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Original Article

High platelet distribution width affects the detection of fetal fraction of cell-free DNA detected by non-invasive prenatal testing

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Abstract: Objective: The aim of this study was to investigate the effect of platelet distribution width (PDW) on fetal fraction (FF) of cell-free DNA (cfDNA) within non-invasive prenatal testing (NIPT) during pregnancy. Methods: The study subjects were pregnant women who voluntarily underwent NIPT and had antenatal examinations at the Affiliated Suzhou Hospital of Nanjing Medical University from January 2016 to January 2021. They underwent routine blood tests before and after NIPT. Univariate and multivariate regression models were used to evaluate the correlation between complete blood count indices and FF. Results: Multivariate linear regression analysis revealed that hemoglobin (Hb), white blood cell count (WBC), platelet count (PLT), red blood cell distribution width (RDW), PDW, and body mass index (BMI) were negatively correlated with FF, while the interval between the two tests was positively correlated with FF. Notably, PDW (standardized β: -0.097) was the second most significant factor after BMI (standardized β: -0.292). Further grouping by PDW showed that compared to PDW ≤ 12.0 fL, FF decreased by 0.070 (95% CI: -0.111 to -0.030; $P = 0.001$) and 0.191 (95% CI: -0.260 to -0.123; $P < 0.001$) in women with PDW levels of 12.1-15.0 fL and > 15.0 fL, respectively, showing a gradual decreasing trend ($P_{trend} < 0.001$). Conclusion: Higher PDW values altered the FF detection, with an observed decrease in FF as PDW levels increased.

Keywords: Platelet distribution width, fetal fraction, pregnant, metaphase, cell-free DNA, non-invasive prenatal testing

Introduction

Internationally, prenatal screening for chromosomal aneuploidy has made extensive use of high-throughput sequencing technology for the detection of cell-free fetal DNA (cffDNA) in pregnant women's peripheral blood, commonly referred to as NIPT (Non-invasive Prenatal Testing) [1-3]. This revolutionary method has proven effective in reducing the incidence of chromosomally aneuploid fetuses through early detection, thereby serving as a critical tool for the secondary prevention of birth defects. The ratio of fetal-derived cell-free DNA (cfDNA) in the overall amount of maternal plasma cfDNA is known as the fetal fraction (FF). However, due to the low FF in pregnant women's peripheral blood, it can result in NIPT detection failure or "no calls", which presents a challenge to the clinical application of NIPT technology [4]. To obtain a greater quantity of cfDNA, current research strategies have focused on two main areas: firstly, fetal free DNA enrichment techniques, including gel electrophoresis (E-Gel)-based, magnetic bead-based enrichment methods and computational simulation (in silico) NIPT techniques. This principle is mainly due to the fact that the size of the fetal fragment is smaller than the maternal piece. There are also more fetal components present in the 143 bp fragment of free DNA compared to the 166 bp fragment of free DNA [5].

In light of this, we and other researchers have developed a unique NIPT method that significantly improves the FF (by 2.3-fold) and analyti-

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cal efficiency of NIPT by enriching shorter cfDNA fragments (< 140 bp) [6-9]. However, the National Medical Products Administration (NMPA) has not yet authorized this technology for clinical use, and it is currently only useful for laboratory quality control. Secondly, several researchers have looked into how FF is affected by experimental variables as well as by maternal and fetal characteristics. Their objective is to modify these elements to increase the quantity of fetal-derived cfDNA. The FF may be influenced by a number of factors, including gestational age (GA), maternal body mass index (BMI), anticoagulant therapy, blood collection and fetal aneuploidy [9-11]. Retesting by sequencing a second sample after the initial failure is a practical strategy to lower screening failure rates, according to prior studies. If the original failure was caused by a low FF, the second sample can be repainted or collected concurrently with the first [12]. This implies that implementing quality control measures prior to testing can minimize maternal DNA interference and enhance fetal components.

Nevertheless, recent studies have indicated that the success rate of repeated NIPT following a second blood draw is only 56%-66%. This finding suggests that additional factors may also influence the FF. Pregnant women are required to undergo a complete blood count (CBC) during prenatal check-ups. CBC is a fundamental examination which is highly significant for reflecting the pathologic and physiologic conditions of pregnant women and can indirectly assess the levels of maternal-derived cfDNA. Therefore, in this study, we investigated the effect of PDW level on FF by analyzing the relationship between whole blood cell characteristics and FF, with the aim of improving the success rate of NIPT by controlling the unfavorable factors before blood draw.

