Observational Study

Anti D injection to Rh negative mothers – a relook

Barunoday Chakraborty[1], Sibapada Mondal[2]

Presence of fetal cells in maternal blood was demonstrated by application of Acid elution wayback in 1957. Woodrow and Finn first produced evidences that transplacental haemorrhage (TPH) occurs during labour, at term and at sensitizing events e.g miscarriage, antepartum haemorrhage, amniocentesis. Maternal alloantibodies (IgG) develop against the RhD positive fetal cells which cross the placenta and destroys the fetal red cells thus causing haemolytic disease of fetus and newborn(HDF/N). Routine post natal administration of anti D immunoglobulin (IgG) to the Mother who has delivered a Rh positive neonate has reduced incidence of alloimmunisation from 16% to 2%; and a further decrease in incidence to 0.07% was observed when Inj. Anti D prophylaxis was given to all Rh- Mothers at 28 weeks and again after delivery should the Neonate is Rh positive. Studies say that a dosage of 300 micrograms (1500 IU) Anti D immuglobulin can neutralize 15 ml of fetal red cells or 30 ml of fetal blood and by this principle when the fetomaternal haemorrhage (FMH) volume is larger than 30 ml an additional dosage of anti D would be required. Accordingly different countries have developed their own protocol of appropriate doses of anti D injection. In our institution we administer 300ug of anti D igG to all Rh negative mothers who has delivered an Rh positive baby within 72 hours of birth. The same procedure is followed after a miscarriage also. But a number of literature has shown that only 0.4% woman have a TPH of more than 15 ml and therefore it is logical to think that in nearly 99% cases a 300µg anti D IgG is excessive and unnecessary and carries not only the burden of huge cost but also an increased risk of parentally transmitted diseases. Hence the present study was undertaken to assess the fetomaternal haemorrhage in cases of normal delivery, caesarean section and miscarriages by kleihauer Betke test (KBT) of maternal blood samples. Fifty cases of normal delivery, 50 cases of caesarean section and 50 cases of late miscarriage were randomly selected and it was found that KBT was negative among 44 to 50 % cases. In majority of cases an FMH of less than 4 ml was seen in 38% after normal delivery, 32% after caesarean section and 38% after late miscarriages. An FMH of 4 to 10 ml occurred in 10% cases of normal delivery and miscarriages and 18% after caesarean sections. Larger volume FMH of 10 to 30 ml was seen only in 2 to 4% of cases and FMH of more than 30 ml was rare. So this study indicated that in nearly 84% cases a dose of 500 IU (100 µg) anti D IgG would be sufficient when administered within 72 hours post delivery or caesarean section or miscarriage. A KBT of maternal blood sample would indicate whether a larger fetal bleed has occurred or not. In that case an additional dose of anti D IgG can be calculated and administered on the following day thus saving a considerable cost and also reducing the risk of blood borne diseases.

[*J Indian Med Assoc* 2018; **116:** 16-20]

Key words **: HDF/N, Anti D IgG, FMH, KBT.**

In 1966 J. C. WOODROW and R. FINN first produced evidence that fetal cells are not uncommon in maternal blood after delivery and that in majority of cases Transplacental haemorrhage (TPH) occurs during labour, often when it was a complicated one. They also said that there occurs progressive increase in the incidence of fetomaternal haemorrhage (FMH) as the pregnancy approaches term[16]. Maternal alloantibodies (IgG) develop against the Rh D positive fetal red cells due to high immunogenicity of D antigen. Alloimmune haemolytic diseases of the fetus and newborns (HDF/N) results from the destruction of fetal red cells by maternal Immunoglobulin (IgG) antibodies that gain access to the fetal circulation during pregnancy especially later weeks and also at sensitising events (eg threatened miscarriage, incomplete miscarriage, antepartum haemorrhage, trauma, external cephalic version, amniocentesis etc)[1-6] . In 1957 Kleihauer, Betke and Braun first demonstrated the presence of fetal cells in the maternal circulation by application of the acid elution principle to identify fetal erythrocytes[7]. Approximately 16% of Rh D–negative women who deliver a Rh D–positive fetus become alloimmunised if RhIG is not administered in due time in appropriate doses. The routine administration of anti-D immunoglobulin to Rh D negative women within 72 hours of delivery of a Rh D positive infant, has decreased the risk of alloimmunisation to approximately 2%.

