Journal of Iranian Medical Council, Volume 8, Issue no. 3
http://dx.doi.org/10.18502/jimc.v8i3.18793

Original Article

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Investigating the Relationship between Fetal DNA Fraction in Cell-Free DNA Test and Adverse Pregnancy Outcomes in Pregnant Women Referring to Perinatology Clinics

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Abstract

Background: Screening is done by examining the Fetal Fraction (FF) in cell-free DNA to find fetal aneuploidy. In this study, the relationship between FF obtained in weeks 10–20 of pregnancy and adverse pregnancy outcomes, as well as demographic variables of mothers were investigated.

Methods: This study included pregnant women (n=685) referred to perinatology clinics in West Azerbaijan from April 2018 to March 2021 who underwent cell-free DNA (cfDNA) screening in weeks 10 to 22 of pregnancy. The demographic variables of the mothers were extracted and recorded at the time of sample collection and the pregnancy outcomes at the time of delivery from their records.

Results: Two patients had cervical insufficiency, 10 participants had preterm Premature Rupture of Membranes (PPROM), and the frequency of NICU Hospitalization was 0.9% (6 person). The distribution estimates FF less than the 25th percentile as less than normal, with 197 (29.5%) individuals having FF less than 4.36. Body Mass Index (BMI) had an opposite relationship with FF (p-value=0.001 and B=0.296). Only the prevalence of Gestational Diabetes Mellitus (GDM) patients was different between the two groups (p=0.032). The area under the diagram was significant for the outcomes of preterm delivery (0.503), pregnancy-related high blood pressure (0.599), and GDM (0.609, according to the ROC diagram).

Conclusion: Based on the present study, FF assessment at 10–20 weeks of pregnancy can predict the possibility of gestational diabetes, pregnancy-induced blood pressure, and premature delivery during pregnancy to a weak to moderate extent.

Keywords: Diabetes, Female, Gestational, Pre-eclampsia, Pregnancy, Pregnancy outcome, Premature birth, Preterm premature rupture of the membranes

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Received: 26 Jul 2024
Accepted: 12 Nov 2024

Citation to this article
Vazifekhah Sh, Wahhabi A, Rouhi Gharehbagh M. Investigating the Relationship between Fetal DNA Fraction in Cell-Free DNA Test and Adverse Pregnancy Outcomes in Pregnant Women Referring to Perinatology Clinics. *J Iran Med Counc.* 2025;8(3):493-502.

Introduction

Prenatal screening for trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome), trisomy 13 (Patau syndrome), and some sex chromosomal aneuploidies can be done using the new generation of cell-free DNA sequencing methods in the maternal bloodstream screening (1). Cell-free DNA originates from the mother, the fetus, or both and is removed from the mother's blood shortly after birth. In cases where sampling is done correctly, screening with cell-free DNA (cfDNA) can detect at least 99% of trisomy 21 pregnancies (2,3). However, due to scattered false positives and negatives, individuals with positive results should be justified for invasive investigations, including amniocentesis or chorionic villus sampling (4). It should be kept in mind that the gold diagnostic standard of fetal aneuploidy disorders is microarray analysis or karyotyping of samples obtained through amniocentesis or chorionic villus sampling (5).

Despite the passage of over 70 years since the discovery of cfDNA, its origin and release mechanism remain largely unknown. However, in this case, various theories have emerged. The most prominent of these theories suggests that in healthy individuals, cfDNA enters the peripheral circulation as a result of the apoptosis of normal hematopoietic cells and other nucleated cells (6). This issue can justify the length of this genetic material, its short half-life, and its' very low levels in healthy people. cfDNA is mainly the result of pathological processes, such as the collapse of tumoral cells and autoimmune processes. However, in 1997, Lo *et al* discovered fetal DNA sequences in the serum and plasma of pregnant mothers and named them cell-free fetal DNA (cffDNA). Early on after its discovery, researchers released cffDNA from the fetus's placental tissue and used it as a non-invasive method for sex determination (6,7). Studies published in 2008 established the clear role of cffDNA in screening for common aneuploidy diseases, leading to the acceptance of cffDNA evaluation for trisomy 21 screening as a non-invasive, nearly safe, and high-accuracy method (8). Various tests, designed and introduced in the following years, evaluated cffDNA to screen for chromosomal disorders, sex-linked diseases, and monogenic diseases. The collection of these screenings is called Non-Invasive Prenatal Tests (NIPT) (8). At the bedside, the circulating fetal cfDNA concentration in the mother's peripheral circulation is known as the Fetal Fraction (FF) and is a fundamental and accurate variable for determining fetal trisomy (9). The minimum FF required to obtain a reliable NIPT response is approximately 4%. It should be noted that FF less than 4% can lead to false negative results due to the low concentration of cffDNA in maternal plasma (10). Low FF can have different reasons, such as low gestational age, improper sampling, maternal overweight, fetal karyotype, and other issues that can lead to low FF, including the use of low molecular weight heparin before the 20th week of pregnancy, pregnancy with in vitro fertilization, and multiple pregnancies (11-16). Other suggested uses for cffDNA include determining genetic disorders and fetal sex. Since cffDNA originates from the placenta, a low FF may indicate a lower placental volume or impaired placental function. Researchers have conducted several mostly small-scale studies to investigate the relationship between low FF and adverse pregnancy outcomes, such as gestational hypertension, pre-eclampsia, intrauterine growth restriction, preterm delivery, gestational diabetes, and invasive placentation (16-18). These studies, which are mostly scattered and old, report an increased risk of adverse pregnancy outcomes for pregnant women with low FF.

