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Cell-free total and fetal DNA in first trimester maternal serum and subsequent development of preeclampsia

Robert Silver, M.D., Rebecca G. Clifton, Ph.D., Leslie Myatt, Ph.D., John C. Hauth, M.D., Kenneth J. Leveno, M.D., Uma M. Reddy, M.D., M.P.H., Alan M. Peaceman, M.D., Susan M. Ramin, M.D., Philip Samuels, M.D., George Saade, M.D., and Yoram Sorokin, M.D. for the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network, Bethesda, Maryland

Departments of Obstetrics and Gynecology of Obstetrics and Gynecology of University of Utah, Salt Lake City, UT (R.S.); University of Cincinnati, Cincinnati, OH (L.M.); University of Alabama at Birmingham, Birmingham, AL (J.C.H.); University of Texas Southwestern Medical Center, Dallas, TX (K.J.L.); Northwestern University, Chicago, IL (A.M.P.); University of Texas Health Science Center at Houston-Children's Memorial Hermann Hospital, Houston, TX (S.M.R.); The Ohio State University, Columbus, OH (P.S.); University of Texas Medical Branch, Galveston, TX (G.S.); Wayne State University, Detroit, MI (Y.S.); and the George Washington University Biostatistics Center, Washington, DC (R.G.C.); and *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, Bethesda, MD (U.M.R.)

Abstract

Objective—To assess the relationship between first trimester cell-free total and fetal DNA in maternal plasma and the subsequent development of preeclampsia.

Study Design—Nested case-control study of patients enrolled in the Combined Antioxidant and Preeclampsia Prediction Studies (CAPPS) prediction study of 175 women who did and 175 women who did not develop preeclampsia. The predictive values of cell-free total and fetal DNA and the subsequent development of preeclampsia were measured using ROC curves.

Results—Cell-free total DNA was higher in African American (median; 25 – 75%; 6.15; 0.14 – 28.73; $p = 0.02$) and Hispanic (4.95; 0.20 – 26.82; $p = 0.037$) compared to white women (2.33; 0.03 – 13.10). Levels of cell-free total DNA was also associated with maternal BMI ($p = 0.02$). Cell-free total DNA levels were similar between women who later developed preeclampsia (3.52; 0.11 – 25.3) and controls (3.74; 0.12 – 21.14, $p=0.96$).

Conclusions—There is no significant difference in levels of cell-free total DNA in the first trimester in women who subsequently develop preeclampsia. Levels of cell-free total DNA in the first trimester are increased in African American and Hispanic compared to white women, and levels increase with increasing BMI.

Corresponding author: Robert M. Silver, M.D., 30 N. 1900 E. 2B200 SOM, Salt Lake City, UT 84132, Ph: 801-581-8425, Fax: 801-585-5156, bob.silver@hsc.utah.edu.

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Keywords

Preeclampsia; cell-free DNA; placenta; fetal fraction

INTRODUCTION

It is desirable to identify patients at risk for the subsequent development of preeclampsia early in pregnancy. This would allow for the use of low dose aspirin as a prophylactic agent¹ and for increased surveillance with the potential for improved outcomes later in pregnancy². In addition, it would provide further insight into the pathophysiology of the condition. Numerous clinical, biochemical, and sonographic markers have been used to identify women at increased risk for preeclampsia^{3,4}. However, none perform well in unselected women such as nulliparas.

Potential biomarkers for the development of preeclampsia include both cell-free total and cell-free fetal DNA in the maternal circulation. Cell-free total DNA is both maternal and fetal in origin, and the cell-free fetal DNA fraction represents approximately 3 – 6% of the total DNA present in maternal plasma⁵. It is unclear how fetal DNA and RNA enter the maternal system but it may occur after apoptotic and necrotic shedding from the placenta⁵. Since preeclampsia is associated with placental damage, dysfunction or abnormal development, as well as increased trophoblast deportation⁶, it is biologically plausible that levels of cell-free fetal DNA correlate with the risk for this condition.