Materials and methods

Study population

This was a retrospective, single-center study of pregnant women who had routine blood draws at 12-26 weeks of gestation and NIPT for trisomy 13, 18, and 21 screening. Between January 2016 and January 2021, information on 4,262 pregnancies with a male fetus was gathered at The Affiliated Suzhou Hospital of Nanjing Medical University. The Ethics Committee of The Affiliated Suzhou Hospital of Nanjing Medical University authorized this study, which complied with all applicable ethical standards (ID: K-2021-032-H01).

Procedure and data collection

All venous blood specimens for routine blood tests were collected in 2 mL EDTA tubes and analyzed within one hour using an automated hematology analyzer (Sysmex XN-20, Japan) for CBC, including Hb, WBC, PLT, RDW, plateletcrit (PCT), platelet large cell ratio (P-LCR), mean platelet volume (MPV) and PDW according to the manufacturer's instructions. This approach was employed to avoid time-dependent ultrastructural morphologic changes in platelets.

Before having NIPT, all patients received pretest counseling and signed an informed consent form. The main topics addressed in genetic counseling included advanced maternal age, prior pregnancies affected by fetal aneuploidy, and the use of ultrasound for detecting aneuploidy or fetal abnormalities. Before 14 weeks of pregnancy, pregnant women who had undergone NIPT screening were examined by ultrasonography. This was done to assess the number of fetuses, chorionicity, GA, and maternal and fetal features. The mother's age, height, weight, number of pregnancies, twin or singleton pregnancy status, GA, and method of conception were all included in the medical history.

Venous blood samples (10 mL) were collected from pregnant women who had provided written informed consent following pre-test counseling from genetic advisors. As directed by the manufacturer, the QIAamp DSP DNA Blood Mini Kit (Qiagen) was used to extract the cfDNA. The polymerase chain reaction (PCR) was used to create the library. The Qubit™ dsDNA HS Kit (Invitrogen, Carlsbad, CA, USA) was used to measure the concentrations of cfDNA and the resultant library. Either the BGISEQ-500 (MGI, China) or the Ion Proton systems were then used to sequence the sequencing libraries. The ratio of Y chromosomal reads was calculated in order to determine the FF. It was found that the FF was more than 4%. A risk evaluation was not generated by the laboratory in cases when the FF was less than 4%. Every sample produced clinical follow-up results or showed a

Table 1. Sample characteristics of the study population (*n* = 4,262) [*M* (*Q*1, *Q*3)]

Characteristic	Value (median and interquartile range)
BMI (kg/m^2)	22.35 (20.55-24.39)
Fetal fraction (%)	10.94 (8.29-13.93)
Interval between two tests (weeks)	4.14 (2.43-5.14)
WBC (10^9/L)	8.72 (7.51-10.03)
Hb (g/L)	128 (122-133)
RDW (%)	13.10 (12.70-13.60)
PLT (10^9/L)	226 (194-259)
PCT (%)	0.23 (0.20-0.26)
P-LCR (%)	25.40 (20.70-31.20)
MPV (fL)	10.10 (9.50-10.70)
PDW (fL)	11.70 (11.60-13.00)

Abbreviations: BMI = body mass index, WBC = white blood cell count, Hb = hemoglobin, RDW = red cell distribution width, PLT = platelets, PCT = plateletcrit, P-LCR = platelet large cell ratio, MPV = mean platelet volume, PDW = platelet distribution width.

fetal karyotype, which was considered pregnant with a positive NIPT result.

Statistical analysis

Descriptive data were presented as median (M) and interquartile range (IQR) [M (P2.5, P97.5)] for non-normal continuous variables. The descriptive data for quantile variables were presented as an interquartile range (IQR) [M (P2.5, P97.5)] and a median (M). For categorical variables, the descriptive data were expressed as absolute values and percentages [n (%)]. Since the FF did not obey the normal distribution, it was transformed into a natural logarithm and then presented as a square root (√) that conformed to the normal distribution. The associations between the FF and a variety of CBC measurements, such as WBC, Hb, RDW, PLT, PCT, P-LCR, MPV, PDW, maternal weight (kg), and the time between the two test sessions (weeks), were examined using univariate analysis and multivariate linear regression models. The variance inflation factor (VIF) was employed to assess the presence of multicollinearity. VIF levels more than 4 called for additional investigation, while VIF levels above 10 signified a significant degree of multicollinearity and necessitated corrective action. The VIFs in the models constructed in this study were all less than 4, indicating that there was no issue with covariance.

