OBSTETRICS

# First-trimester maternal plasma cell-free fetal DNA and preeclampsia

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**OBJECTIVE:** The purpose of this study was to determine whether, in pregnancies that experience preeclampsia, plasma cell-free fetal DNA (cffDNA) at 11-13 weeks of gestation is increased and whether this increase is related to the uterine artery pulsatility index (PI).

**STUDY DESIGN:** Plasma cffDNA and uterine artery PI were measured in 44 cases with preeclampsia, which included 11 cases that required delivery at <34 weeks of gestation and 176 normal control subjects. All fetuses were male, and cffDNA was assessed by amplification of the DYS14 gene. The association between cffDNA and uterine artery PI was assessed by regression analysis.

**RESULTS:** Median cffDNA was higher in early preeclampsia (median, 95.5 genome equivalents/mL; interquartile range, 72.7-140.9 genome equivalents/mL), but not late preeclampsia (median, 50.8 genome equivalents/mL; interquartile range, 25.0-103.8 genome equivalents/mL), than control subjects (median, 51.5 genome equivalents/mL; interquartile range, 31.1-84.9 genome equivalents/mL). There was a significant association between cffDNA and uterine artery PI ($P = .038$) but not in the control subjects ($P = .174$).

**CONCLUSION:** The increase in plasma cffDNA in pregnancies that experience preeclampsia is associated with the degree of impairment in placental perfusion.

**Key words:** cell-free fetal DNA, Doppler, preeclampsia, screening, uterine artery

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Preeclampsia, which affects approximately 2% of pregnancies, is a major cause of maternal and perinatal morbidity and death.[1-3] The underlying mechanism for preeclampsia is thought to be impaired placentation because of inadequate trophoblastic invasion of the maternal spiral arteries, which has been documented by the findings of both histologic and Doppler ultrasound studies of the uterine arteries.[4-7] The differences between pregnancies in which preeclampsia develops and control pregnancies in uterine artery pulsatility index (PI) are marked particularly in severe early onset disease that requires delivery at <34 weeks of gestation.[7] The likelihood of the development of preeclampsia can be predicted by a combination of factors in the maternal history, including black racial origin, high body mass index (BMI), personal or family history of preeclampsia, and the measurement of uterine artery PI at 11-13 weeks of gestation.[7,8]

Several studies reported that, in patients with preeclampsia, the maternal plasma or serum concentration of cell-free fetal DNA (cffDNA) is 2- to 15-fold higher than in normotensive control subjects (Table 1).[9-23] It has been postulated that impaired trophoblastic invasion of the maternal spiral arteries leads to placental ischemia, with release into the maternal circulation of necrotic or apoptotic syncytiotrophoblast fragments that contain fetal DNA.[21,22] In addition to evidence for increased entry of cffDNA into the maternal circulation, there is also evidence that, in preeclampsia, there is reduced clearance of cffDNA from maternal plasma.[13] However, there is controversy as to whether the altered levels precede the onset of the disease (Table 1).[19-23]

The aim of this study was to investigate whether the maternal plasma cffDNA at 11-13 weeks of gestation in pregnancies in which preeclampsia subsequently develops is different from pregnancies without this complication and whether any possible differences are related to the severity of preeclampsia. We also examined the possible association between plasma cffDNA levels and uterine artery PI.

## Materials and Methods

### Study population

This was a case-control study in singleton pregnancies. In our center, we perform screening for hypertensive complications of pregnancy in women who attend for their routine first hospital visit in pregnancy. In this visit, which is held at $11^{+0}$-$13^{+6}$ weeks of gestation, all women have an ultrasound scan (1) to confirm gestational age from the measurement of the fetal crown-rump length, (2) to diagnose any major fetal abnormalities, and (3) to measure fetal

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**TABLE 1**
**Studies that compare maternal plasma concentration of cell-free fetal DNA in pregnancies with preeclampsia and normotensive control subjects**

