

# Flowcytometric assessment of fetomaternal hemorrhage during external cephalic version at term

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## Abstract

External cephalic version (ECV) at term is a safe procedure and reduces the incidence of cesarean sections for breech presentation. One of the known complications, however, is an ECV-related disruption of the placental barrier and a subsequent transfusion of fetal blood into maternal circulation. While the incidence of ECV-related fetomaternal hemorrhage (FMH) has been determined recently in a large trial using a manual Kleihauer-Betke test (KBT), questions remain on the amount of ECV-related FMH. KBT, which detects fetal red blood cells (RBC) on the basis of acidic resistance of fetal hemoglobin (HbF), is known to be a sensitive test, yet prone to procedural errors limiting its accuracy in quantifying FMH. In this study we investigated 50 patients for FMH before and after ECV, using a dual-color flow cytometric test kit with a lower limit of quantification of 0.05% fetal RBC in maternal peripheral blood. Three patients had a quantifiable increase of fetal RBC detected after ECV (0.06%; 0.08%; 0.1%). None of these subtle increments was predictable by ECV-related clinical parameters or translated into fetal compromise. Using a sensitive and accurate flow cytometric test method, our data provide further assurance to mothers on the safety of ECV at term.

**Keywords:** Carbonic anhydrase; external cephalic version; F-cells; fetomaternal hemorrhage; flow cytometry.

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## Introduction

Planned cesarean sections have become the standard mode of delivery for women with breech presentation at term. To reduce the number of cesarean sections for this indication, current evidence recommends external cephalic version (ECV) as a safe procedure, which improves the chance of cephalic birth given that the appropriate selection criteria and safety measures are applied [11]. Although there are solid data on its safety, specific complications of ECV have been reported [5]. Most notable among these complications is the disruption of the placental circulation-barrier, with ensuing fetomaternal hemorrhage (FMH), commonly defined as a transfusion of an equivalent of more than 30 mL fetal blood into the maternal circulation. However, none of the small studies or case reports addressing this issue had the necessary statistical power to provide reliable data on the incidence of FMH. Only recently has a cohort study encompassing a total of 1311 women been published; this work tested the amount of fetal cells in the maternal circulation before and after ECV using a manual Kleihauer-Betke test (KBT) [2]. In this well-powered trial, 3% of all patients had a positive KBT after ECV, with one patient having an equivalent of more than 30 mL fetal blood detectable in the maternal circulation. While providing solid data on the incidence of FMH in ECV, the authors stress that the known limits of the KBT are of concern [2, 17]. KBT, which differentiates fetal from maternal red blood cells (RBC) based on the relative resistance of RBCs containing hemoglobin F (HbF) to acid elution, is inexpensive, but known to be prone to a variety of procedural errors, making the quantification of FMH difficult [8]. Flow cytometric test methods have been evolving rapidly, and, by adding additional parameters to HbF, are able to control for HbF positive maternal RBC (=F-cells) that are present in women with sickle cell disease and thalassemia and in up to 25% of healthy women in the second trimester of pregnancy [14, 17]. Recently, Porra et al. evaluated a dual-color flow cytometric test method that identifies F-cells by additionally staining carbonic anhydrase (CA) [18]. As an inducible enzyme in the respiratory chain, CA is only present in adult RBCs, allowing for the specific quantification of more than 0.05% fetal RBC in maternal blood when analyzing  $2 \times 10^6$  RBCs. In the current study, we quantified the number of fetal cells before and after ECV using this dual-color flow cytometry test kit and correlated the amount of FMH with ECV-related parameters.

