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Fetal and embryonic hemoglobins in erythroblasts from fetal blood and fetal cells enriched from maternal blood in fetal anemia

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Background and Objectives. To determine whether there is a delay or reversal in switch mechanisms from embryonic (ε and ζ) to fetal (γ) hemoglobins accompanying the erythroblastosis of anemic fetuses and whether an increased erythroblast count in fetal blood is associated with an increase in feto-maternal cell trafficking.

Design and Methods. Fetal and maternal blood samples were obtained from 10 cases with rhesus isoimmunization and 2 cases with maternal Parvo-B19 virus at 19-33 weeks' gestation. Blood samples were also taken as controls from 61 fetuses and 86 mothers. Fetal erythroblasts were isolated by triple density gradient centrifugation and magnetic cell sorting with CD71 antibody. Fluorescent antibodies were used to immuno-stain for zeta (ζ), epsilon (ε) and gamma (γ) hemoglobin chains. In the maternal samples, fluorescence *in situ* hybridization (FISH) for X and Y chromosomes was also carried out to confirm the presence and proportion of the enriched fetal cells from maternal blood.

Results. In both fetal and maternal blood the percentage of erythroblasts positive for γ-globin chain was significantly higher in the anemic fetuses compared to the controls (fetal blood, $p<0.001$, R=0.91; maternal blood, $p<0.001$, R=0.56), but there was no significant difference in expression of the ε and ζ-chains. The percentage of cells with Y-signals was also higher in the maternal samples of anemic fetuses compared to normal controls ($p<0.001$, R=0.56).

Interpretation and Conclusions. These findings suggest that the erythroblastosis of anemic fetuses is not accompanied by a delay or a reversal in switch from embryonic to fetal hemoglobin chains. Severe fetal anemia is associated with an increase in feto-maternal cell trafficking.

Key words: fetal and maternal blood ζ, ε, and γ globins, gestational stages, normal and anemic fetuses.

In red blood cell isoimmunized pregnancies, maternal hemolytic antibodies cross the placenta and attach themselves onto fetal red cells, which are then destroyed in the fetal reticulo-endothelial system.[1] In mild to moderate disease there is a compensatory increase in intramedullary erythropoiesis, and in severe disease there is recruitment of extramedullary erythropoietic sites, such as liver and spleen.[2,3] During a normal pregnancy erythropoiesis in the embryo and fetus is achieved in three overlapping anatomical and functional stages: mesoblastic, hepatic, and myeloid, each corresponding to the major hemopoietic organ of the period, which are the yolk sac, liver and spleen, and bone marrow, respectively.[4,5,3] The hepatic period extends from the 10th to the 24th gestational week, but the liver continues to produce red blood cells into the first week of postnatal life. However, from the 16th week onwards there is a rapid increase in medullary erythropoiesis.[4,3] Normally there is an exponential decrease with gestation in erythroblast count and CD71 expression, due to the switch from hepatic to medullary erythropoiesis and maturation of the hematopoietic tissues, respectively.[3,6] Hemoglobin production also involves two developmental *switches*; from embryonic ($\zeta_2\,\varepsilon_2$) to fetal hemoglobin ($\alpha_2\,\gamma_2$) commencing at 6-7 weeks of gestation, and from fetal to adult hemoglobin ($\alpha_2\beta_2$) at birth.[7]