Department of Obstetrics and Gynaecology, Bankura Sammilani Medical College and Hospital, Bankura 722102
[1]DGO, MD, DNB, FICOG, Associate Professor
[2]MBBS, 3rd year MS (Obstet & Gynae) and Corresponding author

Since the introduction of anti-D immunoglobulin prophylaxis at 28 weeks to all D-negative women and again after delivery, the incidence of anti-D isoimmunisation was reduced from 16% to 0.07%[8-11]. Each 300 µg of anti-D immune globulin can neutralize 15 ml of fetal red blood cells or 30 ml of fetal blood[12]. By this principle, when the FMH volume is larger than 30 ml Rh D positive blood, additional doses of anti- D immunoglobulin would be required.

The possibility to accurately detect FMH and precisely determine its volume would enable us a more effective and less costly method of prevention of RhD alloimmunisation. Anti-D immunoglobulin could be administered only in indicated cases and only in doses logically necessary for prevention of RhD alloimmunisation by neutralising the specific amount of FMH[13].

In United Kingdom Blood Transfusion Services, Immunoglobulin Working Party, 1991, stated that at least 500 IU (100µg) anti-D immunoglobulin must be given to every RhD negative women with no preformed anti-D antibody within 72 hours of delivery of a RhD positive infant. This dose will be sufficient to prevent alloimmunisation from a 4 ml of fetal red cells bleed. Only 0.4% of women have a TPH of more than 4 ml and o.3% of women have a TPH of more than 15 ml and will not be protected with 500 IU (100 µg) ant-D Ig. It is therefore important that the amount of any fetomaternal bleed accurately estimated so that if necessary a supplementary anti-D dose can be administered to prevent maternal alloimmunisation[14]. Contrary to what is done in England in our country we give a flat dose of 1500 IU (300 µg) anti-D Ig to all RhD negative mothers who has delivered a RhD positive infant or after a miscarriage. But, kipping in view of the above fact that only around 0.4% of women have a TPH of more than 15 ml, it is logical to think that a large amount of anti-D that is used in our country is redundant or unnecessary in nearly 99% cases. This excessive amount of anti-D carries not only a burden of huge cost but also there is an increased risk of parenterally transmitted diseases like HIV, Hepatitis, Creutzfeldt-jakob disease. Hence the logical thinking is to obviate these risks and to reduce the cost should a rapid and accurate assessment of fetomaternal haemorrhage is done in a laboratory. Therefore the present study is undertaken to assess as far as accurately the amount of fetomaternal haemorrhage caused by a delivery, caesarean section or by a miscarriage by Kleihauer-Betke test of maternal blood sample against a positive control of cord blood diluted in adult blood. In 2013 Cedric Pastoret *et al* after their large study in France opined that correct detection and quantification of FMH is critical for the obstetric management of Rhesus D (RhD) negative women. The amount of fetal red cells (RBCs) in the maternal circulation determines the therapeutic dose of anti-D Ig necessary to prevent alloimmunisation[15].

Rhesus (Rh) D immunoglobulin (anti-D) is a human blood product prepared from plasma obtained from a small group of immunised volunteer donors. It has to be used since the 1960s in women who are RhD negative to prevent RhD alloimmunisation after giving birth to a baby who is RhD positive. Prevention of RhD alloimmunisation has been a major medical achievement, as RhD immunisation is a significant cause of perinatal mortality and morbidity in subsequent pregnancies of affected women. The Kleihauer-betke test (KBT) is the most widely used approach of FMH detection. The test is based on the visual microscopic counting of fetal RBCs on a maternal blood film. In acid condition fetal and adult haemoglobin have differences in solubility properties. Haemoglobin F (HbF) resists to acid elution and fetal RBCs are stained in bright pink, while haemoglobin is eluded from adult RBCs that appear as Ghost cells. The lack of precision is in part due to subjective identification of adult cells with increased content of HbF, also called F cells, physiologically increased during pregnancy[16-17]. Automated detection of fetal cells on blood films stained with KB method was reported to be more precise than KBT decreasing specially the inter-observer variation[18]. Flow cytometry (FCM) is a candidate method of FMH quantification. Indeed FCM assays exhibit a better reproducibility and a more reliable quantification of fetal RBCs than KBT[19]. It could not distinguish fetal RBCs from adult cells in case of RhD immunocompatibility. Latter FCM used monoclonal anti HbF antibody that allowed discrimination of three distinct populations of fetal RBCs, F cells and adult RBCs. The quantification of F cells provided by FCM eliminated a major drawback of KBT. In this context "FMH QuikQuant" become a new CE marked kit (Conformite Europeene), which included a monoclonal HbF antibody and propidium iodide as a specific marker of nucleated cells[20,21]. The analysis of artificial mixtures containing 1-100 fetal RBCs per 10000 adult RBCs together with the investigation of pregnant women samples allowed the author to validate that "FCM QuikQuant kit" to be a reliable and efficient method to screen FMH[22] (Table 1).

