

# Feto-maternal haemorrhage assessment in a woman with a large population of red blood cells containing fetal haemoglobin

Ocena przecieku płodowo-matczynego u kobiety z dużą populacją krwinek czerwonych zawierających hemoglobinę płodową

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## Abstract

**Background:** FMH quantification is necessary to calculate an individual dose of prophylactic anti-RhD immunoglobulin and to diagnose fetal anaemia causes. We encountered a healthy woman with a numerous RBCs containing fetal haemoglobin (HbF).

**Aims:** To investigate the cause of this sign and the correct evaluation of fetal RBCs in maternal circulation.

**Materials and Methods:** Patient's samples and artificial mixtures were tested by microscopic Kleihauer-Betke (KB) and flow cytometric (FC) tests with anti-HbF + anti-CA (carbonic anhydrase), and with anti-D. The patient's blood count with reticulocyte parameters, and concentration of bilirubin, haptoglobin, iron, transferrin, ferritin, hepcidin, sTR, HbF, HbA2 were measured. Genes coding the  $\beta$ - and  $\gamma$ -globin were sequenced.

**Results:** It was impossible to distinguish the population of fetal and maternal HbF positive cells using KBT and FC with anti-HbF. Application of anti-CA and anti-D allowed to separate them.

Maternal blood haematological and biochemical parameters were normal but HbF was 3.3% of total Hb concentration (normal <1%). There were no mutations in the  $\beta$ - and  $\gamma$ -globin genes, but Xmn I polymorphism at -158 position in  $\gamma$ -globin gene was detected in the homozygous state.

**Conclusion:** A very large population of HbF positive cells sometimes can be detect in a healthy woman. Implementation of the various procedures for FMH assessment is necessary in the such case, otherwise, the detection of fetal erythrocytes may not be possible or can give false results.

Key words: **feto-maternal haemorrhage (FMH) / Kleihauer-Betke test (KBT) / flow cytometry tests (FCTs) / F cells / fetal haemoglobin (HbF) / carbonic anhydrase (CA) /  $\beta$ - and  $\gamma$ -globin genes /**

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## Streszczenie

**Wstęp:** Ilościowa ocena przecieku płodowo-matczynego jest niezbędna do obliczenia indywidualnej profilaktycznej dawki immunoglobuliny anti-RhD oraz diagnozowania przyczyny niedokrwistości płodu. U zdrowej kobiety przed i po porodzie wykryto liczne krwinki czerwone zawierające hemoglobinę płodową (HbF).

**Cele:** Zbadanie przyczyn tego zjawiska oraz poprawna ocena płodowych krwinek czerwonych w krążeniu matki.

**Materiał i metody:** Próbkę krwi pacjentki oraz przygotowane mieszaniny krwinek badano przy użyciu mikroskopowego testu Kleihauera-Betke (KB) oraz metodą cytometrii przepływowej (FC) z przeciwciałami anti-HbF+ anti-CA (anhydraza węglanowa) i anti-D. Oceniono morfologię krwi łącznie z parametrami retykulocytów oraz stężenie bilirubiny, haptoglobiny, żelaza, transferyny, ferrytyny, hepcydyny, sTR, HbF, HbA2. Sekwencjonowano geny kodujące  $\beta$ - i  $\gamma$ -globiny.

**Wyniki:** Za pomocą testów KB i FC z anti-HbF nie można było odróżnić populacji HbF dodatnich krwinek czerwonych płodu i matki. Zastosowanie przeciwciał anti-CA i anti-D pozwoliło na rozdzielenia ich. Hematologiczne i biochemiczne parametry krwi matki były w normie, ale stężenie HbF stanowiło 3,3 % z całkowitego stężenia Hb (norma <1%). Nie stwierdzono mutacji w genach  $\beta$ - i  $\gamma$ -globin, ale wykryto polimorfizm Xmn I w pozycji -158 genu  $\gamma$ -globiny, w postaci homozygotycznej.

**Wniosek:** U zdrowej kobiety czasem można wykryć dużą populację komórek HbF dodatnich. W takim przypadku konieczne jest zastosowanie różnych procedur oceny przecieku płodowo-matczynego, w przeciwnym razie wykrycie erytrocytów płodu może być niemożliwe lub można uzyskać fałszywe wyniki.

