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**Title: Occult fetomaternal hemorrhage in women with pathological placenta with respect to permeability**

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**Short running title: Occult FMH**

## ABSTRACT

**Aim:** Women with preeclampsia, placenta previa (PP), placental abruption (PA), and placental mesenchymal dysplasia (PMD) were suggested to have placental permeability dysfunction. This study was performed to determine whether occult fetomaternal hemorrhage (FMH) is common in women with such complications and in women with non-reassuring fetal status (NRFS).

**Methods:** Forty-one antenatal and 39 postnatal blood samples were obtained from 46 women consisting of 11 with complications (5, 3, 2, and 1 with preeclampsia, PP, PA, and PMD, respectively) and 35 controls without such complications. To estimate amount of fetal red blood cells (RBC), flow cytometry was performed using the FCC system with two antibodies against fetal hemoglobin (HbF) and carbonic anhydrase and the  $\beta$ - $\gamma$  system with two monoclonal antibodies against hemoglobin  $\beta$ -chain and hemoglobin  $\gamma$ -chain (Hb- $\gamma$ ). A diagnosis of FMH was made when the fraction size of the isolated cell population on scatter plots expressing HbF alone or Hb- $\gamma$  alone accounted for  $\geq 0.02\%$  of the total cell population on scatter plots.

**Results:** FMH was identified in five women, including one each with preeclampsia, PA, PP, PMD, and no complications. Thus, the prevalence rate of FMH was significantly higher in women with complications than in controls (36% [4/11] vs. 2.9% [1/35], respectively,  $P = 0.009$ ). The FMH occurrence rate did not differ between women with and without NRFS (7.7% [1/13] vs. 12% [4/33], respectively,  $P = 1.000$ ).

**Conclusions:** The risk of fetal RBC trafficking into the maternal circulation may be increased in women complicated with preeclampsia, PA, PP, and PMD.

**Key words:** alpha-fetoprotein, fetomaternal hemorrhage, placental mesenchymal dysplasia, placenta previa, preeclampsia

## INTRODUCTION

Clinical fetomaternal hemorrhage (FMH) is rare, with an occurrence rate of 1 in 3000 – 10000 women<sup>1-4</sup> and is suspected in women exhibiting clinical symptoms due to fetal anemia, such as non-reassuring fetal status (NRFS) on fetal heart rate (FHR) tracing, decreased fetal movement, and/or fetal hydrops. As FMH of  $\geq 30$  mL and  $\geq 80$  mL occur in 1 of 333 women<sup>5,6</sup> and 1 of 1146 women<sup>1</sup>, respectively, the majority of FMH cases of  $\geq 30$  mL occur with minimal clinical signs and symptoms<sup>1</sup>. Thus, occult FMH defined as FMH without any clinical signs may be relatively common among the general pregnant population.

The placenta is the interface between fetal and maternal circulations. Preeclampsia, placenta previa, placental abruption, and placental mesenchymal dysplasia (PMD) are all conditions associated with placental pathology with respect to dysregulated interface function. Cell-free fetal DNA level is elevated in the maternal circulation in preeclampsia<sup>7</sup>. Cell-free human placental lactogen mRNA, which is produced entirely by trophoblastic cells, is increased in the maternal circulation in placenta previa<sup>8</sup>. The level of alpha-fetoprotein (AFP), the majority of which is produced by the fetal liver, is elevated in the maternal circulation before the occurrence of placental abruption<sup>9</sup>. Aneurysmally dilated vessels on the fetal surfaces are characteristic features of PMD placentas<sup>10,11</sup>, in which AFP is markedly elevated in the maternal circulation<sup>10,12,13</sup>. These complications may allow more fetal red blood cell (RBC) trafficking into the maternal circulation compared to uncomplicated pregnancies. However, this issue has not been studied extensively.

The present study was conducted to test our hypothesis that occult FMH is relatively common in women complicated with preeclampsia, placenta previa, placental abruption, and PMD using flow cytometry, which is more sensitive for detection of fetal RBC than high-performance liquid chromatography (HPLC) or the acid elution method (Kleihauer–Betke test)<sup>14</sup>. In addition, we examined whether occult FMH is common in cases with NRFS based on FHR tracing.

## MATERIALS AND METHODS

This study was approved by the Institutional Review Board of Hokkaido University Hospital and written informed consent was obtained from all pregnant participants prior to enrollment.

