ORIGINAL RESEARCH ARTICLE

Hemolysis and Fetal Fraction in Cell-Free DNA Blood Collection Tubes for Noninvasive Prenatal Testing

Renee Stokowski[1] · Karen White[1] · Coleen Hacker[1] · Jigna Doshi[1] · Maximilian Schmid[1]

Abstract

Background Lysis of maternal white blood cells in prenatal cell-free DNA (cfDNA) test samples increases the level of maternal DNA and consequently decreases fetal fraction.

Objective The objective of this study was to determine whether hemolysis, traditionally used as a marker for cell lysis, is correlated with a decrease in fetal fraction in maternal blood samples collected in specialized cfDNA tubes for noninvasive prenatal testing.

Methods In the first part of the study, blood from pregnant women was collected into three Roche Cell-Free DNA Collection Tubes. These replicate specimens from the same subject were evaluated for a visual difference in hemoglobin level as a measure of hemolysis. The specimens were then processed with the Harmony® prenatal test to measure fetal fraction using polymorphic digital analysis of selected regions (DANSR) assays. In a second part of the study, clinical laboratory samples with hemoglobin levels of ≥ 500 mg/dL were tracked through the laboratory and their fetal fraction compared with that of concurrently processed samples with lower hemoglobin levels.

Results There was no significant difference in fetal fraction in 339 paired samples, with a difference in hemoglobin levels ranging from 0 to 1000 mg/dL. There was strong correlation in fetal fraction between tubes, regardless of the differences in hemoglobin concentration. The fetal fraction distribution in 203 tracked clinical samples with hemoglobin levels ≥ 500 mg/dL was statistically equivalent to the distribution in a concurrent series of 12,705 samples.

Conclusion Hemolysis in maternal blood samples collected in specialized cfDNA tubes does not correlate with a decrease in fetal fraction; therefore, it should not be a cause for rejection of samples submitted for prenatal cfDNA testing.

Key Points

Hemolysis is not associated with lower proportions of fetal cell-free DNA in maternal blood samples drawn in cell-free DNA blood collection tubes.

Laboratories performing prenatal cell-free DNA testing do not need to reject samples with visible hemolysis.

✉ Karen White
karen.white.kw2@roche.com

1   Ariosa Diagnostics Inc., Roche Sequencing Solutions Inc., San Jose, CA, USA

1 Introduction

The presence of placenta-derived cell-free DNA (cfDNA) in a pregnant woman's blood makes it possible to obtain fetal genetic information noninvasively. The most common application of this testing currently is fetal trisomy screening. Because most cfDNA originates from the apoptosis of maternal cells of hematopoietic origin, all fetal testing must be performed against a background of maternal cfDNA [1–3]. The cfDNA originating from the conceptus is commonly referred to as fetal cfDNA, and the fetal fraction, or proportion of cfDNA that it represents, is an important quality metric for prenatal cfDNA testing, with most laboratories requiring a minimum threshold [4].

Observational studies in large cohorts have demonstrated significant variation in fetal fraction among blood samples received in clinical laboratories [5, 6]. Patient and pregnancy variables, such as gestational age, maternal weight, twin pregnancy, conception by in vitro fertilization (IVF),

and underlying maternal conditions may contribute to the variation [5–9]. Specimen collection and handling may also be a factor; care must be taken during blood collection and shipping [10]. Inadvertent lysis of maternal nucleated cells releases maternal DNA, which dilutes the placenta-derived cfDNA in the blood tube and effectively decreases fetal fraction. Even with the use of specialized tubes and protective packaging, shipping-related factors such as extreme temperatures can adversely affect fetal fraction [11, 12].

Clinical laboratories performing cfDNA testing can reject potentially compromised samples based on extended transit times, but few other pre-analytic rejection criteria exist. Chemistry laboratories most frequently reject samples because of hemolysis because free hemoglobin from the breakdown of red blood cells is an interfering factor for a wide range of tests [13]. Although the lysis of non-nucleated red blood cells is unlikely to interfere with cfDNA testing, it could be used as a broader indicator of cell lysis. Hemolysis might therefore indicate potential lysis of nucleated maternal cells and identify compromised samples. The objective of this study was to determine whether hemolysis is associated with lower fetal fraction in cfDNA testing samples and should be used as a reason for sample rejection.

2 Methods

2.1 Subjects and Specimens

2.1.1 Part 1

In the first part of the study, 115 subjects were recruited from pregnant women presenting for routine prenatal care at a minimum of 10 weeks' gestation. Pregnancies with more than two fetuses were excluded. Women consented to blood collection and research use of their anonymized specimens at Ariosa Diagnostics Inc. (San Jose, CA, USA). The samples were provided to the investigators in an anonymized format. The study protocol was approved by the Quorum Institutional Review Board (file number 25591).

