelial EVs through gestation. Conversely, the median percentage of platelet derived vesicles was higher in the first (48.7 %, IQR 34.1–58.5) compared to second (34.0 %, IQR 22.7–44.9) and third (9.13 %, IQR 5.01–12.1) trimester of pregnancy, while the median percentage of third trimester (6.01, IQR 2.42–7.34) epithelial derived vesicles was higher than that of the second (1.53 %, IQR 0.65–2.98), but not of the first (4.45 %, IQR 1.44–6.07) trimester.

In Table 2 we report the linear regression exploring the relationship between each extracellular vesicle, neonatal birthweight and fetal or maternal Doppler, including umbilical and uterine arteries. We found no association between the median concentration or percentage of endothelial, epithelial, leukocyte vesicles, neonatal birthweight and fetal or maternal Dopplers (Fig. 1).

4. Discussion

The finding from this study showed that the concentration platelet, leukocyte and endothelial EVs between is stable through pregnancy. Conversely, the median percentage of platelet derived vesicles is higher in the first compared to second and third trimester of pregnancy, while the median percentage of third trimester epithelial derived vesicles was

Table 1
Concentration of extracellular vesicles (EVs) by gestational trimester.

Variables	First trimester (N = 20)	Second trimester (N = 22)	Third trimester (N = 22)	p*
Maternal age, median (IQR)	29.50 (27–35.5)	32.0 (28–36)	32.5 (30–35)	
Maternal body mass index, median (IQR)	22 (19.8–23.9)	22 (19.5–25)	24.5 (22–27.5)	
Parity, median (IQR)	1 (0–1)	1 (0–1)	1 (0–1)	
Assisted reproductive techniques (%)	15 % (3)	13.6 (3)	9.09 (2)	
Gestational age at sampling, median (IQR)	12 (11–13)	21.0 (20–22)	37 (35–36)	
Total EVs/uL, median (IQR)	787 (515–972)	789 (605–1070)	1581 (739–3556)	
Platelet-derived EVs/uL, median (IQR)	242 (169–543)	246 (172–410)	179 (74.2–1058)	
Leukocyte-derived EVs/uL, median (IQR)	35.7 (21.0–49.7)	49.7 (29.4–72.8)	92.4 (51.8–333)	
Endothelial-derived Evs/uL, median (IQR)	91.7 (35.7–151)	145 (78.4–210)	245 (171–627)	
Epithelial-derived Evs/uL, median (IQR)	26.6 (13.3–36.4)	13.3 (4.20–28.0)	82.6 (16.8–82.6)	b, c
DNA-derived Evs/uL, median (IQR)	174 (114–291)	188 (84.0)–336	514 (127–1128)	b, c
% platelet-derived vesicles, median (IQR)	48.7 (34.1–58.5)	34.0 (22.7–44.9)	9.13 (5.01–12.1)	b, c
% leukocyte-derived vesicles, median (IQR)	4.98 (3.72–7.05)	6.82 (4.72–9.66)	7.56 (4.32–10.6)	
% endothelial-derived vesicles, median (IQR)	13.4 (7.36–23.7)	19.3 (12.5–24.3)	20.0 (5.70–34.4)	
% epithelial-derived vesicles, median (IQR)	4.45 (1.44–6.07)	1.53 (0.65–2.98)	6.01 (2.42–7.34)	c
% DNA-derived vesicles, median (IQR)	0.02 (0.01–0.04)	0.02 (0.01–0.04)	0.04 (0.01–2.45)	b, c

IQR: interquartile range.
* One-way ANOVA with Šidák correction; [a]: $p < 0.05$ for the comparison between 1st and 2nd trimester; [b]: $p < 0.05$ for the comparison between 1st and 3rd trimester; [c]: $p < 0.05$ for the comparison between 2nd and 3rd trimester; all p-values that were not reported were > 0.05.

higher than that of the second, but not of the first trimester. Finally, the contrition or percentage of EVs through gestation is not influenced by fetal or maternal Dopplers and neonatal birthweight.