### Participants

During the study period between June 2014 and February 2015, a total of 46 women participated in this study after giving informed consent and gave birth to singletons at our institution. Forty-one antenatal blood samples were obtained from 41 women within 25 days prior to delivery and 39 postnatal blood samples were obtained from 39 women within 3 days after delivery (Table 1). The demographic characteristics of the participants were obtained from medical records. Women were divided into two groups with and without complications, including preeclampsia, placenta previa,

placental abruption, and PMD. During the study period, there were 20, 5, 3, and 1 maternities at our institute complicated with preeclampsia alone, placenta previa, placental abruption, and PMD, respectively. These four complications were designated as placental diseases in this study.

### **Preparation of blood samples for the detection of fetal red blood cells**

The red blood cells (RBC) collected from participants were washed three times in phosphate-buffered saline for 30 minutes in a solution containing formaldehyde, washed once more, and then permeabilized with sodium dodecyl sulfate (SDS) for 3 minutes at room temperature. The flow cytometric study was then performed for detection of fetal RBC by two methods: (1) designated as the FCC test using a Fetal Cell Count™ Kit (IQ Products BV, Groningen, The Netherlands) containing fluorescein isothiocyanate-labeled monoclonal mouse anti-human fetal hemoglobin (HbF) antibody and phycoerythrin-labeled polyclonal rabbit anti-human carbonic anhydrase (CA) antibody; and (2) designated as the  $\beta$ - $\gamma$  test developed in our laboratory containing peridinin-chlorophyll protein complex – cyanine 5.5-conjugated monoclonal anti-human hemoglobin  $\beta$  antibody (hemoglobin  $\beta$  (37-8):PerCP-Cy5.5; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and fluorescein-conjugated monoclonal anti-human hemoglobin  $\gamma$  antibody (hemoglobin  $\gamma$  (51-7): Santa Cruz Biotechnology).

### **Definition of FMH in this study**

Molecules originating from fetal RBC were defined as an isolated cell population on scatter plots expressing HbF alone in the FCC system and/or an isolated cell population on scatter plots expressing hemoglobin  $\gamma$ -chain (Hb- $\gamma$ ) alone in the  $\beta$ - $\gamma$  system. In our preliminary experiments, an isolated cell population on scatter plots expressing HbF alone was identified in adult whole blood containing  $\geq 0.02\%$  (vol/vol) umbilical cord blood (UCB) in the FCC system (Fig. 1, upper right). Similarly, the isolated cell population on scatter plots expressing Hb- $\gamma$  alone was identified in adult whole blood containing  $\geq 0.02\%$  (vol/vol) UCB in the  $\beta$ - $\gamma$  system (Fig. 1, lower right). However, no isolated cell population on scatter plots was identified in adult whole blood containing 0.01% UCB in either system (Fig. 1, middle panels). In adult whole blood containing  $\geq 0.02\%$  UCB, actual readings of fraction sizes of isolated cell populations on scatter plots were correlated with UCB fraction size (Fig. 2). Therefore, a positive test result was defined as a fraction size of the isolated cell population on scatter plots expressing HbF alone  $\geq 0.02\%$  for the FCC system and that expressing Hb- $\gamma$  alone  $\geq 0.02\%$  for the  $\beta$ - $\gamma$  system. FMH was diagnosed in women with at least one positive test result with either antenatal or postnatal FCC or  $\beta$ - $\gamma$  system.

### **Statistical analyses**

Statistical analyses were performed using the JMP® Pro 11 statistical software package (SAS, Cary, NC). Differences in the means were tested using Wilcoxon's rank sum test between each group, and categorical variables were compared using Fisher's exact test. In all analyses,  $P < 0.05$  was taken to indicate statistical significance.

## RESULTS

Of the 46 women included in the study, six had preeclampsia (one with later placental abruption was assigned to the placental abruption group), three had placenta previa, two experienced placental abruption (one also had preeclampsia), and one had PMD. Thus, 11 of the 46 women had placental diseases (Table 1).

Not all of the 46 participants underwent both ante- and postnatal examinations with FCC and  $\beta$ - $\gamma$  systems (Table 2). Nine of the 11 participants with placental diseases (82%) and 24 of 35 without placental diseases (69%) underwent tests with both the FCC system and  $\beta$ - $\gamma$  system antepartum and 5 of 11 with placental diseases (45%) and 23 of 35 without placental diseases (66%) underwent test with both the FCC system and  $\beta$ - $\gamma$  system postpartum. In total, 31 and 111 tests, including both systems for 11 and 35 women with and without placental diseases, respectively, were performed. Positive test results were obtained in 9 cases (29%) for women with placental diseases and only 3 times (2.7%) for women without placental diseases ( $P = 0.001$ ). The frequencies of positive test results were significantly higher in both systems in postpartum women with than without placental diseases (Table 2). Finally, four of the 11 with placental diseases (36%) and one of the 35 without placental diseases (2.9%) exhibited at least one positive test result and were diagnosed with FMH ( $P = 0.009$ ).