PDW levels were categorized as ≤ 12.0, 12.01-15.0 and > 15.0 fL. Additionally, estimations of the mean differences in FF for each PDW category were computed, along with 95% confidence intervals (CI). Three models were used in all. A univariate linear regression assessing the association between FF and PDW levels was model 1. Model 2 was modified to account for BMI. WBC levels were included in model 3 based on model 2. Hb and RDW levels were introduced to model 4 based on model 3. PLT levels were incorporated into model 5 based on model 4. In model 6, the time interval between two tests (weeks) was added on the basis of model 5.

Multivariable logistic regression was used to determine the associations of BMI, WBC, Hb, RDW, PLT, PDW, and the time interval between the two tests (weeks) with the test failure rate. The data was analyzed using SPSS version 26.0 (IBM Corp, Armonk, NY, USA), with a two-sided alpha level of $P < 0.05$.

Results

Sample characteristics

All 4,262 pregnant women in this study were carrying male fetuses. Their BMI was 22.35 (IQR: 20.55-24.39), and the median time between tests was 4.14 weeks (IQR: 2.43-5.14). As shown in **Table 1**, hematological data included a median WBC of 8.72 × 10^9/L, Hb of 128 g/L, RDW of 13.10%, PLT of 226 × 10^9/L, and PCT of 0.23%. Platelet indices included a P-LCR of 25.4% (IQR: 20.7-31.2%) and MPV of 10.1 fL (IQR: 9.5-10.7 fL). In maternal plasma, the median concentration of fetal DNA was 10.94% (IQR: 8.29-13.93%), consistent with previous reports [13]. These findings provide an overview of maternal hematologic characteristics and fetal DNA distribution in this cohort.

Relationship between complete blood count and FF

We examined the relationship between CBC data and FF using linear regression analysis,

Table 2. Regression analysis of factors from maternal characteristics and Complete Blood Count for predicting √ fetal fraction in 4,262 pregnancies with male fetuses

Independent variable	Univariable		Multivariable		
	Regression coefficient (95% CI)	*P*	Regression coefficient (95% CI)	Standardized coefficients	*P*
BMI (kg/m^2)	-0.065 (-0.071 - -0.059)	< 0.001	-0.059 (-0.065 - -0.319)	-0.292	< 0.001
Interval between two tests (weeks)	0.021 (0.012 - -0.030)	< 0.001	0.024 (0.015 - -0.032)	0.081	< 0.001
WBC (10^9/L)	-0.049 (-0.058 - -0.040)	< 0.001	-0.027 (-0.037 - -0.017)	-0.085	< 0.001
Hb (g/L)	-0.005 (-0.007 - -0.003)	< 0.001	-0.004 (-0.006 - -0.002)	-0.059	< 0.001
RDW (%)	-0.027 (-0.042 - -0.013)	< 0.001	-0.020 (-0.035 - -0.006)	-0.041	0.007
PLT (10^9/L)	-0.011 (-0.015 - -0.007)	< 0.001	-0.007 (-0.011 - -0.002)	-0.054	0.002
PCT (%)	-1.625 (-2.037 - -1.218)	< 0.001	-	-	-
P-LCR (%)	-0.005 (-0.007 - -0.002)	< 0.001	-	-	-
PCV (fL)	-0.033 (-0.052 - -0.014)	0.001			
PDW (fL)	-0.019 (-0.028 - -0.011)	< 0.001	-0.028 (-0.037 - -0.019)	-0.097	< 0.001

Abbreviations: BMI = body mass index, WBC = white blood cell count, Hb = hemoglobin, RDW = red cell distribution width, PLT = platelets, PCT = plateletcrit, P-LCR = platelet large cell ratio, MPV = mean platelet volume, PDW = platelet distribution width.

with FF as the dependent variable and BMI, test interval, and eight CBC values as independent factors.