| Study | Marker | Sampling time (wk)[a] | Preeclampsia group, n | Genome equivalent/mL[a] | Control group, n | Genome equivalent/mL[a] |
|---|---|---|---|---|---|---|
| During preeclampsia | | | | | | |
| Lo et al[9] | SRY | 32 (27-41) | 20 | 381 (194-788) | 20 | 76 (54-163) |
| Zhong et al[10] | SRY | — (28-40) | 39 | 1599 (0-7968) | 46 | 333 (0-1608) |
| Smid et al[11] | SRY | — (26-40) | 17 | 256 (59-859) | 38 | 24 (0-138) |
| Swinkels et al[12] | SRY | 33 (27-34) | 7 | 781 (503-1212)[b] | 10 | 128 (96-170)[b] |
| Lau et al[13] | SRY | 32 (30-37) | 7 | 521 (274-3089) | 10 | 277 (34-468) |
| Sekizawa et al[14] | DYS | 35 (29-36) | 9 | 2.1 MoM (0.9-9) | 20 | 1.0 MoM (0.4-2.0) |
| Shimada et al[15] | SRY | — (29-38) | 15 | 173 ± 95[c] | 59 | 22 ± 9[c] |
| Farina et al[16] | SRY | 33.7 ± 3.9[d] | 34 | 2.6 ± 2.7 MoM[d] | 102 | 1.0 ± 1.5 MoM[d] |
| Zhong et al[17] | SRY | 30 (24-33) | 11 | Early preeclampsia: 574 (113-7088) | 18 | 106 (47-269) |
| | | 36 (34-41) | 12 | Late preeclampsia: 536 (61-1324) | 12 | 125 (38-358) |
| Smid et al[18] | SRY | 32.5 (25-38) | 28 | Preeclampsia, no IUGR: 195 (45-990) | 89 | 58 (2-391) |
| | | 33 (23-36) | 15 | Preeclampsia and IUGR: 304.1 (96.5-1682.3) | | |
| Before preeclampsia | | | | | | |
| Leung et al[19] | SRY | 17 (11-22) | 18 | 42 (36-2375) | 33 | 22 (4.2-300) |
| Zhong et al[20] | SRY | 20 (19-25) | 10 | 423 (97-1642) | 40 | 129 (31-318) |
| Levine et al[21] | DYS | — (17-28) | 138 | 36 ± 6[c] | 137 | 16 ± 6[c] |
| Farina et al[22] | DYS | 20 ± 2.08[d] | 6 | 2.4 ± 2.8 MoM[d] | 30 | 1.0 ± 0.8 MoM[d] |
| Crowley et al[23] | SRY | 13 (10-20) | 16 | 31 (0-214)[e] | 72 | 28 (0-1280) |

*IUGR*, intrauterine growth restriction; *MoM*, multiples of the median.

[a] Data are given as median (range), unless otherwise indicated; [b] Data are given as mean (95% confidence interval); [c] Data are given as mean ± SEM; [d] Data are given as mean ± SD; [e] Not significantly different from control subjects.

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nuchal translucency thickness as part of screening for chromosomal abnormalities.[24,25] We record maternal characteristics and medical history, measure the uterine artery PI by transabdominal color Doppler,[7] and store plasma at −80°C for subsequent biochemical analysis. Written informed consent was obtained from the women who agreed to participate in the study, which was approved by the King's College Hospital Ethics Committee. Data from the patients included in this study were included in previous publications on first-trimester screening for hypertensive disorders, but this is the first study on plasma cffDNA.

Patients were asked to complete a questionnaire on maternal age, racial origin (white, black, Indian or Pakistani, Chinese or Japanese, and Mixed), cigarette smoking during pregnancy (yes or no), method of conception (spontaneous, use of ovulation drug, and in vitro fertilization), medical history (ie, chronic hypertension, diabetes mellitus, antiphospholipid syndrome, thrombophilia, human immunodeficiency virus infection, and sickle cell disease), medication (ie, antihypertensive, antidepressant, antiepileptic, antiinflammatory, aspirin, β-mimetic, insulin, steroids, thyroxine), parity (parous or nulliparous, if no delivery beyond 23 weeks of gestation), obstetric history (ie, previous pregnancy with preeclampsia or spontaneous preterm delivery at <34 weeks of gestation), and family history of preeclampsia (mother). The maternal weight and height were measured, and the BMI was calculated in kilograms per square meter.

We measured cffDNA in samples from 44 cases with preeclampsia, which included 11 cases that required delivery at <34 weeks of gestation (early preeclampsia) and from 176 control preg-

nancies that did not experience any complications and resulted in the live birth of phenotypically normal neonates. Each case was matched with 4 control subjects for length of storage of their blood samples; none of the samples was previously thawed and refrozen.

