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*Original Article*

# Maternal blood mitochondrial DNA copy number and placental abruption risk: results from a preliminary study

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**Abstract:** Oxidative stress and impaired placental function – pathways implicated in the pathogenesis of placental abruption – have their origins extending to mitochondrial dysfunction. To the best of our knowledge, there are no published reports of associations of placental abruption with circulating mitochondrial DNA (mtDNA) copy number – a novel biomarker of systemic mitochondrial dysfunction. This pilot case-control study was comprised of 233 placental abruption cases and 238 non-abruption controls. Real-time quantitative polymerase chain reaction (PCR) was used to assess the relative copy number of mtDNA in maternal whole blood samples collected at delivery. Logistic regression procedures were used to estimate adjusted odds ratios (OR) and 95% confidence intervals (CI). There was some evidence of an increased odds of placental abruption with the highest quartile of mtDNA copy number (*P* for trend = 0.09) after controlling for confounders. The odds of placental abruption was elevated among women with higher mtDNA copy number (≥336.9) as compared with those with lower values (<336.9) (adjusted OR = 1.60; 95% CI 1.04-2.46). Women diagnosed with preeclampsia and with elevated mtDNA copy number had a dramatically increased odds of placental abruption as compared with normotensive women without elevated mtDNA copy number (adjusted OR = 6.66; 95% CI 2.58-17.16). Maternal mitochondrial dysfunction appears to be associated with placental abruption in the presence of preeclampsia. Replication in other studies, particularly prospective cohort studies and those that allow for tissue specific assessment of mitochondrial dysfunction (e.g., the placenta) are needed to further understand cellular and genomic biomarkers of normal and abnormal placental function.

**Keywords:** Placental abruption, mitochondrion, mitochondrial DNA, pregnancy, biomarkers

## Introduction

Mitochondria are semi-autonomous cytoplasmic organelles of the eukaryotic system that produce adenosine triphosphate by the coupling of oxidative phosphorylation to respiration, providing a major source of energy to the cell [1, 2]. They are the major source of endogenous reactive oxygen species (ROS) and play an important role in apoptosis. In each cell, several hundreds to thousands of mitochondrial DNA (mtDNA) copies are present [3, 4]. Mitochondrial DNA abundance is positively correlated with the number and size of mitochondria [5], and is modulated by endogenous and exogenous factors such as hypoxemia and steroid hormone exposures [6-8]. Toxicogenomic studies have shown that mtDNA abundance is altered in relation to environmental exposures including low-dose benzene [9] and tobacco smoke [10-12]. In contrast to nuclear DNA (nDNA), mtDNA lacks a protective histone backbone or other specific DNA-binding proteins, replicates faster, and does not have proofreading or an efficient DNA repair mechanism [13-15]. Typically, mtDNA is located near the inner membrane of the mitochondrion and is thus continually exposed to high levels of reactive oxidative species and free radicals from the electron transport chain of mitochondria [12].

Hence, mtDNA is particularly susceptible to oxidative stress-induced damage. Along these lines, investigators have reported that the mitochondrial genome exhibits 17-times higher rates of mutation than does the nuclear genome [16]. Moreover, other investigators have reported that DNA damage persists longer in the mitochondrial genome [17, 18].

Cells exposed to oxidative stress have been shown to synthesize more copies of their mtDNA (a marker of mitochondrial abundance) as a means for compensating for damage and to meet the increased respiratory demand for clearing ROS [5, 19]. On the basis of these observations, alterations in mtDNA copy number in various tissues, including whole blood, has emerged as a possible biomarker of mitochondrial dysfunction and risk factor for diverse cardiometabolic and neurodegenerative disorders, as well as multiple cancers [20-22]. Notably, these diverse disorders have oxidative stress as a common pathophysiological mechanism.