Administration of 100 IU (20 µg) anti-D Ig has been demonstrated to protect against 1 ml of fetal red cells, 500 IU (100µg) should protect against FMH of up to 5 ml of fetal red cells and 1500 IU (300 µg) anti-D Ig against FMH of approximate 15 ml of fetal red cells[23]. Before 20 weeks of gestation 250 IU should be given. After 20 weeks gestation blood should be taken at least for the conventional KBT to estimate the size of FMH and 500 IU of anti-D IgG should be given[24]. **There are differences internationally in the approach to post-partum anti-D prophylaxis not only in the dose of anti-D used but also in whether testing is perform to quantitate the volume of FMH.** In Australia, UK and the United States post-partum

anti-D doses of 600 IU (120 µg), 500 IU (100 µg), 1500 IU (300 µg) respectively are administered to RhD negative women. These doses are sufficient to cover RhD positive fetal RBCs bleeds of 6ml, 5ml and 15 ml respectively. The FMH volume then quantitated and additional anti-D Ig is given if necessary. In other countries (such as Germany and other European countries) a large dose of anti-D (1500 IU) is given. But FMH volume is not quantitated[25,26,27].

Materials and Methods

We have randomly selected 150 mothers admitted to our Department (Obstetrics and Gynaecology B.S Medical College Bankura) of whom 50 cases were after their normal delivery, 50 after caesarean section and 50 were cases of late 1st trimester miscarriage (>10 weeks) & second trimester miscarriages. Cases with early miscarriages, Molar pregnancies, and complicated pregnancy conditions eg Abruptio placenta, pre-eclampsia, preterm or pre labour rupture membrane, multiple pregnancy were excluded.

Feto-maternal haemorrhage was estimated in all cases by using the Kleihauer-betke test (KBT) and the following formula :

FMH (ml of fetal RBC) = No. of fetal cells counted / No. of maternal cells counted X 1800 X 122/100 X 100/92[24].
or, Can be simplified to:

$$\frac{\text{Number of fetal cells per high power field}}{\text{Number of maternal cells per high power field}} \times \text{2400 ml packed fetal red cells.}$$

For example, if the number of fetal cells is 9 and maternal cells 2000, then the fetal bleed will be calculated to be:

$$\frac{9 \times 2400}{2000} = 10.8 \text{ ml packed fetal red cells}^{24}.$$

Study Technique :

Patients were selected randomly. 50 cases of normal delivery, 50 cases of caesarean section and 50 cases of miscarriage were included in the study. Feto-maternal haemorrhage was estimated by using the kleihauer-betke test (KBT). Maternal blood was collected within 24 hours of delivery, miscarriage and caesarean section in an EDTA vial. 3 drops of 0.85% saline was mixed with 2 drops of EDTA blood in the test tube. From one drop of this diluted blood film was drawn on a glass slides. Immediately after drying the film it was put in 80% ethanol in a Coplin jar for 5 minutes. Then the slides were rinsed rapidly in tap water and were kept vertically on a bloating paper for about 10 minutes to get dried. Next the slides were placed in a Coplin jar containing the Elution solution (0.1% erythrosine) for 20 seconds. Then the slides were washed thoroughly in water and finally were placed in the counterstaining solution (1% Eosin) for 2 minutes. Then they were rinsed in tap water and were dried in the air at room temperature. Films prepared from a fresh EDTA cord blood added to 100 times adult whole blood to give a dilution of 1:100 to develop a positive control. Blood film was drawn from normal adult male in the same way so as to provide a negative control. Fetal cells were stained red and adult ghost cells were stained pale pink. Screening of the slides (and positive control) was done by viewing under low power microscope using x10 objective. At least 25 low power fields were screened. If the screening shows fetal cells then fetal erythrocytes were counted in 2000 background maternal red cells under high power field x40 objective where there were at least 100 adult cells present in this high power field. An area of the film was selected where cells were touching but not overlapping.

