Fetal fluid and protein dynamics
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Total blood volume is maintained in nonhydropic fetuses with severe hemolytic anemia.

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Abstract

Objective
Fetal alloimmune anemia is associated with increased blood flow velocities and cardiomegaly. In severe cases, hydrops can develop. We investigated whether the decrease of red blood cell volume is associated with a reduction or expansion of plasma volume.

Methods
In 86 alloimmunized fetuses that received a first intrauterine transfusion, we calculated fetal total blood volumes (i.e. fetoplacental blood volumes) using a dilutional principle of fetal hemoglobin with adult hemoglobin. The relation between total blood volume and estimated fetal weight, severity of anemia and hydrops was analyzed.

Results
Gestational age ranged from 17 to 35 weeks. Mean hemoglobin deficit was 6.8 standard deviations (range 2.1–11.7) below the normal mean. Fetal total blood volume was significantly related to estimated fetal weight (p< 0.001). Mean total blood volume in nonhydropic fetuses was 123 ml/kg (n= 74) and in hydropic fetuses 144 ml/kg (n= 12). There was a significant relation between total blood volume per kg body weight and hydrops (p= 0.035); however, there was no relation with severity of anemia (p= 0.94).

Conclusion
In the human nonhydropic fetus with severe hemolytic anemia, total blood volume is maintained: the decrease in red blood cell volume is thus compensated by an increase in plasma volume. In hydropic fetuses, however, total blood volume seems to be increased. This is in accordance with the hypothesis that congestive heart failure plays a role in the pathophysiology of hydrops in anemic fetuses.
Introduction

In the human fetus, hemolytic anemia is most frequently caused by maternal allo-antibodies against fetal red cells. Intrauterine transfusion of red blood cells has become an established therapy. Nowadays, the diagnosis of fetal anemia is mainly based on ultrasound and Doppler findings, such as cardio-, hepato- and splenomegaly, and increased arterial and venous flow velocities. In severe cases, hydrops can be observed, usually beginning with discrete ascites and pericardial effusion, followed by massive ascites and skin edema in a more advanced stage [1].

Fetal total blood volume, also referred to as fetoplacental blood volume, can be regarded as the combination of red cell volume and plasma volume, circulating in the fetus and the placenta. Theoretically, a considerable decrease of red cell volume may be associated with a similar reduction in plasma volume, or a compensatory increase, or even an overcompensating expansion in plasma volume. Adults with chronic anemia commonly have an increase in plasma volume with a slightly decreased total blood volume [2]. In neonates with alloimmune anemia, the decrease in red cell volume is mostly found to be compensated by the increase in plasma volume [3,4]. In the fetus, however, there are indications that the capillary filtration coefficient and vascular compliance are different from that in the neonate or adult [5]. Thus, the fetus may react differently compared to neonates or adults. Also, in the severely anemic fetus, cardiomegaly, raised umbilical venous flow and hydrops are possible signs of congestive heart failure. High-output cardiac failure is thought to play a role in the pathophysiology of hydrops. Raised cardiac output indeed has been found in anemic fetuses [6–8] and in anemic neonates [9, 10]. In adults, congestive heart failure is often associated with increased total blood volume [11]. Therefore, we sought to determine whether the loss of red cells is associated with a reduction or expansion of total blood volume in the fetus.

Several methods have been used to estimate fetal total blood volume. Most methods used the increase in hematocrit during an intrauterine transfusion to calculate the initial total blood volume. However, these methods either overestimated the blood volume by assuming that the final blood volume equals the initial blood volume [12], or they underestimated the blood volume by assuming that the final blood volume equals the sum of the initial blood volume with the added donor blood volume [13–16]. In animal experiments, both assumptions were shown to be incorrect, as around 30% of the volume given during transfusion immediately leaves the circulation [17]. Rapid plasma loss during transfusion was also suggested in a study in human fetuses.
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[18]. Hoogeveen et al. [19] therefore proposed another method to estimate fetal total blood volume: a calculation based on the dilution of fetal hemoglobin with adult (donor) hemoglobin during an intrauterine transfusion. We calculated fetal blood volume using this formula, with minor adaptations, and investigated the relation between fetal total blood volume and severity of anemia or presence of hydrops.