Determining the FF test's effectiveness and limitations in predicting adverse pregnancy outcomes can help make this method more comprehensive as a primary screening (19-23). To date, no study has examined the correlation between the FF in the cell-free DNA test and adverse pregnancy outcomes such as premature birth, baby birth weight, mother's pregnancy-related blood pressure, and gestational diabetes at the national level, particularly in the North West. Therefore, in this study, the correlation between FF and unfavorable pregnancy outcomes was examined among pregnant mothers referred to the Mendel Genetics Center in Urmia from April 2018 to March 2021.

Materials and Methods

This study was a retrospective cohort in which all pregnant women who underwent FF tests between April 2018 and March 2021 at the age of 10–20 weeks and were referred to the perinatology clinics of Shahid Motahari Hospital in Urmia were investigated.

Pregnant mothers with underlying diseases such as high blood pressure, autoimmune or rheumatological diseases, diseases related to liver and kidney dysfunction, rheumatological and metabolic diseases including overt diabetes, dyslipidemia, history of cancer, the history of organ transplant, recent blood receipt, chemotherapy, multiple pregnancies, positive cfDNA test results, no cell, and reports of disturbances in other pregnancy screenings were excluded from the study. All the mothers signed a consent form when they visited the clinic to allow the use of demographic and clinical data in current and future studies. Once the code of ethics was obtained, the data was extracted from the patients' medical records and inputted into the appropriate software for processing. After presenting the descriptive statistics, analytical statistics and investigating the relationship between FF, adverse pregnancy outcomes (premature birth, high or low birth weight, gestational diabetes, pregnancy-related hypertension, and pre-eclampsia) were evaluated.

Sample size

G*power software was used to determine the required sample size, primarily by comparing the frequency between groups. Based on this, from the menu of the family of tests, choose Chi-square, and from the statistical test menu, choose Goodness of fit tests; contingency Tables; and from the type of power analysis menu, select the option A priori: compute sample size given power, alpha, and effect size, and based on this, the values of 0.05 for alpha, 0.95 for power, and 0.3 for effect size are defined as the sample size of 685 people being considered sufficient to fulfill the objectives of this study.

Data collection

Since the data was extracted from the patients' medical records in this study, validity and reliability have no special status. However, expert and experienced forces performed screening tests and other investigations in a unique and reliable medical and laboratory center, adhering to national guidelines, using updated and calibrated devices, and ensuring the necessary documentation and observations for measurements. All the patients underwent laboratory tests using the same cell-free DNA method in an identical hospital laboratory ward. The researcher prepared a checklist for data collection.

Data analysis

To process the data, SPSS software version 23 for Windows was used. GraphPad Prism and Microsoft Excel software version 2016 for the same operating system were utilized to draw the Figures and Graphs. In the quantitative data, descriptive analysis was expressed as mean and standard deviation, whereas qualitative data was expressed as percentage and frequency. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to check the uniform distribution of the data. An independent samples t-test was used to compare quantitative data, and a chi-squared test was utilized to compare the qualitative and frequency data. The significance level was defined as a p-value <0.05. The descriptive statistics section presented the data according to international standards. The analytical statistics section divided the data into percentiles to define the upper and lower limits of the normal range in the studied population. Following this stage, the Receiver Operating Characteristic (ROC) curve independently identifies the lower and upper limits of the normal range of FF for each variable, and univariate regression analysis assesses the relationship between the lower and higher levels of the normal FF of the studied community and each of the adverse pregnancy outcomes. First, the effect of each variable on FF was investigated through univariate linear regression, then the variables with a $p>0.2$ were entered into the multivariate regression model to investigate the mutual effect.