Table 1 — *Quantification of FMH (Categorized) by KBT*

Procedure	Amount of FMH	No of Cases	Percentage
Normal Delivery (n=50) :			
	Negative	24	48%
	< 4 ml	19	38%
	4-10 ml	5	10%
	10-30 ml	2	4%
	>30 ml	0	0
	Total	50	100%
Caesarean Section (n=50) :			
	Negative	22	44%
	<4 ml	16	32%
	4-10 ml	9	18%
	10-30 ml	2	4%
	>30 ml	1	2%
	Total	50	100%
Miscarriage (n=50) :			
	Negative	25	50%
	<4 ml	19	38%
	4-10 ml	5	10%
	10-30 ml	1	2%
	>30 ml	0	0
	Total	50	100%

Observation and Discussion

The dye we have used for staining the slides is 88% Erythrosine Supra, manufactured and marketed by German Colorcon India, Gujrat (Fig 2). It's a red colour powder from which 0.1% erythrosine solution was made and used for our study purpose. Here somehow the acid elution of the adult cells has worked poorly. However distinct fetal cells were seen as large pale pink cells with multilobed nuclei sparsely scattered over a field of many small pink adult cells. May be that the acid elution worked poorly because of faulty pH of the solution resulting in failure to have a proper ghosting of the maternal cells. This could not be corrected by us even making repeated preparations. However with practice fetal cells could be identified distinctly. Till date to us the matter remains unresolved. However looking at the negative control slides everyday during examination the morphology of the adult cells was reviewed afresh and compared. Any doubt during examination was alleviated looking at the positive control slides

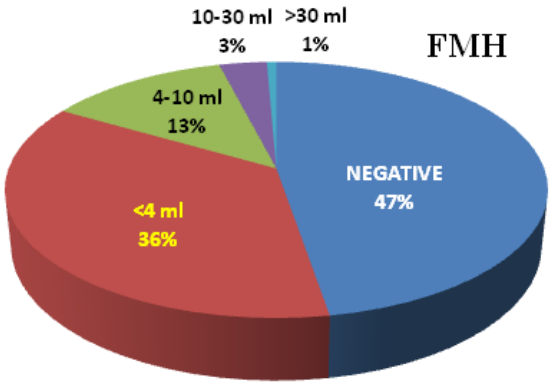

Fig 1

which contain known concentration of fetal cells scattered among the adult cells. And therefore we do agree that there is enough scope of further improvement in the staining process to obviate the interobserver variability. Overall in this study the picture was a negative KBT test, ie absence of fetal cells in the slides was of the order of 48% after normal delivery, 44% after caesarean section and 50% after miscarriage. Majority of the study cases had an FMH < 4 ml in all the three categories; 38% after normal delivery, 32% after caesarean section and 38% after late miscarriage. FMH of 4-10 ml occurred around 10% of cases of normal delivery and miscarriages and 18% after caesarean section. Larger volume FMH of 10-30ml occurred in 2 cases (4%) after normal delivery, 2 cases (4%) after caesarean section and one case (2%) after miscarriage. Only in one case there was a FMH of >30 ml after caesarean section. The details of the case were not known (Fig 1). Augustson *et al* in their study commented that 250 IU (50 µg) dose could be routinely used for 98.5% of RhD negative women post-partum. 25 Urgessa et al in their study concluded that around 92.5% of their study cases by KBT method and 87% by FCM method the calculated FMH volume was < 5ml of fetal RBCs and hence 500 IU (100µg)

Fig 2

anti-D Ig would be sufficient in the majority of the RhD negative mothers.29 This result is also consistent with the study conducted by Lubusky *et al* where they concluded that for the prevention of RhD alloimmunisation a dose of anti-D Ig 100µg should be sufficient in great majority of cases[28] (Figs 3-5).

So, in conclusion the current study indicates that in the great majority of the cases (84%) a dose of 500 IU (100 µg) of anti-D Ig would be sufficient when administered within 72 hours of post-delivery, post-caesarean or post-abortal. A KBT test of maternal blood samples would indicate whether a larger fetal bleed of more than 4ml has occurred or not. Should it happen additional dose of anti-D can be calculated and administered on the following day of initial dose. Because KBT is an easy, simple and rapid laboratory method, with experience and practice the accuracy of the test would increased day by day. Thus a huge quantity of costly RhD Ig can be saved reducing the burden of the government expenditure to a large extent, because the anti-D is freely provided to the Rh negative mothers in our institution.