The definition of *preeclampsia* was from the International Society for the Study of Hypertension in Pregnancy, which requires the development of diastolic blood pressure of ≥90 mm Hg on at least 2 occasions 4 hours apart after 20 weeks of gestation in previously normotensive women and proteinuria of ≥300 mg in 24 hours or 2 readings of at least "++" on dipstick analysis of midstream or catheter urine specimens if no 24-hour collection is available.[26] In preeclampsia superimposed on chronic hypertension, significant proteinuria (as defined earlier) should develop >20 weeks of gestation in women with known chronic hypertension (history of hypertension before conception or the presence of hypertension at the booking visit at <20 weeks of gestation in the absence of trophoblastic disease).

### Quantification of cell-free male fetal DNA

Maternal venous blood samples (5.0 mL) were collected into tubes that contained ethylenediaminetetraacetic acid; within 15 minutes of collection, the plasma was separated (by centrifugation at 2000*g* for 10 minutes) and stored in propylene tubes at −80°C until use. The plasma samples were thawed and centrifuged (≥11,000*g* for 3 minutes) to remove residual cells, cell debris, and particulate matter. DNA was extracted from 240 μL of the supernatant with the use of the NucleoSpin Plasma XS kit (Macherey-Nagel, Duren, Germany) in accordance with the manufacturer's specifications. DNA was eluted in 20 μL final volumes of water to result in highly concentrated DNA; 5 μL of the appropriate dilutions were used for downstream quantitative polymerase chain reaction (qPCR) application.

The Y-chromosome DYS14 locus was used to determine the amount of cell-free male fetal DNA. Real-time PCR amplification of the β-globin gene was performed as a control to confirm the amplifiability of plasma-extracted DNA. For the quantitative analysis of DYS14, we used the Plexor qPCR System (Promega, Madison, WI), as previously described.[27,28] Multiplex real-time qPCR analysis was performed with an Mx3000P Sequence Detector (Stratagene, La Jolla, CA); the raw data were further analyzed by the Plexor qPCR software (Promega). Primer sequences that were specific for multiplexing were designed with the Plexor Primer Design Software, with the use of all the necessary parameters that are required for use with the Plexor qPCR system. The marker sequences that we designed were TSPY1(DYS14): 5′-FAM-iso-dC-GGGCACGTGGGAGGAAAGTC-3′ and 5′-GGTACTCGGAGCCTCTATTTGC-3′ and β-globin: 5-–ROX-iso-dC-GGTCTCCTTAAACCTGTCTTGTAACC-3′ and 5′-GGGCAAGGTGAACGTGGATGAA-3′. No template and positive control reactions were run in parallel to ensure the absence of contamination of the DNA and to verify that the reagents and instrumentation performed consistently. Five microliters of the extracted plasma DNA and dilutions were used for amplification. The thermal profile was carried out using 2-minute incubation at 95°C, followed by an initial denaturation step at 95°C for 5 seconds, followed by 38 cycles of 35 seconds at 60°C. The reaction ended with a Melt-Curve Analysis in which the temperature was increased from 60-95°C at a linear rate of 0.2°C/sec. Each sample was analyzed in duplicate, and the average of the 2 measurements was used for analysis. To determine the number of copies of male DNA present in the plasma sample, we ran a standard dilution curve using a known concentration of a commercially available male genomic DNA (Promega). We used 7 serial 5-fold dilutions, which ranged from 460-0.02944 ng. A conversion factor of 6.6 pg of DNA per cell was used to express the results as copy numbers or genome equivalents (genome equivalents/milliliter), as previously described.[9] Strict precautions were taken to avoid qPCR contamination.

### Statistical analysis

The measured uterine artery PI and plasma concentration of cffDNA were log-transformed to make the distributions Gaussian; the normality of the distributions was confirmed by the Kolmogorov-Smirnov test. In each case and control, the measured uterine artery PI was converted into multiples of the median (MoM) after adjustment for gestation, maternal age, racial origin, BMI, and history of preeclampsia, as previously described.[7] Multiple regression analysis demonstrated that log cffDNA in the control group was not related significantly to fetal crown-rump length, maternal age, racial origin, weight, or smoking; therefore, the values were not converted to multiples of the median. The Bland-Altman analysis was used to compare the measurement agreement and bias for the paired measurements of cffDNA in each case.[29] Mann-Whitney *U* test was used to determine the significance of differences in the median cffDNA and uterine artery PI multiples of the median between the preeclampsia and control groups; the Kruskal-Wallis test with Dunn's procedure was used for comparison of early and late preeclampsia groups with the control groups. Linear regression analysis was then used to determine the significance of association between log cffDNA with log multiples of the median uterine artery PI in each outcome group.

