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A Prospective study to evaluate the demographic variation of gender independent sequences in cell-free fetal DNA (cffDNA) concentration and to predict pregnancy outcomes by non-kit based economical method

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This gender-independent detection of cell-free fetal DNA in maternal plasma using RASSF1A/β-actin has curtained off a new dimension regarding its utility to predict the adverse pregnancy outcomes. Recent efforts have been directed at developing sequences from cell-free fetal DNA (cffDNA) as markers for pregnancy outcomes. The utility of cffDNA using the methylation-dependent DSCR3 and RASSF1A markers along with total cell-free DNA (cf-DNA) in maternal serum by HYP2 marker are useful in predicting adverse pregnancy outcomes. Increased amount (>95th percentile) of cffDNA fraction in the second trimester is associated with preterm birth. Indigenously developed low-cost method of the gender-independent sequence markers from cffDNA was investigated and evaluated with the standardized commercial kits as predictive markers for adverse pregnancy outcomes. Our results indicated that indigenously developed method for detection of geneder-independent cffDNA can be applicable for screening test of adverse pregnancy outcome.

Keyword: Cell-free fetal DNA (cffDNA), Hypermethylated DNA, Preeclampsia, Trophoblast

Cell-free DNA (cffDNA) in maternal plasma can be extracted using various commercially available kits[1,2]. Result of comparisons of various commercially available KITs has been published[3]. However, these kits are expensive[4]. There are few non-kit based methods cited in a few literatures where cffDNA has been extracted by the indigenous method without using any commercial kits[5]. Application of cffDNA could be used widely as a non-invasive prenatal test (NIPT) if the cost can be reduced for the developing countries like India.

Locus specific differences in methylation between placenta trophoblast and maternal blood have been reported. This differential methylation pattern using various reported locus has been used to detect maternal DNA[6]. The promoter of RASSF1A is hypermethylated in trophoblast resulting in resistant to digestion by methylation sensitive restriction endonuclease HhaI, HpaII, Bstu1. On the contrary, the RASSF1A promoter is hypomethylated and sensitive to the digestion of the above restriction endonucleases in maternal blood[7]. Digestion of cell-free DNA purified as stated above with the above restriction enzymes would quantify the cffDNA only on subsequent PCR amplification with specific primers[8]. Specific primers for amplification of β-actin can be used as internal control. Additionally methylation sensitive promoters of ERG and serpin peptidase inhibitor, clade B (ovalbumin), member 5 (SERPINB5) can also be used to detect the maternal DNA irrespective of gender of the fetal baby[9]. Preterm labour (PTL), intrauterine growth restriction (IUGR), intrauterine fetal death, fetal congenital anomalies, *etc*. often complicate a normal pregnancy. Hypertensive disorder in pregnancy is the leading cause of maternal death in developed countries and its incidence is increasing[10,11]. Detection of cell-free fetal DNA (cffDNA) in

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Abbreviations: cffDNA, Cell-free fetal DNA; IUGR, Intrauterine Growth Restriction; NIPT, Non-invasive prenatal test; PE, Preeclampsia; PTL, Preterm Labour

maternal plasma opens the possibility of a non-invasive probe into the health of the fetus. cffDNA has been utilized for prenatal diagnosis of several genetic diseases and screening for trisomy and fetal aneuploidy[12]. In recent years, an increased amount of cffDNA in maternal plasma has been reported in several studies in Preeclampsia (PE) and in few studies for IUGR and PTL[13].

The aim of the present study is toevaluate whether an elevated amount of cffDNA in maternal plasma is associated with adverse outcomes of pregnancy. The demographic variation of cffDNA in maternal plasma will also be tested. To test the hypothesis, we shall detect and quantify cffDNA in maternal plasma among pregnant women. We shall compare the amount of cffDNA obtained among pregnant women having adverse outcomes with that of women without any complications. In addition, selected women will undergo follow up till delivery to determine the outcome.

