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ORIGINAL RESEARCH

Reduced Maternal Circulating Cell-Free Mitochondrial DNA Is Associated With the Development of Preeclampsia

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BACKGROUND: Circulating cell-free mitochondrial DNA (ccf-mtDNA) is a damage-associated molecular pattern that reflects cell stress responses and tissue damage, but little is known about ccf-mtDNA in preeclampsia. The main objectives of this study were to determine (1) absolute concentrations of ccf-mtDNA in plasma and mitochondrial DNA content in peripheral blood mononuclear cells and (2) forms of ccf-mtDNA transport in blood from women with preeclampsia and healthy controls. In addition, we sought to establish the association between aberrance in circulating DNA-related metrics, including ccf-mtDNA and DNA clearance mechanisms, and the clinical diagnosis of preeclampsia using bootstrapped penalized logistic regression.

METHODS AND RESULTS: Absolute concentrations of ccf-mtDNA were reduced in plasma from women with preeclampsia compared with healthy controls ($P \leq 0.02$), while mtDNA copy number in peripheral blood mononuclear cells did not differ between groups ($P > 0.05$). While the pattern of reduced ccf-mtDNA in patients with preeclampsia remained, DNA isolation from plasma using membrane lysis buffer resulted in 1000-fold higher ccf-mtDNA concentrations in the preeclampsia group ($P = 0.0014$) and 430-fold higher ccf-mtDNA concentrations in the control group ($P < 0.0001$). Plasma from women with preeclampsia did not induce greater Toll-like receptor-9–induced nuclear factor kappa-light-chain enhancer of activated B cells-dependent responses in human embryonic kidney 293 cells overexpressing the human *TLR-9* gene ($P > 0.05$). Penalized regression analysis showed that women with preeclampsia were more likely to have lower concentrations of ccf-mtDNA as well as higher concentrations of nuclear DNA and DNase I compared with their matched controls.

CONCLUSIONS: Women with preeclampsia have aberrant circulating DNA dynamics, including reduced ccf-mtDNA concentrations and DNA clearance mechanisms, compared with gestational age–matched healthy pregnant women.

Key Words: cell-free DNA ■ DNase I ■ mitochondrial DNA ■ penalized regression analysis ■ preeclampsia

Preeclampsia is a prevalent, multifactorial complication of pregnancy diagnosed as de novo hypertension after the 20th week of gestation with end-organ damage.[1] This pregnancy-specific syndrome, which is seen in ≈5% to 8% of pregnancies worldwide, results in increased morbidity and mortality for fetus and mother alike. Currently, there is no cure for preeclampsia, and the most common approach to clinical management is delivery of the fetoplacental unit,[2] which often occurs preterm.

During healthy pregnancies, placental cell turnover results in release of cellular constituents and DNA into the maternal circulation in increasing amounts as pregnancy progresses.[3–5] Placentas from pregnancies

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CLINICAL PERSPECTIVE

What Is New

- Cell-free circulating mitochondrial DNA concentrations are reduced in plasma from women with preeclampsia at the third trimester.
- The majority of cell-free circulating mitochondrial DNA is transported encapsulated in vesicles during pregnancy, and this phenomenon is more pronounced in preeclampsia.
- Women with preeclampsia are more likely to have aberrant circulating DNA dynamics, including cell-free circulating mitochondrial DNA concentrations and DNA clearance mechanisms, compared with gestational age–matched healthy pregnant women.

What Are the Clinical Implications?

- Preeclampsia is associated with placental dysfunction, trophoblast cell death, and oxidative stress, which lead to release of proinflammatory factors into the maternal circulation.
- Dysregulation of cell-free circulating mitochondrial DNA concentrations and DNA clearance mechanisms during pregnancy may contribute to the development or establishment of the maternal cardiovascular syndrome in preeclampsia.

Nonstandard Abbreviations and Acronyms

ccf-mtDNA	circulating cell-free mitochondrial DNA
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
PBMCs	peripheral blood mononuclear cells
TLR-9	Toll-like receptor-9

with preeclampsia, however, exhibit signs of damage[6] and exaggerated rates of trophoblast cell death.[7] Subsequently, tissue damage and cell death results in increased release of antiangiogenic factors, proinflammatory cytokines, and DNA fragments[8,9] into the maternal circulation. Thus, concentrations of circulating factors are currently being studied as potential markers of early diagnosis and placental health and predictors of maternal and perinatal outcomes.[10–12]

Circulating cell-free mitochondrial DNA (ccf-mtDNA) is considered a damage-associated molecular pattern molecule, which elicits an immune response via activation of the pattern recognition receptor, Toll-like receptor-9 (TLR-9).[9] We and others have previously reported that activation of TLR-9 during pregnancy induced preeclampsia-like signs in rats and mice.[13,14] In line with these observations, plasma from women with preeclampsia elicited greater TLR-9–mediated inflammatory responses compared with plasma from healthy pregnant women.[15] Cross-sectional and case-control studies have reported increased mitochondrial DNA (mtDNA) copy number in serum and whole blood from women with preeclampsia in the third trimester[16,17] and at time of delivery.[18] On the other hand, ccf-mtDNA was suppressed in the first trimester of women who later developed preeclampsia.[19] Experimental factors such as the choice of biological sample (ie, whole blood versus serum versus plasma[20,21]), DNA extraction methods, and quantification protocols widely varied in these previous studies. This methodological variation raises questions about the biological specificity and interpretation of ccf-mtDNA measurements in pregnant patients.[9,22] For example, quantification of mtDNA in whole blood does not distinguish between cellular and cell-free forms of mtDNA, which correlate with distinct biological functions.[21] Furthermore, studies in maternal blood have not determined the biological forms of ccf-mtDNA, such as quantities of mtDNA encapsulated in vesicular structures versus circulating non–membrane-bound mtDNA.[23]

The main objectives of this study were to determine (1) absolute concentrations of mtDNA in plasma (cell-free fraction of blood, ccf-mtDNA) and mtDNA content in peripheral blood mononuclear cells (cellular mtDNA) and (2) forms of ccf-mtDNA transport (membrane bound versus non–membrane bound) in blood from women with preeclampsia and healthy controls. In addition, we sought to establish the association between aberrance in circulating DNA-related metrics, including ccf-mtDNA and DNA clearance mechanisms, and the clinical diagnosis of preeclampsia using bootstrapped penalized logistic regression.[24,25]

METHODS

Detailed methods are available in Data S1. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Subjects

Deidentified subject information and blood samples were acquired from the Maternal Fetal Tissue Bank (IRB# 200910784) of the Women's Health Tissue Repository at the University of Iowa who obtained informed consent from pregnant women to collect biological samples and clinical data.[26] Cases consisted of 19 pregnant women with preeclampsia and 19 healthy pregnant controls matched for gestational

age (Table S1). As expected, the preeclampsia group exhibited higher mean, systolic, and diastolic blood pressures and a lower gestational age of delivery, neonatal weight, and APGAR scores ($P<0.05$) in comparison to the control pregnancies. Maternal venous blood was collected in the third trimester (mean=33.6 weeks) and separated into plasma and peripheral blood mononuclear cells (PBMCs). Plasma was stored at −80 °C, and PBMCs were stored in liquid N_2 until further processing.

DNA Quantification

DNA was isolated and quantified as published previously,[27] with a few modifications. Previous studies have noted that ccf-mtDNA can be membrane bound or non–membrane bound.[28,29] These data suggest vesicular and nonvesicular transport of ccf-mtDNA within the circulation. To determine the contribution of membrane-bound and -unbound ccf-mtDNA in preeclampsia, DNA from plasma samples was isolated with lysis buffer and without lysis buffer. TaqMan chemistry-based absolute quantification of nuclear DNA (nDNA) and ccf-mtDNA was used as detailed elsewhere.[27] Isolated ccf-mtDNA was quantified using a method modified from Kavlick et al[30] and detailed previously.[27] The target sequence for this analysis was the mitochondrial NADH:ubiquinone oxidoreductase core subunit 5 gene (mitochondrial NADH:ubiquinone oxidoreductase core subunit 5; GenBank Gene ID: 4540), spanning positions 13 288 to 12 392 of the mitochondrial genome.[31] The quantitative polymerase chain reaction primers, probes, and synthetic DNA standards used for ccf-mtDNA quantification are detailed in Table S2. Concentrations of plasma DNA are expressed as pg/mL of plasma. Total DNA was calculated as the sum of ccf-mtDNA and nDNA, in pg/mL plasma. Plasma ccf-mtDNA is expressed in absolute concentrations as a percent of total DNA (%ccf-mtDNA). The DNA content of PBMCs is presented as cell equivalent per µL DNA isolate for nDNA and as ccf-mtDNA genome copies per cell equivalent.

