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Уровень внеклеточной ДНК плода, циркулирующей в сыворотке крови матери, как предиктор преэклампсии

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Резюме

Цель исследования — измерить уровень внеклеточной ДНК плода (вкДНКп), циркулирующей в сыворотке крови матери, и определить связь между уровнем вкДНКп в сыворотке матери и преэклампсией (ПЭ). **Материалы и методы.** В исследование типа случай-контроль включены 30 женщин с ПЭ и 30 сопоставимых здоровых беременных (контроль) в возрасте 18–40 лет. У всех обследуемых был один живой плод на сроке более 20 недель гестации. При включении в исследование у всех женщин были взяты образцы крови для определения уровня вкДНКп. **Результаты.** Уровень вкДНКп значительно отличался у женщин с ПЭ и женщин контрольной группы, более высокие уровни выявлены у женщин с ПЭ. Кроме того, уровень вкДНКп в материнской крови обладал предсказательной ценностью в отношении ПЭ (площадь под кривой 0,979). **Заключение.** Уровень вкДНКп в материнской крови может использоваться для оценки риска развития ПЭ. Средние уровни вкДНКп в сыворотке крови матери коррелируют с развитием осложнений у матери.

Ключевые слова: внеклеточная ДНК плода, преэклампсия, первобеременная

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Circulating maternal serum cell free fetal DNA levels for prediction of preeclampsia

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Abstract

Objective. To measure the maternal serum cell free fetal DNA (CFFDNA) and establish the relationship between preeclampsia (PE) and maternal serum (CFFDNA) levels. **Design and methods.** A nested case-control study was carried out on 30 (PE) subjects and 30 matched controls aged 18–40 years old who were submitted at the Ain-Shams University maternity hospital. All subjects had a single viable fetus beyond 20-week gestational age. Upon enrollment, blood samples were collected, and the CFFDNA was subsequently measured in subjects and controls. **Results.** The CFFDNA level differed significantly between the analyzed groups, showing higher levels in PE diagnosed individuals than in controls. Additionally, the maternal serum CFFDNA level, utilizing an area under the curve of 0,979, predicted PE. **Conclusion.** Maternal serum cell free DNA can be utilized for PE prediction. The mean levels of CFFDNA correlate with developing maternal complications in PE.

Key words: cell free fetal DNA, CFFDNA, preeclampsia, primigravida

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Introduction

Preeclampsia (PE) in the third world countries develops in up to 10 percentage of pregnancies, and emergency care is often nonexistent or insufficient [1].

The sensitivity and specificity of existing tools to predict adverse maternal and fetal outcomes are low, despite the use of proteinuria and blood pressure measurements for PE diagnosing. As a result, there is a need for tests that are both widely applicable and cost-effective, and that are able to precisely detect women at risk and predict which fetuses may show complications thus allowing for timely prenatal care in order to avoid morbidity and improve perinatal outcomes [2].

In Redman's PE three-stage paradigm, the initial stage is defined by the lowered placental perfusion, while the maternal syndrome (inflammatory/oxidative stress) defines the second stage. The initial stage is characterized by the placental bed spiral arteries failure to endure the typical physiological changes [3].

The PE second stage is characterized by an excessive trophoblast embolization, which results in the cell-free fetal DNA (CFFDNA) release in the mother circulation. The purines are catabolized to uric acid by xanthine oxidoreductase in the maternal liver, where the CFFDNA is subsequently degraded. It is hypothesized that the more xanthine oxidase activation, the more xanthine oxidoreductase toxic isoenzyme, and in subjects who subsequently develop PE, it leads to the reactive oxygen species (ROS) formation as byproducts when the hepatocytes are exposed to extreme quantities of purines throughout catabolism. The tissues normal antioxidant capacity is overridden by excessive ROS production. Oxidative stress is probably the key factor in developing the PE second stage. Although CFFDNA is a critical component of PE pathogenesis, as it serves as the initial substrate for ROS production, it is not the primary cause [4].

The non-invasive prenatal screening has been transformed by CFFDNA. In 1997, Y. D. Lo and colleagues first detected the fetal DNA sequences in maternal serum and plasma [5]. The pregnant women cell-free plasma contains substantially higher levels of fetal DNA than maternal blood cells [6].

