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# A PILOT STUDY TO EVALUATE THE CFFDNA AS A PREDICTIVE MARKER OF PREECLAMPSIA

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## ABSTRACT

**Objectives:** Preeclampsia often complicates pregnancy resulting in adverse impact on maternal and fetal health. Early detection helps in intervention and avoiding adverse outcome of pregnancy. Detection of cell free fetal DNA (cffDNA) in maternal plasma opens the possibility of non-invasive probe into health of fetus.

**Methods:** Total 50 pregnant women had been recruited from the Antenatal OPD with and without preeclampsia. The women with preeclampsia were considered as case and without preeclampsia were considered as control. cffDNA had been utilized for prenatal diagnosis of adverse pregnancy outcomes.

**Result:** Among 50 participants, 27 were in preeclampsia group and 23 were in control group. Cell free feta DNA was 55.34 ± 7.232 X 1010 genomic equivalents in preeclampsia group and 11.076 ± 2.345 X 1010 genomic equivalents in control group which is fivefold higher in study group.

**Conclusion:** Elevated amount of cffDNA in maternal plasma is associated with preeclampsia.

**Key words:** Cell free fetal DNA, Maternal plasma, Preeclampsia, Pregnancy outcome

## INTRODUCTION:

Preeclampsia (PE) complicates some 2%-8% of pregnancies worldwide. Hypertensive disorder in pregnancy is the leading cause of maternal death in developed countries and its incidence is increasing. Worldwide approximately 830 pregnant women die every day from preventable causes which accounts for maternal mortality ratio (MMR) 239 per 100,000 live births in developing countries and 12 per 100,000 live births in developed countries.[1] World health organization has demonstrated that 85% maternal death is contributed by the African and South-East Asian countries.[1] The MMR in India is 174 in 2015 and it contributes up to 20% of maternal deaths worldwide due to preventable causes.[2] Sustainable development goals (SDG) after millennium

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development goals (MDG) have been aimed at reducing MMR below 70 per 100,000 live births by the year 2030.[3]

Early detection of pregnancy complications may dictate appropriate intervention. Several randomized controlled trials have been carried out to show that aspirin, an anti-platelet agent, prevents preeclampsia effectively and safely among women with high or moderate risk of PE.[4,5,6] It has been shown that aspirin reduces relative risk of preeclampsia by as much as 53% when administered at 12-16 weeks' of gestation.[7]

Cell Free Fetal DNA (cffDNA) originates from the trophoblastic cells and enters in to the maternal circulation invading the feto-placental barrier. It was detected in maternal plasma in early eighties. Placental origin of cffDNA can be detectable as early as 5 weeks of gestation and it constitutes about 10% of cell free DNA, which is cleared rapidly from maternal circulation after delivery.[8] It thus offers potential source of prenatal diagnosis for various genetic conditions namely achondroplasia, autosomal recessive disorders, fetal thalassemia, aneuploidy, and RHD genotyping.

This gender-independent detection of cffDNA in maternal plasma using RASSF1A/beta-actin has curtained off a new dimension regarding its utility to predict the adverse pregnancy outcomes. Recent studies have shown the utility of cffDNA using the methylation-dependent DSCR3 and RASSF1A markers along with total cell free DNA (cfDNA) in maternal serum by HYP2 marker, are useful in predicting preeclampsia, intrauterine growth restriction.[9] The higher concentration of cffDNA in maternal serum is found in preeclampsia and particularly cffDNA and cfDNA ratio is twofold higher in severe preeclampsia group.[10]

Given the observation that cffDNA in maternal plasma can be detected in early weeks of gestation and elevated in some studies, we have contemplated to test the hypothesis that increased cffDNA is associated with PE. We shall further establish relationship between elevated amount of cffDNA and adverse outcome of pregnancy in terms of preterm birth, low birth weight, stillbirth and spontaneous abortion among pregnant women once the study is completed. The aim of the study was to test the hypothesis that increased amount of cell free DNA in maternal plasma is correlated with preeclampsia (PE) and to determine the outcome of pregnancy with preeclampsia. Outcome was measured in terms of preterm birth, low birth weight, stillbirth and spontaneous abortion was determined.