Several studies have shown that cell-free total DNA^{7–11} and cell-free fetal DNA^{9–13} are associated with an increased risk for the development of preeclampsia as well as small for gestational age (SGA) fetus and even pregnancy loss and spontaneous preterm birth. The levels of total and fetal DNA in the maternal circulation are very attractive as predictive markers of preeclampsia because they are non-invasive, independent of prior pregnancy history, and may identify patients at risk in early pregnancy long before clinical manifestations of the disease are present. Although preliminary studies have been promising they have been conducted in small numbers of patients and the primary outcome of most studies has been the identification of aneuploidy, rather than adverse pregnancy outcomes. Also, a minority of studies did not find an association between cell-free fetal DNA and adverse pregnancy outcomes⁵. Thus, our objective was to assess the relationship between maternal plasma cell-free total and fetal DNA in the first trimester and the subsequent development of preeclampsia.

METHODS

We used a nested case-control study design including patients enrolled in a multicenter, randomized, double blind trial of Vitamins C and E to prevent complications of pregnancy-associated hypertension¹⁴. The vitamins had no effect on the development of preeclampsia or on other adverse perinatal outcomes¹⁴. All data were collected by certified research personnel at the clinical centers and uploaded to a central database. Other methods including the verification of the diagnosis of preeclampsia and its severity are described in detail¹⁴. A subset (N = 2,434 of patients were enrolled in the Combined Antioxidant and Preeclampsia

Prediction Studies (CAPPS) prediction study. Of these, 2,394 had complete outcome data. Inclusion criteria for this analysis included enrollment in the CAPPS prediction study, complete data available regarding pregnancy outcome, gestational age ≥ 13 weeks gestation at the time of enrollment and plasma sample obtained and available ≥ 13 weeks gestation.

Maternal race and ethnicity were self reported. Baseline BMI was calculated from the maternal weight measured by study staff prior to randomization and height either abstracted from the same day clinic visit or measured by study staff. The primary outcome was preeclampsia and included mild and severe preeclampsia, hemolysis, elevated liver enzymes and low platelets syndrome (HELLP) and eclampsia. Mild pregnancy associated hypertension was defined as a systolic blood pressure between 140 and 159 mm Hg or a diastolic pressure between 90 mm and 109 mm Hg on two occasions 2 to 240 hours apart. Severe pregnancy associated hypertension was defined as a systolic blood pressure of 160 mm Hg or more or a diastolic pressure of 110 mm Hg or more on two occasions 2 to 240 hours apart, or a single blood pressure measurement that was severely elevated and that led to treatment with an antihypertensive medication. Mild preeclampsia was defined as mild pregnancy associated hypertension and documentation of proteinuria within 72 hours before or after an elevated blood pressure measurement. Proteinuria was defined as total protein excretion of 300 mg or more in a 24-hour urine sample, 2+ or higher on dipstick testing, or a protein-to-creatinine ratio of 0.35 or more if a 24-hour urine sample was not available for testing. Severe preeclampsia was defined as preeclampsia with severe pregnancy associated hypertension or a protein excretion of 5 grams or more in 24-hours, or mild pregnancy associated hypertension with oliguria (< 500 mL in 24 hours), pulmonary edema (confirmed by radiography), or thrombocytopenia (platelet count $< 100,000$ per cubic millimeter). HELLP and eclampsia were also classified as severe preeclampsia for this analysis. Small for gestational age fetus was defined as a fetus that was less than the 10th percentile for gestational age, adjusted for sex and race or ethnic group¹⁵.