Subsequent investigations have expanded the CFFDNA detection methods with the use of quantitative real-time polymerase chain reaction (PCR) and have concentrated on the clinical implication of this biomarker, with the focus on the pregnancy-associated complications and fetal inherited disorders. The latter approach has drawn significant attention due to its reliability, rapidity, less laborious protocol and low cost, which are all benefits in comparison to the initial fetal cells' isolation [7].

The **purpose of this study** was to measure the maternal serum CFFDNA and determine the correlation between maternal serum CFFDNA levels and PE in the cohort of women examined at Ain Shams University Maternity Hospital (ASUMH). The primary goal of this study was to estimate the predictive role of maternal serum CFFDNA levels in PE. The secondary outcome was the establishing the relationship between maternal serum CFFDNA levels and maternal and fetal morbidities, including intrauterine growth restriction (IUGR), oligohydramnios, and abruptio placentae.

Design and methods

From April 2021 to April 2023, in the nested case-control study 30 PE females and 30 matched controls primigravids examined at ASUMH were recruited. The Research Ethical Committee at the Faculty of Medicine at Ain Shams University approved the study, and all the subjects provided written informed consent.

Participants

The study comprised primigravidas who were initially healthy and aged 18–40 years, and who had been visiting ASUMH. The fetus was a single viable embryo after 20 weeks of gestation. Cases involving any fetal or maternal comorbidities, regardless if they are medical, surgical, or obstetrical (e.g. gestational hypertension, threatened abortion, diabetes mellitus, fetal hydrops, congenital fetal deformity, etc., and abnormal body mass index of the subjects > 25 kg/m^2 or < 18,5 kg/m^2) were excluded from the study.

Blood samples were collected and placed in EDTA sample vacuum tubes. After centrifuging the whole blood at 1600 g for ten minutes, cell-free plasma was collected and frozen at the temperature of –70 degrees Celsius.

To extract DNA, the QIAamp DNA Blood Mini Kit (Qiagen, Germany) was used, and the "Blood and Body Fluid Spin Protocol" recommended by the manufacturer was followed throughout the process.

Amplification of CFFDNA

PCR amplification was carried out with the use of Applied Biosystems' 7500 fast real time PCR (Foster City, United States).

The universal fetal DNA marker RASSF1A Quantification was achieved in both specimen (Qiagen, Germany) and a fully methylated genomic DNA. For real-time PCR the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, USA) was used in conjunction with the following primers and probe:

- RSF-dsgnR5-ACCAGCTGCCGTGTG G-3
- RSF-dsgnT5-FAM–CCAACGCGCTGCGCAT (MGB)-3
- RSF-b151F5-AGCCTGAGCTCATTGAGC TG-3
- RSF-dsgnT5-FAM–CCAACGCGCTGCGCAT (MGB)-3

Methods

We used the real-time 7500 rapid SDS software v.2.05 (Applied Biosystems, Foster City, USA).

Women were monitored until birth and categorized depending on the development of PE. A comprehensive history was obtained for all subjects, including personal, present, and past medical history, as well as family, obstetric, and menstrual history. The clinical investigation encompassed physical examination, including measurement of body temperature, vital signs, respiratory rate, blood pressure, body weight and pulse rate. Laboratory investigations encompassed the following: prothrombin time, concentration, and international normalized ratio (INR), the coagulation profile, Rh type and blood group, liver and renal functions complete blood profile. Detection of CFFDNA was performed in patients who met the study criteria. Abdominal ultrasound (fetal biometry) and ultrasound doppler (umbilical, middle cerebral and uterine artery) were performed utilizing the SONOACEX6MEDISION (USA) ultrasound device.

Sample size calculation

Setting alpha error at 5 %, power at 80 %, taking into account the results from the study PASS11 program [8], a sample size of at least 30 subjects and 30 matched controls was required to achieve the difference in cell free total DNA median (IQR) as following: 8,76 (0,44–48,43) in the main group versus 3,74 (0,12–21,14) among control group. Another study [9] showed that hypertensive disorders prevalence during pregnancy ranged from 2 % to 10 %. Assuming the prevalence of 10 % for hypertensive disorders during pregnancy, blood samples from at least 300 pregnant females were needed to achieve the goal.