Actual scatter plot patterns and clinical details of the five FMH women are shown in Fig. 3 and Table 3, respectively. Two cases (Cases 1 and 5) were complicated with preeclampsia, and each was complicated with placental abruption (Case 1), PMD (Case 2), and placenta previa (Case 3). Only Case 4 did not have placental diseases, but exhibited borderline positive test results three times. Clinical FMH occurred in two women (Cases 2 and 5) giving birth to anemic infants with UCB hemoglobin concentrations of 8.3 and 3.4 g/dL, respectively.

Thirteen of the 46 women were judged as having NRFS according to the Japanese Guidelines for Obstetrical Practice<sup>15</sup> based on antenatal and or intrapartum FHR tracing (Table 1). FMH occurred in one (Case 5 in Table 3) of 13 with NRFS (7.7%), while it occurred in four of 33 women without NRFS (12%) ( $P = 1.000$ ).

## DISCUSSION

Although the population size in the present study was small, the prevalence rate of FMH was significantly higher for women with placental diseases than for those without placental diseases. Consistent with our hypothesis, these results suggested that perinatal fetal RBC trafficking into the maternal circulation was likely to occur in women with four complications, i.e., preeclampsia, placenta previa, placental abruption and PMD.

In this study, the prevalence rate of FMH was 2.9% (1/35) for control women without placental diseases, which was reasonable based on previous reports<sup>1, 5, 6, 16</sup>. We set a cut-off point of 0.02% in this study for both the FCC and  $\beta$ - $\gamma$  test systems. This implied that FCC and  $\beta$ - $\gamma$  systems gave a positive test result when  $\geq$  approximately 1.0

mL of fetal blood was contained in 5000 mL of maternal circulating blood. The prevalence rates of FMH of  $\geq 150$  mL,  $\geq 80$  mL, and  $\geq 30$  mL were estimated to be 0.2, 0.9, and 3 per 1000 births, respectively<sup>1,5,6</sup>. Thus, the incidence of FMH of  $\geq 1.0$  mL was expected to be far more prevalent, reaching several percent among the general population. Indeed, in a study examining the incidence of positive Kleihauer–Betke test results in low-risk, third trimester, and asymptomatic women, as many as five of 98 (5.1%) had a positive test result, indicating FMH of 5 mL and 40 mL in four and another cases, respectively<sup>16</sup>. In another study examining the risk of FMH after external cephalic version (ECV) at term for women with breech presentation using the FCC system<sup>17</sup>, although a higher cut-off point of 0.05% was used compared to our cut-off value of 0.02%, three occurrences of FMH were detected among 50 women (6.0%) after ECV<sup>17</sup>. These three FMH women showed HbF fraction sizes of 0.06%, 0.08%, and 1.0%, respectively<sup>17</sup>.

In contrast to the FMH prevalence rate of 2.9% among control women, one of five women (20%) with preeclampsia alone, one of three with placenta previa (33%), one of two with placental abruption (50%), and one of one with PMD (100%) exhibited FMH. As described in the Materials and Methods, we encountered 20, 5, 3, and 1 maternities complicated with preeclampsia alone, placenta previa, placental abruption and PMD, respectively, during the study period. Thus, 15 of 20 with preeclampsia alone, two of five with placenta previa, and one of three with placental abruption did not participate in this study. However, even on the assumption that these background women with preeclampsia, placenta previa, or placental abruption participated in this study and exhibited exclusively negative test results on FCC and  $\beta$ - $\gamma$  tests, the prevalence rate of FMH would be 5.0% (1/20) for women with preeclampsia, 20% (1/5) for those with placenta previa, and 33% (1/3) for those with placental abruption. Thus, it appeared that perinatal fetal RBC trafficking into the maternal circulation was likely to occur in women with these complications. In these four conditions, the placenta was suggested to have dysregulated function as an interface between fetal and maternal circulation as molecules derived from the fetus, such as cell-free fetal DNA, cell-free placental lactogen mRNA, and AFP, are often elevated in the maternal circulation in cases with these complications<sup>7, 8, 9, 10, 12, 13</sup>. Thus, the present study confirmed dysregulated placental function with respect to permeability in women with such placental diseases.