Słowa kluczowe: **przeciek płodowo-matczyny / test Kleihauera-Betke / cytometria przepływowa / komórki F / hemoglobina płodowa / anhydraza węglanowa / geny  $\beta$ - i  $\gamma$ -globin /**

## Introduction

Detection and quantification of feto-maternal haemorrhage (FMH) in maternal blood sample is necessary to calculate an appropriate individual dose of anti-RhD immunoglobulin in the prophylaxis of haemolytic disease of the fetus / newborn (HDFN) for RhD negative mothers, whose fetuses are RhD positive [1-3]. There are also some non-immune fetal anaemias and/or hydrops fetalis [4, 5]. FMH detection can be helpful in the differentiation of their causes. Several methods based on biochemical or serological differences between the red blood cells (RBCs) of mother and fetus have been introduced for calculation of the percentage of fetal cells in maternal circulation [6-9]. We introduced four methods for assessing FMH: the microscopic Kleihauer-Betke test (KBT) and flow cytometry tests (FCTs) with anti-HbF, anti-HbF+CA, anti-D. They have been validated and used to assess fetal RBCs in maternal blood samples before and after childbirth. We have encountered and studied an exceptional case, which is now present.

## Case presentation

Thirty-year-old woman gave birth to a healthy baby (Apgar score: 10) vaginally. The mother and the newborn were O RhD. The result of the microscopic KBT was doubtful and difficult to quantification. The FCT with anti-HbF detected about 30% of HbF+ cells, clearly separated from the HbF- ones. Morphological parameters of the mother and child were normal. There was no information about any haematological problems in the past. The patient was discharged from the hospital on the third day after birth. She agreed to repeat the tests and was investigated one year later. The study was accepted by the Bioethical Committee of our institution.

## Materials and Methods

Detection and quantification of fetal RBCs was performed in maternal and donor's (control) RhD negative blood samples. Mixtures imitating 1% FMH were prepared with cord O RhD+ RBCs.

### FMH evaluation

- I. **KBT** is based on HbF resistance to acid elution while HbA (adult haemoglobin) is removed from cells. After staining, adult cells are seen as "ghosts" on the blood film. Fetal RBCs remain intact, appearing bright pink. Tests were performed using Fetal Red Cell Kit (Guest Medical, UK). RBCs were counted on two slides (2x 5,000) using Miller square.
- II. **FCTs** with following antibodies: anti-HbF labeled with PE (phycoerythrin) (Becton Dickinson, USA), anti-HbF+anti-CA-FITC (labeled with fluorescein isothiocyanate) (Fetal Cell Count kit, IQ Products, USA), anti-D-PE (IQ Products, USA). In all tests 50,000 of RBCs were analyzed using FACS Canto II and BD FACSDiva Software. RBCs HbF+, CA-, D+ were diagnosed as fetal cells.

Tests were accompanied with appropriate negative control samples without fetal RBCs and positive ones which were mixtures of cord RhD+ and donors RhD- RBCs prepared in our laboratory and also commercial FETALtrol kit (Trillium Diagnostics, IQ Products, USA) with low (<0.2%) and high (>1.5%) concentrations of fetal cells.

### The diagnosis of haemolytic anaemia

The blood morphology: red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, Red Blood Cell Distribution Width and reticulocyte

parameters such as mean corpuscular volume; mean content haemoglobin (CHR); mean corpuscular haemoglobin concentration (CHCMr) were measured by the haematological analyzer, biochemical indicators of haemolysis and iron metabolism: concentration of bilirubin, haptoglobin, iron, transferrin, ferritin by biochemical analyzers, hepcidin and soluble transferrin receptor (sTR) by enzyme linked immunosorbent assay (R&D Systems Europe, Ltd., UK) and percentage of HbF and HbA2 by haemoglobin electrophoresis.

### DNA analysis

Genomic DNA was isolated using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, Germany). Polymerase chain reaction (PCR) was used to amplify the promoter region, entire coding sequence and surrounding sequences of the  $\beta$ -globin and both  $\gamma$ -globin ( $^G\gamma$  and  $^A\gamma$ ) genes. The sequences of all primers and the annealing temperatures used for PCR are available upon request. DNA fragments generated by PCR amplification were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) and directly sequenced with BigDye Terminators and the appropriate primers using an ABI Prism 377 sequencer (Applied Biosystems, CA, USA). The *Xmn* I polymorphism at the -158 site upstream of the  $^G\gamma$  globin gene was detected by PCR amplification and restriction enzyme digestion [10].

### Results

Results of KB and FC tests in the maternal blood sample were the same before and immediately after childbirth and one year later. Using KBT, approximately 10% of the RBCs were well stained, other cells were stained very weakly, and about 70% looked like typical adult "ghost" cells.

Figure 1 shows cytograms of the patient's and newborn's (cord) blood mixture, tested with anti-HbF-PE antibody and compared to the control mixture. Population of about 30% HbF+ cells, separated from the HbF- ones is clearly visible. It was impossible to distinguish the population of fetal and maternal RBCs in the patient sample, while it was visible in the control one. Application of anti-HbF-PE+anti-CA-FITC and anti-RhD-PE allowed to separate maternal and fetal cells (Figure 2).