Statistical analysis

The collected data were coded, tabulated, and statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 28.0, IBM Corp. (Chicago, USA, 2021). Quantitative data were tested for normality using Shapiro-Wilk test, then if normally distributed they were presented as mean ± SD (standard deviation) and compared using independent t-test with Leven's test for equality of variance. In case of non-normal distribution, the data were presented as Median (1^{st}-3^{rd} Interquartiles, IQR) and compared using Mann-Whitney test. Qualitative data summarized as number and percentage and compared using Fisher's Exact test. ROC (Receiver Operating Characteristics) curve was used to evaluate the performance of CFFDNA in diagnosing preeclampsia and its related maternal complications. The differences were considered significant at p-value ≤ 0,050.

Diagnostic characteristics were calculated as follows:

▫ Sensitivity = (True positive test / Total positive golden) × 100

▫ Specificity = (True negative test / Total negative golden) × 100

▫ Diagnostic accuracy =

([True positive test + True negative test] / Total cases) × 100

▫ Youden's index = sensitivity + specificity – 1.

Results

Among 300 samples, we identified 30 subjects who developed PE and 30 matched controls.

Table 1 presents a comparison of baseline characteristics and clinical outcomes between the PE group and the control group. The two groups were matched by age, body mass index (BMI), and gestational age. The average age was 24,4 ± 3,0 years in the PE group and 24,2 ± 2,7 years in the control group (p = 0,719), while the BMI was 35,0 ± 2,4 and 35,2 ± 1,7 kg/m², respectively (p = 0,667). Gestational age at the time of sample collection was also comparable, at 26,2 ± 1,5 weeks in the PE group and 25,8 ± 1,7 weeks in the control group (p = 0,300). Median CFFDNA level was elevated in PE women — 508,0 units (IQR: 360,0–863,0) compared to 43,0 units (IQR: 21,0–70,0) in the control group (p < 0,001).

Table 1

BASELINE CHARACTERISTICS AND MATERNAL AND FETAL OUTCOMES BETWEEN THE STUDY GROUPS

Variables		Preeclampsia group (n = 30)	Control group (n = 30)	p-value
Baseline characteristics				
Age, years		24,4 (3,0)	24,2 (2,7)	^0,719
BMI, kg/m²		35,0 (2,4)	35,2 (1,7)	^0,667
Gestational age, week		26,2 (1,5)	25,8 (1,7)	^0,300
CFFDNA, unit		508,0 (360,0–863,0)	43,0 (21,0–70,0)	⌂**< 0,001***
Outcomes				
Maternal outcomes, n (%)	**All complications**	6 (20,0 %)	0 (0,0 %)	**§ 0,024***
	HELP syndrome	3 (10,0 %)	0 (0,0 %)	§ 0,237
	Eclampsia	2 (6,7 %)	0 (0,0 %)	§ 0,492
	Accidental hemorrhage	1 (3,3 %)	0 (0,0 %)	§ 0,999
Neonatal outcomes, n (%)	**All complications**	7 (23,3 %)	0 (0,0 %)	**§ 0,011***
	IUGR	2 (6,7 %)	0 (0,0 %)	§ 0,492
	IUFD	5 (16,7 %)	0 (0,0 %)	§ 0,052

Note: Data described as Mean (SD); Median (1^{st}–3^{rd} Interquartiles) or number (%); BMI — body mass index; IUGR — intrauterine growth restriction; IUFD — intrauterine fetal death; ^Independent t-test; ⌂Mann-Whitney test; § Fisher's Exact test.

Clinically, PE patients experienced significantly more maternal complications, with 20,0 % (6 out of 30) reporting at least one complication versus none in the control group (p = 0,024). Though HELLP syndrome, eclampsia, and accidental hemorrhage were observed in 3 (10 %), 2 (6,7 %), and 1 (3,3 %) cases, respectively, these individual complications did not reach statistical significance, possibly due to the limited sample size. Neonatal outcomes showed a similar trend: 23,3 % of neonates in the PE group developed complications, compared to 0 % in the control group (p = 0,011). Of these, intrauterine growth restriction (IUGR) occurred in 2 cases (6,7 %) and intrauterine fetal death (IUFD) in 5 cases (16,7 %), with IUFD approaching statistical significance (p = 0,052). These findings indicate that elevated CFFDNA is associated not only with the presence of PE but also with poorer maternal and fetal outcomes.

Table 2 shows the correlation between CFFDNA levels and complications within the PE group alone. Among women diagnosed with PE, those who experienced maternal complications had higher CFFDNA levels: median CFFDNA 1120,5 (IQR: 978,0–1255,0) versus 434,0 (IQR: 191,0–593,0) units in those with and without maternal complications (p < 0,001). This supports the hypothesis that higher CFFDNA concentrations in maternal serum are predictive of more severe disease and associated complications.