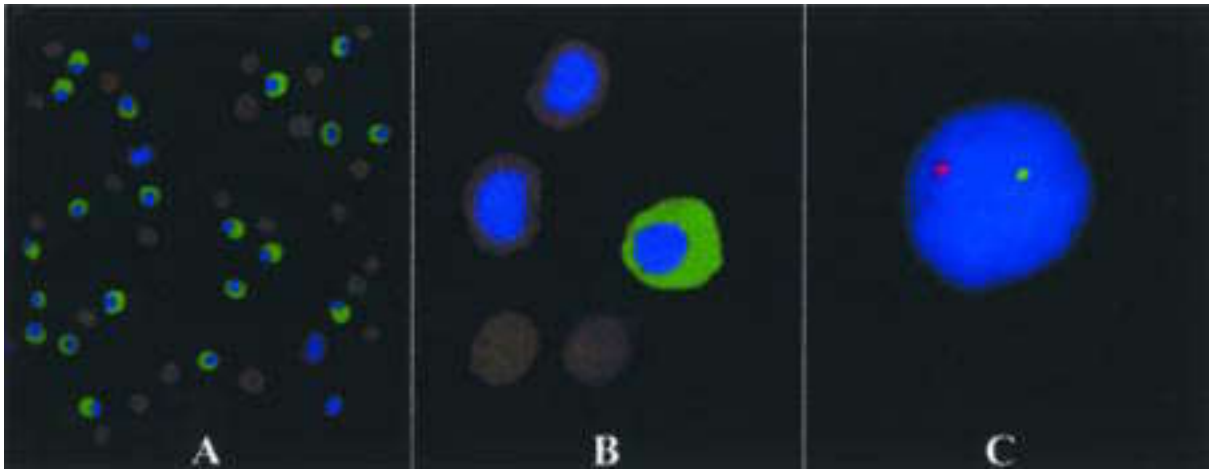

Figure 1. Photograph showing fetal cells after triple density centrifugation and anti-CD71 magnetic cell sorting. (A) Erythroblasts in fetal blood sample positive for γ-hemoglobin green fluorescent stain. (B) Fetal erythroblasts enriched from maternal blood positive for the γ-hemoglobin green fluorescent stain. (C) Fetal erythroblasts enriched from maternal blood positive for X and Y-signals on FISH (red = Y-chromosome, green = X chromosome).

In chromosomally abnormal pregnancies there is a developmental delay in the switch from hepatic to medullary eythropoiesis and this is accompanied by a delay in the switching from embryonic to fetal hemoglobins.[8] However, it is uncertain whether in environmental conditions such as fetal anemia the switch from medullary to hepatic erythropoiesis is accompanied by a delay or reversal in the expression of hemoglobins from embryonic to fetal.

Fetal cells are present in the maternal circulation at a low concentration of 1 fetal cell in 10^3-10^8 maternal cells.[9,10] The proportion of these cells can increase in certain conditions such as pre-eclampsia and intra-uterine growth restriction,[11-13] and is thought to be due to an increase in the passage of these cells across the placenta. In fetal anemia there is an increase in fetal erythroblast count in the fetal blood.[3]

The aims of this study were to establish firstly, whether there is any alteration in the switch mechanisms of expression of embryonic and fetal hemoglobin chains as a result of fetal anemia, and secondly whether the increase in erythroblast count in the fetal blood is accompanied by an increase in feto-maternal cell trafficking.

Design and Methods

Subjects

Fetal blood was obtained by cordocentesis (ultrasound-guided puncture of an umbilical cord blood vessel) from 10 red blood cell isoimmunized pregnancies and from two pregnancies with maternal Parvo-B19 virus infection to determine the degree of fetal anemia. Immediately before cordocentesis maternal blood was obtained by antecubital venipuncture. Fetal blood was also obtained by cordocentesis in 61 pregnancies undergoing fetal karyotyping for prenatal diagnosis. In all cases used for this study the fetal karyotype was normal and the Kleihauer-Betke test (fetal hemoglobin test) confirmed that the samples were fetal. Maternal blood was also obtained from 86 normal pregnancies from women attending for routine antenatal care.

Gestational age was calculated from the date of the last menstrual period and confirmed by ultrasound examination in the first trimester. The patients gave written consent for fetal blood sampling for prenatal diagnosis and for the extra fetal and maternal blood to be used for research.

Isolation of erythroblasts and staining

Fetal blood and maternal blood (20 mL) samples were collected into lithium heparinized bottles (Beckton Dickinson, Franklin Lakes, NJ, USA), stored at 4°C and processed within 24 hours of collection. Fetal erythroblasts were isolated by triple density gradient centrifugation with Histopaque (Sigma Aldrech Ltd., Poole, England) and anti-CD71 magnetic cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany) techniques as previously described.[14] For each sample, three aliquots of the positively selected erythroblasts were cytocentrifuged at 14.3 g for 10 minutes (Shandon, Frankfurt, Germany), and cytospun onto three glass

Table 1. Details and distribution of the percentages of fetal erythroblasts positive for γ-hemoglobin chains for the anemic fetuses in fetal blood samples and maternal samples enriched for fetal cells.

