IJN
Iranian Journal of Neonatology
http://ijn.mums.ac.ir

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Original Article

# Correlation of Maternal Age, Weight, Pregnancy-associated Plasma Protein A, Free Beta-human Chorionic Gonadotropin, Fetal Crown-rump Length, and Fetal Gender with Fetal DNA Fraction in Non-invasive Prenatal Testing: An Experiment on Iranian Pregnant Women

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## ABSTRACT

***Background:*** Non-invasive prenatal testing (NIPT) as a novel screening method has been widely proposed to screen for common fetal chromosomal aneuploidies. The aim of the present study was to examine the possible effects of maternal age, maternal weight, fetal crown-rump length (CRL), serum pregnancy-associated plasma protein A (PAPP-A), free beta-human chorionic gonadotropin (free β-hCG), and fetal gender on cell-free fetal DNA (cffDNA) percentage fluctuations.

***Methods:*** In the present cross-sectional study, 308 singleton pregnant women aged 20-47 years at $11^{+0}$ to $13^{+6}$ weeks of pregnancy referring to the DeNA Laboratory in Tehran, Iran, for NIPT test during a one-month period between July 2018 and August 2018 were selected randomly. The cffDNA was extracted from maternal plasma. Whole exome sequencing by a ion semiconductor sequencer using cffDNA was applied for all participants. The PAPP-A and free β-hCGas biochemical biomarkers were assessed using a closed chemilumine scence immunoassay analyzer. The Shapiro-Wilk test, Pearson analysis, beta regression analysis, and Mann-Whitney U test were performed to analyze the data.

***Results:*** In the screening population, the cffDNA percentage showed no significant correlation with CRL and maternal age (P=0.096 and P=0.881, respectively). However, the cffDNA percentage correlated well with maternal weight, PAPP-A, and free β-hCG (P=0, P=0.009, and P=0.001, respectively). Beta regression between cffDNA percentage and maternal weight, free β-hCG, and PAPP-A was significant (P<0.001). The mean cffDNA percentage between male and female fetal groups showed a significant difference (P<0.001).

***Conclusion:*** This study demonstrated that the cffDNA percentage in the first trimester of pregnancy had a negative correlation with maternal weight and a positive correlation with PAPP-A and free β-hCG values. Furthermore, the cffDNA percentage in male fetuses was higher than that in female fetuses.

***Keywords:*** Cell-free nucleic acids, Chorionic gonadotropin, Pregnancy-associated plasma protein-A, Prenatal diagnosis

## Introduction

The prevalence of chromosomal abnormalities is about 0.6% (i.e., 6 cases per 1,000) in general population (1). These abnormalities are the most important causes of birth defects. There is still no cure for these congenital defects. Although invasive tests, such as karyotype, can diagnose some of these abnormalities accurately, the implementation of karyotype as the first-line

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*Please cite this paper as:*
Dolatkhah M, Rahnamaye Farzami M, Khavari-Nejad RA, Noori S. Correlation of Maternal Age, Weight, Pregnancy-associated Plasma Protein A, Free Beta-human Chorionic Gonadotropin, Fetal Crown-rump Length, and Fetal Gender with Fetal DNA Fraction in Non-invasive Prenatal Testing: An Experiment on Iranian Pregnant Women. Iranian Journal of Neonatology. 2021 Jan: 12(1). DOI: 10.22038/ijn.2020.45840.1765

screening test has been hampered by its high cost, as well as the likelihood of miscarriage (2).

The use of the serum values of pregnancy-associated plasma protein A (PAPP-A) and free beta-human chorionic gonadotropin (β-hCG), as well as nuchal translucency (NT) thickness, during 10-13 weeks of gestation as a combined test has been proven to be the first-line screening method for trisomies 21 (T21), trisomies 18 (T18), and trisomies 13 (T13). The detection rate and false-positive rate of this test are around 85% and 5%, respectively. In T21, T18, and T13 pregnancies, PAPP-A levels are decreased, but the levels of free β-hCG are increased in T21 and decreased in T18 and T13 cases (3).

Among the non-invasive tests for the detection of chromosomal aneuploidies (CA), the use of first trimester screening by serum PAPP-A, serum free β-hCG, and NT thickness has been dramatically developed. However, the use of an available database and efficient system with high performance has led to the utilization of non-invasive prenatal testing (NIPT) as a novel screening method for the evaluation of aneuploidies, like autosomal T21, T18, and T13, as well as some sex chromosomal aneuploidies, such as Turner's syndrome (45, XO), Klinefelter syndrome (47, XXY), supermale disease (47, XYY), and superfemale disease (47, XXX) (4).