Materials and Methods:

Considering the constraint of logistic and time we hereby proposed to screen 100 pregnant women with and without pregnancy related complications in this prospective study, and follow them till delivery. Every participant underwent structured questionnaire, clinical examination, biochemical investigations. Structure questionnaire included age, parity (number of children born), previous history of PE / IUGR/ PTL/ preterm birth, family history of PE/IUGR / PTL, time interval between pregnancies, gestational age at entry and gestational age at delivery. Clinical examination includes Body mass index (BMI), Blood pressure, Proteinuria, Biochemical Investigations, Blood profile (hemoglobin platelet count), Liver function test (LFT), Urea, creatinine (Kidney function), Uric acid, and other antenatal routine investigations. About 5 mL of peripheral blood was drawn into an EDTA tube. Plasma has been separated by centrifugation at 1600 × g at 4°C for 10 min, and re-centrifuged once again at 16000 × g at 4°C for 10 min. The upper layer of the plasma has been distributed into 1.5 mL tubes (300 µL in each tube). About 350 µL plasma either immediately would be used for estimation of cell-free DNA or to be stored at −80°C for future use. Comparisons of various methods for total yield were made. These methods consist of (a) Triton X-100/ Heat/ Phenol Chloroform method (THP) method after SDS and proteinase K lysis, (b) Salting-out protein precipitation method with 6 M sodium chloride, (c) Guanidium isothiocynate-based DNA extraction method and (d) commercial available kits. In our initial experiments, we have used methods (a), (b), (c) and (d). Our initial observation (unpublished) indicates that guanidium isothiocynate -based DNA extraction was convenient and the yield was higher. DNA has been quantified using "Qubit Fluorimeter" and Qubit ds DNA HS assay Kit (Cat no. Q32851). We have also compared the quality and yield of cell-free DNA isolated from the non-kit methods and that from isolated using Kit in the initial phase. The use of various KIT and modified methods as described above has been standardized for recovering consistence quantity cell-free fetal DNA. The concentration of cffDNA was measured in Genomic Equivalent (GE/µL). The study was approved by the Institutional Ethics Committee (No.F-24/ Pr/ COMJNMH/ IEC/16/1210).

Non-Kits Based Methods for isolation of cffDNA

Salting-out protein precipitation method

Maternal plasma of about 350 µL is mixed with equal volume of buffer solution (0.45 *M* NaCl, 10 m*M* TrisHCl, 25 m*M* EDTA in a 1.5 mL Eppendorf tube, 5 µL Proteinase K (20 mg/mL) and 15 µL SDS solution are added and kept at 56°C for 3 h. After that 300 µL NaCl 6 M is added and centrifuged at 10000 rpm/min for 15 min[14]. The supernatant is then transferred to a 5 mL plastic graduated cylinder centrifuge tube and twice the volume of absolute ethanol is added. Transfer the supernatant to a fresh tube and kept at −80°C for 20 min, and re-centrifuged at 12000 rpm/min for 10 min. Discard the supernatant and precipitated DNA using 70% ethanol and then dry it in air closet at 65°C for 3 min[15]. Finally, dissolve it in 20 µL Tris-EDTA (TE) buffer solution and kept it at 4°C.

Guanidiumisothiocynate-based DNA extraction method

Maternal plasma of about 200 µL is added with 500 µL of alanine aminotransferase (ALT/GPT) reagent (6 M guanidine thiocyanate dissolved in 50 mM Tris and mixed with an equal volume Phenol buffered in Tris) in a tube, and mixed. The solution is kept in a boiling water bath for 15 min[16]. The tube is spin for 5 sec and add 250 µL chloroform-isoamaylalcohol (24:1 by volume),centrifuge at 14000 rpm/min for 10 min and the 450 µL liquid phase is transferred in a fresh tube, mixed with 500 µL 100%

isopropanol and 25 mM kept at −20°C overnight. It is centrifuged at 14000 rpm/min for 15 min next day, remove the supernatant was removed without disturbing the nucleic acid pellet. Traces of GPT reagent are to be removed by the addition of ice-cold 500 µL 70% ethanol. Then inverse the tube twice and again centrifuge it at 14000 rpm/ min for 5 min. Ethanol was removed and the pellet was stored.