Of the 2,394 women in the CAPPS prediction study with outcome data, 176 developed preeclampsia and 175 had first-trimester blood samples available. We anticipated that women with preeclampsia would have up to a 5 to 6 fold elevation in free fetal DNA¹⁶. Only 36 patients in each group (cases and controls) are required to achieve 80% power with a two-sided comparison of the mean free fetal DNA between the cases and controls, using previous published estimates (preeclampsia cases: mean $\pm$ SD, 439 ± 299 GE/mL; controls: 284 ± 138)⁷, which are much more conservative than the anticipated 5–6 fold elevation that is anticipated. However, we decided to use all available patients as well as an equivalent number of controls in hopes of providing reliable estimates of test characteristics. Controls were matched by center and gestational age at sample collection and included normotensive, non-proteinuric, non-diabetic women who delivered a non-SGA infant at term.

Maternal plasma was obtained by venipuncture in sodium heparin tubes. Plasma was spun at $2,000 \times g$ for 10 minutes. It was aliquoted into 500 μ L tubes and maintained at -80°C until extraction. Upon thawing, aliquots contained between 250 – 500 μ L and two samples were available for each patient. Thus, there were between 500 – 1,000 μ L of plasma available for each participant. The 2 samples from each individual were combined. Prior to extraction the samples were centrifuged for 15 minutes at $16,000 \times g$ to attempt to remove debris and any

intact fetal cells. DNA was extracted from 600µl of maternal plasma using the QIAamp DNA Mini Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were eluted in 60 µl Qiagen AE buffer and stored at -20°C until use.

In order to differentiate between fetal and maternal DNA, we assessed a gene that is differentially methylated in the fetus/placenta but not in the mother, RASSF1A¹⁷. We also amplified a housekeeping gene, beta Actin (ACTB) to assess total (maternal and fetal) DNA. Digest of the unmethylated maternal DNA would leave only fetal DNA. To differentiate the methylated fetal DNA from the unmethylated maternal DNA a portion of the sample was digested with two methylation sensitive enzymes, HinfII and HhaI (New England Biolabs, Ipswich, MA). These enzymes digest hypomethylated DNA, but not hypermethylated DNA, therefore it is expected that they digest maternal, but not fetal RASSF1A DNA and both maternal and fetal ACTB DNA. Because the expected amounts of DNA are very small a qPCR method is used for detection of the DNA. An absolute quantitation, rather than a relative quantitation, was used for the analysis. Eight standards (4 replicates each) of known quantities were used on each qPCR plate to establish a standard curve.

Quantitative real-time PCR using Taqman chemistry, including a fluorescently labeled minor-groove binding probe, was used to detect the RASSF1A and ACTB signals. Because this was an absolute quantitation assay a standard curve was created and was used to estimate the quantity of DNA in each sample. Each sample was qPCR'd in duplicate for four different scenarios, RASSF1A without digestion (should amplify both fetal and maternal DNA), RASSF1A with digestion (should amplify only fetal DNA), ACTB without digestion (as a control with a gene expected to be similarly methylated in maternal and fetal DNA) and ACTB with digestion (as a control for estimating completeness of the digestion). Because the ACTB gene should not be hypermethylated in either the fetus or the maternal DNA digestion would be expected and no qPCR results expected because the target template would not be intact. The qPCR was performed on an Applied Biosystems 7900 real-time PCR instrument. A standard curve was generated using serial dilutions of a known genomic DNA standard (Affymetrix, Santa Clara, CA). All samples were normalized to have equivalent µl amounts so comparisons of quantity could be made. Using the manufacturer's software a standard curve was generated, an estimation of the quantity of the DNA was calculated, and an average of the replicate samples was calculated. Personnel performing the analyses were blinded to study outcome.

Baseline categorical variables were compared using the chi-square test and continuous variables using the Wilcoxon rank-sum or Kruskal-Wallis test as appropriate. The Kruskal-Wallis and Wilcoxon rank-sum tests (pairwise comparisons) were used to assess the relationship between cell-free total DNA and race. Spearman correlations assessed the relationship between cell-free total DNA and baseline BMI and gestational age. Baseline BMI was categorized into broad WHO categories and assessed using the Jonckheere-Terpstra test. The performance of screening was determined by receiver operating characteristic (ROC) curves and calculating the sensitivity for a fixed 80% specificity. ROC curves were compared using DeLong, DeLong, and Clarke-Pearson. A nominal p value less than 0.05 was considered to indicate statistical significance. Analyses were performed using SAS software (Cary, NC).