A recent study has shown that except the significant difference of specificity (SP) for detecting T13, in which Illumina platform (SP=99.97%) showed better performance when compared to ion proton platform (SP=99.91%), there were no significant differences among sensitivity (ST) testing, SP, or positive predictive values (PPV) in screening T21, T18, and T13 (ST>98%, and SP>99.9% for all CA and PPV>82% for T21, 60.7% for T18, and 18% for T13) (5). The NIPT evaluates the circulating cell-free fetal DNA (cffDNA) which originates from the apoptotic maternal and placental cytotrophoblast cells (6). It has been shown that cffDNA can be early extracted in the maternal bloodstream from the first trimester of pregnancy, and the amount of cffDNA is sufficient from the $9^{th}$ week of pregnancy for clinical diagnostic applications. Hence, it can be sampled in a non-invasive manner and used for studying fetal diseases. Finally, the cffDNA fragments cannot be found in the maternal circulation a few hours after birth because of probable elimination by renal excretion (7).

The ratio of cffDNA concentration to the total concentration of DNA (maternal and fetal) is quantitatively called fetal fraction (FF) (8). The range of FF can vary between < 4% and > 30%. Low FF ratio is responsible for 0.5-3% of no calls. As cell-free DNA (cfDNA) mainly consists of the genomic DNA fragments of maternal and fetal (placental) origins, various variables might affect FF (9). The average percentage of FF was previously reported to be around 10-15% within 10-20 weeks of pregnancy (10, 11). The NIPT result is failed if FF percentage is below 4% because the accuracy of chromosomal analysis is dramatically affected by its own level.

When FF percentage is 10%, the numeric ratio (R) of chromosome 21 fragments in the tested sample and reference disomic fragments increases from 1 to about 1.02 in fetal T21. However, if the FF percentage is less than the threshold value (i.e., 4%), R is less than 1.02 and cannot be statistically distinguished from number 1 consequently. It is also worth noting that some NIPT tests use the FF value in the algorithm to evaluate the trisomy risk, while the other NIPT tests use some predetermined normalization factors that can still achieve high reliability (12-14).

Accurate estimation of gestational age is very crucial for obstetric management. Fetal crown-rump length (CRL) as a more accurate factor in comparison to last menstrual period (LMP) is being used more frequently for the estimation of gestational age (15). It has already been shown that FF ratio is affected by many factors, such as CRL (16, 17), multiple gestation (18), fetal aneuploidy (19-23), serum metabolite levels (24), maternal weight, body mass index (maternal BMI), biochemical biomarkers (e.g., PAPP-A and free β-hCG) (11, 16, 24-26), maternal medical conditions (24, 27, 28), medication use by mothers (29, 30), and placental size and function (31, 32).

According to previous studies about the importance of factors in FF ratio, there is no study to appraise the influential factors in FF ratio in the Iranian population. The aim of the present study was to assess the relationship of maternal age, maternal weight, CRL, PAPP-A, free β-hCG, and fetal gender with the percentage of cffDNA in NIPT test.

## Methods

In the present cross-sectional study, 308 singleton pregnant women aged from 20 to 47 years referring to the DeNA Laboratory in Tehran, Iran, for undergoing NIPT test within $11^{+0}$ to $13^{+6}$ weeks of pregnancy between July 2018 and August 2018 were selected randomly. According to the Clinical and Laboratory Standard Institute

I/LA25-A2, for each analyte, at least 100 pregnant women must be selected. A larger number is recommended whenever practical; therefore, for 3 weeks of pregnancy, 308 women were selected. Cigarette smoker mothers and those who were suffered from insulin-dependent diabetes mellitus or pre-existing maternal illnesses, as well as those with a history of blood transfusion in recent years, bone marrow transplantation, or pregnancy with donated ovum, were excluded. This study was approved by the Ethics Committee of the Islamic Azad University, Science and Research Branch (IR.IAU.SRB.REC.1397.163).

Written informed consent was obtained from all participants, and they were assured that none of the information would be revealed unless for research purposes and in an anonymous form. The participants were allowed to decline participation at any stage of the research. All data were recorded in questionnaires and then filed. Trained personnel undertook blood sampling and collected information on maternal age, maternal weight, CRL, NT thickness, questionnaire data, and ultrasonography parameters (clinical and demographic data). Gestational age was determined by the measurement of CRL.