**MATERIALS & METHODS:**

Pregnant women have been recruited from the Antenartal OPD of College of Medicine & JNM Hospital, WBUHS, Kalyani, Nadia. Being a pilot study with the expected outcome to be relatively uncommon, we were not going in for a formal sample size calculation. Considering the constraint of logistics and time hence we have screened 50 pregnant women with and without preeclampsia. The women with preeclampsia were considered as case and without preeclampsia as control. The exclusion criteria were; pregnancy less than 20 weeks, cervical abnormalities (e.g. excessive friability, malignancy, polyps, and trauma), ectopic pregnancy, molar pregnancy, multiple pregnancies, any medical complication, chronic hypertension, renal disease, autoimmune disease (SLE), antiphospholipid syndrome, and diabetes mellitus. All participants have to sign an informed consent and a standard pro-forma was filled up by the doctor for recording the base line data.

**Laboratory methods:-**

**Quantifying separation of maternal plasma:**

About 5 ml peripheral blood was drawn into an EDTA tube. Plasma was separated by centrifugation at 1600xg at 4ºC for 10 minutes. Plasma was collected and re-centrifuged once again at 16000xg at 4ºC for 10 minutes. Plasma in the upper layer was distributed into 1.5ml tubes (300 µl in each tube, two such). About 300µl plasma could be used either immediately for cell free DNA or stored at -80ºC for future use.

**Methods for detection and quantification of cell free fetal DNA in maternal plasma:**

Cell free DNA in maternal plasma could be extracted using various commercially available Kits as described by many authors. However, these KITs are expensive. Several methods have been described in literature for isolation of fetal DNA from maternal plasma. Comparisons of various methods for total yield were made. These methods consist of (a) Phenol/ chloroform DNA extraction method after SDS (Sodium dodecyl sulfate) and proteinase K lysis, (b) Phenol/ chloroform DNA extraction with addition of a polyacryl DNA carrier, (c) Salting-out protein precipitation method with 6M sodium chloride, (d) Guanidium isothiocynate-based RNA extraction method and (e) Commercially available kits. It was observed that the yield was maximum with guanidium isothiocynate-based RNA extraction method. Our initial result indicates that guanidium isothiocynate-based RNA extraction was convenient and the yield was somewhat higher.

Promoter of RASSF1A is hypermethylated in trophoblast (maternal component) resulting in resistance to digestion by methylation sensitive restriction endonuclease *HhaI, HpaII, Bstu1*. On the contrary, RASSF1A promoter is hypomethylated in fetus (fetal component) and sensitive to digestion of the above restriction endonucleases. Thus digestion of cell free DNA purified as stated above is digested with the above restriction enzymes. Subsequent PCR amplification with specific primers around the promoter would detect the quantity of fetal DNA.

**RESULTS:**

In our study we have screened about 50 women till date which is 27 in preeclampsia group and 23 in control group. In Table 1, baseline parameters are; age (years) 24.37 ± 4.692 in preeclampsia group and in control group 22.87 ± 3.684 (p = 0.220); gravida (number) 1.85±.864 in preeclampsia group and in control 1.70 ± .926 (p = 0.541), Parity (number)1.32 ± .768 in preeclampsia group and in control 1.28 ± .875 (p = 0.398), BMI (kg/m$^2$) 26.53 ± .672 in preeclampsia group and in control 25.42 ± .764 (p = 0.286); gestational age (days) 230.96 ± 18.135 in preeclampsia group and in control 256.91 ± 19.598 (p = 0.452), these all are comparable in both group. But the systolic blood pressure (mmHg) was significantly high in preeclampsia group 149.11 ± 13.993 than the control group 108.52 ± 11.805 (p = 0.000) and similarly diastolic blood pressure (mm Hg) was also higher in preeclampsia group97.19 ± 12.481 than the control group 70.78 ± 9.530 (p = 0.000).

Table 1: Baseline Parameters

| Demographic Characteristics | Case (n= 27) | Control (n=23) | p - Value |
|---|---|---|---|
| Age (years) | 24.37 ± 4.692 | 22.87 ± 3.684 | 0.220 |
| Gravida (number) | 1.85 ± .864 | 1.70 ± .926 | 0.541 |
| Parity (number) | 1.32 ± .768 | 1.28 ± .875 | 0.398 |
| BMI (kg/m2) | 26.53 ± .672 | 25.42 ± .764 | 0.286 |
| Gestational Age (days) | 230.96 ± 18.135 | 256.91 ± 19.598 | 0.452 |
| Systolic Blood Pressure (mm Hg) | 149.11 ± 13.993 | 108.52 ± 11.805 | 0.000 |
| Diastolic Blood Pressure (mm Hg) | 97.19 ± 12.481 | 70.78 ± 9.530 | 0.000 |

In Table 2, laboratory parameters; haemoglobin (g/dl) 10.456 ± 1.1453 in case group and 10.922 ± 1.1156 in control group (p = 0.153), platelet count (per cu mm) 208740 ± 38.731 in case group and 261.65 ± 29.725 in control group (p = 0.000) which is lower in preeclampsia group; creatinine (mg/dl).981 ± .254 in case group and 0.717 ± 0.111 in control group(p = 0.000) which is higher in preeclampsia group; serum bilirubin (mg/dl) 0.822 ± 0.210in case group and 0.761 ±.167in control group (p = 0.265); SGPT (IU/L)28.52 ± 9.283in case group and 22.83 ± 2.674in control group (p = 0.007); SGOT (IU/L) 33.48 ± 10.319 in case group and 26.70 ± 2.771 in control group (p =.004).