The SPSS statistical software package (version 12.0; SPSS, Inc, Chicago, IL) and XLSTAT software package (version 2008.7.01; XLSTAT, Addinsoft SARL, Paris, France) were used for all data analyses.

## RESULTS

Maternal blood was collected between March 2006-March 2007 and analyzed at a median interval of 20.6 months (range, 13.3–25.0 months). The maternal characteristics of each of the outcome groups are compared in Table 2. In the preeclampsia group, compared with the control group, the BMI was higher, and more women had a personal or family history of preeclampsia and chronic hypertension. In the preeclampsia group,

TABLE 2
**Maternal characteristics in the outcome groups**

| Maternal characteristic | Group | | | |
|---|---|---|---|---|
| | Control (n = 176) | Preeclampsia (n = 44) | Early Preeclampsia (n = 11) | Late Preeclampsia (n = 33) |
| Maternal age, y[a] | 32.4 (28.6-36.3) | 31.7 (26.3-36.1) | 32.7 (27.7-38.6) | 31.5 (25.0-36.0) |
| Body mass index, kg/m$^2$,[a] | 24.3 (22.2-27.8) | 26.6 (23.4-32.9)[b] | 26.6 (23.3-33.3) | 26.6 (23.5-32.5)[b] |
| Crown-rump length, mm[a] | 64.6 (60.0-69.7) | 63.5 (58.1-72.0) | 67.0 (56.4-72.8) | 63.1 (58.2-71.8) |
| Racial group, n | | | | |
| White | 120 (68.2%) | 25 (56.8%) | 4 (36.4%) | 21 (63.6%) |
| Black | 32 (18.2%) | 14 (31.8%) | 5 (45.5%)[b] | 9 (27.3%) |
| Indian or Pakistani | 11 (6.3%) | 3 (6.8%) | 1 (9.1%) | 2 (6.1%) |
| Chinese or Japanese | 4 (2.3%) | 0 | 0 | 0 |
| Mixed | 9 (5.1%) | 2 (4.5%) | 1 (9.1%) | 1 (3.0%) |
| Parity, n | | | | |
| Nulliparous | 88 (50.0%) | 32 (72.7%) | 7 (63.6%) | 25 (75.8%) |
| Parous: no previous preeclampsia | 85 (48.3%) | 6 (13.6%)[c] | 1 (9.1%) | 5 (15.2%)[b] |
| Parous: previous preeclampsia | 3 (1.7%) | 6 (13.6%)[b] | 3 (27.3%)[b] | 3 (9.1%) |
| Cigarette smoker, n | 6 (3.4%) | 4 (9.1%) | 0 | 4 (12.1%) |
| Family history of preeclampsia: mother, n | 5 (2.8%) | 6 (13.6%)[c] | 2 (18.2%) | 4 (12.1%)[b] |
| Conception, n | | | | |
| Spontaneous | 170 (96.6%) | 40 (90.0%) | 9 (81.8%) | 31 (93.9%) |
| Ovulation drugs | 5 (2.8%) | 2 (4.5%) | 1 (9.1%) | 1 (3.0%) |
| In vitro fertilization | 1 (0.6%) | 2 (4.5%) | 1 (9.1%) | 1 (3.0%) |
| Medical history, n | | | | |
| None | 175 (99.4%) | 39 (88.64.1%) | 9 (81.8%) | 30 (90.9%) |
| Chronic hypertension | 1 (0.6%) | 3 (6.8%)[b] | 2 (18.2%)[b] | 1 (3.0%) |
| Diabetes mellitus | 0 | 1 (2.3%) | 0 | 1 (3.0%) |
| Thrombophilia | 0 | 1 (2.3%) | 0 | 1 (3.0%) |
| Medication during pregnancy, n | | | | |
| None | 165 (93.8%) | 39 (88.6%) | 9 (81.8%) | 30 (90.9%) |
| Antihypertensives | 0 | 1 (2.3%) | 1 (9.1%) | 0 |
| Insulin | 0 | 1 (2.3%) | 0 | 1 (3.0%) |
| $\beta$-mimetics | 2 (1.1%) | 0 | 0 | 0 |
| Combined asthma | 3 (1.7%) | 1 (2.3%) | 0 | 1 (3.0%) |
| Thyroxine | 2 (1.1%) | 2 (4.5%) | 1 (9.1%) | 1 (3.0%) |
| Antiepileptic | 3 (1.7%) | 0 | 0 | 0 |
| Antidepressants | 1 (0.6%) | 0 | 0 | 0 |

Comparison for categoric variables by $\chi^2$ test or Fisher exact test; comparison between preeclampsia and control groups by *t* test for maternal age and fetal crown-lump length and by Mann-Whitney test for body mass index; comparison between early and late preeclampsia groups with the control groups by analysis of variance test (with the Bonferroni post hoc test) for maternal age and fetal crown-lump length; and Kruskal-Wallis test with Dunn's procedure for body mass index.