Statistical Analysis

Normality tests, outlier removal and univariate analyses were performed using Prism (Version 8, GraphPad, San Diego, CA). Student's *t*-test and Mann-Whitney *U* test were used for group comparisons. The effect of fetal sex on primary outcomes was assessed with a 2-way ANOVA followed by Sidak's correction to adjust for multiple comparisons. DNA outcomes are presented in violin plots showing medians and ranges. Subject characteristics are presented as mean with minimum and maximum unless otherwise indicated. The significance level was set at $\alpha=0.05$ for all comparisons (before multiple testing comparisons).

Penalized Logistic Regression Model Selection

To identify the most important subject characteristics associated with the outcome of preeclampsia, bootstrapped penalized logistic regression was implemented (detailed description in Data S1). All elements of regression analyses were carried out in R software version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria).[32–40] Three penalized regression models (ie, least absolute shrinkage and selection operator, ridge regression, and elastic net regression) were fit, and their performances were compared to select the best model. To reduce the number of covariates and mitigate multicollinearity in regression models while not eliminating potentially important patient characteristics, related parameters were grouped a priori (Table S3), and one single characteristic was selected from each group to generate all possible combinations of independent characteristics (192 total data sets). Bootstrapped ($R=500$) 10-fold cross-validation least absolute shrinkage and selection operator, ridge, and elastic net were then performed on each data set, and best model fit was determined by lowest prediction error (ie, lowest median root mean squared error).[41,42]

Application of Best Model to Explain the Relationship Between Maternal Plasma DNA Metrics and the Diagnosis of Preeclampsia

Predictive accuracy of the final model developed using real data was tested against a simulated naïve prediction data set using the bootstrap estimate ($R=500$) of the area under the receiver operator characteristic curve with 95% CI calculated from standard error. Characteristics selected by the final regression model at least 75% of the time (variable importance probability, 0.75) for all bootstrap samples were regarded as most important.[43] Adjusted odds ratios are reported.

RESULTS

Maternal Plasma mtDNA and nDNA

Figure 1A illustrates the biological forms of mtDNA assessed in this investigation in maternal plasma from women with preeclampsia and gestational age–matched healthy pregnant women. Concentrations of ccf-mtDNA were reduced in preeclampsia compared with healthy controls in both lysis buffer-treated samples (Figure 1B) and in samples that were not treated with lysis buffer (Figure 1C). While the pattern of reduced ccf-mtDNA in preeclampsia remained the same between methods of DNA extraction, the exact quantity of ccf-mtDNA was different between extraction methods. Specifically, concentrations of ccf-mtDNA were increased by 1000-fold in samples

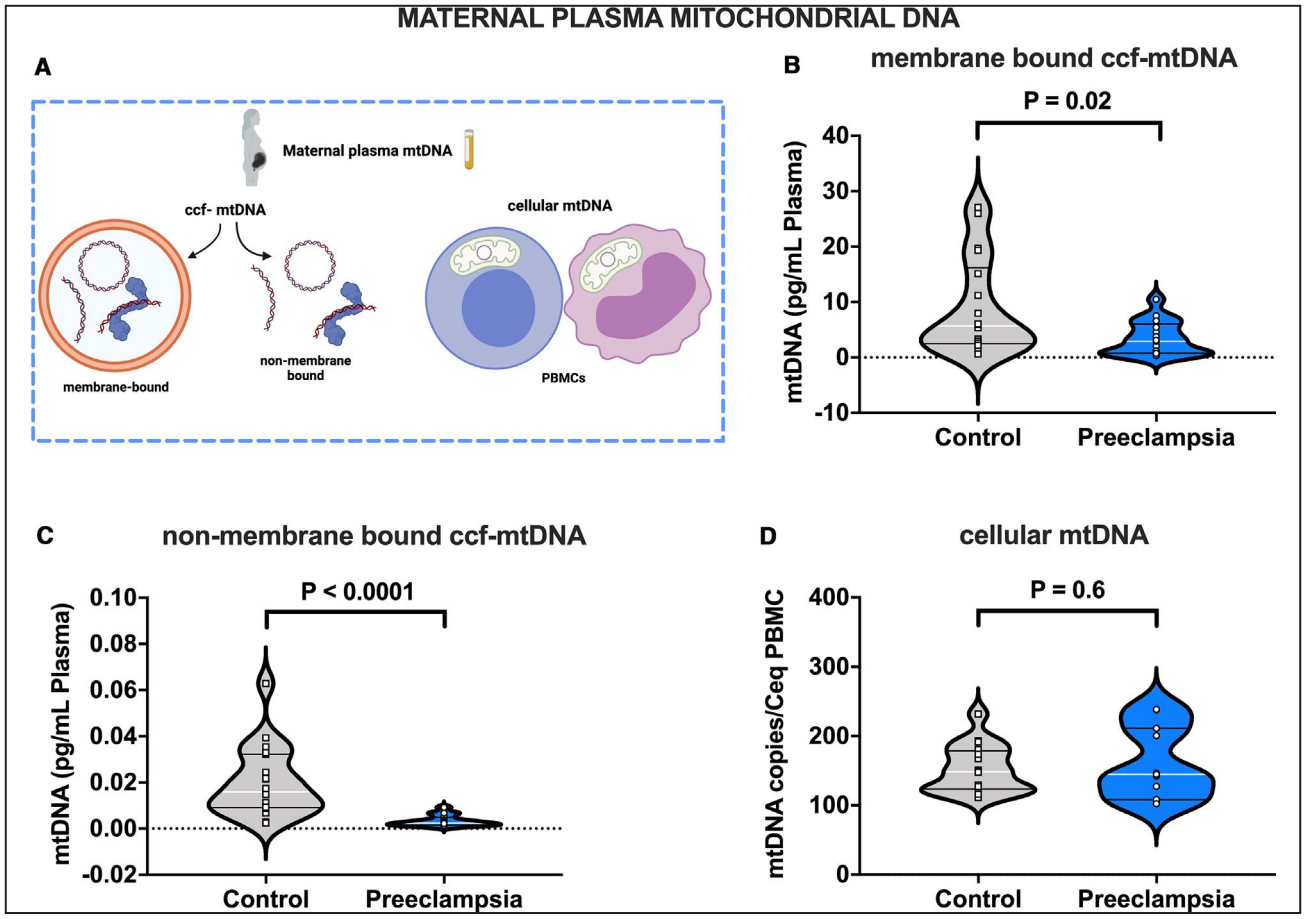

Figure 1. Mitochondrial DNA in cell-free and cellular forms in maternal plasma from pregnancies with preeclampsia and healthy controls.

A, Biological forms of mtDNA assessed in this study, (**B**) ccf-mtDNA concentrations (pg/mL) in plasma isolated using lysis buffer (membrane bound ccf-mtDNA), (**C**) ccf-mtDNA concentrations (pg/mL) in plasma isolated without lysis buffer (non-membrane bound ccf-mtDNA), (**D**) cellular mtDNA copies per estimated number of peripheral blood mononuclear cells (PBMCs), Student's *t*-test (**B** and **D**), Mann-Whitney *U* test (**C**). **B** through **C**, n=18 controls, n=17 preeclampsia; (**D**) n=16 controls, n=11 preeclampsia. All values presented as median (white line), IQR (black lines). ccf-mtDNA indicates circulating cell-free mitochondrial DNA; Ceq, cell equivalent; and PBMC, peripheral blood mononuclear cell. Open squares=gestational age-matched control; open circles=third trimester pregnancies with preeclampsia. **A**, was created with Biorender.com.

that were treated with lysis buffer (3.294±0.828 versus 0.003±0.001 pg/mL, n=15; *P*=0.0014) in the preeclampsia group. On the other hand, concentrations of ccf-mtDNA were increased by 430-fold in samples treated with lysis buffer compared with samples not treated with lysis buffer (9.011±2.043 versus 0.021±0.004 pg/mL, n=18; *P*<0.0001) in the control group. Content of mtDNA in PBMCs did not differ between groups (Figure 1D).

There were no group differences in plasma concentrations of cell-free nDNA in samples treated with lysis buffer (Figure 2A), while plasma concentrations of cell-free nDNA in samples treated without lysis buffer were increased in women with preeclampsia compared with healthy controls (Figure 2B). Content of cell-free nDNA in PBMCs did not differ between groups (Figure 2C).

Plasma DNA concentrations were compared between pregnancies carrying male and female fetuses. There was neither a main effect for sex nor a group-by-sex interaction for plasma ccf-mtDNA concentrations (Figure 3A); however, there was an increase (*P*=0.0003) in circulating nDNA in pregnancies with preeclampsia carrying female fetuses (Figure 3B).