However, relatively few studies have evaluated the correlations between these complications and the risk of FMH. In an earlier study by Christensen<sup>18</sup>, one of three women with relatively severe FMH was complicated with preeclampsia. In a study examining the risk of FMH in 14 women with preeclampsia and 11 control women using the FCC system<sup>19</sup>, the mean volume of FMH was significantly greater in women with preeclampsia than in controls (4.0 mL vs. 1.0 mL, respectively)<sup>17</sup>. Based on theoretical considerations, the prophylactic use of anti-D immune globulin is recommended in placenta previa patients with antenatal hemorrhage<sup>20</sup>. Placental abruption is reported as a strong predictor of FMH<sup>2</sup>. In PMD pregnancies, as an extraordinarily higher risk of fetal death in utero is partially ascribed to FMH<sup>21</sup>, PMD was considered as an apparent risk factor for FMH.

In this study, the prevalence rate of FMH did not differ between women with and without NRFS. However, we expected a higher frequency of FMH in women with NRFS for several reasons, as follows. NRFS occurs frequently during parturition as a result of umbilical cord compression. Theoretically, the cord compression would cause fetal blood congestion within the placenta, as the return of fetal blood from the placenta via the umbilical vein is hampered compared to fetal blood flow into the placenta via the umbilical artery. The assumed fetal blood congestion within the placenta was expected to facilitate FMH. However, our results suggested that such a mechanism hardly caused fetal RBC trafficking into the maternal circulation. Indeed, none of seven intrapartum NRFS women showed positive FCC and  $\beta$ - $\gamma$  test results in this study.

In conclusion, the present study suggested that FMH is common in patients with preeclampsia, placenta previa, placental abruption, and PMD. Most patients with FMH of  $\leq 80$  mL are asymptomatic<sup>1</sup> and the clinical outcomes may not be altered in these patients with minor FMH. However, it may be important to consider that women with preeclampsia, placenta previa, placental abruption, and PMD are prone to FMH.

#### **ACKNOWLEDGEMENTS**

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#### **DISCLOSURE**

No author has any potential conflict of interest.

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## FIGURE LEGENDS

Fig. 1. Flow cytometric study of male adult blood mixed with UCB in final UCB concentrations of 0.01% and 0.02% (vol/vol)

ND, not detectable isolated scatter plot

Male adult blood containing 0.00%, 0.01%, and 0.02% umbilical cord blood (UCB) was used for examinations with the FCC and  $\beta$ - $\gamma$  systems. No isolated scatter plot was identified in the blood containing 0.00% and 0.01% UCB in both FCC and  $\beta$ - $\gamma$  systems. However, the isolated scatter plot was identified in the blood containing 0.02% UCB, and the readings were 0.017% in the FCC and 0.019% in the  $\beta$ - $\gamma$  system.

Fig. 2. Association between actual measurements on flow cytometric systems and actual fractions of UCB in the mixed blood

The whole blood from a male adult volunteer was mixed with UCB obtained from a healthy term infant with parental consent to final UCB concentrations (vol/vol) of 0.01%, 0.02%, 0.05%, 0.1%, 1.0%, and 5.0%. The actual measurements on both FCC and  $\beta$ - $\gamma$  systems were well correlated with actual UCB concentrations of  $\geq 0.02\%$ . Thus, both systems were able to detect fetal RBC in the whole blood containing  $\geq 0.02\%$  UCB.

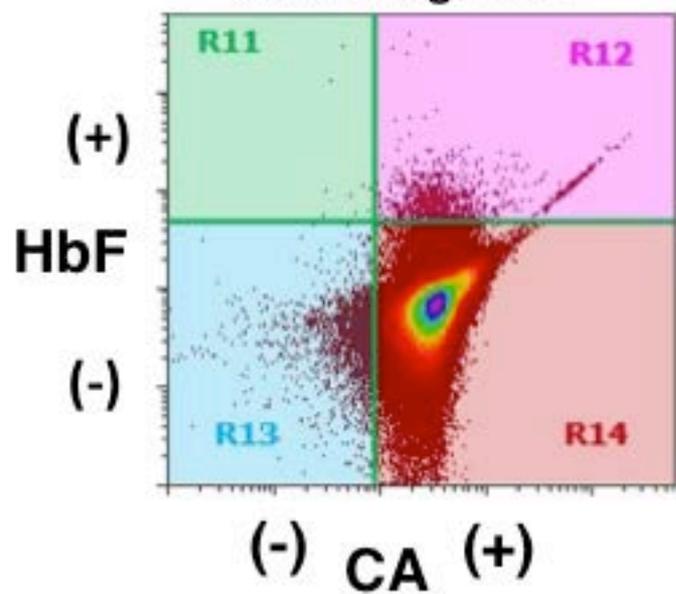
Fig. 3 Five cases that were identified as having FMH