[a] Data are given as median (interquartile range); [b] $P < .05$; [c] $P < .01$.

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the median uterine artery PI was significantly higher than in the control group, and these differences were more marked in early preeclampsia than late preeclampsia (Table 3).

Fetal DNA was detected in all 220 samples. The mean difference between paired measurements of cffDNA was 1.1 genome equivalents/mL (95% confidence interval [CI], -0.8 to 3.0); the 95% limits of agreement were –26.4 genome equivalents/mL (95% CI, -29.6 to -23.2) to 28.6 genome equivalents/mL (95% CI, 25.4-31.8). The mean difference between paired measurements, which are expressed as a percentage of the average between the 2, was 3.0% (95% CI, 0.6-5.4); the 95% limits of agreement were -32.8% (95% CI, -36.9 to -28.6) to 38.8% (95% CI, 34.6-42.9). The difference was >15% in 77 cases (35.0%) and >20% in 31 cases (14.1%).

In the control group, multiple regression analysis demonstrated that the concentration of cffDNA was not affected significantly by any of the maternal or fetal characteristics. The median cffDNA was higher in the early-preeclampsia group than in the control group, but there was no significant difference between late preeclampsia and the control groups (Table 3). There was a significant association between cffDNA and uterine artery PI in the preeclampsia group ($P = .038$) but not in the control group ($P = .174$; Figure). The performance of screening for early preeclampsia by a combination of history and uterine artery PI (area under receiver operating curve, 0.851; 95% CI, 0.792-0.899) was not improved significantly by the addition of serum cffDNA (area under receiver operating curve, 0.918; 95% CI, 0.869-0.953; $P = .169$).

**TABLE 3**
**Comparison of median (interquartile range) of maternal plasma cell-free fetal DNA and uterine artery pulsatility index of preeclampsia and unaffected groups**

| Study group | Maternal plasma cell-free fetal DNA (genome equivalents/mL) | Uterine artery pulsatility index (MoM) |
|---|---|---|
| Unaffected | 51.5 (31.1-84.9) | 1.04 (0.82-1.25) |
| Preeclampsia | 71.2 (30.3-107.4) | 1.26 (0.93-1.50)[a] |
| Early preeclampsia | 95.5 (72.7-140.9)[a] | 1.51 (1.02-1.70)[a] |
| Late preeclampsia | 50.8 (25.0-103.8) | 1.13 (0.91-1.42) |

The Mann-Whitney test was used for comparison between preeclampsia and unaffected groups; the Kruskal-Wallis test with Dunn's procedure was used for comparisons between early and late preeclampsia with the unaffected group.
*MoM*, multiples of the median.
[a] $P < .05$.
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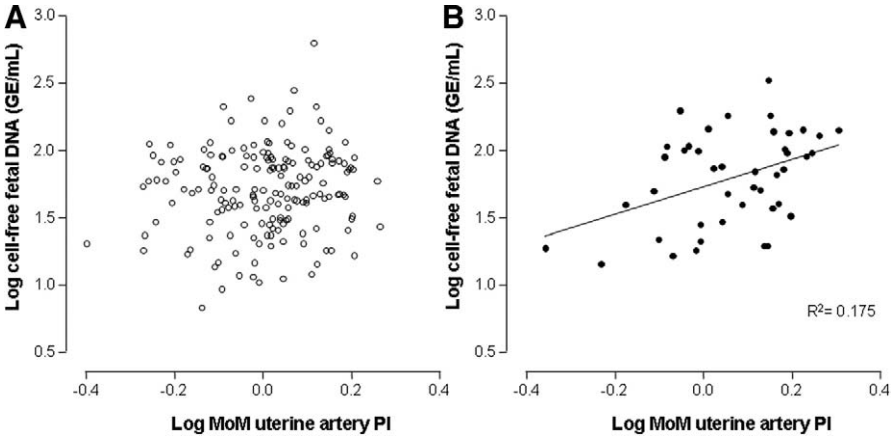
## COMMENT