Collectively, these data demonstrate that the cell-free form of circulating mtDNA and not the mtDNA content of PBMCs is reduced in plasma from women with preeclampsia compared with healthy gestational age–matched pregnant women and that the majority of ccf-mtDNA is encapsulated in vesicular structures.

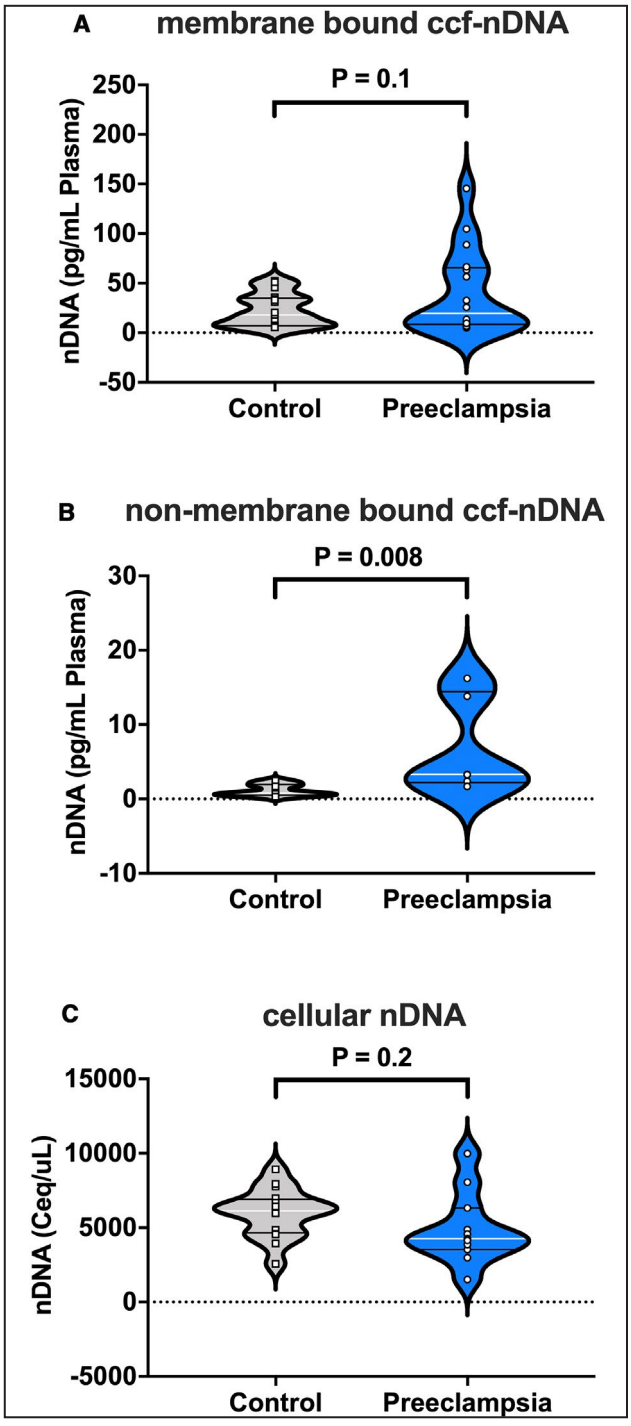

Figure 2. Nuclear DNA in cell-free and cellular forms in maternal plasma from pregnancies with preeclampsia and healthy controls.

A, nDNA concentrations (pg/mL) in plasma isolated using lysis buffer (membrane bound ccf-nDNA); and **B**, nDNA concentrations (pg/mL) in plasma isolated without lysis buffer (non–membrane-bound ccf-nDNA). **C**, nDNA concentration calculated as cell equivalents per microliter of peripheral blood mononuclear cell (PBMC) DNA isolate. Student's *t*-test (**A** and **C**), Mann-Whitney *U* test (**B**). **A**, n=17 controls, n=16 preeclampsia; (**B**) n=8 controls, n=6 preeclampsia; (**C**) n=16 controls, n=11 preeclampsia. All values presented as median (white line), interquartile range (black lines). ccf-nDNA indicates circulating cell-free nuclear DNA; and Ceq, cell equivalent. Open squares=gestational age-matched control; open circles=third trimester pregnancies with preeclampsia.

Effects of Maternal Plasma on TLR-9–Dependent Inflammation

To assess the potential of maternal plasma to induce TLR-9–mediated inflammatory responses, we treated human embryonic kidney 293 cells expressing a secreted embryonic alkaline phosphatase reporter gene (HEK-Blue Null cells, control; Figure 4A) and human embryonic kidney 293 cells expressing a reporter gene and overexpressing the *TLR-9* gene (HEK-Blue hTLR9 cells, Figure 4B) with plasma from women with preeclampsia and healthy controls (detailed methods in Data S1). Plasma from women with preeclampsia did not elicit greater nuclear factor kappa-light-chain enhancer of activated B cells –mediated proinflammatory responses in any of the cell lines ($P \geq 0.096$). To test whether inhibition of TLR-9 in HEK-Blue hTLR9 cells induced

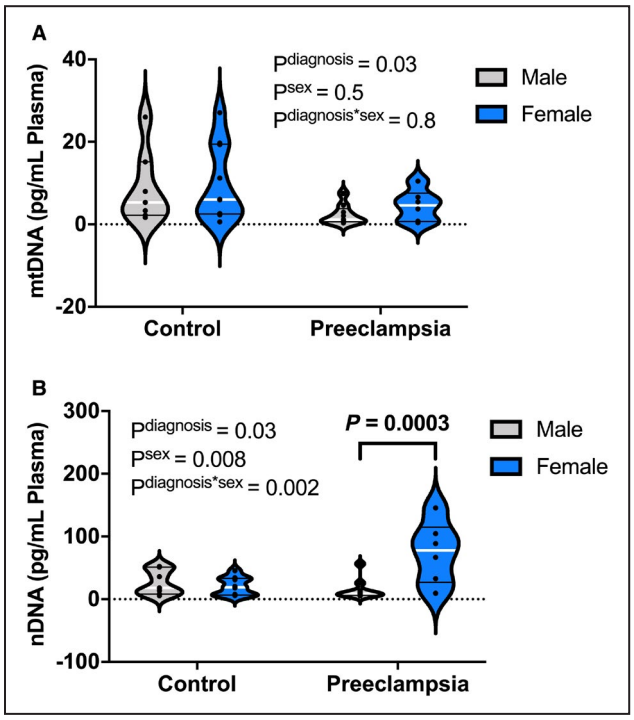

Figure 3. Effect of fetal sex on maternal plasma concentrations of cell-free DNA.

Membrane bound (**A**) ccf-mtDNA (pg/mL) and (**B**) ccf-nDNA (pg/mL) in plasma from pregnant women carrying female (control, n=9; preeclampsia, n=6) and male fetuses (control, n=7; preeclampsia, n=10). Data analyzed by 2-way analysis of variance with Sidak's post hoc analysis. All values presented as median (white line), interquartile range (black lines). nDNA, nuclear DNA; ccf-mtDNA, circulating cell-free mitochondrial DNA; ccf-nDNA, circulating cell-free nuclear DNA.

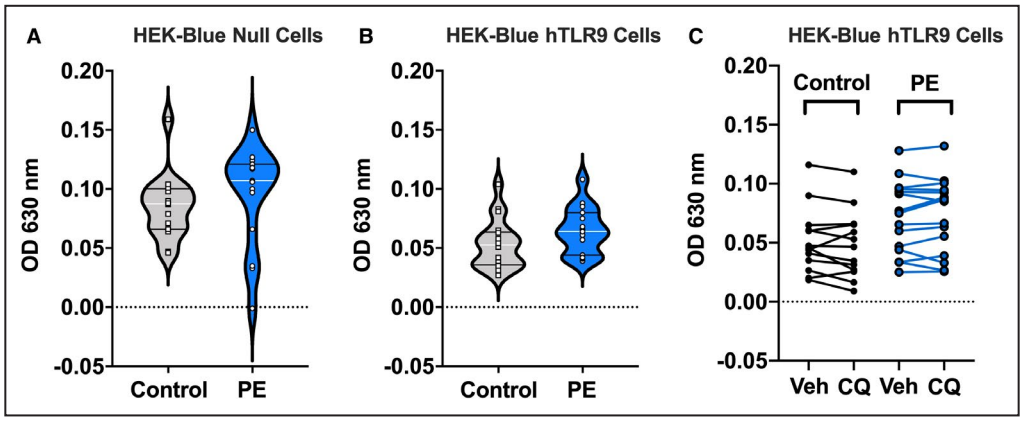

Figure 4. TLR-9–induced NF-κB-mediated inflammation in response to maternal plasma from women with preeclampsia and gestational age–matched healthy controls.
Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated production of secreted embryonic alkaline phosphatase (SEAP) in (**A**) human embryonic kidney (HEK) 293 cells expressing an inducible SEAP reporter gene under the control of an NF-κB inducible promoter (HEK-Blue Null1, control cells; controls, n=16; preeclampsia, n-15), (**B**) HEK293 cells overexpressing the human *TLR-9* gene and expressing an inducible SEAP reporter gene under the control of an NF-κB inducible promoter (HEK-Blue hTLR-9; controls, n=18; preeclampsia, n=15), and (**C**) HEK-Blue hTLR-9 treated with chloroquine (CQ; controls, n=13; preeclampsia, n=15). All cells were exposed to 10% maternal plasma from women with preeclampsia or gestational age-matched healthy pregnant women. Student's t-test. All values presented as median (white line), interquartile range (black lines). OD 630 nm, optical density at 630 nanometer wavelength light. Open squares=gestational age–matched control; open circles=third-trimester pregnancies with preeclampsia.