RESULTS

Demographics and characteristics of cases and controls are shown in Table 1. As expected, women with preeclampsia had a higher BMI, and systolic and diastolic blood pressure at the time of enrollment (9–12 weeks) compared to controls. The groups were similar with regard to race, age, gestational age at sample collection and other potential confounders. Thus, we did not adjust levels based on gestational age of blood draw.

We then assessed the relationship between cell-free total DNA and race/ethnicity (Table 2). Median levels of DNA were higher in black women compared to Caucasian women (6.15 versus 2.33; $p = 0.02$). Levels also were higher in Hispanic compared to Caucasian women (4.95 versus 2.33; $p = 0.04$). Levels were statistically similar between Black and Hispanic women ($p=0.93$). Cell-free total DNA was mildly correlated with maternal BMI ($\rho=0.14$, $p=0.01$). Cell-free total DNA by BMI category is presented in Table 3 ($p=0.02$). Cell-free total DNA was not correlated with the gestational ages tested ($\rho=-0.04$, $p=0.44$).

The median (25% – 75%) cell-free total DNA in preeclamptic cases was 3.52 (0.11 – 25.3) compared with 3.74 (0.12 – 21.14) in controls. There was no significant difference between groups ($p=0.96$). We then assessed the association between race, BMI, cell-free total DNA and preeclampsia using ROC curves. Figure 1a shows the ROC curves for 1) total DNA, 2) BMI and race, and 3) total DNA, BMI and race which included a significant interaction for Black and total DNA. The area under the curve and its 95% confidence interval for total DNA is 0.502 (0.438 – 0.559) with a sensitivity of 21.1% at a specificity of 80%. The ROC for race and BMI had an area under the curve of 0.664 (0.606 – 0.721) with a sensitivity of 46.9% at a specificity of 80% which was significantly different from total DNA ($p<0.001$). The characteristics of the ROC did not appreciably change when total cell free DNA was added to race and BMI (AUC 0.682, 95% CI 0.626–0.739, sensitivity 51.4% at 80% specificity) although this was significant improvement from total DNA ($p<0.001$). Given the significant interaction of Black women and total DNA, we also assessed the ability of total DNA alone, BMI alone and total DNA and BMI together to predict subsequent preeclampsia in the subset of Black women (Figure 1b). In Black women, total DNA and BMI had an area under the curve of 0.693 (95% CI 0.596 – 0.789) with a sensitivity of 60.7% (95% CI 47.3–72.9%) at a specificity of 80% which was significantly different from total DNA ($p=0.003$). However, this was primarily due to the association between BMI and preeclampsia rather than total DNA.

Cell free total DNA was not different between women who developed severe preeclampsia ($N=71$) versus controls (median and (25%–75%) 8.76 (0.44–48.43) vs 3.74 (0.12–21.14), $p=0.11$, Figure 2). Similarly, total cell free DNA was not associated with the development of preeclampsia and SGA fetus ($N = 38$) (Figure 3).

There was no association between cell-free fetal DNA and preeclampsia. However, meaningful levels of free fetal DNA were not detected in a majority of both cases and controls. Thus, we could not perform valid comparisons of cell free fetal DNA between groups.