In contrast, the association between CFFDNA levels and neonatal complications was not statistically significant: median CFFDNA 524,0 (IQR: 408,0–978,0) versus 492,0 (IQR: 150,0–863,0) units in women with neonates with and without complications (p = 0,624).

Table 3 presents the diagnostic utility of CFFDNA for both identifying PE and predicting maternal complications. The performance of CFFDNA as a diagnostic biomarker was exceptional. When used to diagnose PE, the area under the ROC curve (AUC) was 0,978 (95 % CI: 0,950–1,000), indicating good discrimination (Fig. 1). A cut-off level of ≥ 81,0 units showed 100 % sensitivity and 86,7 % specificity, with an overall diagnostic accuracy of 93,3 %. Therefore, CFFDNA was able to detect every true case of PE with the minimum rate of false positives.

In predicting maternal complications among PE women, CFFDNA performed remarkably well, with an AUC of 0,965 (95 % CI: 0,901–0,999). At a higher

Table 2

CFFDNA (UNIT) IN PREECLAMPSIA GROUP DEPENDING ON MATERNAL AND FETAL OUTCOMES

Variables	Positive	Negative	p-value
Maternal complications	1120,5 (978,0–1255,0)	434,0 (191,0–593,0)	⌂**< 0,001**
Neonatal complications	524,0 (408,0–978,0)	492,0 (150,0–863,0)	⌂0,624

Note: Data are presented as Median (1st–3rd Interquartiles); ⌂ Mann–Whitney test; positive — presence of complications, negative — absence of complications.

Table 3

DIAGNOSTIC PERFORMANCE AND CHARACTERISTICS OF CFFDNA IN DIAGNOSING PREECLAMPSIA AND RELATED MATERNAL COMPLICATIONS

Characteristics	Diagnosing preeclampsia		Any maternal complication in subjects with preeclampsia	
	Value	95 % CI	Value	95 % CI
AUC	0,978	0,950–1,000	0,965	0,901–0,999
p-value	< 0,001		< 0,001	
Cut point	≥ 81,0 units		≥ 811 units	
Sensitivity	100,0 %	88,4–100,0 %	100,0 %	54,1–100,0 %
Specificity	86,7 %	69,3–96,2 %	83,3 %	62,6–95,3 %
Diagnostic accuracy	93,3 %	83,8–98,2 %	86,7 %	69,3–96,2 %
Youden’s Index	86,7 %	74,5–98,8 %	83,3 %	68,4–98,2 %

Note: AUC — area under curve; CI — confidence interval.

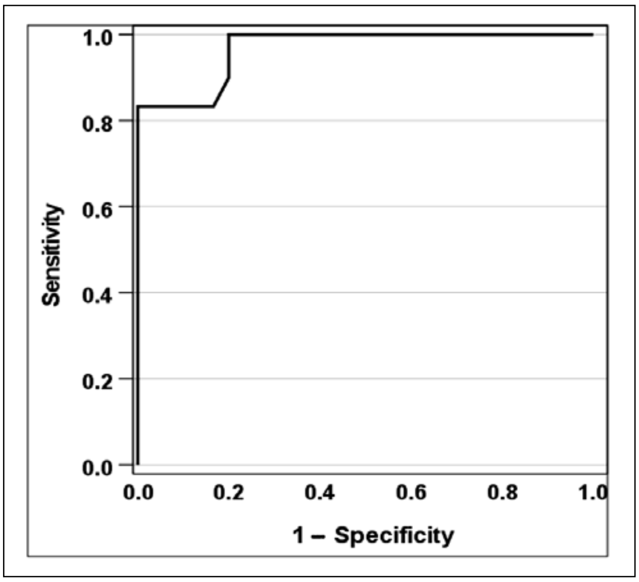

Figure 1. ROC curve for CFFDNA in diagnosing preeclampsia

cut-off ≥ 811 units, the sensitivity remained 100 %, while specificity was slightly lower — 83,3 % (Fig. 2). Diagnostic accuracy in this scenario was 86,7 %, with a Youden's index of 83,3 %. These findings suggest that CFFDNA can serve as both a screening and risk stratification tool, offering valuable predictive insight into not only the diagnosis of PE but also the likelihood of its progression to more severe maternal complications.