differential inflammatory responses when cells were treated with plasma from preeclamptic patients versus controls, we pretreated HEK-Blue hTLR9 cells with chloroquine (an endolysosomal acidification inhibitor). Chloroquine treatment did not affect inflammatory responses of HEK-Blue hTLR9 cells (Figure 4C) to either maternal plasma from preeclamptic patients (P=0.92) or controls (P=0.78). In summary, plasma from women with preeclampsia does not elicit greater TLR-9–dependent inflammatory responses compared with plasma from healthy pregnant women.

DNA Clearance in Maternal Blood

Univariate analysis showed no differences (P=0.14) in plasma DNase I concentrations between control pregnancies and pregnancies with preeclampsia (Figure 5A). Moreover, there was no correlation (P=0.5) between DNase I concentrations and membrane-bound ccf-mtDNA in either group (Figure 5B). Plasma DNase I was not correlated to nDNA (P=0.2) or %ccf-mtDNA (P=0.3) in control pregnancies (Figure 5C and 5D). In contrast, there were significant correlations (P≤0.04) between DNase I and nDNA and %ccf-mtDNA in preeclampsia (Figure 5C and 5D). These data suggest that in preeclampsia, circulating DNase I concentrations are associated with cell-free nDNA but not with membrane-bound ccf-mtDNA.

Contribution of Maternal Plasma DNA Metrics to the Clinical Diagnosis of Preeclampsia

A total of 192 data sets (all possible combinations of independent observations) were analyzed to determine the best combination of variables for modeling the relationships between clinical characteristics, circulating DNA metrics, and the diagnosis of preeclampsia. The best performing combination of characteristics and corresponding coefficients and odds ratios are listed in Table S4. After outlier removal via Mahalanobis distance,[44] the final data set used in model building included 35 patients (preeclampsia, n=17; control, n=18). Assessing model performance between ridge, least absolute shrinkage and selection operator, and elastic net penalized regression of the best-performing data set over R=500 bootstraps showed that elastic net penalized regression produced the lowest prediction error (Table S5).[41,42] This model demonstrated an accuracy of ≈100% (95% CI, 0.9977–1.00; Table S6), and an area under the curve of the receiver operator characteristic value of ≈1 (95% CI, 0.9983–1.00; Table S6, Figure S1).

Application of the variable importance probability approach with 75% threshold (variable importance probability, 0.75) indicated that 9 of the subject characteristics were most important to the diagnosis of preeclampsia (Figure 6A). Characteristics with the

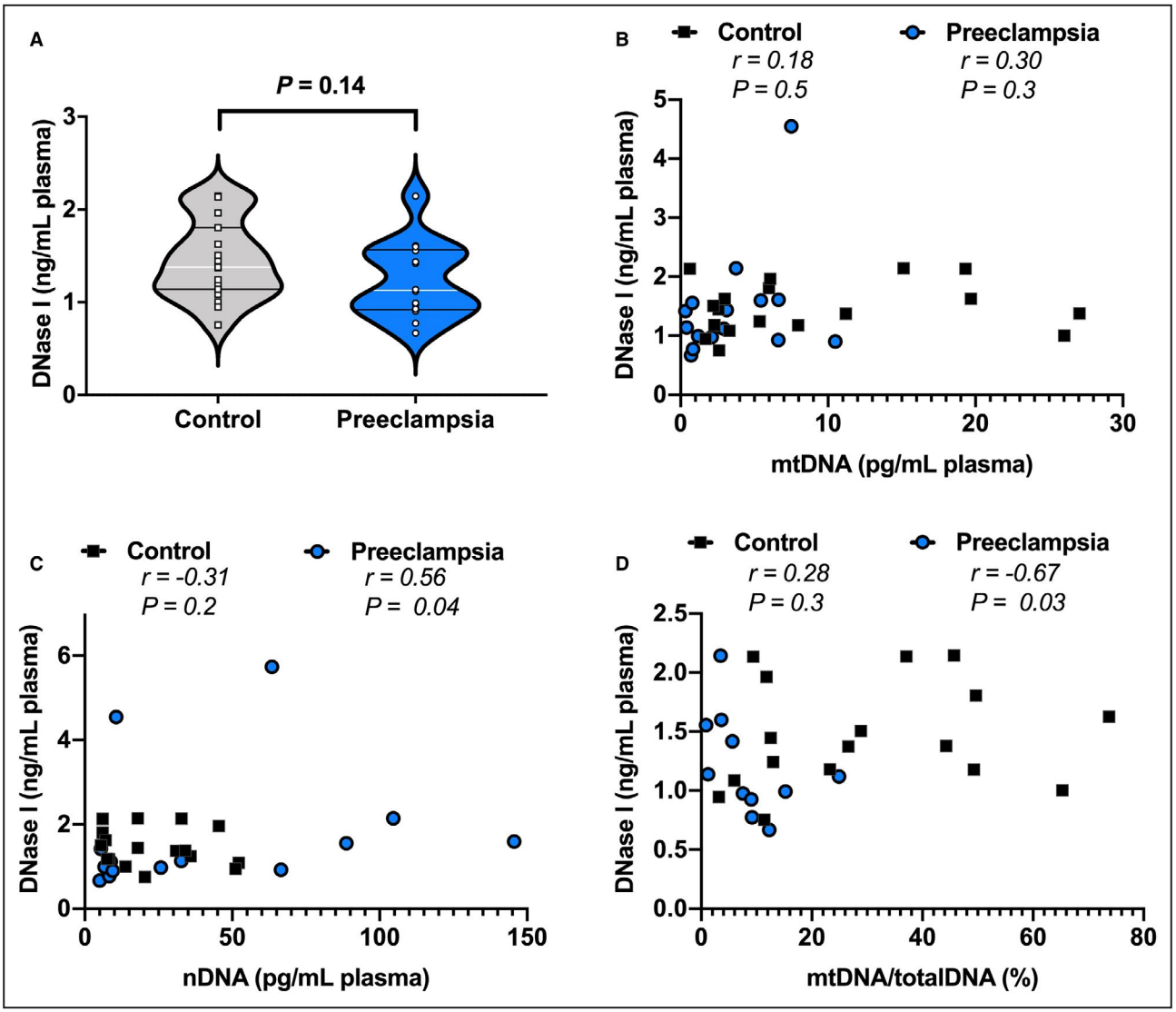

Figure 5. Maternal plasma concentrations of DNase I measured in healthy pregnancies and pregnancies with preeclampsia. **A**, DNase I concentrations (ng/mL) in maternal plasma (controls, n=19; preeclampsia, n=14), and DNase I plotted as a concentration against (**B**) ccf-mtDNA concentration (pg/mL) in plasma, (**C**) nDNA concentration (pg/mL), and (**D**) ccf-mtDNA as a percent of total DNA. **A**, Student's *t*-test, values presented as median (white line), IQR (black lines). **B** through **D**, Spearman correlation with *r* and *P* values presented for each analysis. ccf-mtDNA and nDNA used for Spearman correlations were derived from samples treated with lysis buffer to extract DNA. nDNA, nuclear DNA; ccf-mtDNA, cell-free circulating mitochondrial DNA; totalDNA, sum of ccf-mtDNA and nDNA. Open squares=gestational age-matched control; open circles=third trimester pregnancies with preeclampsia.

largest effect (odds ratios) describing the diagnosis of preeclampsia were higher levels of nDNA and DNase I and having a history of preeclampsia (Figure 6B). Preeclampsia was also associated with lower concentrations of non–membrane-bound ccf-mtDNA, higher likelihood of having a cesarean delivery, higher body mass index at first obstetric visit, elevated mean arterial pressure, and higher maternal age. In summary, maternal circulating DNA metrics have made a significant contribution to the clinical diagnosis of preeclampsia.