DISCUSSION

We found no association between cell-free total DNA in the first trimester and the subsequent development of preeclampsia. This is in contrast to some other reports. Farina and coworkers found higher levels of total DNA in patients developing preeclampsia compared to controls using GAPDH and beta globin genes as markers of total DNA concentration. These differences were noted at approximately 20 weeks of gestation⁷. In a larger study, Sekizawa and colleagues quantified the beta globin concentrations in the plasma of 207 pregnant women. They found that adjusted multiple of the median values were significantly higher in the 43 women with preeclampsia⁸. Indeed, these women had a fourfold increase compared to controls. In the same study, beta globin DNA levels also were increased by twofold in pregnancies complicated by SGA. This study was conducted in the third trimester⁸. On the other hand, Crowley and colleagues found no difference in total cell free DNA prior to 20 weeks gestation in a large cohort of women⁹. They used β -actin to assess total cell free DNA⁹. Poon and colleagues also did not find an association between total (or fetal) cell free DNA and preeclampsia¹⁸. Differences among studies are likely due to the gestational age at assessment, populations studied and methods used to detect total cell free DNA. One interpretation of these data is that total cell free DNA (reflecting mostly maternal DNA) is elevated once the patient manifests the phenotype of preeclampsia, perhaps due to endothelial cell activation and damage. However, it is not elevated prior to the clinical onset of disease.

Our data do not permit us to make conclusions regarding the association between free fetal DNA and preeclampsia since we did not detect free fetal DNA in a majority of our specimens. There are several possible reasons for this. Assessment of cell free fetal DNA was not a primary goal of the parent study and the plasma collection was not ideal for evaluation of free fetal DNA. First, the anticoagulant heparin was not as desirable as EDTA for obtaining plasma¹⁹. Second, the samples were not centrifuged twice after collection to remove maternal cells, a step considered critical to the optimal detection of free fetal DNA^{19–22}. The centrifugation helps to get rid of maternal cells, increasing the fetal:maternal ratio of cell free DNA. Third, the quantity of plasma may have been inadequate given the gestational age. Most studies have used 800–1,500 μ L of plasma for extraction^{11,17,23,24}. We were only able to use 500 – 1000 μ L. Free fetal DNA increases over gestation and is easier to assay later in pregnancy¹⁷. Nonetheless, free fetal DNA is clearly present in the first trimester^{22,25}. Hence, this aspect of the study needs to be repeated using optimal methods of plasma collection.

Also, there are newer methods of quantifying free fetal DNA in the first trimester that are extremely sensitive and specific. These have been developed primarily for prenatal diagnosis of fetal aneuploidy and include massively parallel sequencing and digital analysis of selected regions^{22,25,26}. It is likely that these methods can more accurately answer the question of whether elevations in free fetal DNA in the first trimester are associated with the subsequent development of preeclampsia. Such approaches are ideal since it is thought that cell free fetal DNA is derived from trophoblast, providing an early marker of abnormal placental growth and development or placental damage. Also, it can be performed early in pregnancy and is independent of fetal gender.

An important finding of our study was the relationship between total cell free DNA and BMI. On balance, levels increased with increasing maternal BMI. Lapaire and co-workers also noted a correlation between total cell free, but not fetal cell free DNA and maternal BMI at both 20 – 21 weeks gestation and at the end of pregnancy in a cohort of over 400 women²⁷. Similar findings were noted in a recent study from Great Britain¹⁸. Another study found no correlation between maternal weight and free fetal DNA²⁸ and a third found an inverse correlation between free fetal DNA and maternal weight in the second trimester²⁹. Thus, total but not fetal cell free DNA appears to be influenced by BMI.

We also noted substantial differences in total cell free DNA based on race. Specifically, total cell free DNA was higher in Black and Hispanic compared to white women. Gerovassili and coworkers also noted increased levels of total cell free DNA in the first trimester in women of African and African-Caribbean descent compared to Northern Europeans³⁰. The same group found lower levels of cell free fetal DNA in African and African-Caribbeans compared to Europeans³⁰. Poon and colleagues also noted increased levels of cell free DNA in women of Afro-Caribbean descent¹⁸. Wataganara et al. did not find any relationship between race/ethnicity and cell free fetal DNA levels. As with BMI, total cell free DNA is influenced by race/ethnicity. However, an association between free fetal DNA and race/ethnicity is less clear.