Morning blood sampling was conducted owing to fasting requirements. About 10 ml of peripheral blood was drawn into a BCT tube (Streck, Omaha, NE, USA) and a clot activator blood collection tube (Improve Medical Corp., Guangzhou, Guangdong China). Plasma fractions were separated by double centrifugation, 15 min at a relative centrifugal field (RCF) of 1,600 and then 10 min at 16,000 RCF. Serum fractions were separated by 2,000 RCF centrifugation for 10 min immediately after collecting. Then, DNA was extracted from 600 µL plasma using the BGI nucleic acid (DNA) extraction kit (BGI, Shenzhen, Guangdong, China) within an hour after separation.

Library construction was performed by the BGI Library Construction Kit (BGI, Shenzhen, Guangdong, China). Quantitative detection and quantification were performed by the Qubit 3.0 fluorometer (Life Technologies, USA). Libraries from 13 samples were pooled in equimolar ratios to the actual concentration (pM) of DNA, following the ion proton protocol. Emulsion polymerase chain reaction (PCR) was performed by the ion one-touch2 instrument (Thermo Fisher, USA), and ion-sphere particle enrichment was accomplished by the ES machine (Thermo Fisher Scientific, Wilmington, DE, USA). Finally, cffDNA testing was performed using next-generation sequencing (NGS) ion semiconductor platform by means of a ion proton sequencer (Thermo Fisher Scientific, Wilmington, DE, USA). All NGS procedures were performed on the sampling day, and specimens were kept in a refrigerator till analyzing. All of the data were analyzed by the BGI company (China) to detect CA (T21, 18 and 13), FF, and fetal gender. The PAPP-A and free β-hCG of the sera were measured on the same day using a fully automated CLIA analyzer, MAGLUMI 4000 (Snibe Company, Shenzhen, Guangdong, China).

### *Statistical analysis*

The SPSS software (version 22, IBM Corp., Armonk, NY, USA) and Microsoft Excel (version 2013, Microsoft Corp., Redmond, WA, USA) were used for statistical analysis. The data were expressed as mean±standard deviation (SD), and a p-value of $< 0.05$ was considered significant. The Shapiro-Wilk test was performed to investigate the normality of distribution. Pearson analysis was utilized to assess the bivariate correlation. Beta regression analysis was used to determine which of the factors among maternal age and maternal weight, CRL, PAPP-A, and free β-hCG were significant with cffDNA percentage. Mann-Whitney U test was also used to compare the mean cffDNA percentage between the male and female fetal groups.

## Results

The population was comprised of 308 Iranian pregnant women aged 20-47 years with $11^{+0}$ to $13^{+6}$ weeks of pregnancy referring to the DeNA Laboratory for NIPT test. Fetal population consisted of 157 male fetuses and 151 female fetuses. Maternal and fetal characteristics of all participants are depicted in Table 1.

**Table 1.** Maternal and fetal characteristics of the research population (n=308)

| Characteristic | Values |
|---|---|
| Maternal age (year) | 34±4.8 |
| Maternal weight (kg) | 67±11.1 |
| Fetal CRL (mm) | 58.6±8.76 |
| Fetal NT thickness (mm) | 1.6±0.47 |
| Male (% fetal fraction) | 10±4.22 |
| Female (% fetal fraction) | 7±2.5 |
| PAPP-A (MoM) | 0.97±0.71 |
| PAPP-A (mIU/L) | 3192±156 |
| Free β-hCG (MoM) | 1.03±0.8 |
| Free β-hCG (ng/ml) | 49.1±16.5 |

Data are presented as mean±standard deviation.
Free β-hCG: free beta-human chorionic gonadotropin, NIPT: non-invasive prenatal testing, CRL: crown-rump length, NT: nuchal translucency, PAPP-A: pregnancy-associated plasma protein-A, free β-hCG: free beta-human chorionic gonadotropin