Morphological and biochemical parameters are shown in Table I. The concentration of HbF accounted for 3.3% of total haemoglobin. Hepcidin and sTR concentration, markers of haemolysis compensation by increased erythropoiesis, were normal. Slightly increased haemoglobin content in reticulocytes (CHCMr and CHR) was noted.

Direct sequencing showed no mutations in the  $\beta$ - and  $\gamma$ -globin genes. However, *Xmn* I polymorphism at -158 position in  $\gamma$ -globin gene was detected in the homozygous state.

### Discussion

It is estimated that in healthy adults usually less than 1% of HbF is present among total Hb concentration and values from 1% up to 5% are considered as high [11]. Very high level of HbF concentration, from 50% to 100% can be detected in some congenital diseases such as sickle cell anaemia, haemoglobinopathies or  $\beta$ -thalassaemia, and it compensates the lack of normal adult HbA (HbA1 + HbA2) [12-14]. In such cases, HbF usually is homogenously distributed among the red blood cells and it is named pancellular hereditary persistence

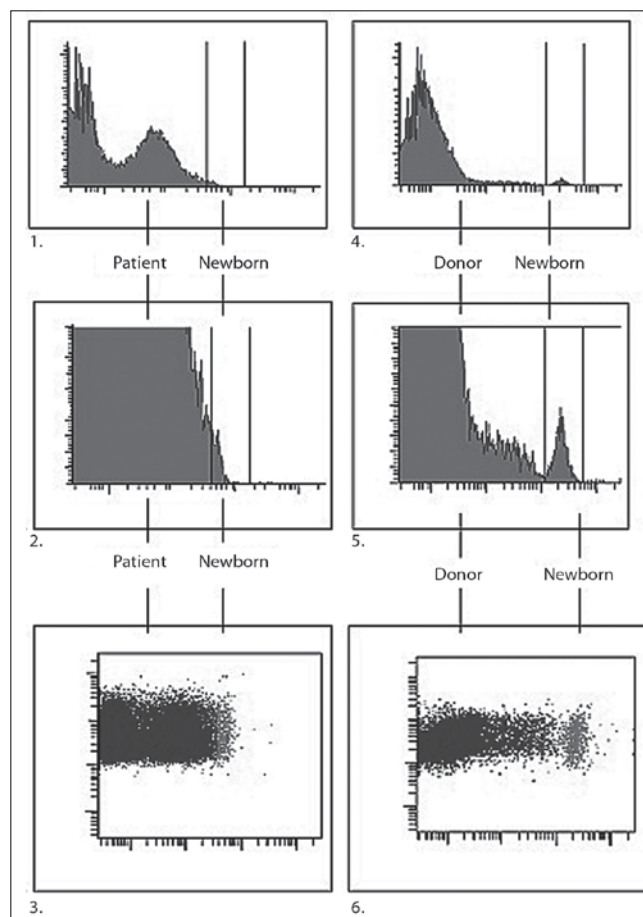


Figure 1. Cytograms of patient's and newborn's (cord) RBCs mixture, imitating 1% FMH (1, 2, 3) compared to the control mixture (4, 5, 6), tested with anti-HbF-PE.

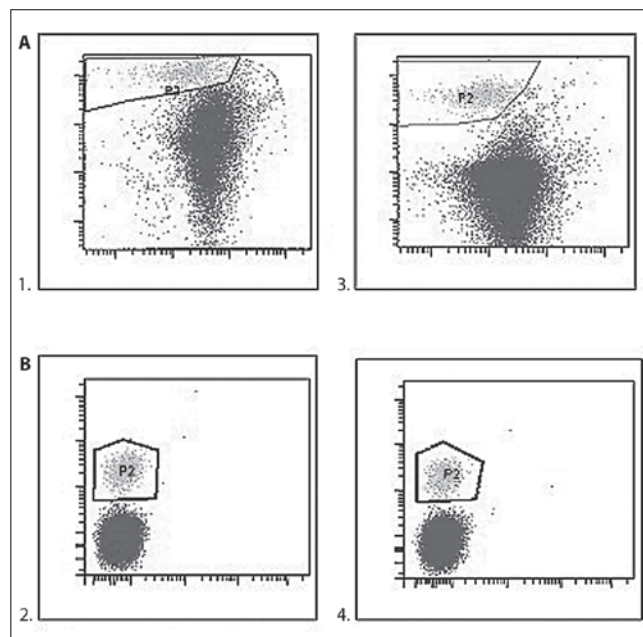


Figure 2. Cytograms of the patient's and newborn RBCs mixture (1, 2) compared to the controls (3, 4), tested with anti-HbF-PE+anti-CA-FITC (A: 1, 3) and anti-D (B: 2, 4) /P2: newborn's RBCs/.