Triton X-100/Heat/ Phenol Chloroform method (THP) method

Maternal plasma of about 500 µL is mixed with 5 µL Triton X-100 solution and heated at 98°C for 5 min. Keep the sample over ice for 5 min, mixed with equal volume of phenol, chloroform, isoamayl alcohol (25:24:1, *v:v:v*) and centrifuged at 14000 rpm/min for 10 min. The aqueous phase is transferred to a separate tube, allowed to precipitate overnight by 1/10 volume of 3 *M* sodium oxalo-acetate (NaOAc) and 2.5 times volume of 100% ethanol. DNA pellets are washed with ethanol, air dried and re-suspended in 50 µL double-distilled water[17].

Locus specific differences in methylation between placenta topoblast and maternal blood have been reported[18,19]. We have utilized this differential methylation pattern using various reported locus and has been used to detect maternal DNA[20]. The promoter of RASSF1A is hypermethylated in trophoblast resulting in resistant to digestion by methylation– sensitive restriction endonuclease HhaI, HpaII, Bstu1. On the contrary, the RASSF1A promoter is hypomethylated and sensitive to the digestion of the above restriction endonucleases. Thus digestion of cell-free DNA purified as stated above will be digested with the above restriction enzymes. Subsequent PCR amplification with specific primers around the promoter would detect the quantity of fetal DNA[21].Specific primers for amplification of β-actin will be used for internal control. Additionally, mehylation sensitive promoters of ERG and serpin peptidase inhibitor, clade B (ovalbumin), member 5 (SERPINB5) will also be used to detect the maternal DNA irrespective of the gender of the fetal baby as described[22,23]. PCR product will be detected by real time PCR using SYBER green.

Different available commercial kits

Three spin column-based kits for isolation of cffDNA [DNA Blood Mini Kit (DBM), DSP Virus Kit (DSP) and Circulating Nucleic Acid (CNA) Kit are available. Presently it is very difficult to find out the best available kit for isolation of cffDNA from plasma in an accurate manner[24].

Investigation of Gender Independent Sequences in cffDNA

Quantitative changes of cell-free fetal cffDNA in maternal plasma as an indicator for impending preeclampsia have been reported in different studies, using real-time quantitative PCR for the male-specific SRY or DYS 14 loci[25]. Recent efforts have been directed at developing gender-independent sequences from cffDNA as markers for preeclampsia Several studies showed the utility of cffDNA using the methylation dependent DSCR3 and RASSF1A markers along with total cell-free DNA (cf-DNA) in maternal serum by HYP2 markers either alone or in combination with biochemical marker like PAPP-A[26] are potentially effective in early prediction of preeclampsia. The higher concentration of cffDNA, cfDNA along with soluble endoglin in maternal serum are found in preeclampsia and particularly cffDNA and cfDNA are twofold higher in severe preeclampsia group than the mild group[27]. Variation of the standardized protocol is a major issue for meta– analysis reports to opine about the efficacy of cffDNA[28]. However, no such studies have been performed in the Indian context and it is necessary to investigate and evaluate the gender-independent sequence markers from cffDNA as predictive markers for preeclampsia in pregnant women in India.

Statistical analysis

Results were expressed as mean ±SE (standard error). All statistical analysis was performed by one-way analysis of variance (ANOVA) with bivariate correlation tests and Student's '*t*' test using the Statistical Package for Social Sciences, version 25 (SPSS, Chicago, Illinois). A '*P*' value of <0.05 was considered significant.

Result

We have found in our study that the concentration of cffDNA increases with age, gravida, BMI (Table 1). But there is a typical variation in corresponding with the gestational age. Though with the increase in gestational age, cffDNA concentrations increase but at term (after 37 weeks) it again decreases in the maternal serum. The rise of blood pressure increases cffDNA concentration in the maternal serum enormously particularly when blood pressure is more than 140/90 mm Hg. The concentration of cffDNA

Table 1 — Comparison of variation of cffDNA concentration[#] in different demographic, and baseline parameters (n=100)