Table 2: Baseline Laboratory Parameters:

| Laboratory Parameters | Case (n= 27) | Control (n=23) | p - Value |
|---|---|---|---|
| Haemoglobin (g/dl) | 10.456 ± 1.1453 | 10.922 ± 1.1156 | 0.153 |
| Platelet Count X 1000(cu mm) | 208.74 ± 38.731 | 261.65 ± 29.725 | 0.000 |
| Creatinine (mg/dl) | 0.981 ± 0.254 | 0.717 ± 0.111 | 0.000 |
| Bilirubin (mg/dl) | 0.822 ± 0.210 | 0.761 ± 0.167 | 0.265 |
| SGPT (IU/L) | 28.52 ± 9.283 | 22.83 ± 2.674 | 0.007 |
| SGOT (IU/L) | 33.48 ± 10.319 | 26.70 ± 2.771 | 0.004 |

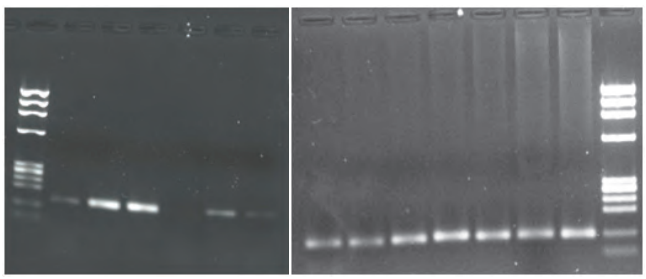

*Figure 1:* PCR amplification using Y chromosome specific locus DYS14 (for primers used please see Table 2). Lanes 1 (from left to right) ϕX174 digested with restriction enzyme Hae III; from lowest to highest bands were of sizes 72bp, 118bp, 194bp, 234bp (other are not mentioned), lane 2, (3 and 4), 6 and 7 were from different samples. Lane 5 shows negative control (without template). The band intensities visibly vary indicating that different samples had different amount of cell free fetal DNA as mothers do not have Y chromosomes. PCR amplified DNA using markers (NQO1) for total cell free DNA (Panel B). First lane (from right to left) was maker as described for panel A, 2-5 same samples as that of in panel A. Negative control was not shown in the panel B. Lanes 6-8 other samples from mothers who did not deliver male child.

The outcome of pregnancy depicted in table 3 where case is n=23 as four patients were lost in follow up and similarly control group is n=20 where three patients were lost in follow up. The outcome parameters are gestational age (days) at birth 232.46 ± 18.932 in case group and 267.56 ± 15.958 in control group (p = 0.000) which is lower in preeclampsia group, birth weight (kg) 2.327 ±.6672 in case group and 2.581 ±.4520 in control group (p = .171), APGAR Score at birth 5.71 ± 1.654 in case group and 6.31 ± .602 in control group (p = .191). The total numbers of still born were five in preeclampsia group and only one in control group.

Table 3: Pregnancy Outcomes Parameters

| Outcomes Parameters | Case (n= 23) | Control (n=20) | p - Value |
|---|---|---|---|
| Gestational Age (days) at birth | 232.46 ± 18.932 | 267.56 ± 15.958 | 0.000 |
| Birth weight (kg) | 2.327 ± 0.6672 | 2.581 ± 0.4520 | 0.171 |
| APGAR Score at birth | 5.71 ± 1.654 | 6.31 ± .602 | 0.191 |
| Still Born | 5 | 1 | 0.000 |

Regarding the quantification of cell free fetal DNA in preeclampsia group as shown in figure 2A & 2B, it was 55.34 ± 7.232 X 1010 genomic equivalents in preeclampsia group and 11.076 ± 2.345 X 1010 genomic equivalents in control group which is fivefold higher in study group.