Placental abruption, the premature separation of the placenta, is a life threatening obstetrical condition that complicates roughly 1-2% of all pregnancies [23-25]. The condition occurs in much higher frequencies among women with multi-fetal gestation, coagulopathies, acquired forms of thrombophilia, uterine anomalies, abdominal trauma, hypertension, premature rupture of membranes and maternal-fetal hemorrhage, and intrauterine infections [26-30]. Young and advanced maternal age, grand-multiparity, and maternal cigarette smoking have been identified as placental abruption risk factors [28-31]. Pathophysiologic mechanisms involved in placental abruption, and related perinatal disorders – preterm birth, preeclampsia, and intrauterine growth restriction, include uteroplacental ischemia, underperfusion, chronic hypoxia, and infarctions. On this basis, we and other investigators have begun to conceptualize abruption as an “ischemic placental disorder” characterized by acute and chronic pathophysiological features [24, 32].

Several lines of evidence suggest that oxidative stress may be involved in the pathogenesis of placental abruption [33-35]. Moreover investigators have observed elevated risks of preeclampsia, one of the strongest risk factors for placental abruption, in a family with clinically diagnosed mitochondrial dysfunction [36]. Building upon this initial observation, other investigators [37] proposed that defects in the mitochondria of trophoblasts may be the initiating step in the pathophysiological cascade of preeclampsia. These seminal observations, an emerging literature supporting mitochondrial abundance, a biomarker of oxidative stress and oxidative stress-related disorders, and findings from three studies which suggest that mitochondrial abundance may be associated with placental insufficiency, intrauterine growth restriction and preeclampsia [38-40], we sought to expand the current literature by investigating associations of alterations in mitochondrial copy number in maternal blood with the risk of placental abruption.

We hypothesized that maternal whole blood mtDNA copy number, a novel biomarker of systemic cellular oxidative stress and mitochondrial dysfunction, would be elevated in women with pregnancies complicated by placental abruption as compared with unaffected controls. We further hypothesized that the odds of placental abruption would be particularly elevated among women with both high mtDNA copy number and a diagnosis of preeclampsia during the index pregnancy. We used information from a large case-control study of Peruvian women [30] to test our study hypotheses.

## Materials and methods

This case-control study was conducted at the Hospital Nacional Dos de Mayo, Instituto Especializado Materno Perinatal, and Hospital Madre-Niño San Bartolomé in Lima, Peru, from August 2002 through May 2004. The procedures used in this study were in agreement with the protocols approved by participating institutions. All participants provided written informed consent.

Placental abruption cases were identified by daily monitoring of all new admissions to antepartum, emergency room, and labor and delivery wards of participating hospitals. Study subjects were recruited during their hospital stay. Hospital medical records were reviewed so that clinical diagnostic signs, symptoms and physical characteristics of placental abruption could be objectively confirmed; and so that other clinical diagnoses associated with late pregnancy vaginal bleeding could be excluded. During the

study period, there were a total of 41,175 deliveries of which 289 were complicated by placental abruption. The diagnosis of placental abruption was based on routine clinical examination performed by the attending physician. For the research diagnosis of placental abruption, we required evidence of blood clot behind the placenta accompanied by at least two of the following signs and symptoms: 1) vaginal bleeding in late pregnancy that was not associated with placenta previa or cervical lesions; 2) uterine tenderness and/or abdominal pain; and 3) fetal distress or death. Fifteen cases were missed because of inadequate staffing. Of the remaining 274 cases approached, 260 (95%) elected to participate in the study. Controls were selected from eligible women who delivered at the participating institutions during the study period. Eligible controls were women who did not have a diagnosis of placental abruption and whose medical record review later confirmed this fact. Of the 285 controls approached, 262 (92%) agreed to participate in the study.

During the in-patient admission period (after delivery), enrolled subjects were interviewed by trained research personnel using a standardized and structured questionnaire. Information collected during the interview included maternal age, marital status, employment status during pregnancy, medical history, and smoking and alcohol consumption before and during pregnancy. At the time of the interview, study personnel completed a brief physical exam to measure maternal standing height, weight, and mid-arm circumference. At the time of the interview, maternal non-fasting blood samples, collected in 10 ml vacutainer tubes after the interview, were frozen at -80°C until analysis.