This is, to the best of our knowledge, the first study exploring the peripheral blood concentration of different EV cell-types in uncomplicated pregnancies during in the first, second and third trimester of pregnancy. Prospective design, inclusion of uncomplicated pregnancies and evaluation of the potential influence of fetal or maternal Dopplers or neonatal birthweight of EVs concentration are the main strengths of the present study. The relatively small sample size represents the main limitation of this study. Furthermore, we could not stratify the analysis according to the type of conception, gestational age at assessment and other pregnancy characteristics such as parity.

EVs have been recently proposed as promising biomarkers in several obstetric complications, including miscarriage, pre-eclampsia and fetal growth restriction. Women with pre-eclampsia show higher concentration of EVs from syncytiotrophoblast compared to normotensive women, suggesting a crucial role of inflammatory, anti-angiogenic and pro-coagulant function mediators in triggering the disease (Hadley et al., 2018; Gilani et al., 2016; Xiao et al., 2017). EVs also play a key role in vascular functions, and in particular in nitric oxide (NO) mediated vasodilation of the uterine arteries (Donker et al., 2012; Tan, 2014).

Increased levels of anti-vascular growth factors (e.g., soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng)) in the maternal circulation are believed to be important in the pathogenesis of PE, as they reduce angiogenesis, and are known to be present even before the onset of PE (Escudero et al., 2016).

Recent studies also reported that proteins and microRNAs (miRNAs)

Table 2
Linear regression exploring the relationship between each extracellular vesicle (EV) and (1) right uterine artery pulsatility index (PI); (2) left uterine artery PI; (3) umbilical artery PI; (4) birthweight, by gestational trimester.

Variables	Mean UtA PI	UmA PI	Birthweight
Total EVs/uL	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	-5494 (− 18,938; 5885)	–	–
- Second trimester	36,234 (− 26,143; 98,634)	–	–
- Third trimester	-7609 (− 16,411; 13,567)	10,168 (− 27,000; 47,344)	2.73 (− 10.57; 16.03)
Platelet-derived EVs/uL	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	-1815 (− 5167; 1537)	–	–
- Second trimester	6584 (− 3730; 17,930)	–	–
- Third trimester	-1768 (− 4075; 359)	-1451 (− 4938; 2035)	0.36 (− 0.89; 1.62)
Leukocyte-derived EVs/uL	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	**22.3 (4.32; 37.1)**	–	–
- Second trimester	11.67 (− 127; 321)	–	–
- Third trimester	-1450.1 (− 5129; 3463)	1948 (− 3796; 7691)	0.11 (− 1.97; 2.18)
Endothelial-derived Evs/uL	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	-29.2 (− 77.6; 31.8)	–	–
- Second trimester	2946 (− 2055; 7946)	2504 (− 13,455; 18,463)	–
- Third trimester	-739 (− 1152; 129.5)	239 (− 1213; 1691)	-0.10 (− 0.42; 0.62)
Epithelial-derived Evs/uL	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	5.82 (− 101; 17.4)	–	–
- Second trimester	-5.95 (− 21.6; 16.2)	–	–
- Third trimester	-395.5 (− 2312; 1522)	1213 (− 1720; 4147)	0.23 (− 0.83; 1.29)
DNA-derived Evs/uL	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	61.1 (− 42.25; 164)	–	–
- Second trimester	53.1 (− 187; 322)	–	–
- Third trimester	-4789 (− 10,883; 1306)	741 (− 9368; 10,851)	1.31 (− 2.23; 4.87)
% platelet-derived vesicles	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	-8.78 (− 16.6; 7.67)	–	–
- Second trimester	-2.28 (− 17.5; 21.0)	–	–
- Third trimester	-22.5 (− 666; 21.5)	-30.6 (− 99.5; 38.2)	0.01 (− 0.02; 0.03)
% leukocyte-derived vesicles	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	0.36 (− 1.21; 3.37)	–	–
- Second trimester	-2.78 (− 16; 10.5)	–	–