References

1 Greer JP, Foerster J, Lukens J, Rodgers G, Paraskevas F, Glader B — Autoimmune Hemolytic Anemia. In Wintrobe's Clinical Hematology. 11th edition. Edited by Neff A. USA: Lippincott Williams & Wilkins Publishers; 2003: 2363-72.
2 AABB — Technical Manual. 15th edition. AABB: Bethesda; 2005.
3 Pourazar A, Homayouni A, Rezaei A, Andalib A, Oreizi F — The Assessment of Feto-Maternal Hemorrhage in an Artificial Model Using Anti-D and Anti-Fetal Hemoglobin Antibody by FCM, Iran. *Biomed J* 2008, **12:** 43-8.
4 Lafferty JD, Raby A, Crawford L, Linkins LA, Richardson H,

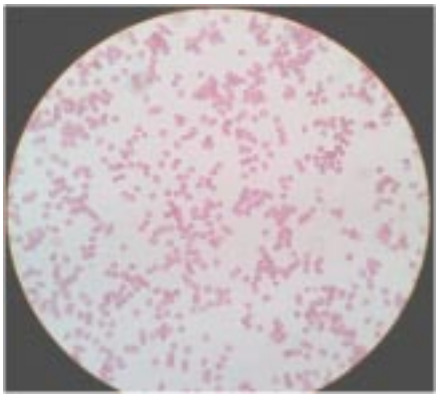

Fig 3 — Negative Control (only fetal cells)

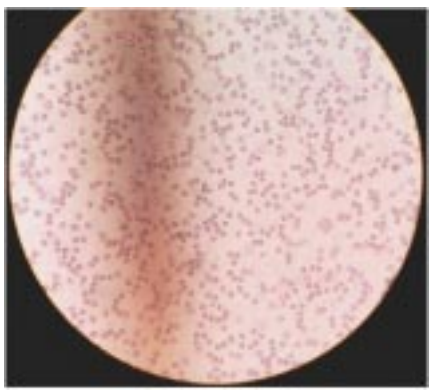

Fig 4 — Positive Control (known percentage of fetal cells within adult cells)

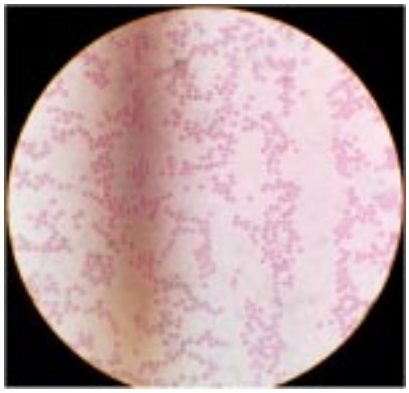

Fig 5 — Fetal Cells at 4 & 6 O'clock Position

Photographs are directly taken from the microscope under x40 objective during the study by a 12mp Canon Digital Camera