The findings of this study confirm the association of preeclampsia with increased cffDNA in maternal blood and demonstrate that (1) the increased cffDNA is evident from 11-13 weeks of gestation and (2) the level of cffDNA is associated with the degree of impairment in placental perfusion and severity of preeclampsia. Significantly increased levels of cffDNA and uterine artery PI were observed in severe early-onset preeclampsia that required delivery at <34 weeks of gestation rather than in late-onset disease. Previous studies demonstrated that it is early preeclampsia rather than late preeclampsia that is associated with an increased risk of perinatal death and morbidity and both short-term and long-term maternal complications.[30-32]

In this study we used the DYS14 gene to characterize and quantify cffDNA in maternal blood. Although most previous studies used the single copy SRY gene as their fetal marker, there is recent evidence that a multicopy sequence on the Y chromosome (such as DYS14) is more sensitive, accurate, and efficient than SRY in the assessment of cffDNA, which is particularly important in the first trimester, when the copy numbers of fetal DNA are low.[33,34]

In preeclampsia there is placental ischemia and maternal endothelial dysfunction. The finding of an association between cffDNA level and uterine artery PI is compatible with the hypothesis that impaired trophoblastic invasion of the

**FIGURE**
**Relationship between maternal plasma cell-free fetal DNA concentration and uterine artery pulsatility index (PI)**




Relationship between maternal plasma cell-free fetal DNA concentration and uterine artery pulsatility index (log genome equivalents [GE]/milliliter) and uterine artery pulsatility index in the **A,** unaffected and **B,** preeclampsia groups.
*MoM*, multiples of the median.
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maternal spiral arteries leads to placental ischemia and damage, with the consequent release of apoptotic syncytiotrophoblast fragments that contain fetal DNA into the maternal circulation.[21] The extent to which the cffDNA in maternal blood is a mere marker of impaired placentation or the necrotic placental products are involved in the pathogenesis of preeclampsia by causing maternal endothelial dysfunction and the development of the clinical symptoms of the disease remains to be determined.

Established disease is associated certainly with a substantial increase in cffDNA in maternal blood[9-18] that could be the consequence of both increased passage from the placenta and decreased clearance from maternal blood.[13] Fetal DNA could be liberated directly from dying cells in the placenta and/or fetal cells[35] and cytotrophoblast microvilli,[36] the escape of which into the maternal circulation is increased in preeclampsia. Studies that have examined women before the clinical onset of preeclampsia have reported conflicting results concerning levels of cffDNA. Because liver and renal functions are not impaired before the onset of preeclampsia, it is unlikely that women who are destined to experience preeclampsia would have reduced clearance of cffDNA.[13,19] In contrast, studies during the second trimester demonstrated that increased fetal erythroblast trafficking into the maternal circulation predates the clinical onset of preeclampsia.[37,38] The contradictory results concerning cffDNA could be a consequence of a difference in the study populations, both in terms of the severity of preeclampsia and gestation at sampling. In our study, significantly increased cffDNA was observed in the cases of early preeclampsia but not in cases with late preeclampsia. In the studies that have reported a 2- to 3-fold increase in cffDNA in women who are destined to experience preeclampsia, the mean gestation at sampling was 17-20 weeks,[19,20,22] compared with 13 weeks of gestation in the study with no significant difference between the preeclampsia and control groups.[23] Levine et al[21] measured cffDNA longitudinally from 8 weeks of gestation and reported that, compared with control subjects, in women who subsequently experienced preeclampsia, the levels were similar before 17 weeks of gestation, increased but not significantly at 17-24 weeks of gestation, and significantly increased at 25-28 weeks of gestation.

Effective screening for early detection of pregnancies that are destined to experience preeclampsia is provided by a combination of maternal characteristics with uterine artery PI.[7] Although plasma cffDNA was increased significantly in cases that experienced early-onset preeclampsia, inclusion of this metabolite is unlikely to improve the performance of screening because of the high association between uterine artery PI and plasma cffDNA. Additionally, the potential value of cffDNA in screening for preeclampsia by comparison with other biochemical markers (such as pregnancy-associated plasma protein-A and placental growth factor[39]) is limited presently by cumbersome methods of detection, to its applicability only to pregnancies with male fetuses, and to poor reproducibility for its measurement. The difference in levels between paired measurements was >15% in 35% of the cases.

In pregnancies that subsequently experience early-onset preeclampsia, the maternal plasma cffDNA concentration at 11-13 weeks of gestation is increased, which provides further support for impaired placentation in the pathogenesis of the disease. ■

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