(*continued on next page*)

Table 2 (*continued*)

Variables	Mean UtA PI	UmA PI	Birthweight
- Third trimester	1,71 (− 17.4; 21.2)	9.65 (− 20.3; 39.6)	0.00 (− 0.01; 0.01)
% endothelial-derived vesicles	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	-2.70 (− 11.7; 6.28)	–	–
- Second trimester	4.44 (− 5.09; 14.0)	7.76 (− 22.0; 37.6)	-0.01 (− 0.10; 0.06)
- Third trimester	10,87 (− 31.0; 40.3)	11.7 (− 43.3; 66.7)	-0.00 (− 0.02; 0.02)
% epithelial-derived vesicles	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	0.69 (− 1.29; 2.66)	–	–
- Second trimester	-0.60 (− 3.48; 2.78)	–	–
- Third trimester	3.48 (− 10.30; 17.2)	8.64 (− 12.5; 29.8)	-0.01 (− 0.01; 0.00)
% DNA-derived vesicles	*Regression coefficient for 1-unit increase (95 % CI)*		
- First trimester	0.00 (− 0.01; 0.03)	–	–
- Second trimester	0.00 (− 0.02; 0.03)	–	–
- Third trimester	-23.7 (− 61.5; 14,1)	-1.96 (− 63.2; 59.3)	0.01 (− 0.12; 0.03)

UtA: Uterine artery; UmA: Umbilical artery; CI: confidence interval. Significant results ($p < 0.05$) are reported in bold.

delivered by EVs in pregnancies regulate inflammatory responses and the invasion of trophoblasts through intercellular delivery in the placental microenvironment. EV-derived miRNAs have different functions, including control of the key signaling pathways in the maternal-fetal circulation for pregnancy disorders (Yang et al., 2020; Tan et al., 2020; Salomon and Rice, 2017; Birò et al., 2019).

However, assessing the role of EVs as potential biomarkers for different pregnancy complications implies comparison with apparently uncomplicated pregnancy. However, previous studies did not specifically report gestational age at comparison of EVs and it may be entirely possible that the concentration of the different SVs is related to pregnancy. More importantly, such studies did not report the strength of association between EVs and ultrasound parameters, such as fetal or maternal Dopplers or neonatal birthweight. We have previously reported that the concentrations of EVs is different in pregnant compared to non-pregnant women and that such different may reflect maternal adaptation to the physiological changes occurring in pregnancy. In the present study, we reported a relatively stable concentration of examined EVs during the three trimesters of pregnancy and no influence of neonatal birthweight or maternal and fetal Doppler on such concentration. This allows a more objective interpretation of studies comparing EVs between women with compared to those without obstetric complications from different trimester of pregnancy. The main limitations of the present study are that not all EV populations were examined, with a relatively small sample size of patients. Furthermore, pregnancy-specific EVs was not detected More importantly, the lack of association between the concentration of EVs and maternal or fetal Doppler's or neonatal birthweight suggest that the distribution is similar in uncomplicated pregnancies irrespective of the birthweight of the different values in umbilical or uterine arteries.

5. Conclusions

Distribution of examined EVs does not change during the three trimesters of pregnancy and is not influenced by neonatal birthweight or maternal and fetal Dopplers. The findings from this study allows a more objective interpretation of studies comparing EVs in pregnancies with compared to those without obstetric complication.

EVs identification, count and subtyping may play a promising role in the establishment of the liquid biopsy, especially in the context of the maternal-fetal cross-talk studies. Further analyzes exploring the role of EVs in different pathological conditions complicating pregnancy are needed in order to elucidate whether they may be integrated with the already existing diagnostic algorithms in order to more accurately predict the occurrence and stratify the severity of pregnancy-related complications.

Condensation

Extracellular vesicles (EVs) are cell-derived particles that circulate in the maternal blood and given that they cross the placenta, may play a promising role in the establishment of the liquid biopsy in pregnancy.