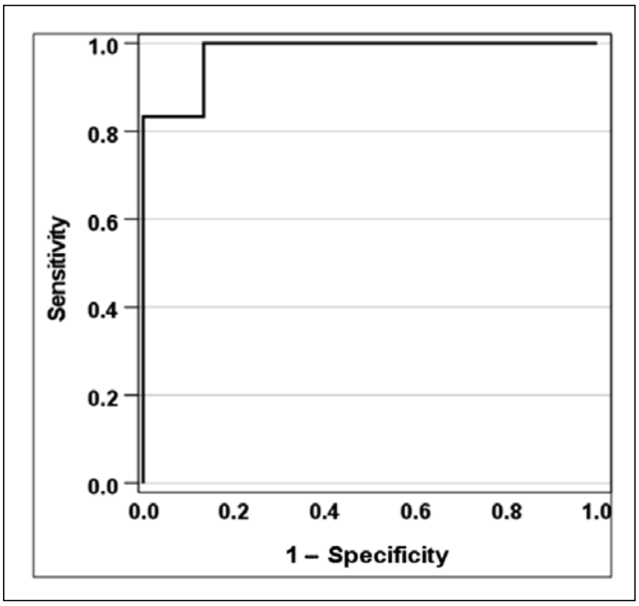

Figure 2. ROC curve for CFFDNA in diagnosing having any preeclampsia related maternal complication in preeclampsia

Discussion

In developed world, PE is one of the most significant perinatal and maternal morbidity and mortality causes. Despite the fact that its pathogenesis is not completely understood, vascular endothelial cell dysfunction is generally considered the main underlying mechanism. The pathological changes that underlie endothelial cell dysfunction are still not fully elucidated; however, poor placentation has been suggested as a significant contributing reason. Placental ischemia results from spiral arteries incomplete or failed trophoblastic invasion, which leads to a diffusion of one or more factors associated with the impairment of the maternal vascular endothelium [10].

The release of CFFDNA into the mother's bloodstream is a consequence of excessive trophoblast embolization throughout the PE second stage. The maternal liver degrades the CFFDNA, and xanthine oxidoreductase catabolizes the purines to uric acid. The isoenzyme of xanthine oxidoreductase — xanthine oxidase — is believed to produce ROS as a byproduct. This enzyme is activated in hepatocytes that receive excessive number of purines in PE [11].

The natural antioxidant capacity of the tissues is overpowered by excessive ROS production, resulting in oxidative stress. Most likely, the primary factor contributing to the PE second stage development is oxidative stress. As the initial substrate for ROS formation, CFFDNA plays a significant role in PE pathophysiology [12].

The primary objective of the study was to investigate the levels of maternal serum CFFDNA and its relationship with PE. A nested case-control design was used, involving 30 PE subjects and 30 matched controls. Participants ranged in age from 18 to 40 years and were beyond 20 weeks of gestation with a single viable fetus. Blood samples were collected upon enrollment for CFFDNA levels evaluation. PE group showed significantly higher CFFDNA and more frequent maternal and neonatal complications.

Our results were supported by T. R. Kolarova and colleagues (2021) [13]: in their cohort, significantly higher total CFFDNA was observed in the PE group compared to controls (1235 versus 106,5 pg/μL, $p < 0{,}001$).

Also, M. Soroura and colleagues (2022) [7], who evaluated the maternal serum CFFDNA levels in primigravidas at 10–20 weeks of gestation as a predictive test for the PE development, demonstrated a statistically significant increase in the median CFFDNA (GE/ml) in severe PE compared to non-severe PE and the control group.

Moreover, H. Yu and colleagues (2013) [14] found that the serum CFFDNA level was significantly higher in PE mothers than in the controls. Additionally, the higher CFFDNA may be the consequence of placental apoptotic and/or necrotic materials increased discharge and reduced clearance from maternal blood, which is induced by fetoplacental hypoxemia.

Additionally, J.H. Kang and colleagues (2008) [15] conducted a prospective cohort investigation. In 32 gravidas that ultimately encountered PE, the CFFDNA level was significantly higher than in normal pregnancies, as discovered by the testing of 3076 gravidas. This discovery is in accordance with our findings.