The analytical population for the study was derived from the 260 placental abruption cases and 262 controls enrolled in the study. A total of 32 women with insufficient whole blood DNA (15 cases and 17 controls) were excluded. An additional eight women with twin or higher order pregnancies (5 cases and 3 controls) were excluded from analysis. We further excluded 11 women with a history of chronic hypertension (7 cases and 4 controls). Hence a total of 233 cases and 238 controls remained for analysis.

DNA, from aliquots of maternal whole blood, was extracted using standard salting-out procedures [41]. Total DNA was used as a template in real-time quantitative polymerase chain reaction (PCR) experiments using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The RNase P gene was used as an endogenous control (catalog # 4316844; Applied Biosystems) and Applied Biosystems MT-7S (catalog # Hs02596861_s1) encoding the D-loop of the mitochondrial DNA as the target gene. RNase P is a single-copy nuclear gene and MT-7S is the replication start site for mtDNA. Experiments were performed using 50 ng total DNA in a 20 μL reaction made up of 10 μL 2X TaqMan universal PCR master mix, 1 μL primer, and nuclease-free water in a 96-well reaction plate. MT-7S and RNase P reactions were run in duplicate in separate wells. Cycling conditions were: 50°C for 2 min; 95°C for 10 min; followed by 40 cycles of 95°C, 15 s and 60°C, 1 min. Data were analyzed using the comparative Ct method, where Ct is defined as the cycle number in which fluorescence first crosses the threshold. ΔCt was found by subtracting the RNase P Ct values from the MT-7S Ct values. The result was applied to the term $2^{(-\Delta Ct)}$. All assays were performed without knowledge of pregnancy outcome.

We examined frequency distributions of maternal socio-demographic, medical characteristics, and medical and reproductive histories according to placental abruption case-control status. Since the distribution of whole blood mtDNA copy number was skewed for both cases and controls, differences in median values of mtDNA copy number between placental abruption cases and control groups were compared based on the Mann-Whitney *U* test.

To estimate the relative association of placental abruption with maternal whole blood mtDNA copy number, we categorized each subject according to quartiles determined by the distribution of mtDNA relative copy number among controls. We used the lowest quartile as the referent group, and we estimated odds ratios (OR) and 95% confidence intervals (95% CI) for each of the upper three quartiles. We then contrasted the highest quartile with the lowest 3 quartiles combined since we observed no evidence of association of placental abruption with mtDNA across the lower 3 quartiles. We also evaluated linear trends in risk by treating the

four quartiles as a continuous variable after assigning a score to each quartile [42].

Since preeclampsia is a risk factor for placental abruption (30) and since the odds of preeclampsia were positively associated with maternal blood mtDNA copy number (40), we assessed the joint and independent effect of mtDNA and preeclampsia status on the odds of abruption. We categorized women into four groups based on combinations of whether their mtDNA copy number and the diagnosis of preeclampsia during the index pregnancy. For this analysis, elevated mtDNA was defined as copy number ≥336.9 (determined using the top quartile value observed among controls). The four resulting categories were as follows: no elevations of mtDNA and no preeclampsia, elevations of mtDNA only, preeclampsia only, and both elevations of mtDNA and preeclampsia. Women with no elevations of mtDNA and no preeclampsia comprised the reference group, against which women in the other three categories were compared.

To assess confounding, covariates were entered into a logistic regression model one at a time, then the adjusted and unadjusted odds ratios were compared [42]. Final logistic regression models included covariates that altered unadjusted odds ratios by at least 10%, as well as those covariates of *a priori* interest (e.g., maternal pre-pregnancy BMI). We considered the following covariates as possible confounders in this analysis: maternal age, parity, smoking during pregnancy, no prenatal care, and gestational age at delivery. All analyses were performed using STATA 9.0 statistical software. All continuous variables were presented as mean ± standard deviation (SD) or median [interquartile range, IQR].

## Results

The socio-demographic, medical and reproductive characteristics of the two study groups are summarized in **Table 1**. Median values of maternal mtDNA copy number were similar between the two study groups (262 vs. 250; *P*-value = 0.37). mtDNA copy number were slightly higher among abruption cases with a concurrent preeclampsia diagnosis versus controls (median 269 versus 250), although this difference did not reach statistical significance.