The percentages of scatter plot populations with HbF expression alone and Hb- $\gamma$  expression alone were 0.04% and 0.01%, respectively, in the postpartum blood of Case 1. Corresponding values were 0.04% and 0.06%, respectively, in the postpartum blood of Case 2; 0.11% and 0.09%, respectively, in the antepartum blood of Case 3; 0.02% and 0.03%, respectively, in the antepartum blood of Case 4; and 3.03% and 2.45%, respectively, in the antepartum blood of Case 5 (this case was described previously in a case report<sup>12</sup>).

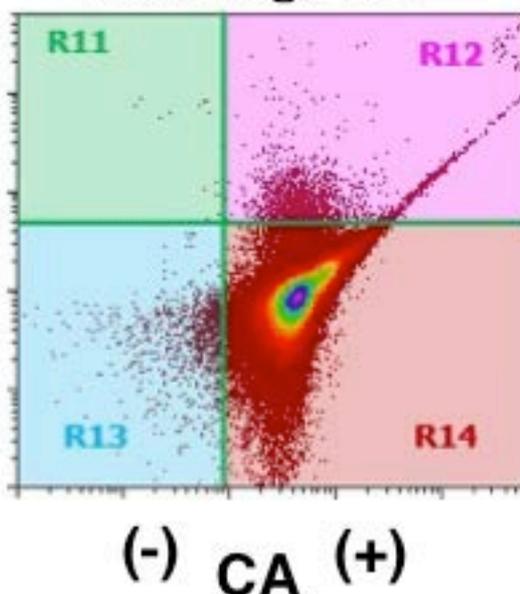
Fig. 1

# FCC kit

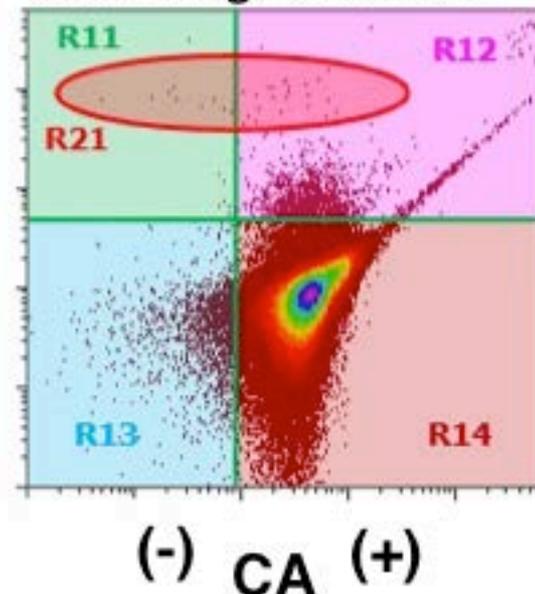
UCB 0.00%  
Reading, ND



UCB 0.01%  
Reading, ND