Case no.	Antibody type	Antibody level (i.u.)	GA wks.	Fetal sex	Hb level (g/dL)	γ-positive erythroblasts in cord blood (%)	γ -positive erythroblasts in maternal sample (%)	Y-signal positive erythroblasts in maternal blood (%)
1*	D	217	23	M	1.6	97	8	7
2*	D	23	26	M	3.3	83	6	6
3	D	22	26	M	5.8	84	0.9	0
4	D	19	27	F	5.5	80	0.6	–
5	D	23	29	M	11.8	72	1.1	0
6	D	38	33	F	7.9	76	0.6	–
7	D	16	22	F	2.9	88	2.4	–
8	D	41	25	M	5.2	82	2.3	0
9	D	70	19	M	3.3	95	6.6	5
10	K	15	31	F	11.7	69	1.7	–
11*	Parvo virus	–	22	M	1.9	92	6	3
12*	Parvo virus	–	22	F	1.7	94	7.5	–

**Hydrops present, Hb: hemoglobin; GA: gestational age; wks: weeks; F: female; M: male.*

slides. Cells were fixed and permeabilized, as previously described,[8] using commercial *Fix and Perm* reagents (Caltac Burlingame, CA, USA). Slides were then washed in phosphate buffered saline (PBS) solution and incubated with monoclonal fluorescein isothiocyanate (FITC) conjugate fluorescent antibody for the ζ, ε and γ chain respectively[8] (Figure 1). After antibody incubation, the slides were washed in PBS solution, mounted with 4,6-diamidino-2-phenylindole (DAPI) and visualized under a fluorescence microscope (Zeiss Axioskop microscope, Carl Zeiss, Gottingen, Germany). Nucleated cells that showed specific staining above the DAPI background stain were counted as positive. At least 100 nucleated cells were counted.

In the maternal samples, the remaining cells in the positive fraction were treated with KCl, fixed with methanol/glacial acetic acid and frozen at –20°C. Fluorescence *in situ* hybridization (FISH) was subsequently carried out as previously described,[11,12,14] using dual chromosome-specific DNA probes (Vysis Inc., Downers Grove, Illinois, USA) to screen for X and Y chromosomes (Figure 1). At least 100 nucleated cells were examined on each slide and the percentages of cells with one signal for the Y chromosome probe, and one, two and three signals for the X chromosome probe were calculated. Only intact cells that were not overlapping were chosen for the analysis. The slides were examined under a fluorescence microscope (Zeiss Axioskop microscope, Carl Zeiss, Gottingen, Germany), using a DAPI/FITC/TRITC triple band pass filter set. Image capture and processing was by a Microsoft computerized system (Vysis, USA). Enrichment of fetal cells and analysis were carried out without knowledge of the clinical details of the patients.

Statistical analysis

For each hemoglobin chain, a comparison was made between the study group (red cell isoimmunized and Parvo-B19 viral infection) and the controls using a Mann-Whitney U-Wilcoxon rank sum W test in relation to the fluorescent antibody positive erythroblasts (as a percentage of the total nucleated cells). Multiple regression analysis was carried out to compare the percentage of positive cells for these three different globin chains between the study and control groups, taking into account the gestational age. In the study group the individual values were subtracted from the appropriate normal mean for gestation to derive delta (Δ) values. The normal gestational means of fetal hemoglobin and fetal erythroblast percentage were obtained from previously published values.[1,8] For maternal samples the values obtained from the control group in the present study were used. In the maternal samples, a comparison was made between the study and control groups for the percentage of positive cells with Y-signals using a Mann-Whitney U-Wilcoxon rank sum W test and multiple regression analysis. The association between the percentage of erythroblasts positive for γ-chain and the percentage of cells positive for Y-signals on FISH was also determined using Spearman's correlation coefficient.

Results

The median gestation in the study group was 25 weeks (range19-33, Table 1) and in both the fetal

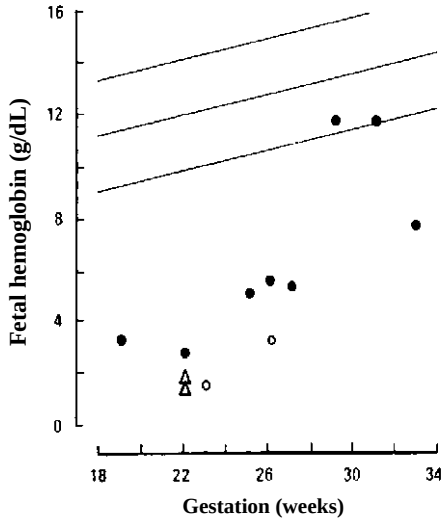

Figure 2. Fetal hemoglobin measurements (g/dL) plotted on the normal range for gestation (95th, 2.5 and 97th centiles).[1] The closed circles indicate the non-hydrops rhesus isoimmunized fetuses, the open circles indicate those fetuses with rhesus-hydrops, and the open triangles indicate the two cases with maternal Parvo-B19 virus hydrops.