The reasons for the variations in total cell free DNA with BMI and race/ethnicity are uncertain. It is noteworthy that increased BMI is associated with an increased risk of a variety of adverse pregnancy outcomes including preeclampsia³¹. Similarly, African American race also is associated with an increased risk for preeclampsia as well as virtually all adverse pregnancy outcomes³². Increased first trimester cell free DNA could reflect increased inflammation or endothelial activation. These data provide rationale for further research into the pathophysiology that underlies the increased risk of adverse pregnancy outcomes in African Americans and women with increased BMI.

Our study had several limitations. These focused on the methods for plasma collection that were suboptimal for evaluation of free fetal DNA including the use of sodium heparin tubes, lack of additional centrifugation to remove maternal cellular debris, and limited amounts of plasma in some cases. Overall the experimental procedure performed well. DNA extraction was confirmed by amplification of both the undigested RASSF1A and ACTB sequences. Completion of digestion was confirmed by absence of amplification in the ACTB digested qPCR. Thus, the results are quite reliable for total cell free DNA. Another limitation is the generalizability of our results. Most of our hospitals are tertiary care academic medical centers and the patient population may not reflect the population at large. In addition, being willing to participate in a clinical trial is another source of potential bias.

There also were numerous strengths of the study. Patients were enrolled prospectively without a priori knowledge of outcome. Rigorous definitions of clinical outcomes were used and medical and obstetric data were ascertained by certified study nurses. All cases of preeclampsia were reviewed by three reviewers not associated with the clinical site of origin using a standardized protocol. There were a very large number of well-characterized patients

with preeclampsia and appropriate controls. In addition, the cohort was racially and ethnically diverse. Finally, the laboratory was blinded to patient status.

In summary, total cell free DNA in the first trimester is not associated with the subsequent development of preeclampsia. Total cell free DNA in the first trimester is increased with increasing BMI and in African American and Hispanic women compared to Caucasians. These relationships need to be considered when using assays of cell free DNA during pregnancy.

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Appendix

In addition to the authors, other members of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network are as follows:

University of Utah, Salt Lake City, UT – M. Varner, P. Reed, S. Quinn (LDS Hospital), V. Morby (McKay-Dee Hospital), F. Porter (LDS Hospital), J. Miller (Utah Valley Regional Medical Center), K. Hill

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University of North Carolina at Chapel Hill, Chapel Hill, NC – J. Thorp, K. Boggess, K. Dorman, J. Mitchell, K. Clark, S. Timlin

Case Western Reserve University-MetroHealth Medical Center, Cleveland, OH – B. Mercer, J. Bailit, C. Milluzzi, W. Dalton, C. Brezine, D. Bazzo

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Northwestern University, Chicago, IL – M. Dinsmoor (NorthShore University HealthSystem), T. Matson-Manning, G. Mallett

University of Texas Health Science Center at Houston-Children's Memorial Hermann Hospital, Houston, TX – S. Blackwell, K. Cannon, S. Lege-Humbert, Z. Spears

Brown University, Providence, RI – M. Carpenter, J. Tillinghast, M. Seebeck

The Ohio State University, Columbus, OH – J. Iams, F. Johnson, S. Fyffe, C. Latimer, S. Frantz, S. Wylie

Drexel University, Philadelphia, PA – A. Sciscione, M. Talucci, M. Hoffman (Christiana), J. Benson (Christiana), Z. Reid, C. Tocci

Wake Forest University Health Sciences, Winston-Salem, NC – M. Harper, P. Meis, M. Swain

Oregon Health & Science University, Portland, OR – J. Tolosa, W. Smith, L. Davis, E. Lairson, S. Butcher, S. Maxwell, D. Fisher

University of Texas Medical Branch, Galveston, TX – J. Moss, B. Stratton, G. Hankins, J. Brandon, C. Nelson-Becker, G. Olson, L. Pacheco