Ethical considerations

During the visit, all the clients of the perinatology clinics at Shahid Motahari Hospital signed the consent form to allow the use of medical and demographic information for current and future studies. In addition, the data related to the patients was coded and only available to the research team. Carrying out the project required obtaining a code of ethics from the ethics committee of Urmia University of Medical Sciences (IR.UMSU.REC.1400.360). This plan was implemented while adhering to the guidelines of the Declaration of Helsinki.

Results

This descriptive and analytical study examined all pregnant women in Urmia City who underwent FF examinations from April 2018 to March 2021. The study included a total of 685 females. In the study population, the average age of the patients was 33.91 years. On average, the gestational age at the time of delivery was 38.11 weeks. Maternal pregnancy Body Mass Index (BMI) and maternal BMI before delivery were 23.63±1.2 and 27.21±1.4, respectively. In the present population, the frequency of preterm delivery, gestational diabetes, pre-eclampsia, and pregnancy-related blood pressure were 3.6, 1.5, 0.3, and 9%, respectively (Table 1). Other outcomes are presented in table 1.

From the point of view of the baby's weight, the frequency of babies with low birth weight (less than 2500 *gr*), normal (between 2500 and 4000 *gr*), and high birth weight (more than 4000 *gr*) is equal to 7.3% (49 people), 4.4 80% (536 people), and 12.3% (82 people). To investigate the relationship between the FF and each of the adverse outcomes of pregnancy, FF less than the percentile, between the 25-75th percentile, and more than the 75th percentile were defined as low, normal, and high FF, respectively (Table 2 and Figure 1). Figure 2 presents the distribution of FF in the study population.

The mother's BMI showed the only significant difference in the one-way ANOVA test, which aimed to compare the mother's BMI and the neonate's birth weight with FF. Linear logistic regression was used to determine this relationship, and it was found that a

Table 1. Frequency of the adverse pregnancy outcomes

Adverse pregnancy outcome	Number	Percentage
Premature birth	24	3.6%
Gestational diabetes	10	1.5%
Pre-eclampsia	2	0.3%
Pregnancy-related hypertension	60	9%
Cervical insufficiency	2	0.3%
Preterm premature rupture of membranes (PPROM)	10	1.5%
Neonatal intensive care unit (NICU) hospitalization	6	0.9%

Table 2. Division of the Fetal Fraction (FF) based on low, normal, and high percentiles

Fetal fraction status	Interval (%FF)	Number (percentage)
Down	0-24% (≤4.36)	197(29.5%)
Normal	25-75% (4.37-10.25)	293(43.9%)
High	76-100% (>10.26)	177(23.5%)

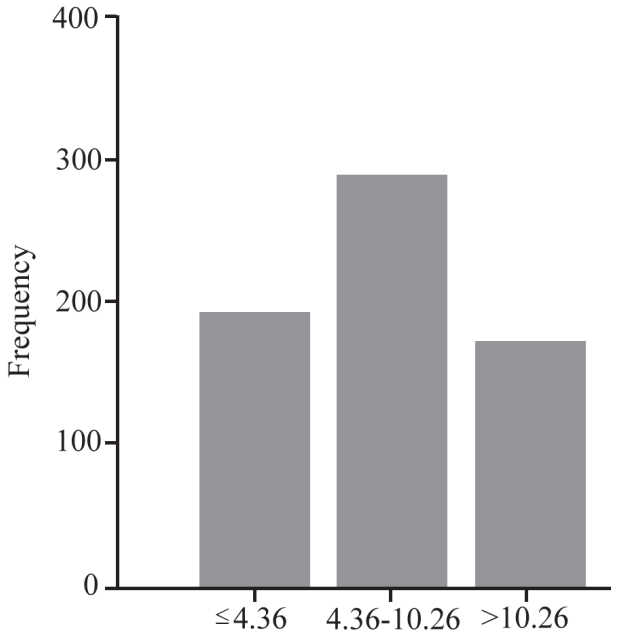


Figure 1. Distribution of different groups according to quartiles.