Crowther M — Fetal-Maternal Hemorrhage Detection in Ontario. *Am J Clin Pathol* 2003, **119:** 72-7.
5 Blood BCSH — Transfusion and General Haematology Task Forces. The estimation of fetomaternal haemorrhage, GUIDELINES. *Transfus Med* 1999, **9:** 87-92.
6 Quinley E — Immunohematology, principles and practice. 2nd edition. 1998: 14.
7 Bowman JM, Pollock JM, Penston LE — Fetomaternal transplacental hemorrhage during pregnancy and after delivery. *Vox Sang* 1986; **51:** 117-21.
8 Bowman JM — Controversies in Rh prophylaxis: who needs Rh immune globulin and when should it be given? *Am J Obstet Gynecol* 1985; **151:** 289 -94.
9 Stedman CM, Baudin JC, White CA, Cooper ES — Use of the erythrocyte rosette test to screen for excessive fetomaternal hemorrhage in Rh-negative women. *Am J Obstet Gynecol* 1986; **154:** 1363-9.
10 Ness PM, Baldwin ML, Niebyl JR — Clinical high-risk designation dose not predict excess fetal-maternal haemorrhage. *Am J Obstet Gynecol* 1987; **156:** 154-8.
11 Bowman JM, Pollock JM — Antenatal Rh prophylaxis: 28 week gestation service program. *Can Med Assoc J* 1978; **118:** 627-30.
12 Pollack W, Ascari WQ, Kochesky RJ, O Connor RR, Ho TY, Tripodi D — Studies on Rh prophylaxis. I. Relationship between doses of anti-Rh and size of antigenic stimulus. *Transfusion* 1971; **11:** 333-9.
13 Lubusky M — Prevention of RhD alloimmunization in RhD negative women. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2010, **154:** 3-8.
14 Blackwell Science Ltd — Estimation of Fetomaternal Haemorrhage; *Transfusion Medicine* 1999; **9:** 87-92.
15 Cedric Pastoret, Jerome Le Priol, Thierry Fest, and Mikael Roussel — Evaluation of FMH QuikQuant for the Detection and Quantification of Fetomaternal Haemorrhage. *Cytometry Part B (Clinical Cytometry)* 2013; **84B:** 37-43.
16 Woodrow JC, FINN R — Transplacental Haemorrhage. *Brit J Haemat* 1966; **12:** 297-309.
17 Popat N, Wood WG, Weatherall DJ, Turnbull AC — Pattern of maternal F-cell production during pregnancy. *Lancet* 1977; **2:** 377-9.
18 Pelikan DMV, Mesker WE, Scherjon SA, Kanhai HHH, Tanke HJ — Improvement of the Kleihauer–Betke test by automated detection of fetal erythrocytes in maternal blood. *Cytometry Part B Clin Cytom* 2003; **54B:** 1-9.
19 Bromilow IM, Duguid JK — Measurement of feto-maternal haemorrhage: A comparative study of three Kleihauer techniques and two flow cytometry methods. *Clin Lab Haematol* 1997; **19:** 137-42.
20 Igout J, Fretigny M, Vasse M, Callat MP, Silva M, Willemont L, Gelle M, Lenormand B — Evaluation of the coulter LH 750 haematology analyzer compared with flow cytometry as the reference method for WBC, platelet and nucleated RBC count. *Clin Lab Haematol* 2004; **26:** 1-7.
21 Tsuji T, Sakata T, Hamaguchi Y, Wang FS, Houwen B — New rapid flow cytometric method for the enumeration of nucleated red blood cells. *Cytometry* 1999; **37:** 291-301.
22 Cedric Pastoret, Jerome Le Priol, Thierry Fest, Mikael Roussel — Evaluation of FMH QuikQuant for the Detection and Quantification of Fetomaternal Haemorrhage; *Cytometry Part B (Clinical Cytometry)* 2013; **84B:** 37-43.
23 National Health and Medical Research Council: Guidelines on the prophylactic use of Rh D immunoglobulin (anti-D) in obstetrics. Australia: *National Blood Authority*; 2003: 1-36.
24 Austin E, Bates S, Silva M, Howarth D, Lubenko A, Rowley M, Scott M, Thomas E, White J, Williams M — BCSH Blood Transfusion and General Haematology Task Forces. The estimation of fetomaternal haemorrhage, *Guidelines* 2009, 1-23.
25 Bradley M Augustson, Elizabeth A Fong, Dianne E Grey, Janine I Davies, Wendy N Erbe — Postpartum anti-D: can we safely reduce the dose? ; *MJA* 2006; **184:**12;awery67890 611–613/a 5
26 Royal College of Obstetricians and Gynaecologists. Clinical green top guidelines. Use of anti- D immunoglobulin for Rh prophylaxis (22) — revised May 2002. Available at: http://www.rcog.org.uk/index.asp?PageID=512 (accessed May 2006).
27 National Blood Authority. Guidelines on the prophylactic use of Rh D immunoglobulin (anti- D) in obstetrics — June 2003. Canberra: NBA, 2003. Available at: http://www.nba.gov.au/pubs.htm (accessed May 2006).
28 Lubusky M, Simetka O, Studnickova M, Prochazka M, Ordeltova M, Vomackova K — Fetomaternal hemorrhage in normal vaginal delivery and in delivery by cesarean section. *Transfusion* 2012, **52:** 1977-82.
29 Fekadu Urgessa, Aster Tsegaye — Yirgu Gebrehiwot and Asaye Birhanu; Assessment of feto-maternal hemorrhage among rhesus D negative pregnant mothers using the kleihauer-betke test (KBT) and flow cytometry (FCM) in Addis Ababa, Ethiopia. *BMC Pregnancy and Childbirth* 2014, **14:** 358.