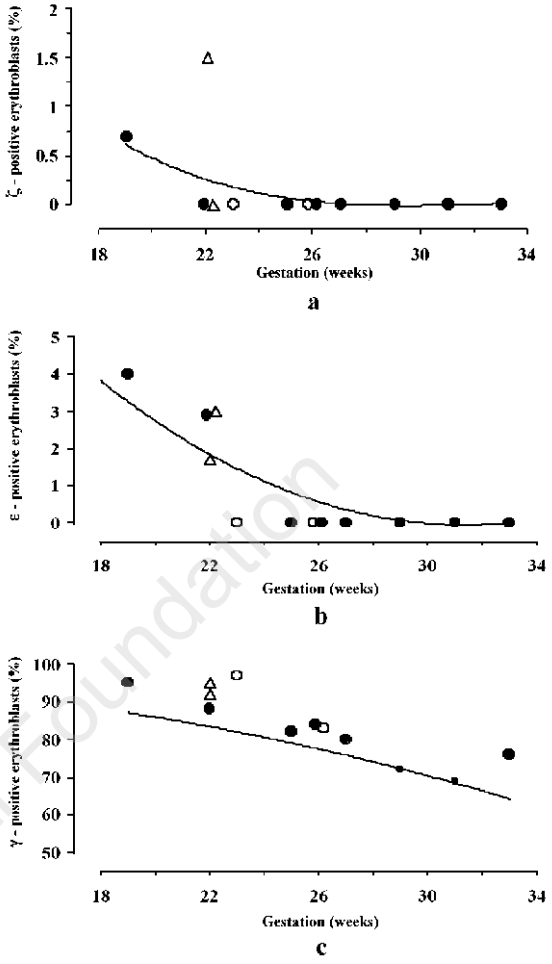

Figure 3. Percentages of erythroblasts positive for the three hemoglobin chains in the blood of anemic fetuses in relation to gestational age for (a) ζ-chain, (b) ε-chain, and (c) γ-chain. The black curved line is the regression line of the association of these hemoglobin chains with gestation in the normal fetuses. The closed circles indicate non-hydrops rhesus isoimmunized fetuses, the open circles indicate rhesus hydrops fetuses, and the open triangles indicate maternal Parvo-B19 virus hydrops fetuses.

and maternal controls it was 24 weeks (range 19-33 weeks).The fetal hemoglobin in the study group was below the 2.5 centile of the normal range in 10 of the 12 cases and the lowest hemoglobin was noted in the hydropic fetuses (Figure 2, Table 1). The percentages of erythroblasts positive for γ, ε and ζ hemoglobin chains were calculated in the normal and study groups in both the fetal blood samples and maternal samples.

Fetal blood samples

There was no significant difference in the proportion of cells that stained positive for ζ and ε-globin chains in both groups. In 2 of 12 fetuses in the study group (0.7%, 2%) and 6 of 61 fetuses in the control group (median 1.5, range 1.4-2%), there were erythroblasts that positively stained for the ζ-globin chain (Mann-Whitney, p=0.49; multiple regression, p=0.541, R= 0.345). In the control group, the percentage of cells positive for ζ-globin chains decreased from 0.6% at 19 weeks to zero % at 21 weeks gestation (Figure 3a, $Y= -0.00029849X^3 + 0.0289716X^2 - 0.9301231X+9.87495$). The ζ-globin chain in the study group was similar to the normal control group, with the percentage of cells in the study group expressing the ζ-globin chain at 19 weeks being 0.6% (Figure 3a, $Y= -0.000067576552355X^3 + 0.010823898985073X^2 - 0.469587540356254X + 6.155161021324490$).

There were 4 cases in the study group and 35 in the control group with erythroblasts that stained positive for the ε-chain. The median proportion of ε-positive erythroblasts in the study group was 3% (range 1.7-3) and that in the control group was 2% (range 1.2-6; Mann-Whitney, p=0.36; multiple regression, p=0.610, R=0.70). In the control group, the