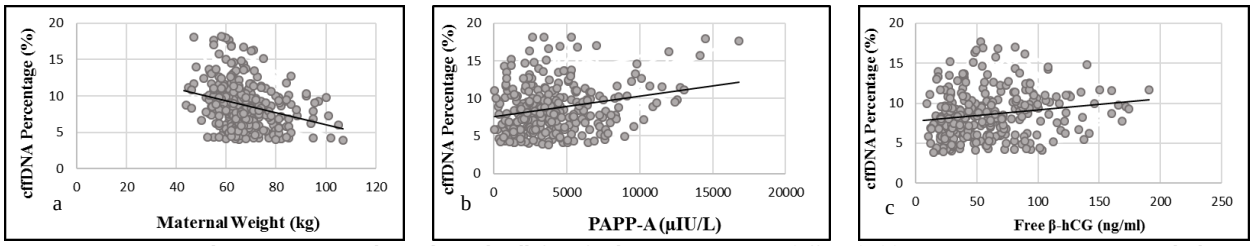


**Figure 1.** Association between maternal weight and cell-free fetal DNA percentage (cffDNA) (a), serum pregnancy-associated plasma protein A and cffDNA percentage (b), and serum free beta-human chorionic gonadotropin and cffDNA percentage (c)

**Table 2.** Beta regression

| Items | Coefficients | Standard error | Z-value | P-value |
|---|---|---|---|---|
| Intercept | -2.364 | 0.0224 | -105.5 | 2e-16*** |
| Maternal weight | -0.085 | 0.0235 | -3.612 | 0.0003*** |
| $Log_{10}$-free β-hCG | 0.043 | 0.0219 | 1.993 | 0.021* |
| $Log_{10}$-PAPP-A | 0.047 | 0.0230 | 2.043 | 0.02* |

*** P-value<0.001, * P-value<0.05

Free β-hCG: free beta-human chorionic gonadotropin, PAPP-A: pregnancy-associated plasma protein-A

The percentage of cffDNA was normally distributed in the study population (P=0.08). Maternal age and CRL did not have a significant correlation with cffDNA percentage (P=0.881 and P=0.096, respectively). On the other hand, maternal weight (Pearson's r=-0.9, P<0.001), serum PAPP-A (Pearson's r=0.16, P=0.009), and free β-hCG (Pearson's r=0.15, P=0.001) had a significant correlation with cffDNA percentage (Figure 1).

Maternal weight, serum free β-hCG, and PAPP-A values had significant beta regression with cffDNA percentage ($R^2$=0.1; Table 2). The Mann-Whitney analysis showed that the distribution of cffDNA percentage was not the same in the fetal genders (P<0.001).

In conclusion, the beta regression model obtained through this study was introduced as follows:

$$cffDNA = -2.364 - 0.085\,(Maternal\ Weight) + 0.043\,(Log10 - free\ \beta - hCG) + 0.047\,(Log10 - PAPP - A)$$

## Discussion

In our screening population, maternal age and CRL showed no significant correlation with cffDNA percentage. However, maternal weight, PAPP-A, and free β-hCG showed a significant correlation with cffDNA percentage. Furthermore, maternal weight, PAPP-A, and free β-hCG values showed significant betaregression with cffDNA percentage. The percentage of cffDNA in male fetuses was significantly higher than that in female fetuses.

The present study outlined the possible factors influencing the cffDNA percentage. Recently, a significant shift has taken place from the second to the first trimester of screening (33). In high-risk pregnancies, it is probable that NIPT screening is applied at 11-13 weeks of gestation. Furthermore, fetal screening at 11-13 weeks of pregnancy is not only vital for the screening of CA but also significantly considerable for many pregnancy complications, such as preterm birth, pre-eclampsia, and fetal growth restriction (34). At 11-13 weeks of pregnancy, the mean cffDNA percentage in maternal plasma was 8.86%, but this percentage was different between male and female fetuses.

In addition, cffDNA percentage was decreased with increasing maternal weight and increased with elevating PAPP-A. Free β-hCGand did not correlate well with maternal age in our screening population. A considerable contribution to cffDNA percentage was provided by maternal weight as its coefficient was significant and negative (r=-0.085). The negative correlation of fetal fraction with maternal weight showed that cffDNA was decreased by increasing maternal weight. This finding is consistent with the results of previous NIPT studies regarding high-risk pregnancies (18, 25, 34) and has also been reported for other fetoplacental products in maternal circulation (35-37). Besides, there is an accelerated turnover of adipocytes in obese women, which can release some cffDNAs of maternal origin into the circulation, resulting in a lower proportion of fetal cffDNA percentage in obese women in comparison with that in other pregnant women with a normal weight (26).