As shown in **Table 2**, there was some evidence of an increased odds of placental abruption with the highest quartile of mtDNA copy number (*P* for trend = 0.09) after controlling for maternal pre-pregnancy body-mass index, no prenatal care and gestational age at delivery. The adjusted ORs of placental abruption for the successive quartiles of mtDNA copy number, compared with the referent (first quartile) were 0.85 (95% CI 0.47-1.51), 0.99 (95% CI 0.56-1.73) and 1.51 (95% CI 0.88-2.59) (*P* for trend = 0.09). After collapsing the lower 3 quartiles, we noted that the odds of placental abruption were 1.6-fold higher among women with higher mtDNA copy number as compared with those with lower values (adjusted OR = 1.60; 95% CI 1.04-2.46). Additional adjustment for preeclampsia did not change the OR (**Table 2**), thus indicating that the observed association of mtDNA copy number with placental abruption was not driven by maternal preeclampsia status.

We examined the joint associations of mtDNA copy number and concurrent preeclampsia status with risk of placental abruption (**Table 3**). Compared with normotensive women without mtDNA copy number elevations, preeclamptic women with elevated mtDNA copy number had a 6.7-fold increased odds of placental abruption (OR = 6.66, 95% CI 2.58-17.16). The markedly higher odds of placental abruption associated with preeclampsia and elevated mtDNA copy number was greater than the sum of the excess risks for each factor considered independently. Hence, in this population, there appears to be some evidence of a greater-than-additive effect between the two risk factors and placental abruption. It is important to note, however, that the test for effect modification did not reach statistical significance (P = 0.50).

## Discussion

The placenta, a highly complex organ which serves as the site for nutrient, water, and waste exchange between the mother and her fetus, plays an important role in modulating placental function and fetal growth. In addition, as an immune-endocrine organ that constitutes the intrauterine environment, the placenta plays an important role in mediating the impact of environmental and metabolic insults on fetal growth. Placental mitochondria, cellular structures that provide most of the energy produc-

**Table 1.** Socio-Demographic and Reproductive Characteristics and Infant Outcomes in the Study Population, Lima, Peru

| Characteristics | Placental Abruption | | | | *P*-value |
|---|---|---|---|---|---|
| | Cases (N = 233) | | Controls (N = 238) | | |
| | N | % | N | % | |
| Maternal Age at Delivery (years) | 27.2±6.5[1] | | 27.2±6.9 | | 0.97 |
| <20 | 23 | 9.9 | 31 | 13.0 | 0.64 |
| 20-29 | 129 | 55.4 | 122 | 51.3 | |
| 30-34 | 46 | 19.7 | 45 | 18.9 | |
| ≥35 | 35 | 15.0 | 40 | 16.8 | |
| Parity | 1.10±1.58[1] | | 0.95±1.17 | | 0.26 |
| 0 | 111 | 47.6 | 108 | 45.4 | 0.44 |
| 1 | 62 | 26.6 | 67 | 28.1 | |
| 2-4 | 49 | 21.0 | 58 | 24.4 | |
| ≥5 | 10 | 4.3 | 4 | 1.7 | |
| missing | 1 | 0.4 | 1 | 0.4 | |
| Education ≤ high school | 186 | 79.8 | 202 | 84.9 | 0.18 |
| Single Marital Status | 43 | 18.5 | 35 | 14.7 | 0.32 |
| Employed during Pregnancy | 86 | 36.9 | 85 | 35.7 | 0.37 |
| Planned Pregnancy | 108 | 46.4 | 117 | 49.2 | 0.80 |
| No Prenatal Care | 39 | 16.7 | 23 | 9.7 | 0.03 |
| No Prenatal Vitamin | 106 | 45.5 | 111 | 46.6 | 0.54 |
| Smoked during Pregnancy | 10 | 4.3 | 5 | 2.1 | 0.15 |
| Alcohol Use during Pregnancy | 0 | 0.0 | 0 | 0.0 | --- |
| Illicit Drug Use during Pregnancy | 1 | 0.4 | 0 | 0.0 | --- |
| Pre-Pregnancy Body Mass Index (kg/m$^2$) | 23.5±3.5[1] | | 23.9±4.2 | | 0.19 |
| <18.5 | 17 | 7.3 | 10 | 4.2 | 0.01 |
| 18.5-24.9 | 142 | 60.9 | 150 | 63.0 | |
| 25.0-29.9 | 50 | 21.5 | 46 | 19.3 | |
| ≥30.0 | 9 | 3.9 | 25 | 10.5 | |
| Missing | 15 | 6.4 | 7 | 2.9 | |
| Preeclampsia | 70 | 30.0 | 28 | 11.8 | <0.001 |
| Gestational Age at Delivery (weeks) | 35.5±4.3[1] | | 37.9±3.4 | | <0.001 |
| Infant Birth Weight (grams) | 2423±895[1] | | 3041±794 | | <0.001 |
| Whole Blood Mitochondrial DNA Copy Number Median [interquartile range] | 262 [180-366] | | 250 [180-337] | | 0.37 |