Wayne State University, Detroit, MI – G. Norman, S. Blackwell, P. Lockhart, D. Driscoll, M. Dombrowski

The George Washington University Biostatistics Center, Washington, DC – R. Clifton, T. Boekhoudt, L. Leuchtenburg, T. Williams

National Heart, Lung, and Blood Institute, Bethesda, MD – G. Pearson, V. Pemberton, J. Cutler, W. Barouch

Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD – C. Spong, S. Tolivaisa

MFMU Steering Committee Chair (*University of Texas Medical Center, Galveston, TX*) – G.D. Anderson, M.D.

Figure 1a

Figure 1b

Figure 1.

Receiver operating characteristic curves for race, BMI, and total cell free DNA as a predictor of preeclampsia. 1a: Race, BMI and total cell free DNA as predictors of preeclampsia, 1b: BMI and total cell free DNA as a predictor of preeclampsia in the subset of African American women.

Figure 2. Receiver operating characteristic curve of race, BMI and total cell free DNA as a predictor of severe preeclampsia.

Figure 3. Receiver operating characteristic curves for race, BMI and total cell free DNA as a predictor of preeclampsia in pregnancies with preeclampsia and SGA fetus (< 10%).

Table 1

Patient demographics and characteristics

Characteristic	Preeclampsia (N = 175)	Controls (N = 175)	P value
Maternal age (yrs)	22.5 ± 4.4	22.7 ± 4.4	0.65
Gestational age at sample collection (wks)	11.3 ± 1.1	11.4 ± 1.1	0.90
Education (yrs)	12.3 ± 2.5	12.6 ± 2.6	0.23
Smoking	28 (16.0)	41 (23.4)	0.08
Prior pregnancy < 20 weeks	35 (20.0)	40 (22.9)	0.51
Vitamins (randomized)	94 (53.7)	85 (48.6)	0.34
Baseline BMI	28.3 ± 6.9	25.0 ± 5.2	< 0.001
Baseline systolic blood pressure	112 ± 10	108 ± 10	< 0.001
Baseline diastolic blood pressure	67 ± 8	64 ± 8	0.002
Race/Ethnicity			0.23
Black	61 (34.9)	56 (32.0)	
Caucasian/Other	61 (34.9)	76 (43.4)	
Hispanic	53 (30.3)	43 (24.6)	

Table 2

Total cell free DNA and race/ethnicity

Race/Ethnicity	N	Median	25 – 75%
Black	117	6.15	0.14 – 28.73
Hispanic	96	4.95	0.20 – 26.82
Caucasian/Other	137	2.33	0.03 – 13.10

Black versus Caucasian/other; P = 0.024. Hispanic versus Caucasian/other; P = 0.037. Black versus Hispanic; P = 0.93.

Table 3

Total cell free DNA and BMI

BMI	N	Median	25 – 75%
< 18.5	16	0.02	0.00 – 3.39
18.5 – 24.9	146	4.03	0.21 – 16.08
25.0 – 29.9	104	2.70	0.10 – 24.71
30	84	7.60	0.24 – 28.78

P = 0.020

Table 4

Total cell free DNA, BMI, race and preeclampsia

Model	Area under the ROC curve [95% CI]	Sensitivity at 80% specificity % [95% CI]
Total DNA	0.502 [0.438 – 0.559]	21.1 [15.3–27.9]
BMI, race	0.664 [0.606 – 0.721]	46.9 [39.3–54.5]
Total DNA, BMI, race	0.682 [0.626 – 0.739]	51.4 [43.8–59.0]

Table 5

Free fetal DNA in preeclamptics and controls

Percentile free fetal DNA	Preeclampsia	Controls
25%	0.0	0.0
50%	0.0	0.0
75%	0.0	0.0
90%	0.028	0.011
95%	0.115	0.033
99%	4.855	0.187

P = 0.69.