The PAPP-A and free β-hCG as fetoplacental circulating products at 11-13 weeks of gestation had a significant and positive relationship with

cffDNA percentage as their coefficients were 0.047 and 0.043, respectively. The results showed that cffDNA percentage was risen by increasing the measurements of PAPP-A and free β-hCG. The PAPP-A and free β-hCG are produced by the placenta, and the serum concentration of these metabolisms probably provides an indirect measure of placental mass. In a study, placental volume was reported to be correlated with maternal serum PAPP-A and free β-hCG levels (38). This is the most likely explanation for correlation and beta regression between cffDNA percentage and serum level of fetoplacental products since the source of cffDNA in maternal plasma is the placenta. However, these findings seemed apparently contradictory to the results of previous studies which reported that the cffDNA percentage of maternal serum was higher in pregnancies with impairment than in those with normal placentation.

The results showed a positive beta regression between cffDNA and PAPP-A, as well as between cffDNA and free β-hCG, which is consistent with the results of studies addressing high-risk pregnancies. Since these biochemical biomarkers are produced by the placenta, an indirect measurement of placental mass is caused by maternal serum concentration. Death of cells in the placenta is a potential source of cffDNA in maternal plasma, as a result of which the number of apoptotic cells would be proportional to the placental mass (24).

Unlike a study addressing high-risk pregnancies at 8-22 (average 15) weeks of gestation (18), we did not find a significant correlation between cffDNA percentage and CRL. On the other hand, there are studies reporting an even small contribution to cffDNA percentage from CRL (18). The detection of no correlation between cffDNA percentage and CRL seems to be a contradictory result, but it is affected by multiple factors, such as maternal age, lack of a constant increase in FF across gestational age (9), accelerated turnover of adipocytes in high-weight pregnant women by raising CRL, gestational circumstances, ethnic and biochemical biomarkers, fetal gender, and pre-analytical and analytical errors in the measurement of variables, like ultrasound examinations. These factors can have effects on the consequences; therefore, it seems that here is a need for more investigations to evaluate other factors. Since the values of maternal height were not available to calculate the BMI, it would be far preferable to evaluate this subject with BMI and more data in the future.

Furthermore, the current study showed that cffDNA percentage was significantly higher in male fetuses than in female fetuses. Differences in cffDNA percentage between male and female fetuses might be mediated by placental functions like fetal-placental ratio. Previous studies showed that male fetuses had a higher fetal-placental ratio than female fetuses in normal pregnancies (39). Since the placental growth and ratio are higher in male fetuses, it must be reasonable that cffDNA percentage is higher in male fetuses than in female fetuses. Regarding the findings, it seems that knowledge gaps still exist regarding what affects the production, metabolism, and clearance of fetoplacental DNA. Accordingly, it is required to perform more investigations with more data and variables.

The present study is the first survey in the Iranian population in which the possible factors influencing cffDNA percentage in NIPT test have been evaluated. R-squared obtained for beta regression showed that there might be other factors affecting cffDNA. The salient limitations of our study are the expenses of NIPT tests and shortage of the immunoassay reagents due to commercial sanctions, as well as the lack of further information about the history of fetal death, chronic diseases, preterm birth, and maternal-fetal blood incompatibility of our screening population.

## Conclusion

This study demonstrated that cffDNA percentage in the first trimester of pregnancy had a negative correlation with maternal weight and positive correlation with PAPP-A and free β-hCG values. Furthermore, the cffDNA percentage of male fetuses was higher than that in female fetuses. Nevertheless, it is highly recommended to assess other considerable variables like BMI, placental volume, and ethnic factors in the future studies.

***Abbreviations***

NIPT: Non-Invasive Prenatal Testing
CRL: Crown-Rump Length
PAPP-A: pregnancy-associated plasma protein-A
Free β-hCG: free beta-human chorionic gonadotropin
cffDNA: cell-free fetal DNA
CLIA: chemilumine scence immunoassay
NT: Nuchal Translucency
T21: trisomies 21
T18: trisomies 18
T13: trisomies 13

FPR: false positive rate
SP: specificity
ST: sensitivity
PPV: positive predictive values
CA: chromosomal aneuploidies
FF: fetal fraction
LMP: last menstrual period
GA: gestational age
BMI: body mass index
ISP: ion-sphere particle
NGS: next-generation sequencing
SD: standard deviation

## Acknowledgments

We would like to thank Dr. Alireza Abadi for performing statistical analysis and Mohammad Alinejad for his participation in the survey. We are also grateful to Dr. Mohammadreza Hekmat and members of DeNA Laboratory (Tehran, Iran) and Farvardin pathobiology Laboratory (Tehran, Iran) for providing us with all necessary facilities for the research.

## Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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