Parameters	Number of Patients	Concentration of cffDNA (GE/ µL)*
Age (yrs)		
18-23	35	7.74 ± 0.21
>23-28	42	7.92 ± 0.92
>28-33	15	9.44 ± 0.32
>33	8	14.56 ± 1.12 (< 0.001)
Gravida		
Primi (1st)	22	5.34 ± 0.34
Second (2nd)	59	8.66± 0.42
Third (3rd)	16	9.42 ± 0.48
>3rd	3	12.76 ± 1.24 (0.342)
Gestational Age		
20 week (at entry)	100	7.82± 0.28
<37 week (preterm)	12	132 ± 3.28 (<0.001)
>37 week (term)	88	14.32 ± 1.91
Systolic blood pressure (mmHg)		
<120	67	6.53 ± 0.46
120-130	19	8.40± 0.93
>130-140	10	24.68 ± 3.11
>140	4	546.78 ± 72.68 (<0.001)
Diastolic blood pressure (mmHg)		
<80	72	7.43± 0.36
80-85	16	8.20± 0.56
>85-90	9	26.43± 4.16
>90	3	692 ± 84.72 (<0.001)
Community		
Tribal (Mandi,Tudu, Murmu)	32	116.76 ± 2.25 (<0.001)
Others	68	7.34± 0.62
Body Mass Index (BMI)		
18.5–24.9	38	7.82± 0.48
25.0–29.9	42	7.96± 0.63
30.0–34.9	17	32.76 ± 3.26
35.0–39.9	3	287.64 ± 36.52 (<0.001)
Birth Weight (gm)		
>2500	82	5.98± 0.53
2500 – 1500	16	40.66 ± 2.41
<1500	2	482.44 ± 39.72 (<0.001)

*All statistical analysis was performed by one-way analysis of variance (ANOVA), *P* values <0.05 were considered as statistically significant

#cffDNA extracted by guanidiumisothiocynate-based DNA extraction method where maternal plasma with alanine aminotransferase (ALT/GPT) reagent (6 M guanidine thiocyanate dissolved in 50 mM Tris and mixed with an equal volume Phenol buffered in Tris)

depends on the birth weight of the baby also, as it increases when the birth weight of the baby is less than 2500 g and when it is <1500 g there is an abrupt rise of cffDNA concentration. In our study we have observed in longitudinal follow up that out of 100 mothers, 12 mothers delivered by preterm labour, 4 women developed preeclampsia and 18 women delivered low birth weight baby, out of whom two were severe low birth weight (<1500 g).

A comparison of different methods of isolation of cffDNA was done by analysis. Descriptive analysis of cffDNA (Table 2) has revealed that the interquartile range of cffDNA in guanidine isothiocyanate was 7.665 - 52.2 (GE/ µL) (95% CI for the median = 7.74 - 44.07) and in commercial kit (QIAamp MinElute cffDNA mini kit (Qiagen), the interquartile range was 8.32 - 49.56 (95% CI for the median = 8.12 – 56.76) and both are comparable (Table 2).

Table 2 — Comparison between different methods of cffDNA isolation

Variables	Salting out	Guanidine Isothiocyanate	Phenol/Chloroform	QIA ampmin Elute ccfDNA mini kit (Qiagen)
Sample size	10	70	10	10
Lowest value	3.66	2.18	2.7600	1.19
Highest value	156.12	619.36	262.46	676.46
Median	12.21	29.98	17.42	32.34
95% CI for the median	2.0907 - 6.1124	7.74- 44.07	5.56 - 8.94	8.12 - 56.76
Interquartile range	2.5 - 17.5	7.665 - 52.2	3.56 - 16.82	8.32 - 49.56

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[29]. It has also been observed that elevated total cell-free DNA and cffDNA were also significantly higher among women with preterm labor and adverse fetal outcome groups compared with the term and favorable outcome groups[30]. These three markers were almost equivalent about the area under the curve for predicting adverse fetal outcome in the severe PE group. No significant difference in levels of cffDNA was observed in the first trimester in women who subsequently develop preeclampsia[31]. One study suggested that pre-diabetes is associated with the feature of metabolic syndrome including BMI[32]. Our study showed that levels of cell-free total DNA being increased in tribal population compared with white women with increasing BMI[33]. Interestingly, total cell-free DNA in pregnant women is dependent on ethnicity. In a study 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 with white women (2.33; 0.03-13.10). This result shows that cell-free DNA in maternal plasma may depend on ethnic background[34]. No systematic study has been carried out so far and requires further studies. In a study with 8 women with preeclampsia and 8 normotensive control with singlet on 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[35,] along with alteration of other novel markers (*e.g.* hepcidin, serum iron) as suggested in other study[36].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 related to maternal weight and uterine artery pulsatility index and maternal cfDNA increased with maternal weight. It cannot be ruled out that whether the cffDNA increased in the advanced stage of gestation[37].