DISCUSSION

The main findings of this study are (1) plasma ccf-mtDNA concentrations are reduced in women with preeclampsia compared with gestational age–matched healthy pregnant women, while there are no differences in cellular mtDNA copy number between groups; (2) the majority of ccf-mtDNA is encapsulated in vesicles during pregnancy, and this phenomenon is more pronounced in preeclampsia; and (3) aberrance in circulating DNA dynamics,

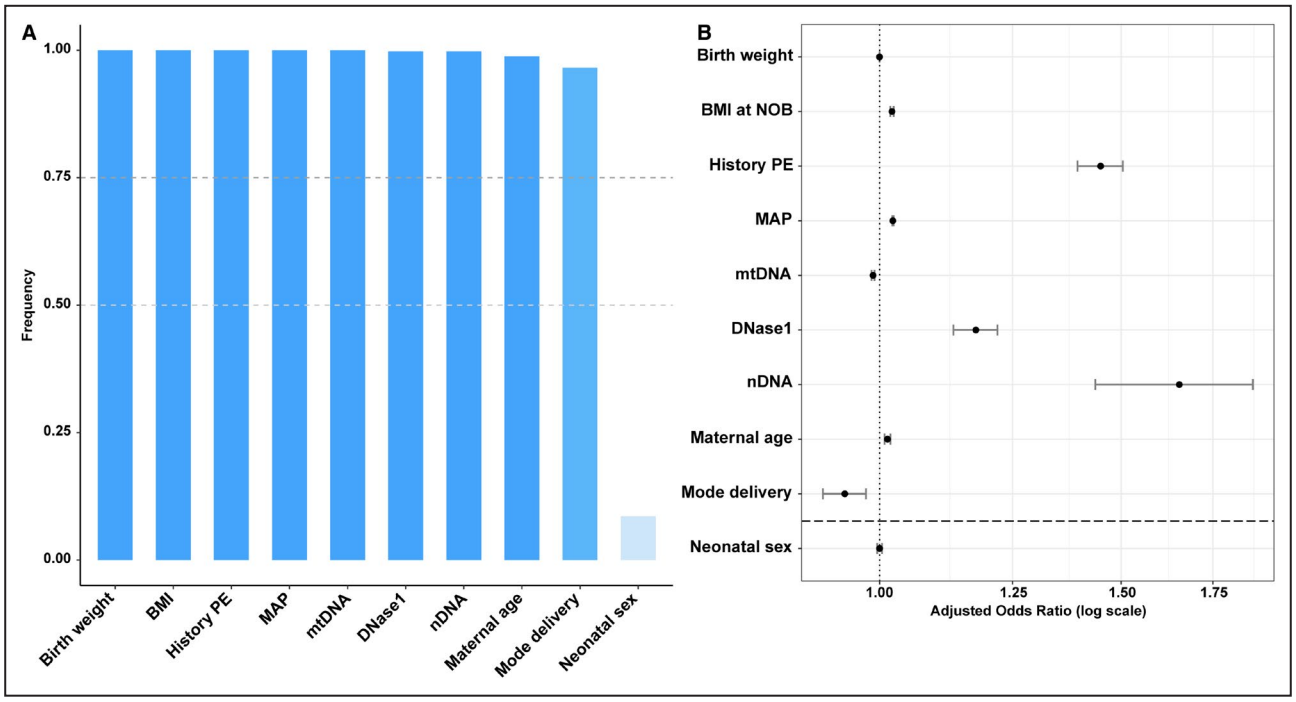

Figure 6. Circulating DNA dynamics and patient characteristics most important to accurately determine preeclampsia diagnosis via elastic net penalized regression.

A, Subject characteristics whose variable importance probabilities (VIP) reached at least 75%. **B**, Adjusted odds ratios (OR) of selected subject characteristics. Vertical line represents OR=1 (no effect); horizontal line represents VIP 0.75 cutoff. BMI, body mass index; MAP, mean arterial pressure; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; NOB, time of first (new) obstetric appointment.

including reduced ccf-mtDNA, is related to the clinical diagnosis of preeclampsia.

ccf-mtDNA has been studied in various pathological conditions as a marker of disease-associated changes in easily accessible body fluids such as blood samples.[45,46] The prognostic value of ccf-mtDNA has been evaluated in patients with cancer,[47] trauma,[48] neurodegenerative diseases,[49,50] and multiorgan failure.[51] Furthermore, ccf-mtDNA downstream signaling mediated by TLR-9 activation has been implicated in cardiovascular disease,[52] diabetes,[53] and autoimmune disorders.[54] Here, we found reduced ccf-mtDNA in plasma from women with preeclampsia compared with controls. These findings are in agreement with previous studies that also reported a reduction in ccf-mtDNA in the first trimester of pregnant women who later developed preeclampsia.[19] We speculate that the amount of ccf-mtDNA is correlated with the amount of placental mtDNA content. However, when preeclampsia was studied separately from intrauterine growth restriction, there was no consensus on the change in mtDNA content in placentas from these pregnancies. One study reported increased placental mtDNA content,[55] while another study showed no difference in placental mtDNA content[56] between pregnancies with preeclampsia and healthy controls. Interestingly, a reduction in mtDNA has also been observed with aging,[57] reflecting cellular metabolism and generalized catabolism of mitochondria in tissues. Although placental cell senescence increases with advancing gestational age as a function of normal placental growth in healthy pregnancies, greater levels of biomarkers of cell senescence have been reported in placentas from women with preeclampsia compared with gestational age–matched controls, suggesting accelerated aging.[58–60] While we speculate that the majority of ccf-mtDNA is of placental origin in preeclampsia, maternal organ damage secondary to high blood pressure may also contribute to release and modification of mtDNA fragments into the maternal blood.

In this study, quantities of ccf-mtDNA were determined in blood samples collected at the third trimester but before labor and delivery. In contrast, others noted an increase in ccf-mtDNA in the serum of pregnant women (third trimester) suffering from preeclampsia when compared with controls.[16] Methodological differences in the protocol used to quantify ccf-mtDNA may explain discrepancies between the results of our study and those from previous investigations. One of the major challenges of measuring ccf-mtDNA is the specificity of current quantification protocols in determining absolute concentrations of ccf-mtDNA in biological samples.[9] Most studies in pregnant patients have used relative polymerase chain reaction

quantification approaches,[16,18,19,61] which rely on a nuclear housekeeping gene. However, we have recently reported that concentrations of nDNA increase with advancing gestational age during pregnancy,[27] making quantification of ccf-mtDNA relative to nDNA liable to produce spurious results. In the present study, we overcome this challenge by using our previously published absolute polymerase chain reaction quantification protocol,[27,30] which uses a reference standard curve and is highly sensitive for detecting minute quantities of ccf-mtDNA.[30] A second challenge is that a significant part of the mitochondrial genome is duplicated in the nuclear genome,[62] and therefore it can be coamplified by primers targeting ccf-mtDNA.[9] Here, we address this challenge by amplifying a mitochondrial genome region (position 13,288-12,392) that has few known mutations and is absent from the nuclear genome.[30,63] This approach protects against the effects of hypervariability and pseudogene contamination, respectively. To the best of our knowledge, this is the first study to use standardized conditions to isolate and quantify ccf-mtDNA in plasma from women with preeclampsia.

While the pattern of reduced ccf-mtDNA in preeclampsia was similar between methods of DNA extraction, DNA isolation with membrane lysis buffer resulted in 1000-fold higher ccf-mtDNA quantification in the preeclampsia group and 430-fold higher ccf-mtDNA quantification in the control group. Membrane-bound and non–membrane-bound cell-free nDNA was also determined in these samples, and although the non-membrane-bound fraction was greater than the membrane-bound nDNA, the magnitude of increase was much smaller than what we observed for ccf-mtDNA (control, 27-fold increase; preeclampsia, 15-fold increase). Our observation that maternal blood contains ccf-mtDNA packaged in vesicles as well as membrane-unbound ccf-mtDNA has been previously reported in conditions other than pregnancy or preeclampsia.[9,22,64,65] To the best of our knowledge, this is the first study to demonstrate that the vast majority of circulating cell-free DNA, and particularly ccf-mtDNA, is membrane bound in healthy pregnant women and more so in patients with preeclampsia. This observation can lead future studies to the identification of the source of ccf-mtDNA in preeclampsia using cell-surface markers and to the investigation of ccf-mtDNA downstream signaling relative to its biological form and type of transport in the maternal circulation.