Replicate specimens of venous blood were collected from each subject into three Roche Cell-Free DNA Collection Tubes (Roche, Pleasanton, CA, USA) at a single blood draw. Before use, the tubes were aged by storing for the recommended 18-month shelf life or longer, up to 26 months, at a mean temperature of 21.8 °C (range 20.5–27.4). For each subject, a standard specimen collection kit for the Harmony® prenatal test was used for shipping by overnight courier to the Ariosa Diagnostics Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP)-accredited clinical laboratory (San Jose, CA, USA). A 30 °C WarmMark or 10 °C ColdMark temperature indicator (ShockWatch Indicators, Fountain Inn,

SC, USA) was included in the kit, depending on seasonal temperatures, to determine whether tubes were exposed to temperatures outside the recommended range of 18–25 °C. Shipping temperature was not used as an exclusion criterion.

2.1.2 Part 2

The second part of the study was observational and based on clinical testing of patient specimens submitted to the Ariosa Diagnostics Clinical Laboratory for noninvasive prenatal testing. Submitting healthcare providers and laboratories are supplied with a standard sample collection kit identical to the one used for Part 1, which contains two Roche Cell-Free DNA Collection Tubes. The laboratory also accepts specimens received in Cell-Free DNA BCT tubes (Streck, Omaha, NE, USA). The measures included in this study are determined as part of standard laboratory procedure. The data were deidentified before being provided to the authors. Because this study involved deidentified existing data with no personally identifiable information, no additional consent was obtained.

2.2 Laboratory Methods

Blood was stored at room temperature and processed to plasma within 7 days of specimen collection. The laboratory only processes specimens with at least 2 mL of plasma. Trained personnel used a standard hemolysis chart to visually evaluate each plasma specimen for approximate hemoglobin concentration (Mayo Medical Laboratories, Rochester, MN, USA) (Online Resource 1). Hemoglobin levels were classified as 0 (minimal), 20, 50, 100, 250, 500, and 1000 mg/dL.

Specimens were analyzed with the Harmony prenatal test [14, 15]. The test, which is intended for determination of the probability of fetal trisomy, uses non-polymorphic digital analysis of selected regions (DANSR) assays on chromosomes 21, 18, and 13 and polymorphic DANSR assays for fetal fraction determination [16].

2.3 Data Analysis

2.3.1 Part 1

For each set of replicate tubes from the same subject, pairwise comparisons for difference in hemoglobin concentration between the tubes were determined. These pairs were binned into four groups based on the level of hemoglobin difference. Within each group, the coefficient of determination (r^2) was calculated for fetal fraction between paired tubes, and the mean fetal fractions were compared with t test analysis. Agreement of Harmony test results (trisomy probability for chromosomes 21, 18, and 13, and fetal sex) was

also determined for all tubes from the same subject. Agreement with pregnancy outcome was not determined because the study protocol did not include clinical follow-up.

2.3.2 Part 2

Plasma specimens showing ≥ 500 mg/dL hemoglobin concentration were considered to have significant hemolysis. By conventional classification, this is a standard threshold for specimen rejection for most tests performed by clinical chemistry laboratories [17]. These specimens were processed by the clinical laboratory, and their mean fetal fraction was compared with that of all other specimens processed through the laboratory during the same time period.

3 Results

3.1 Part 1

In the 115 subjects, mean maternal age was 26 years (range 19–41) and mean gestational age was 16 weeks (range 10–32). The temperature indicator showed exposure to temperatures outside the recommended range during shipment of the specimens from the clinic to the clinical laboratory, with approximately 9% exposed to temperatures < 10 °C and 41% exposed to temperatures > 30 °C. Of 115 subjects, 56 (49%) exhibited differential hemolysis between specimens drawn at the same time. This was likely because of the intentional and unintentional suboptimal handling, i.e., aged tubes, exposure to temperature extremes, phlebotomy issues such as blood draw order, vein collapse, and differential mixing of blood tubes after collection.

For each set of replicate tubes from the same subject, pairwise comparisons for difference in hemoglobin concentration were determined. In total, 342 tubes met the specimen volume requirement, generating a total of 339 comparisons. Average plasma volume was 5 mL (range 2–6). The pairs were divided into four groups for analysis: 210 that showed no difference in hemoglobin concentration

between tubes (group A); 64 with a difference between 50 and 200 mg/dL (group B), 42 with a difference between 250 and 450 mg/dL (group C), and 23 with a difference between 500 and 1000 mg/dL (group D). For each of these groups, the mean fetal fractions for the tubes with the highest and lowest hemoglobin levels are shown in Table 1. There were no significant differences in mean fetal fraction between tubes in any of these groups as determined by paired t test for a hypothesized mean difference of zero (Table 1).