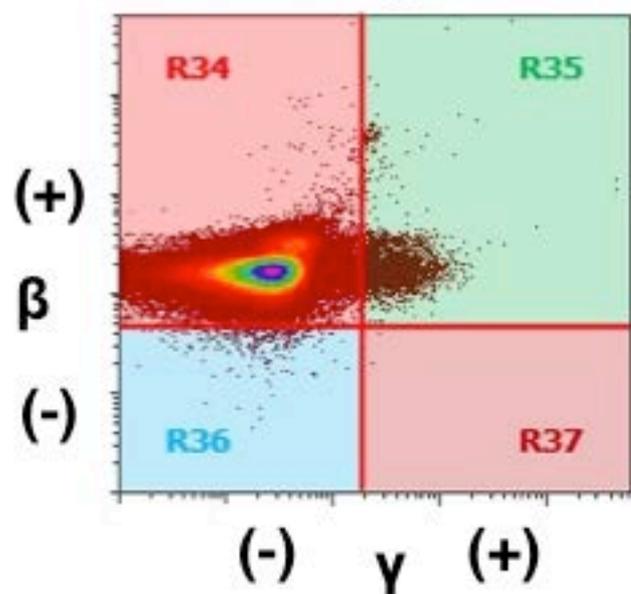


UCB 0.02%  
Reading, 0.017%

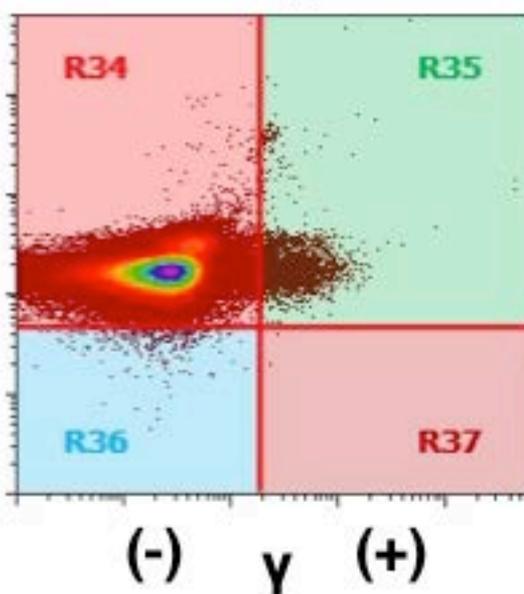


# $\beta$ - $\gamma$ system

UCB 0.00%  
Reading, ND



UCB 0.01%  
Reading, ND



UCB 0.02%  
Reading, 0.019%

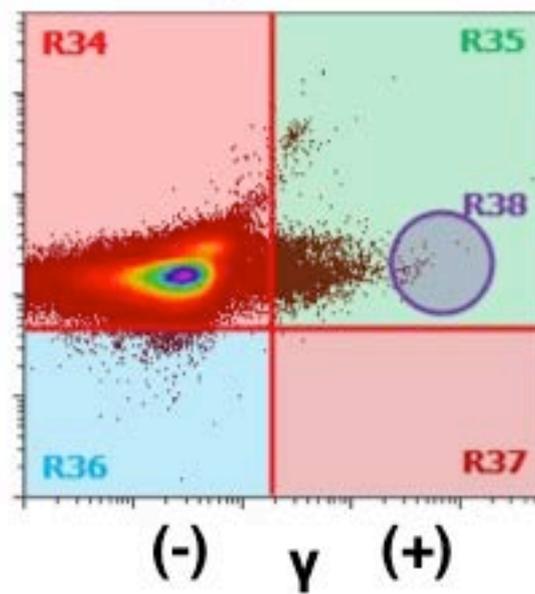


Fig. 2

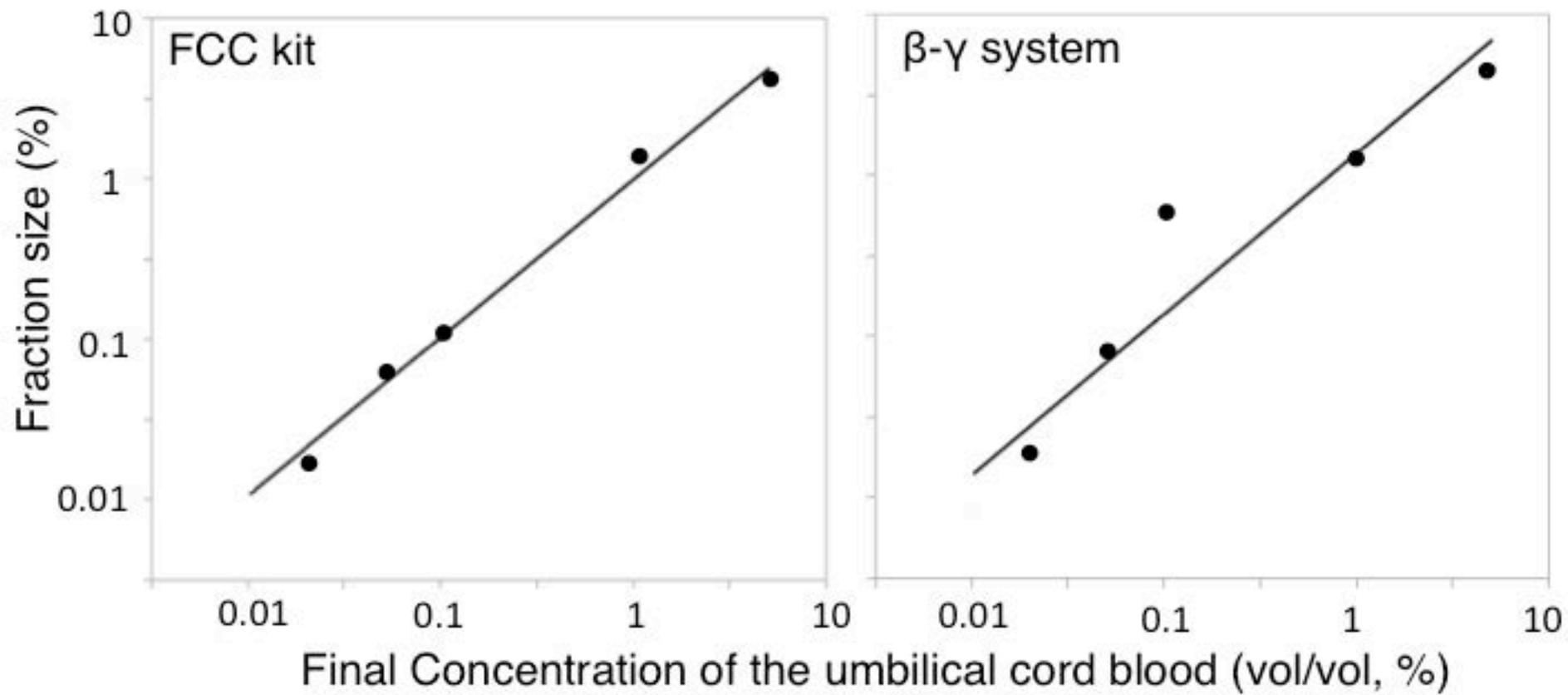
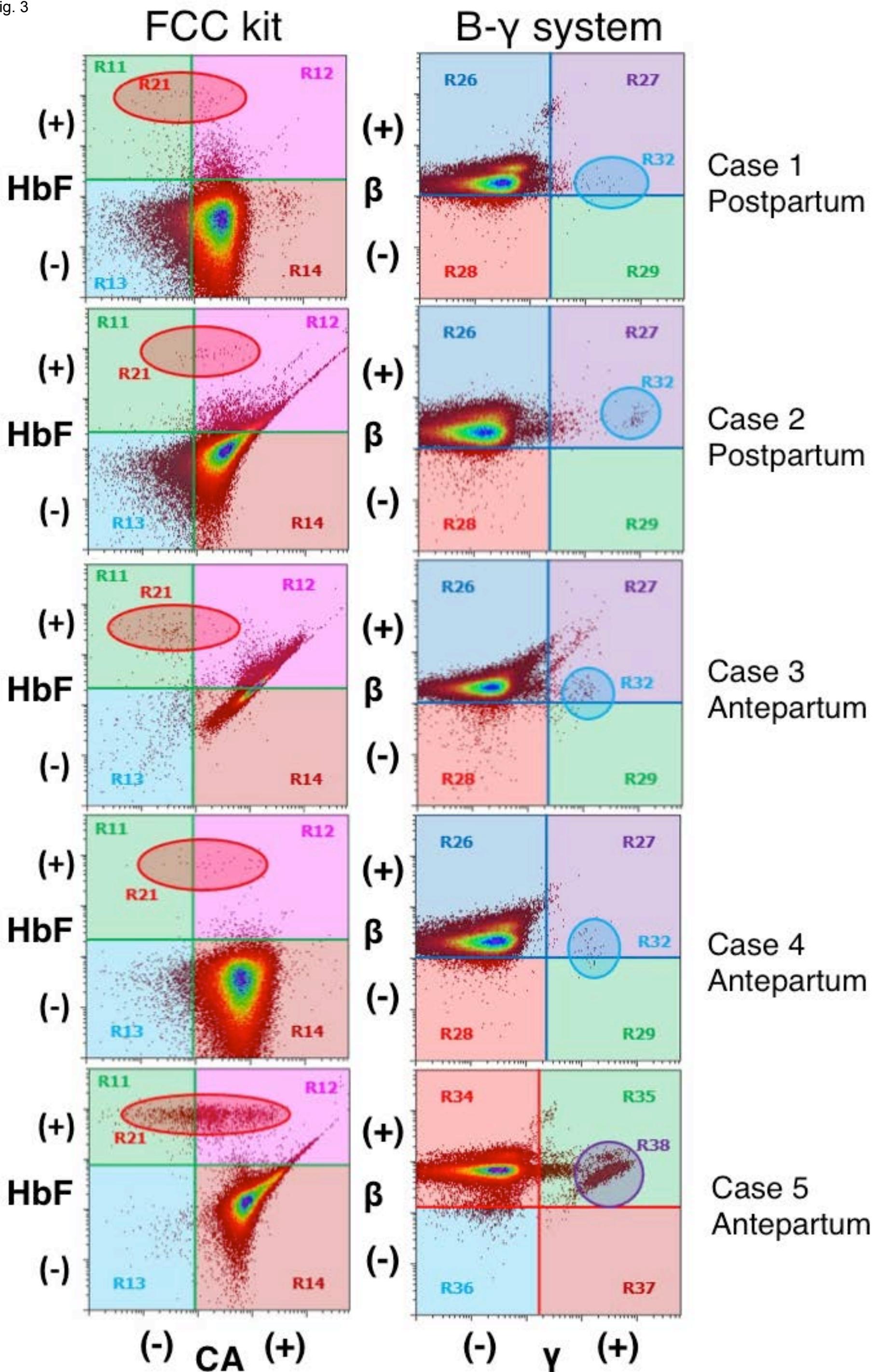