## DISCUSSION:

In a recent study with 107 pregnant women having clinically established PE at their third trimester and 93 normotensive pregnant women, it has been shown that total cell free DNA, cell free fetal DNA and soluble endoglin (sEng) increased significantly among women with PE. It has also been observed that elevated total cell free DNA and cffDNA were also significantly higher among women with preterm labour and adverse fetal outcome groups compared to the full- term and favourable outcome groups. These three markers were almost equivalent with regard to the area under the curve for predicting adverse fetal outcome in the severe PE group.[11] No significant difference in levels of cffDNA was observed in the first trimester in women who subsequently developed preeclampsia. Levels of cell-free total DNA in the first trimester are increased in African American and Hispanics compared to the white women, and levels increase with increasing BMI. Interestingly, total cell free DNA in pregnant women has been shown to be dependent on the ethnicity. 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). This result shows that cell free DNA in maternal plasma may depend on ethnic background. No systematic study has been carried out so far, hence requires further studies. In a study with 8 women with preeclampsia and 8 normotensive control with singleton male pregnancy between 28 and 32 gestational weeks, it has been shown that cell free fetal DNA concentrations were higher in early preeclamptic women than control subjects.[12] To determine relationship between maternal and fetal characteristics and pregnancy outcomes on fetal and maternal cell-free DNA in maternal plasma at 11-13 weeks' gestation, it has been observed that cell free DNA in maternal plasma was not significantly altered in pregnancies complicated by preeclampsia, early spontaneous preterm birth (SPB) delivery of small for gestational age (SGA) neonates. However, fetal cfDNA level has been seen to be inversely related to maternal weight and uterine artery pulsatility index while maternal cfDNA has been seen to be increased with maternal weight.[13] It cannot be ruled out whether the cffDNA increases in the advance stage of gestation. In a meta-analysis of several studies elevated cffDNA was observed in preeclampsia.

## CONCLUSION:

In summary, increased cffDNA was identified in many studies; while in some studies negative result was also

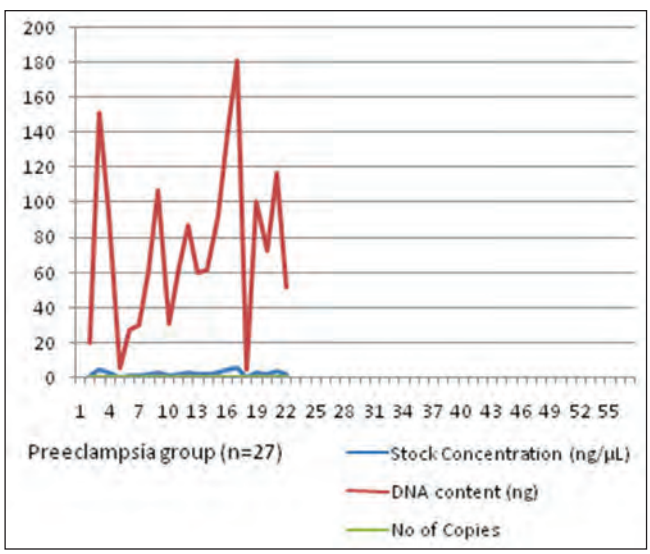


Fig 2A: Cell free fetal DNA quantification in preeclampsia group

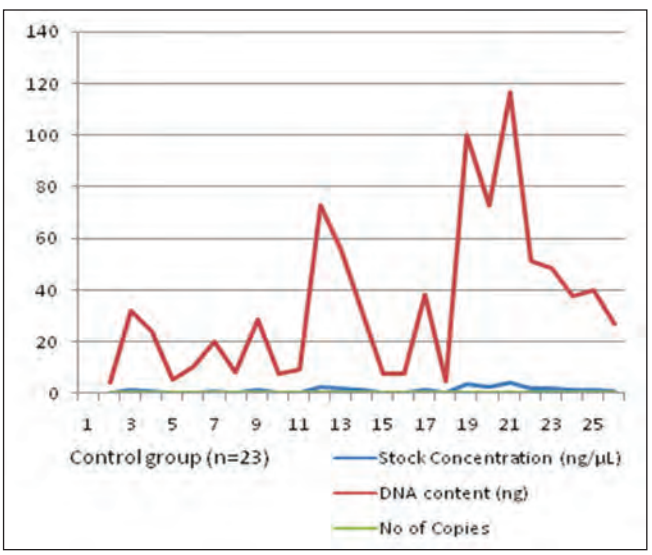


Fig 2B - Cell free fetal DNA quantification in control group

reported. In most of the studies, small numbers of samples were used and one or two loci were used to detect cffDNA. Amount of cffDNA may depend on the gestational period, parity, obesity, ethnicity and age of women. Though in our study we have found fivefold higher cffDNA in the study group, but we have planned to screen about 500 women in future for larger study.

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