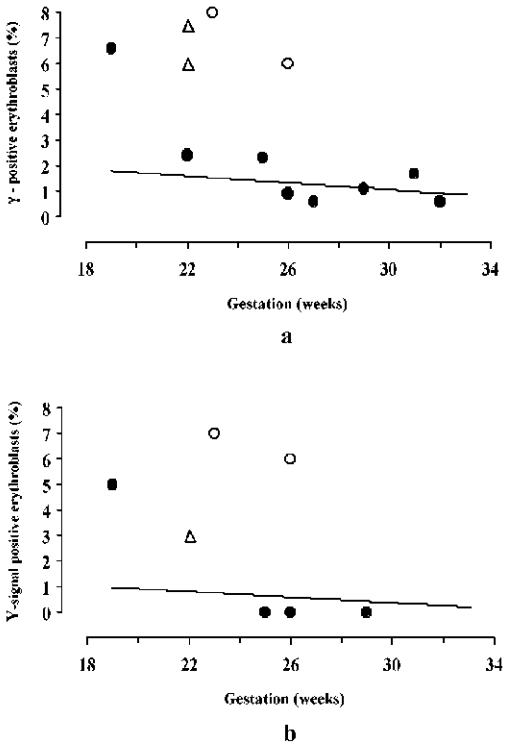

Figure 4. Percentage of fetal cells enriched from maternal blood in relation to gestation for (a) erythroblasts that stain positive for γ-hemoglobin chain in all cases studied, and (b) erythroblasts positive for Y-signal FISH in the male pregnancies. The curved line is the regression line of the association of the γ-hemoglobin chain and of the Y-signals with gestation, respectively. The closed circles represent non-hydrops rhesus isoimmunized fetuses, the open circles represent rhesus-hydrops fetuses, and the open triangles represent maternal Parvo-B19 virus hydrops fetuses.

percentage of cells positive for ε-chain decreased from 3% at 19 weeks to less than 1% at 25 weeks (Figure 3b, $Y= 0.0221978518X^2 - 1.3854861356X + 21.5723577868$). In the study group the exponential regression line for the ε-globin chain also decreased from 4% at 19 weeks' gestation to less than 1% at 25 weeks' gestation (Figure 3b, $Y = -0.001787289135590X^3 + 0.175789584946335X^2 - 5.745121274913070X + 62.395657351940500$), i.e., there was no delay or reversal in expression and switching from embryonic to fetal hemoglobins in the fetal anemia group compared to controls.

The median percentage of erythroblasts that stained positive for the γ-globin chains in the study group was 83 (range 69-97), and the median for the control group was 82 (range 61-89). Within the 12 fetal samples of the study group there were 10 cases with anemia. The median percentage of γ-positive erythroblasts in these 10 cases was 86 (range 76-97), which was significantly higher than in the control group (Figure 3c, Mann-Whitney $p<0.01$; multiple regression analysis $p<0.001$, R= 0.910, regression line equation for control group: $Y=-0.019966X^2-0.589462X+105.726987$).

Maternal blood samples

There were 3 out of the 12 study group cases that showed ε-positive erythroblasts of 0.1-0.2% at 19-22 weeks' gestation, the remaining 9 cases were ε-negative. This was similar to the range of percentages and gestations of ε-positive erythroblasts in the control group (Mann-Whitney $p=0.98$; multiple regression $p=0.717$, R=0.41). For the ζ-globin chain all cases had zero % at all gestations in both the isoimmunized-hydropic and normal groups.

The median percentage of the fetal erythroblasts enriched from maternal blood that were positively stained for γ-globin chain in the study group was 2 (range 0.6-8), which was significantly higher than in the control group (median 1, range 0.3-4, $p< 0.02$). In these 12 cases of the study group, the percentage of γ-positive erythroblasts was increased in 5 cases only in which severe anemia and hydrops were present. The median percentage of positive cells in these 5 cases was 7 (range 6-8), and this was significantly higher than in the maternal control group (Mann-Whitney $p<0.001$; multiple regression $p<0.001$, R= 0.56; Figure 4a).

There were 7 of the 12 pregnancies in the study group and 57 of the 86 pregnancies in the control group carrying male fetuses. Of the 7 study group males, 4 (57%) cases had positive Y-signals. These 4 cases of positive Y-signals were the cases of severe anemia and hydrops. In the 57 males of the control group, Y-signals were detected in 30 (53%) cases. The median percentage of Y-signal positive cells in the study group was 5.5 (range 3-7), which was significantly higher than the percentage in the control group (median 1, range 0.4-3, Mann-Whitney $p<0.002$; multiple regression $p<0.001$, R= 0.56; Figure 4b). In none of the 5 female pregnancies of study group and the 29 female pregnancies of control groups, were any Y-signals detected. There was an association between the percentage of erythroblasts positive for γ-globin chain and cells positive for Y-signals in the male cases of both the study and control groups (r=0.73, $p<0.001$).