of fetal haemoglobin (HPFH) syndrome. In thalassemias the concentration of HbA2 is often increased. In HPFH cases without congenital haemoglobinopathies or thalassemias HbF usually appears as 15% - 30% of total haemoglobin and it also can be pancellular. In healthy individuals with elevated HbF concentration it is irregularly distributed among erythrocytes and correlated with low percentage (< 1%) of so-called F cells [8]. Munde *et al* indicated the correlation between concentration of HbF and percentage of F cells. However, they noted that the concentration of HbF in F cells may vary from 10% to 40% [6]. Our patient showed different result. Her number of F cells was unexpectedly high (30%) in relation to the level of her HbF (3.3%). Thorpe *et al* described four donors with large population of F cells and moderately increased HbF concentration, but they did not study them in relation to FMH assessment [15]. In three described cases of pregnant women, in which F cells complicated assessment of FMH, the concentration of HbF was 2%, 5.4% or 15% of total HbF and F cells were <4% among all erythrocytes in those samples [16, 17]. Results in those patients varied from day to day, while in our patient they were stable even after a year. In contrast to the report by Janssen and Hoffman or Kush *et al* [9, 17] our FCT with anti-HbF was insufficient to distinguish fetal from maternal RBCs. Our opinion is rather consistent with Leers *et al*, Iyer *et al*, Porra *et al*, and Kumpel *et al* [16, 18-20] that adult F cells in some cases can be differentiated from the fetal cells when additional antibodies are used. Prus and Fibach have just presented study of 12 thalasemic patients, women and men, tested with anti-HbF+CA. They observed additional population of RBCs resembling fetal cells because of the lack of CA, and concluded the coexistence of F cells from two types of stem cell, adult and fetal, lineages. For such women anti-HbF+CA may lead to misinterpretation [21]. For our patient these antibodies were useful, as we have examined a mixture of her and cord blood.

Our patient did not have any thalassemia  $\beta$  signs, including concentration of HbA2, which was normal, as well as morphological and biochemical results. Molecular analyses have not showed the presence of possible mutation responsible for thalassemia  $\beta$  and HPFH syndrome [22-24]. Slightly increased haemoglobin content in reticulocytes (CHCMr and CHr) could be due to a genetic variant, C→T at position -158 of the  $\gamma$  globin gene detected in our patient. The *Xmn* I polymorphism is widespread in all populations and is present at frequency of 0.32 - 0.35 [24]. It has been shown that this variant is associated with elevated HbF level [25].

## Summary

In a healthy mother we sometimes can detect a large population of RBCs containing HbF. It is important to implement different FMH assessment procedures, otherwise the distinction between HbF positive maternal and fetal erythrocytes in such cases may not be possible or may give false results.

### Oświadczenie autorów

1. Agata Gieleżyńska – współautor koncepcji badań, wykonanie testów cytometrycznych i mikroskopowych oraz opracowanie wyników.
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**Table 1.** Patient's morphology of RBCs and parameters of haemolysis and erythropoiesis /\*slightly increased, \*\*increased/

Parameter	Unit	Reference range	Patient's result
Red Blood Cell count	x 10 <sup>12</sup> /l	3.5-5.4	4.71
Haemoglobin concentration	g/dl	12.0-15.0	14.2
Haematocrit	%	37.0-47.0	42.2
Mean Corpuscular Volume	fl	81.0-99.0	89.6
Mean Corpuscular Haemoglobin	pg	26.0-34.0	30.1
Mean Corpuscular Hemoglobin Concentration	g/dl	31.0-36.0	33.0
Red Blood Cell Distribution Width	%	11.5-14.5	12.5
Reticulocyte count	%	0.50-2.50	1.87
Mean Corpuscular Volume r (reticulocyte)	fl	101-119	104.6
Mean Corpuscular Hemoglobin Concentration r	g/dl	23-29	29.9*
Content of Haemoglobin r	pg	25-30	31.2*
Haemoglobin F	%	< 1	3.3 **
Haemoglobin A <sub>2</sub>	%	1.9-3.5	2.2
Bilirubin	mg/dl	< 0.70	0.28
Haptoglobin	mg/dl	> 30	224.9
Iron	µg/dl	37.0-145.0	101.6
Transferrin	mg/dl	200.0-360.0	256.00
Ferritin	ng/dl	13.0-150.0	64.50
Hepcidin	ng/ml	13.3-54.4	20.70
Soluble Transferrin Receptor	nmol/l	8.7-28.1	16.42

3. Jadwiga Fabijańska-Mitek – współautor koncepcji badań przygotowanie manuskryptu i piśmiennictwa – autor zgłaszający i odpowiedzialny za manuskrypt.
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### Konflikt interesów:

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