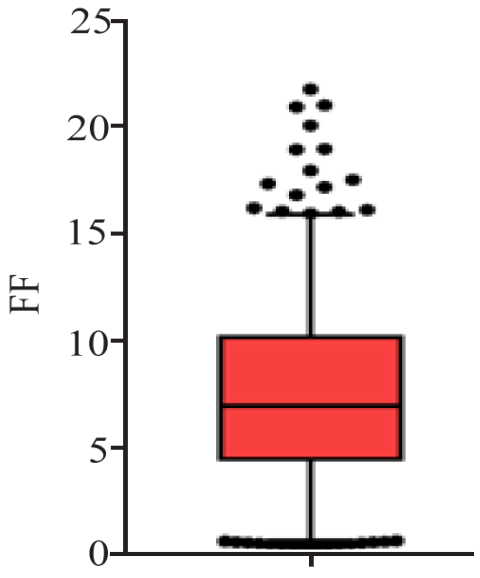


Figure 2. A box diagram of FF distribution in the studied community.

higher BMI is associated with a lower FF (p=0.026) (Table 3). Pearson's correlation coefficient test was used to determine the relationship between mother's age and FF, and no significant relationship was found between these two variables. The Fisher's exact test was performed to compare adverse pregnancy outcomes with FF. This study revealed that the only difference between the two groups was the frequency of Gestational Diabetes Mellitus (GDM) (p=0.032). Similarly, the difference in the prevalence of high blood pressure during pregnancy between the two groups is borderline significant (p=0.043).

To investigate the performance of FF in predicting adverse pregnancy outcomes, the ROC curve was drawn. Table 4 and tables 3-5 mention the area under the graph for each variable. According to the area under the graph, FF taken at 10–20 weeks of pregnancy can be used to diagnose pregnancy-related hypertension (AUC=0.599) and GDM (AUC=0.609). From a statistical perspective, this relationship is on the verge of being moderate in terms of predictive power. Preterm delivery showed a significant area under the ROC FF chart at 10–20 weeks of pregnancy (AUC=0.503), indicating a weak and minimal statistical relationship. Other adverse pregnancy outcomes did not exhibit this relationship. In terms of the baby's birth weight, ROC analysis demonstrates a weak correlation between higher FF and lower birth weight (AUC=0.461).

To investigate the relationship between adverse pregnancy outcomes and demographic characteristics with FF, variables were added separately to the binary logistic regression model. Then, to obtain the adjusted model, the variables that had a significance level of less than 0.2 in the first model were entered into multiple regression analysis. Of these, GDM, pregnancy-related blood pressure, the mother's BMI, and preterm delivery were included in the multivariate regression analysis. Except for the mother's BMI at the time of sampling, none of the mentioned items had a significant relationship with FF in the multivariate analysis. Considering all the possible variables investigated in this study, the increase in the mother's weight is significantly associated with a decrease in FF (Table 3).

Table 4. Area under the graph for each adverse pregnancy outcome

Variable	AUC
HTN	0.599
GDM	0.609
Pre-eclampsia	0.018
PTL	0.503
Variable	AUC
HTN	0.599
GDM	0.609
Pre-eclampsia	0.018
PTL	0.503

Discussion

Unfavorable pregnancy outcomes are one of the crucial challenges for healthcare systems around

Table 3. The relationship between adverse pregnancy outcomes and demographic characteristics

Variables	Univariate logistic regression analysis		Multivariate logistic regression analysis		p-value
	OR	Interval	OR	Confidence interval 95%	
GDM	-1.547	-0.059	-1.325	0.072-0.988	0.032
Pregnancy-related hypertension	-0.466	-0.356	-0.523	0.327-1.056	0.043
Mother's BMI	0.001	-0.862	-0.104	0.863-0.940	0.026
Premature birth	-1.11-1.269	-	-	-	-
Neonate weight at birth	0	-	-	-	-