## Materials and methods

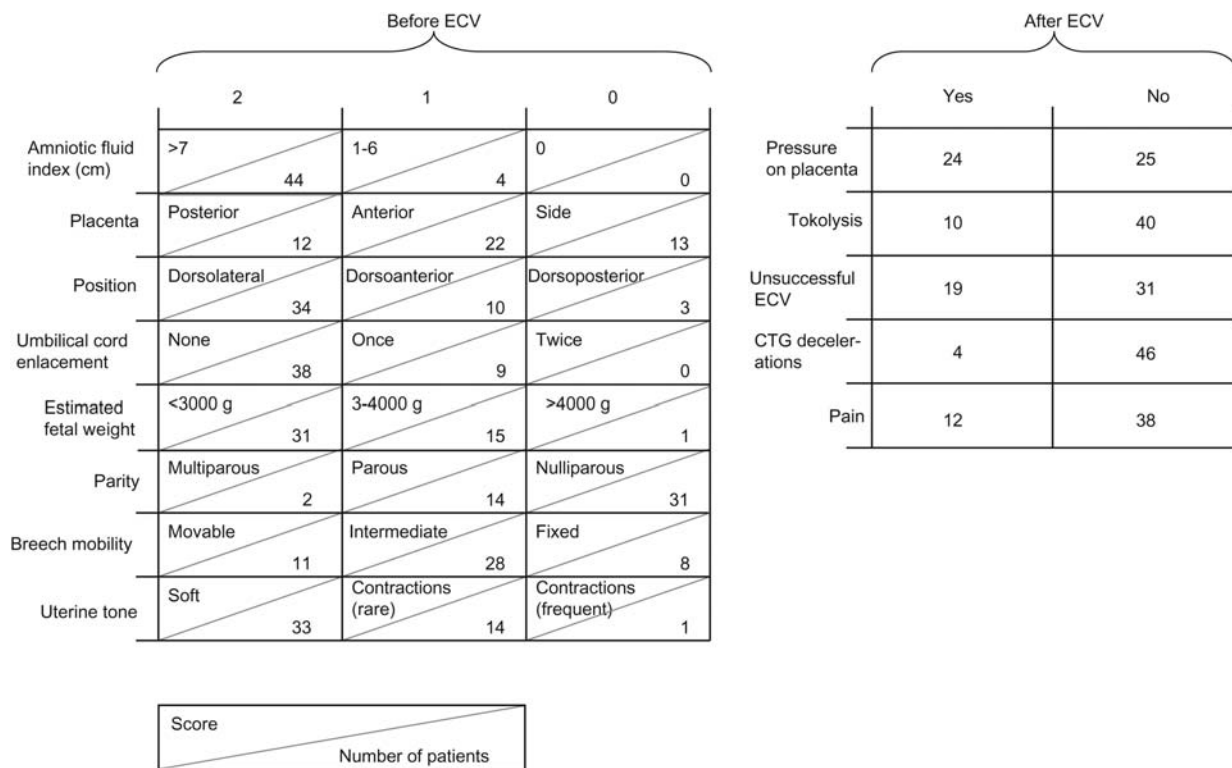
A total of 50 women were included in this prospective observational study of all singleton breech pregnancies during the year 2007 that were subject to ECV in the department of obstetrics and gynecology Klinikum – Innenstadt of the Ludwig-Maximilians University in Munich. All women gave their informed consent, and the institutional Review Board approved the protocol. All women with breech presentation at term were evaluated with a scoring system before ECV. We integrated data regarding amniotic fluid index, fetal and placental position, enlacement of the umbilical cord, estimated fetal weight, parity, uterine tone and breech mobility (Figure 1). ECV was attempted among women with a score of  $>8$  points and was performed according to the current German guideline on delivery in breech presentation [10]. During an initial 30-min non-stress test, an i.v. line was inserted and 5 mL of total venous blood were collected into ethylenediaminetetraacetate (EDTA) anticoagulant. ECV was performed by an experienced consultant and attempted no more than three times. After a post-ECV CTG of 30 min we collected five additional mL of peripheral venous blood and labeled them as “post ECV”.

The procedure was documented with special reference to presumed risk factors for FMH: pressure on placenta, use of tocolytic agent, failure of ECV, number of attempts, CTG decelerations and perceived pain (Figure 1).