We discovered that FF and the time between the two tests had a positive correlation by the univariate linear regression study (β = 0.021, *P* < 0.001). On the other hand, FF showed negative correlations with BMI and several CBC-related indices. Specifically, FF was negatively correlated with WBC, Hb, RDW, PLT, PCT, P-LCR, MPV and PDW, with β-values of -0.065, -0.049, -0.005, -0.027, -0.011, -1.625, -0.005, and -0.019, respectively (*P* < 0.001 for all). Additionally, FF was negatively correlated with PCV (β = -0.033, *P* = 0.001).

The findings from the univariate study were validated by a multivariate linear regression analysis. There was still a favorable correlation between FF and the time between the two tests (β = 0.081). Moreover, FF showed significant negative correlations with BMI, WBC, Hb, RDW, PLT, and PDW, with β-values of -0.292, -0.085, -0.059, -0.041, -0.054, and -0.097, respectively. These results suggested that both clinical and CBC data were significantly associated with FF, as summarized in **Table 2**.

Association between PDW and FF

The analysis revealed that PDW (standardized β = -0.097) was the most significant factor influencing FF after BMI (standardized β = -0.292). Based on PDW quartiles, we categorized all pregnant women into three groups: PDW ≤ 12.0 fL, 12.1-15.0 fL, and > 15.0 fL, with 2495, 1378, and 389 participants, respectively. The median FF values in these groups were 11.17% (8.35%, 14.18%), 10.79% (8.31%, 13.73%), and 10.25% (7.83%, 12.87%). As PDW increased from ≤ 12.0 fL to > 15.0 fL, FF showed a gradual decrease, and there was a significant difference (*P* < 0.001) (**Figures 1**, **2**).

The regression coefficients (B values) and 95% CIs for every PDW group are shown in **Figure 3**, along with the findings of the trend tests for every model. In all six models, group 1 (PDW ≤ 12.0 fL) was used as the control group. In the univariate linear regression model (model 1), the FF in group 2 and group 3 decreased by 0.044 (95% CI: -0.086 to -0.003; *P* = 0.035) and 0.133 (95% CI: -0.200 to -0.066; *P* < 0.001), respectively, showing a significant, gradual decline with increasing PDW (P_{trend} < 0.001).

In the multivariate model adjusted for BMI (model 2), the FF values in group 2 and group 3 decreased by 0.056 (95% CI: -0.095 to -0.017; *P* = 0.005) and 0.155 (95% CI: -0.219 to -0.091; *P* < 0.001), respectively, with the trend test also showing statistical significance (P_{trend} < 0.001). When WBC was added to the model (model 3), the FF values in group 2 and group 3 decreased by 0.063 (95% CI: -0.102 to -0.024; *P* = 0.002) and 0.161 (95% CI: -0.224 to -0.098; *P* < 0.001), with the trend test remaining significant (P_{trend} < 0.001).

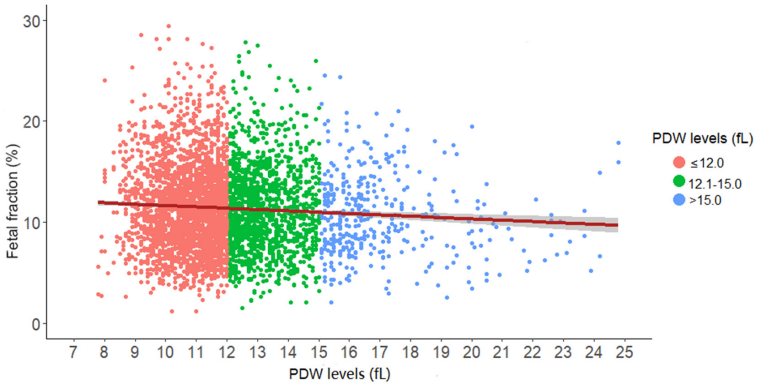

Figure 1. Scatterplots of fetal fraction according to different groups of PDW levels with lines of best fit from the linear regression.

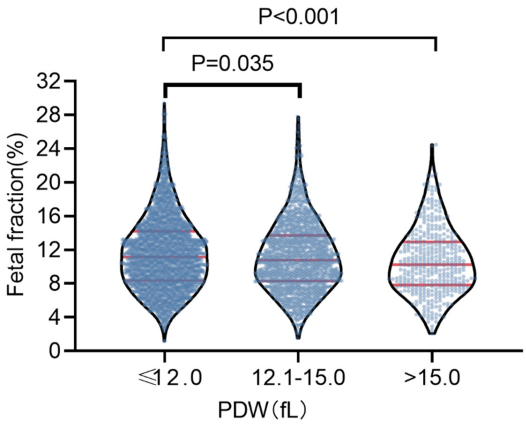

Figure 2. Mean fetal fraction across PDW categories.