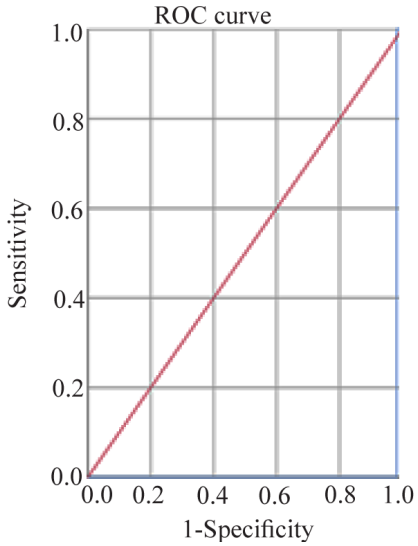
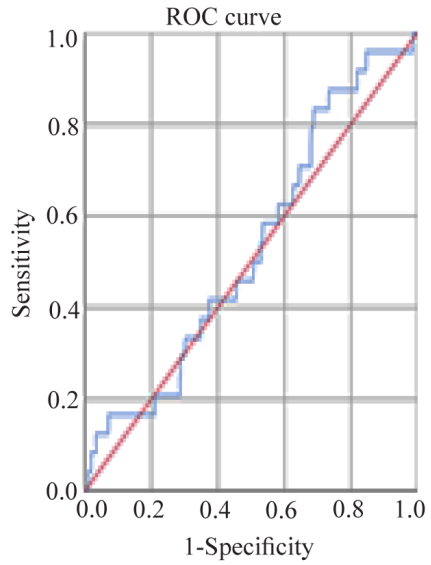
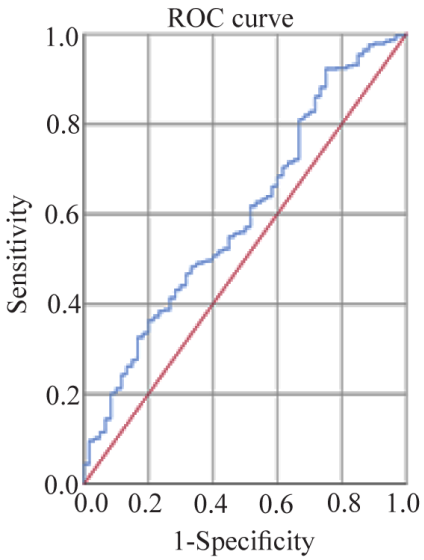


Figure 3. ROC diagram for pre-eclampsia.



Diagonal segments are produced by ties.

Figure 5. ROC diagram for PTL.



Diagonal segments are produced by ties.

Figure 4. ROC diagram for HTN.

the world. The ability to predict such consequences is invaluable, and due to the multitude of cases and possible variables involved in their occurrence, non-invasive, accessible, and cheap screening methods are very important. Despite previous identification of a strong relationship between some of these variables and adverse pregnancy outcomes, the lack of appropriate screening methods poses a challenge in identifying and monitoring patients at increased risk for these complications until symptoms manifest (24). Additionally, this leads to the inefficient use of financial and human resources within health and treatment systems. The incidence of adverse pregnancy outcomes in developed societies is significantly lower than in developing societies, with most studies on the burden of such cases conducted in these countries (25-27).

Examining the FF is one of the proposed non-invasive methods to assess the risk of adverse pregnancy outcomes. Researchers have conducted several studies to date on the effectiveness of examining the FF as a screening method to predict the risk of adverse pregnancy outcomes. However, the obtained results are not consistent with each other, and the debate on the effectiveness of this method is still ongoing (28,29). Therefore, in the present study, 685 pregnant women who underwent NIPT in weeks 10 to 20 of pregnancy were examined. The average age of women participating in the study was 33.91 years, and the average gestational age at delivery was 38.11 weeks. The frequency of preterm delivery, gestational diabetes, pre-eclampsia, and pregnancy-related blood pressure were 3.6, 1.5, 0.3, and 9%, respectively. The present study observed only the relationship between the mother's BMI at the time of sampling and FF, showing a significant decrease in FF with an increase in BMI.

Yuan *et al* conducted a study on 2191 women with singleton pregnancies, observing that FF lower than the 5th percentile increased the probability of having a baby with a lower birth weight, and FF lower than the

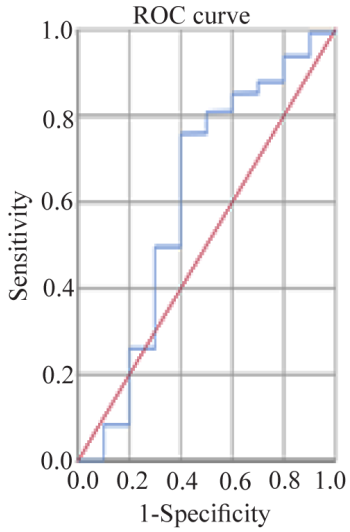


Figure 6. ROC diagram for GDM.