Detection and quantification of fetal erythrocytes was performed with the Fetal Cell Count Kit II (IQ-Products, Groningen, The Netherlands). In brief, RBCs were washed three times in

phosphate-buffered saline (PAA-Laboratories, Pasching, Austria) for 30 min in a formaldehyde-containing solution, washed once more, and then permeabilized with sodium dodecyl sulfate solution for 4 min at room temperature. RBCs were then stained with a fluorescein isothiocyanate-labeled monoclonal mouse anti-human fetal hemoglobin (HbF) antibody (recognizing the HbF alpha-chain) and a phycoerythrin-labeled polyclonal rabbit anti-human CA antibody (recognizing the CA-II isoform). The additional antibody for CA removed the need to determine HbF threshold values, as it distinguished between the physiological maternal F-cells (HbF<sup>+</sup>/CA<sup>+</sup>) and the genuine fetal RBCs (HbF<sup>+</sup>/CA<sup>-</sup>). Prior to flow cytometry, optimal detector amplifications and compensations were determined using unstained or single-stained controls as well as cord-blood samples. Data acquisition was performed on a FACSCalibur flow cytometer (Becton-Dickinson, Heidelberg, Germany). For quality control, spiking experiments were performed using a mixture of peripheral blood of nulliparous non-pregnant individuals and cord blood at various dilutions. Concomitant test controls to validate and monitor the quality of flow cytometric procedures were performed using FetalTrol<sup>®</sup> test samples (IQ-Products, Groningen, The Netherlands). In concomitant test controls and spiking experiments, we were able to confirm the level of quantification of 0.05% fetal RBC when screening  $2 \times 10^6$  maternal erythrocytes, as described by Porra et al. (2007) [18].

Data were acquired and analyzed using the Statistical Package for the Social Sciences (SPSS, Munich, Germany) software version 15.0. Pre-ECV data as well as post-ECV data were acquired in a dichotomous fashion and statistically analyzed using the  $\chi^2$ -test.

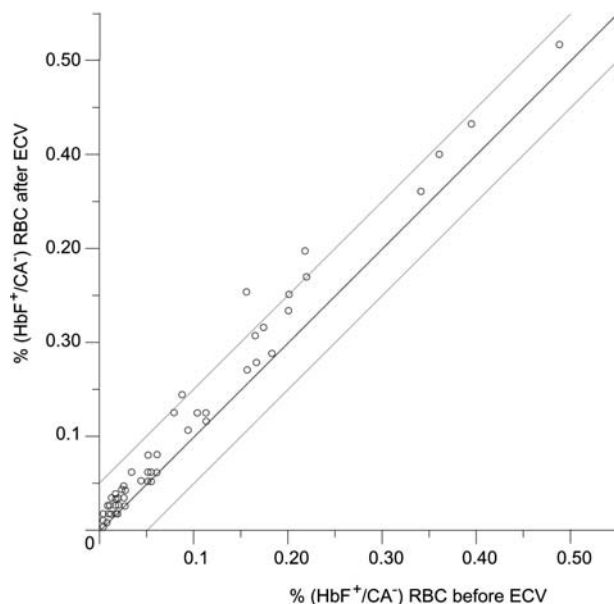


**Figure 1** Clinical and sonographic parameters surveyed before external cephalic version (ECV) and procedure related risk-factors documented after ECV.

## Results

FMH volumes were evaluated for all 50 women with breech presentation at term that opted for a trial of ECV, both before and after the procedure. For technical reasons, clinical pre-ECV data were not available for three women, all of whom had  $<0.05\%$  fetal RBC (HbF<sup>+</sup>/CA<sup>-</sup>) before and after ECV. A percentage of fetal RBC of 0.05% defined the lower limit of quantification of our flow cytometric test method. This was determined in 124 spiking experiments performed by Porra et al. and confirmed in our laboratory in five independent experiments [18]. When screening  $2 \times 10^6$  erythrocytes via flow cytometry with this method out of 50 blood samples, 26 had quantifiable amounts of fetal RBC before ECV, with 0.49% being the maximal percentage.

Whereas all of our blood samples contained equal or greater amounts of fetal RBC after ECV, as compared to before the procedure, only three procedure-related increases were elevated above 0.05% (0.06%; 0.08%; 0.1%) (Figure 2). No procedure-related parameter predicted this event, and no fetal compromise was noted in spite of an estimated FMH of 80 mL. In our study population, none of the five predefined procedure-related risk factors or the eight parameters surveyed before ECV (Figure 1) turned out to be an independent risk factor for the small amount of FMH in the three cases with  $>0.05\%$  fetal RBC, as calculated by a  $\chi^2$ -test.