Why was the study conducted?

This study wants to describe the different EV phenotypes and concentrations in peripheral blood of uncomplicated pregnant women during the three trimesters of pregnancy.

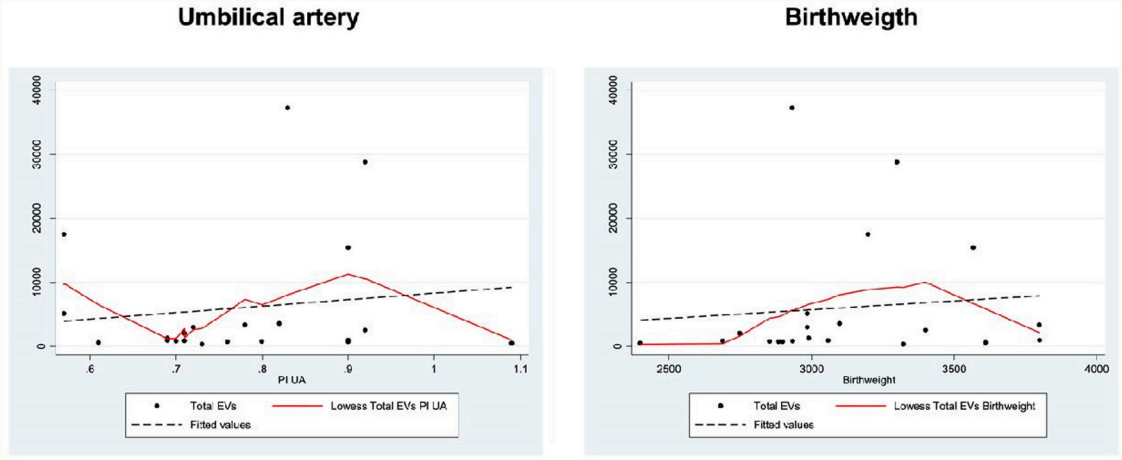

Fig. 1. Scatter plot (and regression lines) of the total extracellular vesicles (EVs) concentration versus the Pulsatility Index (PI) of the umbilical artery (UA) and birthweight in the third trimester.

Key findings

Distribution of EVs is stable through pregnancy and is not influenced by neonatal birthweight or maternal and fetal Dopplers. The findings from this study allows a more objective interpretation of studies comparing EVs in pregnancies with compared to those without obstetric complication. EVs in future can be used for "liquid biopsy" for the early diagnosis of pathological pregnancies up to the development of possible screening protocols.

What does this add to what is known?

This is, to the best of our knowledge, the first study exploring the peripheral blood concentration distribution of different EV cell-types in uncomplicated pregnancies during the three trimester of pregnancy. Prospective design, the inclusion of only asymptomatic cases and EV analysis with a new validated polychromatic flow cytometry protocol which ensures the sensitivity and ability of processing multiple parameters in a short period of time represent the main strengths of this study. The relevance of EVs in pregnancies has been largely demonstrated, given that placental-derived EVs are released into the maternal circulation and that their cargoes (i.e. proteins and miRNAs) target maternal cells, producing hormone-like effects and mediating the communication between the mother and the fetus. It is also known that EVs significantly increase during pregnancy, reaching their maximum level at term. These cell-derived particles circulating in all body fluids and involved in many pathophysiological processes, such as cell growth, activation, proliferation, apoptosis, or senescence may therefore represent promising biomarkers of different pathological conditions complicating pregnancies, such as from pre-eclampsia or to preterm birth.

CRediT authorship contribution statement

D.B., F.D.A., F.D.S. and P.L. conceived and planned the experiments. D.B., A.L., E.D.A. and D.V.B. carried out the experiments. S.V. and A.L. contributed to sample preparation. D.B. contributed to the interpretation of the results. F.D.A. and D.B. wrote the manuscript in consultation with F.D.A. and A.L, P.L., S.V., E.D.A. and M.L. reviewed the manuscript. All authors discussed the results and contributed to the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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