There was an association between the deficit in fetal hemoglobin (Δ value) and the increase in γ-

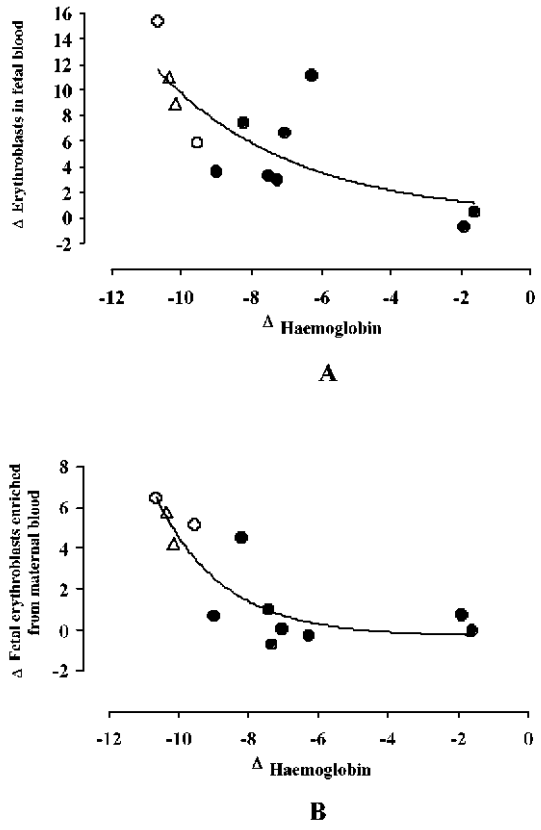

Figure 5. The relation between the delta (Δ) values of fetal hemoglobin measurements and the Δ values of percentage of erythroblasts that stain positive for γ-globin chains in (A) fetal blood, and (B) fetal erythroblasts enriched from maternal blood.

positive erythroblasts (Δ value) in both fetal and maternal blood (Figure 5; Spearman's correlation coefficient, fetal blood, r=0.64, $p<0.03$; maternal blood, r=0.8, $p<0.003$).

Discussion

Our study demonstrated an increase in the proportion of erythroblasts in the blood of anemic fetuses from red cell isoimmunization or Parvo virus B19 infection. This confirms the previous reports of increased erythropoiesis in these fetuses.[3] In severe anemia, the fetus is subjected to varying degrees of hypoxia which stimulates extramedullary erythropoiesis and consequent development of erythroblastemia. The findings of this study that there is no change in the expression of the embryonic (ε and ζ) hemoglobin chains suggest that the switch from medullary to hepatic erythropoiesis is not accompanied by a delay or reversal in the switch mechanism of expression from embryonic to fetal hemoglobins. The percentage range and regression line for these ε-positive cells was similar between the groups suggesting that there was no increase in the production or expression of this hemoglobin chain.

The finding of an increase in the proportion of γ-positive erythroblasts in the blood of anemic fetuses is due to increased extramedullary erythropoiesis. In marrow erythropoiesis the nucleated erythroid precursors are confined to the parenchyma in which hematopoiesis takes place. In the presence of severe anemia, there is a failure and breakage of the threshold and filtering mechanism occurring in the intramedullary system and parenchyma to compensate for the changed environment, and this leads to erythroblastosis, producing erythroblasts containing fetal hemoglobin.

Our study demonstrated an increase in fetal cells enriched from maternal blood that are positive for γ-globin chain and for Y-signals on FISH in male cases. It is possible that this finding is a mere consequence of the increased number of erythroblasts in fetal blood. Alternatively, in severe anemia and fetal hydrops, the placenta, which is also hydropic, becomes more *leaky* allowing for further feto-maternal cell trafficking.

We have established that the increased erythropoiesis in fetal anemia is not accompanied by a delay or reversal in the switching mechanisms from embryonic to fetal hemoglobins and that environmental factors such as hypoxia result in increased erythropoiesis without disturbance in the hemoglobin gene expression. In addition the increased proportion of these erythroblasts in the fetal blood results in increased feto-maternal cell trafficking into the maternal circulation.

Contributions and Acknowledgments

AMR: contributor of the concept and design of the study, principle investigator, performed all the experimental work, data analysis and statistical work, and wrote the manuscript; HH: supervised the experimental work, contributed to the revision of the manuscript; FF: contributed to the revision of the manuscript; KHN: main supervisor of the study, supplied blood samples, reviewed data analysis, contributed to the writing and revision of the manuscript.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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Potential implications for clinical practice

These findings do not have any implication for clinical practice at the moment, although they may allow a better understanding of the pathophysiology of fetal anemia.

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