Further analyses incorporating Hb and RDW into model 3 (model 4), followed by PLT in model 4 (model 5), and adjusting for the time interval between tests (in weeks) in model 5 (model 6), continued to show the greatest reduction in FF in group 3. The B values and 95% CIs for group 3 in these models were -0.155 (95% CI: -0.219 to -0.092; $P < 0.001$), -0.189 (95% CI: -0.257 to -0.121; $P < 0.001$), and -0.191 (95% CI: -0.260 to -0.123; $P < 0.001$), with trend tests consistently showing significance ($P_{trend} < 0.001$).

Discussion

After Lo et al. [14] discovered a small amount of free fetal DNA in the plasma of pregnant women, noninvasive prenatal genetic testing using next-generation sequencing (NGS) rapidly evolved, becoming a prevalent method for screening for chromosomal abnormalities during pregnancy [15-19]. Accurate fetal aneuploidy detection relies on a sufficient FF in NIPT samples, which typically ranges from 10% to 15% during the 10th to 20th week of gestation (the most prevalent period for NIPT) [13]. In most cases, a FF below 3.5% to 4% may result in false-negative or "no call" results in NIPT assays [19]. Accordingly, the avoidance of factors that may prove detrimental to the FF prior to the test is likely to result in a higher FF and an enhanced success rate for the test.

As a proportion, FF varies dynamically and is influenced by various factors. In this study, the median FF was 10.84%, with a negative correlation observed between this value and WBC, Hb, RDW, PLT, and BMI. First, the effect of BMI, a significant negative correlate, on FF was attributed primarily to the higher amount of plasma cfDNA from maternal adipose tissue or the dilution of FF due to the relatively high blood volume of pregnant women with high body mass. This is in accordance with previous studies [20-22]. A positive correlation between the interval between the two tests and FF, on the other hand, reflects the dynamic changes of fetal free DNA in pregnant women. As gestation time progresses, an increase in placental weight and the release of fetal free DNA by apoptosis contribute to an increase in FF, a finding that aligns with those of Alberry and Cao [20, 23].

Furthermore, this study revealed a negative correlation between FF and PDW, which remained significant even after stratifying PDW, adjusting for potential confounding factors such as BMI, WBC, Hb, RDW and PLT, and constructing six distinct models. These findings indicate a potential correlation between certain hematologic values in pregnant women and the release, circulation, and clearance of fetal free DNA. Some physiologic changes that occur during pregnancy are influenced primarily by alterations in the hormonal milieu. For instance, white WBC counts are physiologically elevated during pregnancy [24-26]. Catabolism or death of WBCs is a major source of maternal plasma DNA [27], which results in a negative correlation between WBC counts and