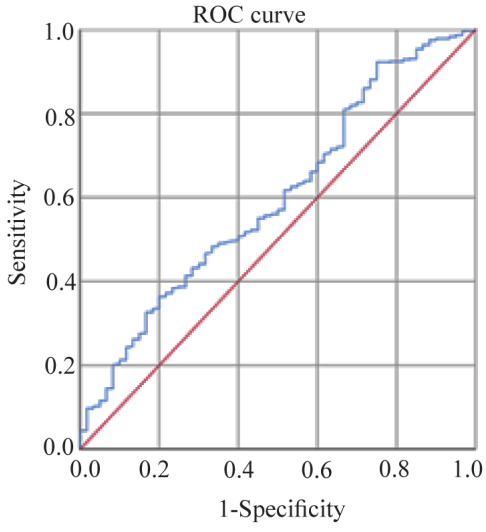


Figure 7. ROC diagram for low birth weight fetus (<2500 *gr*).

10th percentile increased the probability of preterm birth, associated with 34 weeks of pregnancy, which is not consistent with the findings of the current study. This could be due to the larger study volume and the increase in identified cases for each of the defined adverse outcomes. On the other hand, similar to what is observed in the present study, there was no significant relationship between FF and gestational diabetes and blood pressure caused by pregnancy (30). Jiang *et al*'s study, which involved 3534 pregnant mothers, found no significant correlation between FF and premature birth, low birth weight, or gestational diabetes. However, in this study, the association between pregnancy-related blood pressure and lower FF was significant. However, the current study found that FF could predict GDM, and with less power, blood pressure due to pregnancy and preterm delivery, based on univariate analysis and AUC (31). The discrepancy between Jiang's study and the present study may stem from the first study's higher FF cut-off of 15.15%, which differs from this study's defined cut-offs of 4% and the 25th percentile (4.37%). Also, similar to Yuan *et al*'s study, the sample size examined in this study is significantly larger than the current study.

Suzumori's study investigated the FF's predictive ability for pregnancy-related hypertension diseases. This study examined 5582 pregnant women who had a negative NIPT test. According to the observations, women with hypertension-related pregnancy diseases had a lower FF than other women. In this study, the area under the graph was equal to 0.608, which indicates the weak predictive ability of this variable for this outcome (31). In the current study, the area under the graph was equal to 0.599, which is similar to the findings of Suzumori *et al*, which indicates the predictive ability of the average FF to predict pregnancy-related blood pressure. The main difference between these two studies is the healthiness of the pregnant women examined by Suzumori *et al* examined the health of pregnant women. In addition, the number of patients examined in the first study is significantly higher than other studies conducted in this field.

In a prospective study conducted by Kikhaei *et al* in Zabul, 450 pregnant women underwent NIPT between 11 and 16 weeks of gestation (28). The average FF in this study was 8.3%, which is similar to the present study (7.29%). The frequency of low, normal, and high FF was 17.3, 73.2, and 9.5%, respectively, which has a different distribution compared to this study. According to their findings, the high FF, despite its high specificity, lacks sufficient positive predictive value. In the current study, based on AUC calculations, the FF demonstrated a moderate predictive ability for GDM and a weak predictive ability for gestational hypertension and preterm delivery. Contrary to the majority of studies conducted in this field, in a study of 77 pregnant women, Bennett *et al* reported that a FF lower than the 10th percentile is associated with

an increased risk for lower birth weight, gestational diabetes, and hospitalization in the ICU. Similar to Kikhaei *et al*'s study, the FF has a very low sensitivity (14.9%) but a high specificity (32).

Conclusion

Even though the present study found no significant relationship between the FF and any of the mothers' demographic variables or adverse pregnancy outcomes, conducting studies with a more consistent methodology can help better clarify the issue.

Suggestions

Conducting multi-center studies, sampling during different weeks of pregnancy, and following up with patients in the form of a prospective study with a much larger sample size are suggested for subsequent studies.

The study's problems and limitations

One of the problems and limitations of the project is the limited number of patients studied. Although there was adequate ethnic and geographic coverage due to the spread of medical centers, conducting larger studies can be associated with an increase in the number of observations (adverse pregnancy outcomes) and an increase in the study's power.

Acknowledgement

We want to express our gratitude to the Vice Chancellor for Research and Technology of Urmia University of Medical Sciences for their financial support of the research and to the colleagues of Kosar Women's General Hospital of Urmia University of Medical Sciences and all the patients of this study.

Conflict of Interest

Authors declare no conflict of interest.

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