[1]Mean ± SD (SD: standard deviation).

**Table 2.** Odds Ratio (OR) and 95% Confidence Interval (CI) for Placental Abruption in Relation to Categories of Maternal Whole Blood Mitochondrial DNA Copy Numbers

| Whole Blood Mitochondrial DNA Copy Number | Placental Abruption Cases (N = 233) | Controls (N = 238) | Unadjusted OR (95% CI) | Adjusted OR* (95% CI) | Adjusted OR** (95% CI) |
|---|---|---|---|---|---|
| Quartile 1 (<179.5) | 58 | 59 | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| Quartile 2 (179.5-249.8) | 45 | 60 | 0.76 (0.45-1.30) | 0.85 (0.47-1.51) | 0.89 (0.49-1.63) |
| Quartile 3 (249.9-336.8) | 55 | 59 | 0.95 (0.57-1.59) | 0.99 (0.56-1.73) | 0.97 (0.54-1.75) |
| Quartile 4 (≥336.9) | 75 | 60 | 1.27 (0.77-2.09) | 1.51 (0.88-2.59) | 1.54 (0.88-2.70) |
| *P-value for linear trend* | | | *0.23* | *0.09* | *0.11* |
| Quartiles 1-3 (<336.9) | 158 | 178 | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| Quartile 4 (≥336.9) | 75 | 60 | 1.41 (0.95-2.10) | 1.60 (1.04-2.46) | 1.61 (1.03-2.52) |

*Adjusted for maternal pre-pregnancy body mass index, no prenatal care and gestational age at delivery. **Adjusted for maternal pre-pregnancy body mass index, no prenatal care and gestational age at delivery and preeclampsia status.

**Table 3.** Odds Ratio (OR) and 95% Confidence Interval (CI) for Placental Abruption in Relation to the Joint Effect of High Maternal Whole Blood Mitochondrial DNA Copy Numbers and Preeclampsia Status

| Whole Blood Mitochondrial DNA Copy Number & Preeclampsia | Whole Blood Mitochondrial DNA Copy Number [median (IQR)] | Placental Abruption Cases (N = 225) | Controls (N = 238) | Unadjusted OR (95% CI) | Adjusted OR* (95% CI) |
|---|---|---|---|---|---|
| Low & No | 209.8 (155.4-268.0) | 107 | 156 | 1.00 (reference) | 1.00 (reference) |
| High & No | 420.9 (366.0-528.9) | 48 | 54 | 1.30 (0.82-2.05) | 1.51 (0.92-2.48) |
| Low & Yes | 228.4 (146.8-262.6) | 45 | 22 | 2.98 (1.69-5.25) | 3.11 (1.70-5.67) |
| High & Yes | 431.2 (382.0-517.1) | 25 | 6 | 6.07 (2.41-15.31) | 6.66 (2.58-17.16) |
| *P-value for interaction* | | | | *0.43* | *0.56* |