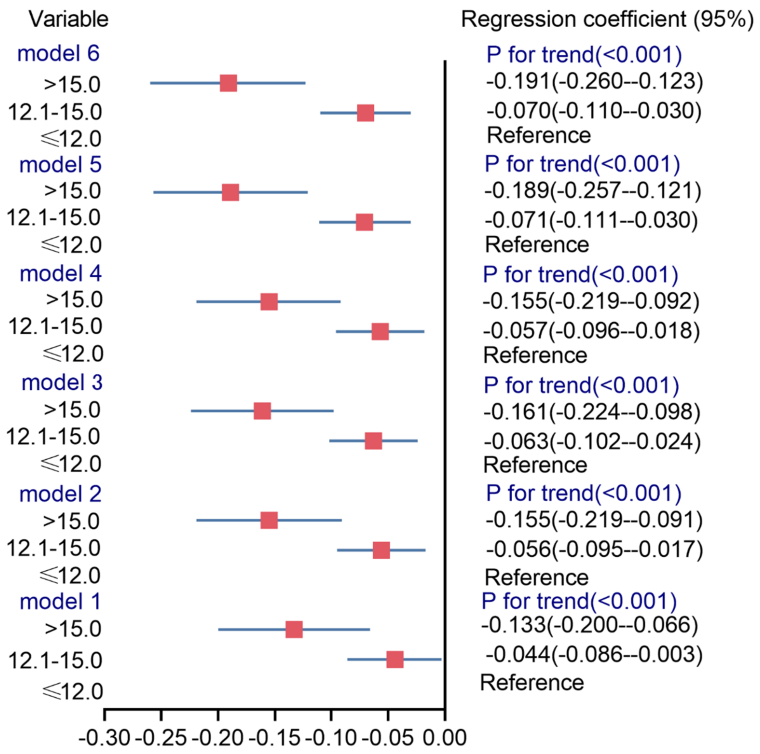

Figure 3. Differences in √ fetal fraction according to PDW categories. Model 1 was a univariate linear regression of the relationship between PDW level and √ fetal fraction. Model 2 was adjusted for BMI. In model 3, WBC was added on the basis of model 2. In model 4, Hb and RDW were added on the basis of model 3. In model 5, PLT was added on the basis of model 4. In model 6, the time interval between two tests (weeks) was added on the basis of mode 5. Platelet distribution width (PDW), white blood cell count (WBC), red blood cell distribution width (RDW).

FF. During the second trimester, the placenta produces and secretes more progesterone and estrogen, which stimulates the kidneys to release renin. This, in turn, causes sodium retention and a rise in plasma volume through stimulation of the renin-angiotensin-aldosterone system. This allows for the increased metabolic demands of the mother and fetus to be met, as well as for the acute blood loss that occurs at delivery to be tolerated. Consequently, maternal production of erythropoietin (EPO) during pregnancy stimulates red blood cell (RBC) production; however, its effect is less pronounced than that of plasma dilution, resulting in a secondary dilutional reduction in RBCs, Hb and platelets [25, 28, 29]. It has been established that placental trophoblast cells represent the primary source of cell free fetal DNA [30]. The maternal-placental blood supply becomes active at the beginning of the 11th week of gestation. The RDW is a marker for the variability of red blood cell size and is commonly used in clinical practice to differentiate anemia. Decreased levels of RDW and Hb may affect oxygen transport in the blood and numerous tissues, including the placenta. Hypoxia is a primary trigger of apoptosis in the placenta, where trophoblast cells constitute the majority of apoptotic cells (> 50%). Additionally, placental trophoblast cells are the primary source of fetal plasma-free DNA. There is a negative correlation between Hb and RDW levels and FF.

It is noteworthy that PDW is a more stable and specific platelet parameter than MPV [31, 32], PDW reflects changes in platelet volume and activation status and is also a risk factor for pregnancy-related complications [33-36]. However, increased platelet activation and clearance may be a cause of pregnancy-associated thrombocytopenia [37], and platelet activation induces platelet swelling and pseudopod formation, which increases MPV and PDW [29, 38]. Concurrently, activated platelets release a plethora of cytokines and chemokines, including 5-hydroxytryptamine (serotonin) and arachidonic acid (AA), which enter the liver and contribute to hepatocyte injury through a cascade of signaling pathways. Additionally, they recruit leukocyte aggregates to facilitate the inflammatory response [39, 40]. It was therefore postulated that the effect of PDW on FF may be a reduction in FF resulting from an increase in maternally derived background cfDNA of leukocyte and liver tissue origin [27]. Typically, these adaptive alterations occur concurrently, thereby maintaining a relatively stable FF. Conversely, an excessively high or low FF may indicate maternal or fetal pathology. The mechanism of interaction of these changes is highly intricate, and the specific biologic mechanisms remain to be further elucidated. Moreover, it is important to take into account the following factors given

the limitations imposed by the clinical data: first, the association between complete blood count and FF was studied using data received from a single center. It is advised that larger samples be used for further evaluation. Second, it is possible that some pertinent variables were unintentionally left out even after accounting for a number of contributing factors, such as biochemical placental markers (PAPP-A, free β-hCG, and PlGF) and *in vitro* fertilization conception.

In conclusion, our findings indicate that FF was inversely related to PDW, WBC, RDW, PLT, Hb, and BMI levels, but that FF was positively related to the time between the two tests. Higher levels of PDW may be detrimental to obtaining adequate FF, thus improving test success by controlling for unfavorable factors prior to blood collection.

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Disclosure of conflict of interest

None.

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