In a previous study by S.Y. Kim and colleagues (2016) [16], the fetal CFFDNA, total CFDNA, and pregnancy-associated plasma protein-A combination was shown to be a valuable PE predictor throughout the first trimester. They utilized the HYP2 gene as total CFFDNA marker to document that the total CFFDNA levels were significantly higher in subjects who later developed PE at 6–14 and 15–23 gestational weeks.

Our study demonstrated that PE was strongly associated with higher maternal and neonatal complication rates. In addition, CFFDNA was significantly higher in subjects with maternal complications, but there was no significant association with neonatal complications. These results were supported by T.N. Leung and colleagues (2001) [17]: a five-fold increase in the mean circulating fetal DNA was observed in subjects with complicated pregnancies compared to control pregnant women ($p < 0{,}001$).

Also, X.Y. Zhong and colleagues (2002) [18] also demonstrated in preeclamptic mothers with maternal complications a significant increase in the fetal DNA copies number.

R. Darghahi and colleagues (2019) [19] showed a direct and significant correlation between GA and CFFDNA in both cases and controls. The serum CFFDNA levels are reduced as gestation progresses.

Using ROC-curves, a cut-off value of CFFDNA $\geq$ 81,0 units demonstrated excellent diagnostic performance with 100,0 % sensitivity, 86,7 % specificity for diagnosing PE. For identifying maternal complications in PE, a higher threshold of $\geq$ 811 units achieved perfect sensitivity (100,0 %) and moderate specificity (83,3 %). These findings underline the potential of CFFDNA as a highly sensitive biomarker for early detection and risk stratification in PE.

These findings are consistent with M. Soroura and colleagues (2022) [7], who identified optimal thresholds using ROC analysis. For distinguishing PE from controls, a cutoff of 222 units achieved 70,2 % sensitivity and 84,4 % specificity, while a cutoff of 378 units effectively discriminated non-severe from severe PE with 85,7 % sensitivity and 83,3 % specificity. H. Yu and colleagues (2013) [14] similarly applied ROC curve analysis and identified a log-transformed CFFDNA threshold of 2.62 for PE prediction, with high sensitivity and specificity metrics

Moreover, M.Y. Divon and colleagues (2001) [20] reported a five-fold increase in plasma CFFDNA levels among pregnancies with maternal complications compared to healthy pregnancies, reaffirming its value as a clinical marker.

Strengths of the study

One of the key strengths of the study is the innovative use of CFFDNA as a biomarker for predicting PE and maternal complications. Our study demonstrates significant diagnostic accuracy, with high sensitivity (100 %) and moderate specificity for both PE and maternal complications. This highlights the potential for CFFDNA to revolutionize early detection and risk stratification in obstetrics, providing a reliable tool for clinical use.

The study design adds to its robustness. By employing a nested case-control methodology, the researchers ensured a focused comparison between PE subjects and matched controls. The inclusion of relevant variables, such as age, BMI, gestational age, and outcome measures, allows for a comprehensive analysis of the relationship between CFFDNA levels and complications. Additionally, ROC curves analysis enhances the reliability of the diagnostic performance assessments.

Limitations of the study

Despite its strengths, the study has several limitations that should be acknowledged. First, the sample size is relatively small, with only 30 participants in each group. This limited number may reduce the statistical power of the findings and restrict the generalizability of the results to broader populations. Larger, multicenter studies would be necessary to validate these outcomes.

Second, the study focused exclusively on a single population at Ain-Shams University Maternity Hospital. This may involve selection bias and limit the applicability of the findings to diverse demographic or geographic populations with varying baseline risks of PE and different clinical practices.

Another limitation is the lack of longitudinal data. While the study effectively establishes associations between CFFDNA levels and PE, it does not track changes in CFFDNA levels over time or explore their predictive value earlier in pregnancy. Furthermore, the inability to conclusively link CFFDNA with neonatal complications suggests that additional studies are needed to explore this area. This gap underscores the need for a more comprehensive evaluation of neonatal outcomes and potential confounders.

Conclusion

This study establishes that circulating maternal serum CFFDNA levels are significantly elevated in PE and correlated with maternal complications, making it a promising diagnostic biomarker. With a sensitivity of 100 % for both PE and maternal complications, CFFDNA can play a crucial role in early identification and management. However, while the association with neonatal complications remains inconclusive, the findings highlight the need for further research to refine CFFDNA predictive capabilities and to integrate it into clinical practice.

Конфликт интересов / Conflict of interest

Авторы заявили об отсутствии конфликта интересов. / The authors declare no conflict of interest.

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