Note: 8 placental abruption cases had missing data for preeclampsia status and were thus excluded from this analysis. High mtDNA was defined (≥336.9, upper quartile); and low mtDNA was defined as (<336.9, lower three quartiles). *Adjusted for maternal pre-pregnancy body mass index, no prenatal care, and gestational age at delivery.

tion in cells, control a number of important cellular processes, (e.g., fat metabolism, steroid synthesis, and apoptosis) and are likely to play a central role in placental implantation, growth and development. To date, there has been little research directed towards the study of maternal mitochondrial function status and risk of adverse pregnancy outcomes. Notably, two investigative teams have suggested that mitochondrial dysfunction may contribute to the pathogenesis of preeclampsia [36, 37]. Widschwendter et al. suggested that defects in trophoblastic mitochondria may be the initiating step in the pathophysiological cascade of preeclampsia [37]. Inspired by these early observations, we examined the association of maternal whole blood mtDNA copy number with the odds of preeclampsia [40] and found that the odds of preeclampsia was positively associated with maternal blood mtDNA copy number. In this present study, we sought to expand the existing literature by further evaluating the extent to which, if at all, maternal whole blood mtDNA copy number may be associated with the odds of placental abruption. We found evidence of a modest increased odd of placental abruption with elevated mtDNA copy number. The observed 1.6-fold increased odds of placental abruption for mothers with blood mtDNA copy number ≥336.9, as compared to those with values <336.9 remained even after adjustment for preeclampsia status. We also found that women with pregnancies complicated by preeclampsia and who also had a high mtDNA copy number had a 6.7-fold increased odds of placental abruption as compared with those women with neither risk factor.

There are several reasons why mitochondrial dysfunction assessed based on mtDNA copy number, may be a useful risk marker of adverse placental function, including preeclampsia, IUGR and placental abruption. First, mtDNA is highly susceptible to oxidative damage. This vulnerability to oxidative damage has been attributed to a number of factors including: the absence of histone or DNA-binding proteins; a limited DNA repair mechanism; genes consisting only of exons without introns and; and replicating rapidly without a sufficiently accurate proofreading system [13-15]. Mitochondrial DNA damage, particularly deletions in the control regions of the circular mitochondrial genome, as reflected by alterations in mtDNA copy number may alter mitochondrial gene expression and lead to a deficiency in oxidative phosphorylation and enhance generation of ATP by glycolysis [5]. Second, oxidative stress may contribute to alterations in mitochondrial function and increased mtDNA copy numbers through several mechanisms involving reactive oxygen species (ROS)-induced damage to cellular structural elements, including the lipid membranes of mitochondria [43]. ROS may also affect mitochondrial function by damaging DNA and impairing election chain transport; and a compensatory response to this cellular stress is thought to increase in mtDNA copy number [5]. This latter hypothesized mechanism is consistent with empirical evidence from experimental animal studies documenting increased mitochondrial damage and mtDNA copy numbers with increasing exposure to prooxidants [43].

Several limitations should be considered when interpreting the results of our study. First, given the retrospective study design, we cannot establish the temporal relationship between elevated mtDNA copy numbers and the onset

of placental abruption. Prospective cohort studies with serial longitudinal follow-up are needed to further clarify the temporal relationship of mtDNA copy number with the occurrence of placental abruption. Second, differential misclassification of maternal mtDNA copy number is a possibility, though unlikely, as all laboratory analyses were conducted without knowledge of participants' case-control status. Lastly, although we controlled for multiple confounding factors, we cannot exclude the possibility that the odds ratios reported are unaffected by residual confounding.

Our results suggest that mtDNA copy number in maternal blood may be associated with placental abruption, particularly when abruption occurs in a pregnancy that is also complicated by preeclampsia. Future research will be needed to confirm our preliminary observations and expand the scope of research to include assessment of placental mtDNA copy number, direct measure of placental and maternal whole body mitochondrial function. Finally, analyses that interrogate the influences of variation in the mitochondrial genome in maternal, fetal and uteroplacental compartments are also warranted as such studies will elucidate the molecular mechanisms of maternal, fetal and uteroplacental bioenergetics during pregnancy.

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## Disclosure of conflict of interest

There is no conflict of interest to declare.

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