Extracellular mtDNA can elicit a proinflammatory response driven by activation of TLR-9.[9,66,67] In this study, plasma from women with preeclampsia did not elicit greater TLR-9–induced inflammation compared with plasma from healthy controls. Our findings contrast previous studies showing increased TLR-9 activity in response to plasma from women with preeclampsia.[15] These previous studies monitored TLR-9 activity using HEK-Blue TLR-9 reporter cells as we did in the present study.[15] Further, they used maternal blood from pregnant women at the third trimester, during which mtDNA in maternal plasma was increased in preeclampsia compared with healthy pregnancy.[15] However, control and preeclamptic patients were not matched for gestational age (control, 40±0.38 weeks versus preeclampsia, 37.29±0.83 weeks; $P<0.01$). ccf-mtDNA increases with advancing gestational age,[27] and thus, assessing group differences between healthy pregnancies and pregnancy complications requires control of temporal variability by matching subjects for gestational age. In addition, circulating mtDNA was measured using a relative quantification protocol, and studies on TLR-9 activity did not include control experiments (ie, HEK-Blue Null cells, parental cell line of HEK-Blue hTLR9).

Previous studies have suggested that plasma ccf-mtDNA and cellular mtDNA copy number reflect different cellular processes.[68] Intracellular mtDNA copy number in PBMCs is thought to reflect variations in mitochondrial bioenergetics and biogenesis,[69] while ccf-mtDNA is mostly associated with cellular stress and tissue damage.[9,21] In contrast to our findings in plasma samples, we detected no group differences in PBMC mtDNA copy number, suggesting that dysregulation occurred only in extracellular ccf-mtDNA dynamics and not in cellular respiratory capacity of circulating cells in this cohort of women with preeclampsia.

We then examined the relationship between aberrance in circulating DNA dynamics and clinical diagnosis of preeclampsia using elastic net penalized regression. Our analysis demonstrated that dysregulation in circulating DNA dynamics, reflected by higher nDNA and DNase I concentrations, and lower non–membrane-bound ccf-mtDNA, were associated with the clinical diagnosis of preeclampsia. Well-known relationships, such as a higher likelihood of experiencing preeclampsia if a subject has a history of preeclampsia,[70,71] and higher mean arterial blood pressure as part of the diagnostic criteria,[1] were also corroborated by the elastic net regression model.

DNase I is a significant component of cell-free DNA degradation and DNase I activity has been shown to be increased in pregnant women with intrauterine growth restriction,[72] a common feature of pregnancies with preeclampsia. It has been speculated that ccf-mtDNA can be degraded by circulating DNases when it is non–membrane bound but when it is encapsulated in vesicles may be protected from DNases.[73,74] Although this hypothesis has not been investigated in preeclampsia, it is noteworthy that in the present study concentrations of DNase I were correlated with cell-free nDNA but not with ccf-mtDNA, the majority of which was encapsulated in vesicular structures. Collectively, we have demonstrated the

utility of penalized logistic regression in determining the association of circulating DNA-related data with preeclampsia diagnosis.

Limitations of this study include a lack of diversity in subject race and ethnicity and small sample sizes, which may limit the generalizability of our results. Nonetheless, the demographics of the included subjects are representative of the Iowa population, where the samples were collected. Despite the small sample size, we were able to make well-powered conclusions based on our statistical method. Furthermore, our assessment of DNA degradation mechanisms was limited to DNase I, while additional mechanisms may contribute to elimination of circulating cell-free DNA, including filtration by the kidney or spleen, differential flux in DNA release and uptake by tissues, and degradation by other DNA hydrolases.[75] Despite these limitations, our study is the first to (1) provide a rigorous and accurate measurement of exact concentrations of ccf-mtDNA; (2) determine ccf-mtDNA forms of transport in healthy pregnancy and preeclampsia; and (3) identify an association between circulating cell-free DNA dynamics, including ccf-mtDNA and DNA clearance mechanisms, and clinical diagnosis of preeclampsia.

CONCLUSIONS

Previous studies have suggested that ccf-mtDNA may be a potential pathogenic trigger or a contributor to the maintenance of the maternal syndrome in preeclampsia.[16,18,19] The present study demonstrates dysregulation of ccf-mtDNA dynamics in preeclampsia. Findings from the present study set the foundation for future investigations to examine the role of biological forms of ccf-mtDNA (ie, membrane bound versus non–membrane bound) in the pathogenesis of preeclampsia.

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Disclosures

Drs D. Santillan and M. Santillan hold patents related to the prediction and treatment of preeclampsia: US 293 #9 937 182 (April 10, 2018), EU #2 954 324, and PCT/US2018/027152. The remaining authors have no disclosures to report.

Supplemental Material

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Subjects and Experimental Design

This is a cross-sectional, case-control study. De-identified samples and subject information were acquired from the Maternal Fetal Tissue Bank of the Women's Health Tissue Repository at the University of Iowa[26]. Tissue bank inclusion and exclusion criteria have been previously published[26]. The Maternal Fetal Tissue Bank has been approved by the Institutional Review Board of the University of Iowa (IRB#200910784). Details about sample collection for the tissue bank are published elsewhere[26]. The present study was reviewed by the Institutional Review Board of the University of North Texas Health Science Center, which determined the protocol to meet criteria for exempt status (IRB#2017-065, exempt category 4).

Cases consisted of 19 pregnant women clinically diagnosed with preeclampsia. Nineteen healthy pregnant controls were matched to cases for gestational age at sampling. Subject characteristics can be found in Table S1. Maternal blood was collected in the third trimester (28-41 weeks of gestation) during routine venipuncture. Blood was collected into ACD-A tubes (Becton Dickinson) containing: 22.0 g/L trisodium citrate, 8.0 g/L citric acid, and 24.5 g/L dextrose, and stored in 4°C until further processing. Blood was then separated into plasma and peripheral blood mononuclear cells (PBMCs). PBMCs were stored in cryopreservation media (RPMI media [40% v/v],

FBS [50%], and DMSO [10%]). Plasma samples were snap frozen and stored at -80°C and PBMCs were snap frozen and maintained in liquid nitrogen.

DNA Measurements – Absolute quantification polymerase chain reaction (qPCR)

DNA was isolated and quantified as published previously[27], with a few modifications. Briefly, DNA from plasma and PBMCs (200 µL) was isolated using a magnetic bead-based extraction method (Omega Bio-tek) with a final elution volume of 360 µL. DNA from plasma samples was isolated in the presence and absence of lysis buffer (AL Buffer, Omega Bio-tek, Inc., AL-1000; proprietary formulation, guanidine hydrochloride and proteinase k are known components) to elucidate the contribution of the membrane bound component of plasma, as this has been noted previously to contain mtDNA[28, 29].

TaqMan™ chemistry-based absolute quantification of nuclear DNA (nDNA) and mtDNA are detailed elsewhere[27]. Briefly, isolated nDNA (2 µL) was quantified on a 7500 Real-Time PCR System (Applied Biosystems) using the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, Waltham, MA, USA; Cat. No. 4482910) in 18 µL of master-mix for a total reaction volume of 20 µL. PCR settings were as follows: 95 °C for 2 minutes and 40 cycles of 95 °C for 9 seconds with 60°C at 30 seconds. Cycle threshold (C_T) was compared to five 1:10 serial dilutions of male, genomic reference DNA in order to calculate a concentration [nDNA/µL$_{\text{DNA isolate}}$] according to the manufacturer's directions.

Isolated mtDNA was quantified using a method modified from Kavlick et al.[30] and detailed previously[27]. The target sequence for this analysis is the *MT-ND5* gene

(mitochondrial NADH:ubiquinone oxidoreductase core subunit 5; GenBank Gene ID: 4540), spanning positions 13,288-12,392 of the mitochondrial genome (based on revised Cambridge Reference Sequence positions)[31]. Isolated mtDNA (2 µL) was added to 23 µL of master-mix for a total reaction volume of 25 µL. Quantification was performed on a 7500 Real-Time PCR System (Applied Biosystems), with the following settings: 9600 emulation, 50 °C for 2 minutes, 95 °C for 10 minutes, and 40 cycles of 95 °C for 15 seconds with 60 °C for 1 minute. C_T of samples was compared to eight, 1:10, serial dilutions of double-stranded, synthetic, reference DNA (gBlocks® gene fragment; Integrated DNA Technologies, Coralville, IA, USA) in order to calculate a concentration of mtDNA in the isolate [mtDNA/µL$_{\text{DNA isolate}}$]. The qPCR primers, probes, and synthetic DNA standards employed for mtDNA analysis are detailed in Table S2.