For each tube pair, Fig. 1 plots the linear correlation in fetal fraction between the tube with the highest hemoglobin and the tube with lowest hemoglobin. In all four groups, the fetal fractions were strongly correlated, and no significant changes in the linear relationship between tubes were observed. If there were to be an association between increasing hemoglobin concentration and decreasing fetal fraction, we would expect a difference between the groups in mean fetal fraction and in the
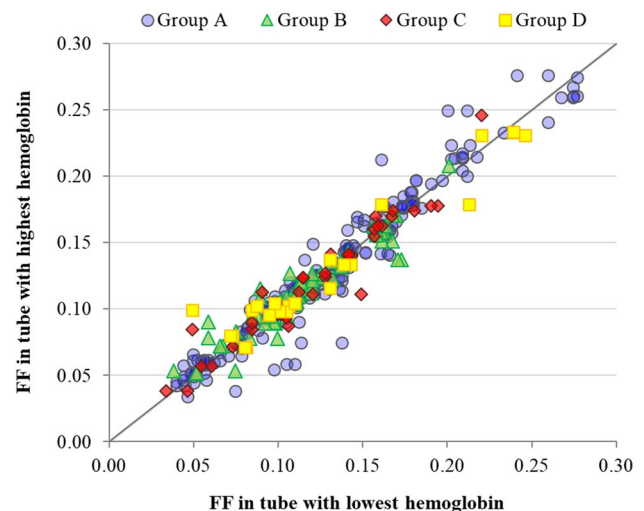

Fig. 1 Correlation in fetal fraction (FF) between replicate specimens based on change in hemoglobin mg/dL: group A (0 mg/dL) coefficient of determination (r^2) = 0.94; group B (50–200 mg/dL) r^2 = 0.90; group C (250–450 mg/dL) r^2 = 0.92; group D (500–100 mg/dL) r^2 = 0.93. The equivalence line is plotted

Table 1 Mean fetal fraction between replicate samples with different hemoglobin levels

Variable	Hemoglobin difference (mg/dL)							
	500–1000		250–450		50–200		No change	
Hemoglobin level	Lowest	Highest	Lowest	Highest	Lowest	Highest	Tube 1	Tube 2
Mean fetal fraction	0.13	0.13	0.12	0.12	0.11	0.11	0.13	0.13
Variance	0.004	0.003	0.002	0.002	0.001	0.001	0.003	0.003
Observations	23		42		64		210	
Two-tailed p value[a]	0.93		0.93		0.97		0.73	

[a] t test: paired two sample for means with hypothesized mean difference of zero
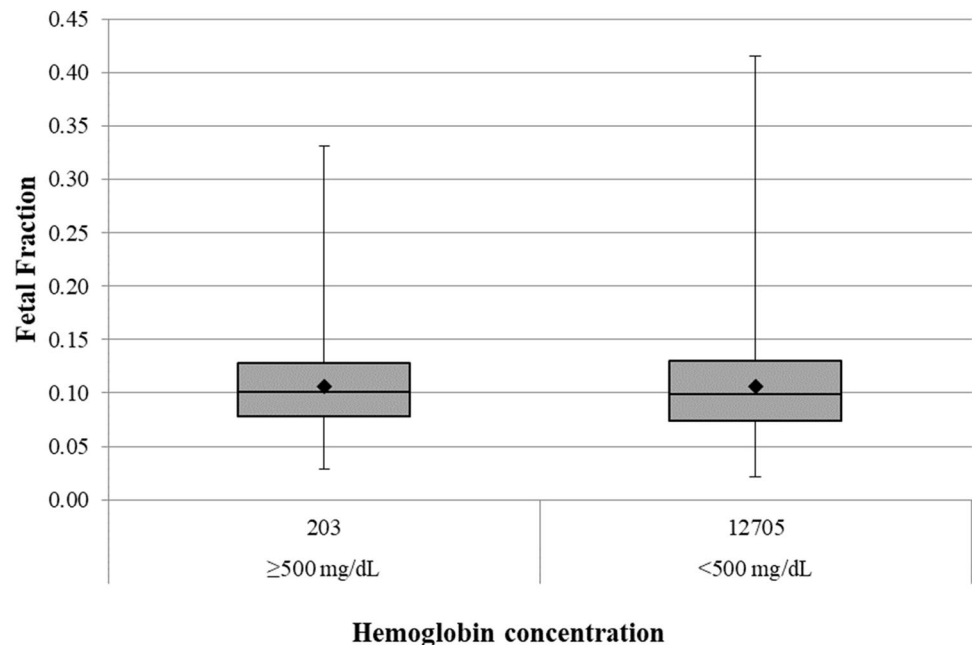
slopes of the linear relationship between tubes as changes in hemoglobin concentration increases.