Fig. 3



**Table 1. Demographic characteristics of 46 participants**

	Placental diseases		<i>P</i> -value
	Present ( <i>n</i> =11)	Absent ( <i>n</i> =35)	
Age (years)	36.5±4.3	33.9±5.3	0.171
Antepartum NRFS	2 (18%)	4 (11%)	0.619
Intrapartum NRFS	0 (0%)	7 (20%)	0.171
Nulliparous	7 (64%)	22 (63%)	1.000
Placental diseases			
Preeclampsia*	5 (55%)	NA	
Placenta previa	3 (27%)	NA	
Placental abruption	2 (18%)	NA	
PMD	1 (9%)	NA	
Gestational week at delivery	32.4±4.0	38.3±1.7	0.165
<37	8 (73%)	1 (3%)	<0.001
<34	7 (64%)	1 (3%)	<0.001
Caesarean delivery	11 (100%)	20 (57%)	0.009
Birth weight (g)	1911±923	2916±571	0.002
1-min Apgar score <8	8 (73%)	6 (17%)	0.001
5-min Apgar score <8	0 (0%)	6 (17%)	0.311
[Hb] in the UCB (g/dL)	12.5±4.0	14.6±2.0	0.097
Blood sampling			
Antepartum	9 (82%)	32(91%)	0.580
Postpartum	8 (73%)	31 (89%)	0.333
Fetomaternal hemorrhage	4 (36%)	1 (2.9%)	0.009

NRFS, non-reassuring fetal status based on FHR tracing; PMD, placental mesenchymal dysplasia; [Hb], hemoglobin concentration; UCB, umbilical cord blood. \*, There were six women with preeclampsia, but one with later onset of placental abruption was assigned to placental abruption.

**Table 2. Results of flow cytometric study**

Placental diseases	Present ( <i>n</i> =11)	Absent ( <i>n</i> =35)	<i>P</i> -value
Positive test result with FCC system			
Antenatal blood	2/9 (22%)	1/33 (3%)	0.111
Postnatal blood	3/8 (38%)	1/31 (3%)	0.022
Positive test result with $\beta$ - $\gamma$ system			
Antenatal blood	2/9 (22%)	1/24 (4%)	0.174
Postnatal blood	2/5 (40%)	0/23 (0%)	0.027
No. of women with FMH*	4/11 (36%)	1/35 (2.9%)	0.009

\*, FMH was diagnosed in women with at least one positive test result.

Antenatal blood was examined in 42 and 33 of the 46 women with FCC and  $\beta$ - $\gamma$  systems, respectively. Postnatal blood was examined in 39 and 28 of the 46 women with FCC and  $\beta$ - $\gamma$  systems, respectively.

**Table 3. Details of five patients detected to have FMH**

Case (yrs)	Age	Placental disease	Delivery		AFP (ng/mL)		Hb F fraction				UCB [Hb] (g/dL)
			GW	mode	Antep	Postp	FCC		$\beta$ - $\gamma$		
							Antep	Postp	Antep	Postp	
1*	38	PE/PA	27	CS	348	86	0.01%	0.04%	0.01%	0.01%	13.2
2*	26	PMD	30	CS	10786	4990	NA	0.04%	NA	0.06%	8.3
3	36	Previa	36	CS	447	258	0.11%	0.00%	0.09%	NA	14.5
4	37	None	39	TV	141	84	0.02%	0.02%	0.03%	NA	14.3
5*†	43	PE/HELLP	31	CS	23000	NA	3.03%	1.74%	2.45%	2.71%	3.4

\*, Nulliparous woman; †, NRFS on FHR tracing; PE, preeclampsia; PA, placental abruption; PMD, placental mesenchymal dysplasia; GW, gestational week at delivery; CS, caesarean section, TV, transvaginal delivery, AFP,  $\alpha$ -fetoprotein; Antep, antepartum data; Postp, postpartum data; NA, not available blood samples.