For DNA quantification, amplification efficiency >80% and R^2 >99% was considered adequate. Concentration of plasma DNA ([DNA]$^{\text{plasma}}$) was determined by relating the calculated concentration of the DNA lysate ([DNA]$^{\text{isolate}}$) multiplied by the volume of elution buffer (0.360 mL) to the known volume of isolated plasma (0.200 mL) (equation 1a-b), and it was expressed as picograms (pg) per mL of plasma. Total DNA was calculated as the sum of mtDNA and nDNA, in pg/mL plasma.

$$[DNA]^{plasma}\ pg/mL \times\ 0.2\ \text{mL}\ =\ [DNA]^{isolate} pg/mL\ \times\ 0.36\ \text{mL} \qquad \text{(1a)}$$

$$[DNA]^{plasma}\ pg/mL\ =\ \frac{0.36\ \text{mL} \times [DNA]^{isolate}\ pg/mL}{0.2\ \text{mL}} \qquad \text{(1b)}$$

The DNA content of PBMCs is presented as cellular equivalents (Ceq) per microliter DNA isolate [pg/µL$_{\text{DNA isolate}}$] for nDNA and as mtDNA genome copies per Ceq. Ceq was calculated based on the estimated molecular weight of nDNA per human diploid cell of 6.7pg (equation 2a). mtDNA copies were calculated by relating number of mitochondrial

genomes that has a mass of 1 pg, where 1 pg is equal to 58,800 mitochondrial genome copies (equation 2b).

$$Ceq^{PBMC}/\mu L \; = \; [nDNA]^{PBMC}\, pg/\mu L \times \frac{1\, Ceq}{6.7\, pg} \qquad (2a)$$

$$[mtDNA]^{PBMC}\, copies/\mu L \; = \; [mtDNA]^{PBMC}\, pg/\mu L \times \frac{58{,}800\, copies}{1\, pg} \qquad (2b)$$

DNase I measurement in maternal plasma

DNase I concentrations in maternal plasma were measured using an ELISA (MyBioSource, Ca MBS763541). Plasma samples were diluted 1:10 before performing the ELISA per manufacturer's instructions.

TLR-9-induced NF-κB-dependent inflammatory responses

To determine the immunostimulatory potency of plasma from pregnancies with preeclampsia in relation to TLR-9 activation, we used an engineered cell line of human embryonic kidney (HEK) 293 cells transfected with a human *TLR-9* gene (HEK-Blue™ hTLR-9 cells, Invivogen) and control cells (HEK-Blue™ Null1 cells, Invivogen). HEK-Blue™ hTLR-9 cells overexpress the human *TLR-9* gene and also express an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of an NF-κB inducible promoter comprised of an IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites. Stimulation of HEK-Blue™ hTLR-9 cells with a TLR-9 ligand activates NF-κB and AP-1, which induce the production of SEAP. HEK-Blue™ Null1 cells weakly express TLR-9 and express the SEAP reporter gene under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites. HEK-Blue™ Null1 and hTLR-9 cells were cultured and maintained according to the manufacturer's

instructions. TLR-9 stimulation was assessed in response to 10% plasma from women with healthy pregnancies or pregnancies complicated by preeclampsia by monitoring SEAP production in a cell-culture detection medium (HEK-Blue™ Detection, Invivogen). TLR-9 agonists (ODN 2006, CpG-ODN of class B, and ODN 2395, CpG-ODN of class C, Invivogen) in the presence and absence of a TLR-9 antagonist (ODN 2088, Invivogen) were used as positive and negative controls of SEAP production, respectively. TLR-9 stimulation in HEK-Blue™ hTLR9 cells was inhibited by pretreating cells with 100 µM chloroquine (Sigma, Ca C6628) 30 min prior to incubating with plasma samples or controls. After 24 hours of incubation, SEAP production was quantified by reading the optical density (O.D.) of samples at 630 nm using a BioTEK Synergy HTX spectrophotometer. Data of TLR-9 activity are presented as OD at 630 nm.

Penalized logistic regression analysis

To identify the most important patient characteristics associated with the outcome of preeclampsia, bootstrapped penalized logistic regression was implemented. All elements of regression analyses were carried out in R software version 4.0.2[32]. The following penalized regression models were fit and their performances were compared to select the best model: 1) least absolute shrinkage and selection operator (LASSO) regression; 2) ridge regression; and 3) elastic net regression. These models perform optimally under different conditions: LASSO performs best when few predictors influence the outcome[76]; ridge performs best when many predictors have a small effect on the outcome[76, 77]; and elastic net performs best when the dataset is an intermediate

between the two[78]. To reduce the number of covariates and mitigate multicollinearity in regression models while not eliminating potentially important patient data, related characteristics were grouped *a priori* (Table S3) and one single characteristic was selected from each group to generate all possible combinations of independent characteristics (192 total datasets). Bootstrapped (R = 500) 10-fold cross-validation LASSO, ridge, and elastic net were then performed on each dataset and best model fit was assigned by lowest prediction error (specifically, lowest median root mean squared error; RMSE[41, 42]). Predictive accuracy of the final model developed using real data was tested against a simulated naïve prediction dataset using by the bootstrap estimate (R = 500) of the area under the curve of the receiver operator characteristic (AUC ROC) with confidence intervals (CI 95%) calculated from standard error. The R package 'fabricatr' was used during this step because of its ability to retain relationships present within real data when creating a simulated dataset[79]. Because of the retrospective nature in this study, models included known associations with preeclampsia (e.g., BMI, history of preeclampsia), in addition to diagnostic criteria (e.g., blood pressure) and factors affected by preeclampsia (e.g., mode of delivery and neonatal characteristics). Therefore, the final model describes the association between preeclampsia diagnosis, clinical data, and DNA metrics and does not predict preeclampsia occurrence. Characteristics selected by the model at least 75% of the time (variable importance probability; VIP 0.75)[43] for all bootstrap samples were regarded as most important. Adjusted odds ratios are reported. Using this approach, the optimized characteristic combination and model were selected to best explain the relationship between patient characteristics and the diagnosis of preeclampsia.

Statistical analysis

Statistical analyses were performed using Prism (Version 8, GraphPad, San Diego, CA, USA). Data distribution was assessed using the D' Agostino-Pearson omnibus test and the robust regression and outlier removal (ROUT) method was used to identify and remove outliers. Non-parametric statistics were used for non-normally distributed data sets. Group differences in DNA quantities and DNase I concentrations were determined using Student's t-test or Mann-Whitney *U* test. A two-way analysis of variance (ANOVA) followed by Sidak's correction to adjust for multiple comparisons was used determine the effect of fetal sex on group differences in DNA quantities. Spearman correlation was applied to evaluate relationships between DNA quantities and DNase I for each group. DNA outcomes are presented as mean ± standard error of the mean (SEM). Subject characteristics are presented as mean with minimum and maximum unless otherwise indicated. Exact P values are presented for each analysis.

Table S1. Subject characteristics.

	Control (n = 19)	*Preeclampsia* (n = 19)	*P value*
Race (%)			
Caucasian	19 (100)	18 (95)	
Non-Hispanic	19 (100)	19 (100)	
BMI (kg/m^2)	27 (18, 46)	31 (18, 50)	0.1
Overweight (BMI: 25-29.9) (%)	3 (16)	7 (37)	
Obese (BMI: $\geq$ 30) (%)	4 (21)	8 (42)	
MAP (mmHg)	90 (76, 101)	108 (86, 129)	<0.0001
SBP (mmHg)	124 (105, 140)	146 (122, 170)	<0.0001
DBP (mmHg)	73 (57, 87)	89 (67, 112)	<0.0001
History of chronic hypertension (%)	0 (0)	8 (42)	
History of preeclampsia (%)	0 (0)	4 (21)	
Medications (%)			
Aspirin	0 (0)	3 (16)	
Magnesium	0 (0)	3 (16)	
Nifedipine	0 (0)	2 (11)	
Gestational age at sample (weeks)	33 (28, 39)	34 (28, 41)	0.7
Gestational age delivery (weeks)	39 (37, 42)	36 (31, 41)	0.0002
Mode of delivery (%)			
NSVD	14 (74)	8 (42)	
VAVD	0 (0)	2 (11)	

Cesarean section	5 (26)	9 (47)	
Neonatal weight (kg)	3.3 (1.5, 4.6)	2.7 (1.5, 3.9)	0.0076
Neonatal sex (F:M)	(12:7)	(7:12)	
Apgar, 1 minute	7.58 (3,9)	7.05 (4, 9)	0.4
Apgar, 5 minutes	8.95 (8, 9)	8.42 (6, 9)	0.028

Maternal BMI, MAP, SBP, DBP, gestational age at delivery, and neonatal weight were analyzed using unpaired t-test. Gestational age at sample, Apgar (1 minute), and Apgar (5 minute) were analyzed with Mann-Whitney *U* test. Values presented as mean with minimum and maximum unless otherwise noted. BMI, body mass index at first obstetric visit; MAP, mean arterial blood pressure at time of blood sample; SBP, systolic blood pressure at time of blood sample; DBP, diastolic blood pressure at time of blood sample; NSVD, normal spontaneous vaginal delivery; VAVD, vacuum-assisted vaginal delivery.