There are several studies to show that cffDNA is increased in IUGR. An increase in cffDNA was observed for the cases with a growth-restricted fetusin comparison with the controls[38]. Quantification of cffDNA using SRY sequences in 64 male-bearing pregnant women with IUGR and 89 controls revealed a significant increase of cffDNA in women with IUGR compared with control[39]. Significantly higher cffDNA was detected in fetal growth restriction groups than in normal pregnancy[40].

Women having elevated cffDNA in mid-trimester are at risk of spontaneous preterm delivery[41]. Women with episodic preterm labor and higher concentration of cffDNA are at increased risk for preterm delivery[42].

Conclusion

cffDNA concentration is variable in the maternal plasma in relation to the number of pregnancy, age of the mother, period of gestational age, ethnicity particularly in tribal population and BMI. Indigenously developed low-cost method for isolation and quantification of cffDNA to predict various adverse pregnancy outcomes is equally effective as commercially available kits. Qualitative analysis by the epigenetic approach to identify individual genetic components for particular condition is the future way.

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Conflict of Interest

All authors declare no conflict of interest.

References

1 Nahum O, Thomas A & Levy B, DNA Extraction from Various Types of Prenatal Specimens. *Methods Mol Biol*, 1885 (2019) 105.

2 Jain M, Balatsky AV, Revina DB & Samokhodskaya LM, Direct comparison of QIAamp DSP Virus Kit and QIAamp Circulating Nucleic Acid Kit regarding cell-free fetal DNA isolation from maternal peripheral blood. *Mol Cell Probes*, 43 (2019) 13.

3 Akbariqomi M, Heidari R, Gargari SS, Omrani MD, Rigi G, Sanikhani NS, Kooshki H, Mahmoudian F, Mazlomi MA & Tavoosidana G, Evaluation and statistical optimization of a method for methylated cell-free fetal DNA extraction from maternal plasma. *J Assist Reprod Genet*, 36 (2019)1029.

4 Bayón JC, Orruño E, Portillo MI & Asua J, The consequences of implementing non-invasive prenatal testing with cell-free foetal DNA for the detection of Down syndrome in the Spanish National Health Service: a cost-effectiveness analysis. *Cost Eff Resour Alloc*, 17 (2019) 6.

5 Lim JH, Lee BY, Kim JW, Han YJ, Chung JH, Kim MH, Kwak DW, Park SY, Choi HB & Ryu HM, Evaluation of extraction methods for methylated cell-free fetal DNA from maternal plasma. *J Assist Reprod Genet*, 35 (2018) 637.

6 Leontiou CA, Hadjidaniel MD, Mina P, Antoniou P, Ioannides M & Patsalis PC, Bisulfite Conversion of DNA: Performance Comparison of Different Kits and Methylation Quantitation of Epigenetic Biomarkers that Have the Potential to Be Used in Non-Invasive Prenatal Testing. *PLoS One*, 10 (2015) e0135058.

7 White HE, Dent CL, Hall VJ, Crolla JA & Chitty LS, Evaluation of a novel assay for detection of the fetal marker RASSF1A: facilitating improved diagnostic reliability of noninvasive prenatal diagnosis. *PLoS One*, 7 (2012) e45073.

8 Rodríguez-Martínez A., Sarasola-Díez E, Achalandabaso E & García-Barcina MJ, Optimized short digestion protocol for free fetal DNA detection using methylation-dependent markers. *J Lab Med*, 41 (2017) 195.

9 Tounta G, Kolialexi A, Papantoniou N, Tsangaris GT, Kanavakis E & Mavrou A, Non-invasive prenatal diagnosis using cell-free fetal nucleic acids in maternal plasma: Progress overview beyond predictive and personalized diagnosis. *EPMA J*, 2 (2011) 163.