**Figure 2** Dot plot of the percentage of fetal hemoglobin (HbF) positive/carbonyl anhydrase (CA) negative red blood cells (RBC). Every circle represents one patient and the results of two blood samples drawn before and after external cephalic version (ECV). Gray lines indicate  $\pm 0.05\%$  change, black line  $\pm 0$  change after ECV.

## Discussion

Several methods to quantify FMH are currently available [15]. KBT is a time-honored method that is still used in many laboratories, but has been shown to have a considerable intra- and inter-test variability and to lack accuracy to quantify FMH, especially at concentrations of  $<1\%$  fetal cell in maternal peripheral blood [4, 8, 13]. Furthermore, KBT is prone to overestimate FMH due to the persistence of HbF, which is especially prominent in patients with hemoglobinopathies, but is also present in up to 25% of all healthy women in the second trimester of pregnancy [14, 17]. Monoclonal fluorochrome-labeled antibodies have been implemented, either in fluorescence microscopy or in flow cytometry, which has been shown to be more sensitive compared to KBT [9]. Fluorescence microscopy has been shown to be equally precise compared to flow cytometry – which remains the gold standard for quantification of low concentrations of FMH, but utilizes less expensive equipment [12, 15]. Other techniques like the gel agglutination test (GAT) have a role in determining an amount of fetal erythrocytes in maternal circulation that exceeds the standard dose of anti-D immune globulin administered in clinical practice but are less suitable to detect low concentrations of FMH [6]. In the same context, dual-color flow cytometry using anti-HbF and anti-D monoclonal antibodies has been recently evaluated to simultaneously D-type and assess optimal doses of anti-D immunoglobulin in a single assay [19]. In our experiment, we chose a test kit that uses anti-HbF and anti-carbonic anhydrase Typ II (CA) antibodies within an established and tested dual-color flow cytometric approach to estimate as precisely and sensitively as possible the low concentrations of fetal erythrocytes expected in our study population [18]. Staining HbF, as in our test method, instead of the D-antigen for discriminating fetal erythrocytes, offers the advantage of being applicable regardless of the blood group of the mother or the fetus. A subpopulation of adult erythrocytes, however, contains HbF. These so-called F-cells are usually discriminated by their slightly lower expression of HbF compared to fetal erythrocytes. This somewhat arbitrary cut-off level can be difficult to define, especially in cases with large amounts of F cells [7]. To add specificity and to circumvent the need for an arbitrary cut-off point, a second monoclonal antibody was added to the test kit used in our study, staining for CA, which is part of the respiratory chain and is fully expressed only after birth [1, 3]. In their study of 124 spiking experiments, Porra et al. were able to confirm a lower level of quantification of 0.05%, which we were able to reproduce in concomitant controls. With this test kit the percentage of women with  $>0.05\%$  fetal RBC even before ECV was higher than previously reported [18]. Given that we confirmed our level of quantification at 0.05% in preliminary experiments as well as in concomitant controls, it is highly

unlikely that any subtle alterations made by the manufacturer to improve the test kit might account for this difference. Since all of our controls confirmed that the test method yielded accurate results, we must assume that our study population happened to have physiologically elevated levels of fetal RBC. There is some scientific debate on how to estimate transfused fetal blood volume from the percentage of fetal RBC in maternal circulation, and where to draw the line between physiological values and clinically relevant FMH [16]. For flow cytometric analysis, 0.85% fetal RBC is considered to be equivalent to 30 mL fetal whole blood, which is the volume commonly used to define clinically relevant FMH [16]. All of the blood samples tested in this study were substantially below this threshold before and after ECV. These data, generated by screening  $2 \times 10^6$  erythrocytes via flow cytometry, were in line with the findings of Boucher et al., who found a single FMH after ECV among 1311 women screened by single-slide manual KBT with  $400 \times$  magnification [2]. Boucher et al. reported a total of 40 out of 1311 women as having an increase in RBC volume after ECV. Given the large study population, their data are likely to represent the true incidence of FMH after ECV. Our cytometry data provide further assurance about the safety of ECV, given that quantifiable percentages of fetal RBCs were only found in three of 50 women, even in a population with rather high physiologic levels of fetal RBC before ECV and with a highly precise test method. Blood samples of these three women barely crossed the lower limits of quantification.

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