Table S2. **Primer, probe, and synthetic standard nucleotide sequences for absolute qPCR of mitochondrial DNA.**

mtDNA (*MT-ND5*) F:	5'- GGC ATC AAC CAA CCA CAC CTA -3'
mtDNA (*MT-ND5*) R:	5'- ATT GTT AAG GTT GTG GAT GAT GGA -3'
TaqMan probe:	5'- **6FAM** CAT TCC TGC ACA TCT G **MGBNFQ** -3'
SS (gBlock) F:	5'- TG TTC TGT TCA TTG TTA AGG TTG TGG ATG ATG GAC CCG GAG CAC ATA AAT AGT CGT TAT TTG AAG AAG GCG TGG GTA CAG ATG TGC AGG AAT GCT AGG TGT GGT TGG TTG ATG CCG ATT GGA TTG -3'
SS (gBlock) R:	5'- CAA TCC AAT CGG CAT CAA CCA ACC ACA CCT AGC ATT CCT GCA CAT CTG TAC CCA CGC CTT CTT CAA ATA ACG ACT ATT TAT GTG CTC CGG GTC CAT CAT CCA CAA CCT TAA CAA TGA ACA GAA CA -3'

F, forward. R, reverse. *MT-ND5*, mitochondrial NADH:ubiquinone oxidoreductase core subunit 5. 6FAM, 6-Carboxyfluorescein. MGBNFQ, minor groove binder non-fluorescent quencher. SS, synthetic standard

Table S3. Variable groupings used for dataset generation.

Included in all datasets	**Group 2: Neonatal characteristics**	**Group 4: Extracellular mtDNA**
nDNA ng/ml plasma	Neonatal birth length (cm)	**signaling**
DNase I ng/ml plasma	Neonatal head circumference (cm)	mtDNA pg/ml plasma (membrane-
Mode of delivery	Gestational age at delivery (days)	bound)
Maternal age at delivery	Neonatal birth weight (g)	mtDNA pg/ml plasma (non-membrane
Neonatal sex	Apgar1 score	bound)
BMI at NOB	Apgar5 score	
Group 1: Maternal blood pressure	**Group 3: Maternal reproductive**	
Systolic blood pressure (SBP)	**history**	
Diastolic blood pressure (DBP)	History preeclampsia	
Mean arterial pressure (MAP)	Maternal gravidity	
Chronic hypertension	Maternal parity	
	Number spontaneous abortions	

Datasets were comprised of select variables included in all datasets and one variable randomly chosen from Groups 1-4. Groupings of related variables determined *a priori*; variables included in all datasets were those that were independent from all other variables in the original dataset. BMI, body mass index; NOB, time of first obstetric appointment.

Table S4. Coefficients and odds ratios for patient characteristics (elastic net).

	Coeff	Coeff SE	OR	OR SE	OR CI (95%)
Intercept	-2.5034	0.1410	0.0818	0.0184	(0.0457, 0.1179)
nDNA, ng/ml plasma	0.5032	0.0822	1.6540	0.1110	(1.4365, 1.8716)
mtDNA, pg/ml plasma (membrane-unbound)	-0.0111	0.0013	0.9890	0.00131	(0.9864, 0.9915)
DNase I, ng/ml plasma	0.1617	0.0199	1.1755	0.0221	(1.1322, 1.2188)
BMI	0.0208	0.0015	1.0210	0.0015	(1.018, 1.024)
History preeclampsia (Yes)	0.3710	0.0206	1.4492	0.0281	(1.3941, 1.5043)
Neonatal sex (Male)	0	0.0020	1	0.0020	(0.9961, 1.0039)
Mode delivery (Vaginal)	-0.0584	0.0182	0.9433	0.0174	(0.9092, 0.9774)
Maternal age	0.0132	0.0025	1.0133	0.0026	(1.0083, 1.0183)
Birth weight	-0.0001	7.55e-06	0.9999	7.55e-06	(0.9999, 0.9999)
MAP	0.0222	0.0006	1.0225	0.0006	(1.0214, 1.0236)

Values in parentheses indicate reference state for coefficients and odds ratios. Values are the result of 500 bootstraps.

BMI, body mass index; CI, confidence interval (95%); Coeff, coefficient; MAP, mean arterial pressure; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; OR, odds ratio; SE, standard error

Table S5. Model comparison.

	RMSE	SE	CI (95%)
Ridge	0.2627	0.0179	(0.2276, 0.2978)
LASSO	0.3200	0.0193	(0.2822, 0.3578)
Elastic Net	0.2438	0.0196	(0.2054, 0.2823)

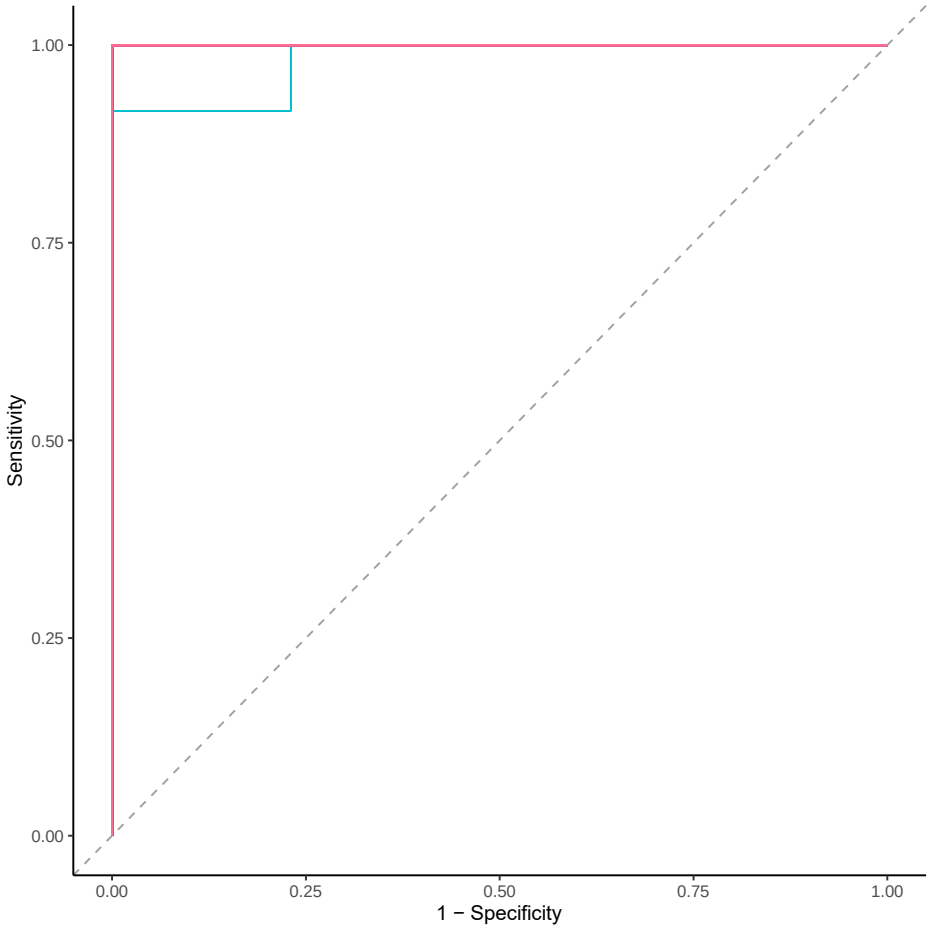
RMSE: median root mean square error; SE: standard error; CI: confidence interval (95%)

Table S6. Model accuracy and receiver operating characteristic.

	Boot Stat	SE	CI (95%)
Model Accuracy	1	0.0012	(0.9977, 1.00)
AUC ROC	1	0.0009	(0.9983, 1.00)

Boot stat: bootstrap summary statistic; SE: standard error; CI: confidence interval (95%); AUC ROC: area under curve of the receiver operating characteristic

Figure S1. Accuracy of elastic net penalized regression in the current study.



AUC ROC plot R = 500 simulations (supplementary)