For the 115 subjects, 100% agreement was observed in the fetal trisomy probability for chromosomes 21, 18, and 13 determined from replicate tubes from the same subject. The subject set contained a single trisomy 18 high probability result; all other subjects had results consistent with a euploid fetus. The results for replicate tubes from the same subject also gave 100% agreement for fetal sex.

3.2 Part 2

In a consecutive series of specimens received in the clinical laboratory, trained clinical laboratory staff identified 203 specimens with significant hemolysis. During the same time period, 12,705 specimens without significant hemolysis were analyzed in the laboratory. The specimens processed in the clinical laboratory reflect standard intended use pregnancies and include singleton and twin pregnancies as well those with both natural and IVF conception. Of all specimens received, > 99% were in Roche Cell-Free DNA Collection Tubes. Average plasma volume was 5 mL (range 2–7). There was no difference in the mean fetal fraction between the specimens with and without significant hemolysis ($p = 0.091$); both groups had mean fetal fractions of 11% (Fig. 2). The number of collection tubes received from other manufacturers was not sufficient to extend the analysis by brand.

4 Discussion

This study looked for a possible association between hemolysis and decreased fetal fraction in specimens drawn in specialized tubes for prenatal cfDNA testing. The intent was to determine whether visual assessment of hemoglobin concentration would identify prenatal test specimens that are potentially compromised because of maternal white blood cell lysis. Because of the significant variation in fetal fraction between pregnancies and the change in fetal fraction with gestational age, the first part of the study used replicate samples drawn from the same pregnant woman at the same time for comparison. The second part of the study observed the mean fetal fraction in specimens received for clinical testing with significant hemolysis (≥ 500 mg/dL) compared with the mean fetal fraction in specimens without significant hemolysis (< 500 mg/dL). We found no association between hemoglobin concentration and fetal fraction in either part of the study.

The lack of an association between the presence of hemoglobin and fetal fraction suggests that maternal red blood cell lysis is not a reliable indicator of white blood cell lysis in this study group. Our observations are consistent with those of Zhao et al. [18], who performed a comparison between blood collection tubes by studying the characteristics of blood specimens drawn from six healthy, non-pregnant individuals and stored over a period of 0–14 days before plasma processing. They found differences in characteristics between the tubes but, overall, hemoglobin concentration as measured by spectrophotometry did not synchronize with release of DNA from white blood cell lysis. We now add a

Fig. 2 Box plot depicting fetal fraction in 203 specimens with significant hemolysis compared with 12,705 specimens without significant hemolysis. The whiskers represent minimum and maximum values. Black diamonds represent the mean. There was no difference in the mean fetal fraction between the groups ($p = 0.091$)

systematic evaluation in a large cohort of pregnant women, 115 subjects with replicate tubes and 12,908 subjects in the clinical group, in a real-world setting.

4.1 Study Limitations

The current study relied on visual assessment of hemoglobin concentration rather than using automated spectrophotometric instrumentation, which has been shown to be more reproducible and sensitive for measurement of hemoglobin [19]. However, the lack of an effect on fetal fraction, even with gross differences in hemoglobin concentration (400–950 mg/dL), indicates that the precision of this sophisticated instrumentation would not have been required for this study. Furthermore, some of the high hemoglobin concentrations observed in this study are likely to be beyond the upper limit for measurement by some equipment. In contrast, since fetal cfDNA makes up only a minor fraction of the cfDNA present in maternal plasma, detection of subtle changes in fetal fraction is important. Rather than quantitative polymerase chain reaction, which has been used in previous studies [11], we used single nucleotide polymorphism-based fetal fraction determination, which has been shown to be highly accurate and reproducible [16]. It should be noted that the conclusion that hemolysis is not associated with lower fetal fraction and should not be a reason for specimen rejection was clearly demonstrated in the context of Roche Cell-Free DNA Collection Tubes and the Harmony prenatal test. The Harmony prenatal test extracts cfDNA from the plasma before enzymatic manipulations that could be sensitive to the presence of hemoglobin. One could assume these data would be generalizable to other specialized cfDNA collection tubes and testing platforms that extract the cfDNA before sensitive enzymatic processing; however, given differences in methodology, further studies would be needed to demonstrate this.

The technical and biological complexity of prenatal cfDNA testing necessitates rigorous quality control, such as the measurement of fetal fraction, which means that a proportion of tests will not yield a genetic result on the first draw. Refining the quality control measures used by the laboratory will best serve patients by both ensuring quality results and minimizing the number of specimens needing to be redrawn. Based on our experience in this study, in which approximately 1.6% of clinical samples were received with significant hemolysis, eliminating hemolysis as a cause for specimen rejection will significantly decrease the number of specimens needing to be redrawn.

Compliance with Ethical Standards

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