10 Bilano VL, Ota E, Ganchimeg T, Mori R & Souza JP, Risk factors of pre-eclampsia/eclampsia and its adverse outcomes in low- and middle-income countries: a WHO secondary analysis. *PLoS One*, 9 (2014) e91198.

11 Saha MM, Mukherjee D, Ghosh U & Das SK, Application of cell-free fetal DNA for early evaluation of preeclampsia to reduce maternal mortality by low-cost method – A prospective cohort study. *Indian J Biochem Biophys*, 55 (2018) 334.

12 Prefumo F, Paolini D, Speranza G, Palmisano M, Dionisi M & Camurri L, The contingent use of cell-free fetal DNA for prenatal screening of trisomies 21, 18, 13 in pregnant women within a national health service: A budget impact analysis. *PLoS One*, 14 (2019) e0218166.

13 Yu H, Shen Y, Ge Q, He Y, Qiao D, Ren M & Zhang J, Quantification of maternal serum cell-free fetal DNA in early-onset preeclampsia. *Int J Mol Sci*, 14 (2013) 7571.

14 Angelo F, Santillo A, Sevi A & Albenzio M, Technical note: A simple salting-out method for DNA extraction from milk somatic cells: investigation into the goat CSN1S1 gene. *J Dairy Sci*, 90 (2007) 3550.

15 Tan SC & Yiap BC, DNA, RNA, and protein extraction: the past and the present. *J Biomed Biotech*, (2009) Article ID 574398.

16 Keshavarz Z, Moezzi L, Ranjbaran R, Aboualizadeh F, Behzad-Behbahani A, Abdullahi M & Sharifzadeh S, Evaluation of a modified DNA extraction method for isolation of cell-free fetal DNA from maternal serum. *Avicenna J Med Biotechnol*, 7 (2015) 85.

17 Beránek M, Vlčková J, Hypiusová V, Živný P & Palička V, Comparison of various methods used for extraction of cell-free genomic DNA from human plasma. *Clin Biochem Metab*, 14 (2006) 21.

18 Jacob RR, Saxena R & Verma IC, Noninvasive Diagnosis of Fetal Gender: Utility of Combining DYS14 and SRY. *Genet Test Mol Biomarkers*, 19 (2015) 505.

19 Jin S, Lin XM, Law H, Kwek KY, Yeo GS & Ding C, Further improvement in quantifying male fetal DNA in maternal plasma. *Clin Chem*, 58 (2012) 465.

20 Tsui DW, Chiu RW & Lo YD, Epigenetic approaches for the detection of fetal DNA in maternal plasma. *Chimerism*, 1 (2010) 30.

21 Saraswathy S, Sahai K, Arora D, Krishnan M, Mendiratta SL, Biswas S & Abraham KM, Fetal-specific hypermethylated RASSF1A quantification in pregnancy. *J Matern Fetal Neonatal Med*, 30 (2017) 849.

22 Chen X, Xiong L, Zeng T, Xiao K, Huang Y, Guo H & Ren J, Hypermethylated ERG as a cell-free fetal DNA biomarker for non-invasive prenatal testing of Down syndrome. *Clin Chim Acta*, 444 (2015) 289.

23 Lee E, Kim SY, Lim JH, Park SY & Ryu HM, Non-invasive prenatal testing of trisomy 18 by an epigenetic marker in first trimester maternal plasma. *PLoS One*, 8 (2013) e78136.

24 Larrabee PB, Johnson KL, Pestova E, Lucas M, Wilber K, Le Shane E, Tantravahi U, Cowan JM & Bianchi DW, Microarray analysis of cell-free fetal DNA in amniotic fluid: a prenatal molecular karyotype. *Am J Hum Genet*, 75 (2004) 485.

25 Hahn S, Rusterholz C, Hösli I & Lapaire O, Cell-free nucleic acids as potential markers for preeclampsia. *Placenta*, 32 (2011) Suppl. S17.

26 Kim SY, Kim HJ, Park SY, Han YJ, Choi JS & Ryu HM, Early prediction of hypertensive disorders of pregnancy using cell-free fetal DNA, cell-free total DNA, and biochemical markers. *Fetal Diagn Ther*, 40 (2016) 255.

27 Abdel-Halim RM, Ramadan DI, Zeyada R, Nasr AS & Mandour IA, Circulating maternal total cell-free DNA, cell-free fetal DNA and soluble endoglin levels in preeclampsia: Predictors of Adverse Fetal Outcome? A Cohort Study. *Mol Diagn Ther*, 20 (2016) 135.

28 Konečná B, Vlková B & Celec P, Role of Fetal DNA in Preeclampsia (Review). *Int J Mol Med*, 35 (2014) 299.

29 Nabiel Y, Mosbah A, Maternal Serum sEndoglin and Cell-Free Fetal DNA as Probable Markers of Preeclampsia: A Study in Single Center, Egypt. *Immunol Invest*, 48 (2019) 608.

30 Seval MM, Karabulut HG, Tükün A & Koç A, Cell-free fetal DNA in the plasma of pregnant women with preeclampsia, *Clin Exp Obstet Gynecol*, 42 (2015) 787.

31 Silver RM, Myatt L, Hauth JC, Leveno KJ, Peaceman AM, Ramin SM, Samuels P, Saade G, Sorokin Y, Clifton RG & Reddy UM, Cell-free total and fetal DNA in first trimester

maternal serum and subsequent development of preeclampsia. *Am J Perinatol*, 34 (2017) 191.

32 Agarwal A, Hegde A, Yadav C, Ahmad A, Manjrekar PA & Rukmini MS, Interleukin-6 in impaired fasting glucose. *Indian J Biochem Biophys*, 55 (2018) 424.

33 Kruckow S, Schelde P, Hatt L, Ravn K, Petersen OB, Uldbjerg N, Vogel I & Singh R, Does maternal body mass index affect the quantity of circulating fetal cells available to use for cell-based noninvasive prenatal test in high-risk pregnancies? *Fetal Diagn Ther*, 45 (2019) 353.

34 Barrett AN, Xiong L, Tan TZ, Advani HV, Hua R, Laureano-Asibal C, Soong R, Biswas A, Nagarajan N & Choolani M, Measurement of fetal fraction in cell-free DNA from maternal plasma using a panel of insertion/deletion polymorphisms. *PLoS One*, 12 (2017) e0186771.

35 Martin A, Krishna I, Badell M & Samuel A, Can the quantity of cell-free fetal DNA predict preeclampsia: a systematic review, *Prenat Diagn*, 34 (2014) 685.

36 Kumar T, Zahra K, Dey T, Singh A, Pandey U & Mishra SP, Correlative study of serum hepcidin levels and serum iron reserve parameters in preeclampsia and HELLP syndrome. *Indian J Biochem Biophys*, 55 (2018) 420.

37 Poon LC, Musci T, Song K, Syngelaki A & Nicolaides KH, Maternal plasma cell-free fetal and maternal DNA at 11-13 weeks' gestation: relation to fetal and maternal characteristics and pregnancy outcomes. *Fetal Diagn Ther*, 33 (2013) 215.

38 Sifakis S, Koukou Z & Spandidos DA, Cell-free fetal DNA and pregnancy-related complications (review). *Mol Med Rep*, 11 (2015) 2367.

39 Smid M, Galbiati S, Lojacono A, Valsecchi L, Platto C, Cavoretto P, Calza S, Ferrari A, Ferrari M, Cremonesi L, Correlation of fetal DNA levels in maternal plasma with Doppler status in pathological pregnancies, *Prenat Diagn*, 26 (2006) 785.

40 Yin A, Ng EHY, Zhang X, He Y, Wu J & Leung KY, Correlation of maternal plasma total cell-free DNA and fetal DNA levels with short term outcome of first-trimester vaginal bleeding, *Hum Reprod*, 22 (2007) 1736.

41 Van Boeckel SR, Davidson DJ, Norman JE & Stock SJ, Cell-free fetal DNA and spontaneous preterm birth. *Reprod*, 155 (2018) R137.

42 Dugoff L, Barberio A, Whittaker PG, Schwartz N, Sehdev H & Bastek JA, Cell-free DNA fetal fraction and preterm birth. *Am J Obstet Gynecol*, 215 (2016) 231.e1.