Methods

Measurements and Inclusion Criteria

Leiden University Medical Center is the national referral centre for the treatment of fetal anemia in the Netherlands. Our methods for diagnosis and treatment of severe fetal alloimmune anemia with intrauterine transfusion have been described previously [20]. In short, a pre-transfusion sample is taken to measure the hemoglobin concentration and hematocrit, to determine the required amount of donor blood. After the transfusion and a 2-min waiting period to allow even distribution of the donor blood, a post-transfusion sample is taken to check if the desired level of hematocrit is reached. The hemoglobin concentration and the hematocrit of the fetal blood samples are measured using a Sysmex XE 2100 hematology analyzer (Sysmex, Kobe, Japan). The hematocrit of the donor blood is determined by capillary high-spin centrifugation. Also, fetal total blood volume is routinely calculated at every transfusion. In the initial and the final sample, the percentage of fetal hemoglobin is measured using high-performance liquid chromatography (HPLC, Primus Ultra 2; Siemens, The Netherlands). The measurement is the same as for routine HbA1c determination. The fetal hemoglobin and the derived (glycated or acetylated) fetal hemoglobin peak are combined to determine the total amount of fetal hemoglobin. During the study period, from January 2002 to April 2006, we included all first intrauterine transfusions for fetal anemia due to red cell allo-immunization. At subsequent transfusions, there usually is only a small amount of fetal hemoglobin left, making measurement of the dilution of fetal hemoglobin less accurate.

Estimated fetal weight was determined with the formula of Hadlock et al. [21], using sonographically measured biparietal diameter, head circumference, abdominal circumference and femur length, within 2 days before or at the time of transfusion. Hydropic fetuses were classified as mild or severe using criteria described by van Kamp et al. [22]. Briefly, mild hydrops was defined as the presence of a distinct rim of ascites, with or without pericardial effusion, while severe hydrops was defined as
the presence of a more abundant amount of fluid collection, usually ascites, with skin edema. In severely hydropic fetuses, fetal weight was also estimated using the formula of Hadlock et al. [21], but with a measurement of the abdominal circumference that excluded the abundant amount of ascites. For this purpose, the tracing ellipse, in the usual transverse plane, included all fetal organs, except the intra-abdominal collection of fluid and the anterior abdominal wall. In this way an attempt was made to estimate the nonhydropic size of the fetus. We excluded fetuses with non-immune hydrops, structural or chromosomal anomalies or congenital infection.

**Calculations**

The initial total blood volume was calculated with a formula based on the dilutional principle as described by Hoogeveen et al. [19]. Basically, with the known amount of adult hemoglobin in the donor blood, and the dilution of fetal hemoglobin with adult hemoglobin, the initial red cell volume can be calculated (formula 1). The assumption herein is that the mean corpuscular hemoglobin concentration (MCHC) in the fetal and the donor blood is approximately the same. In that case, the change in hemoglobin concentration during transfusion is representative for the change in red cell volume during transfusion.

\[
(1) \quad \text{RCV}_{\text{initial}} = \frac{(V_{\text{donor}} \times \text{Ht}_{\text{donor}}) \times \text{HbF}_{\text{final}}}{(\text{HbF}_{\text{initial}} - \text{HbF}_{\text{final}})}
\]

RCV \(_{\text{initial}}\) is the initial red cell volume, \(V_{\text{donor}}\) is the volume of transfused donor blood, \(\text{Ht}_{\text{donor}}\) is the hematocrit of the donor blood and \(\text{HbF}_{\text{initial}}\) and \(\text{HbF}_{\text{final}}\) are the pre-transfusion and post-transfusion percentages of fetal hemoglobin. With the initial red cell volume and the initial hematocrit (Ht \(_{\text{initial}}\)), the initial total blood volume (FBV \(_{\text{initial}}\)) can be determined (formula 2). The volume of the initial sample (V \(_{\text{sample}}\)) is taken into account, to determine the entire initial total blood volume.

\[
(2) \quad \text{FBV}_{\text{initial}} = \frac{(V_{\text{sample}} \times \text{Ht}_{\text{initial}}) + \text{RCV}_{\text{initial}}}{\text{Ht}_{\text{initial}}}
\]

**Data Analysis**

First, severity of anemia was expressed as the standardized hemoglobin deficit, defined as the number of standard deviations that an actual value deviated from the normal mean for gestational age. Reference values for hemoglobin were derived
from the literature [23]. Then, the variation in MCHC was analyzed and a paired samples t test was performed to rule out the possibility of a large difference between MCHC before and after transfusion. Further, it was possible to estimate the measurement error of our calculation, since in some cases an interim blood sample was taken during transfusion. This gave us the opportunity to compare 2 measurements of total blood volume in the same fetus. These 2 measurements were compared in a Bland-Altman plot. Next, linear regression was performed to study the relation between estimated fetal weight and fetal total blood volume. The standard deviations of fetal total blood volume were given per weight category. Pearson’s correlation coefficient was calculated after log transformation of estimated fetal weight and total blood volume, since these variables both grow exponentially during gestation, to analyze the correlation between estimated fetal weight and total blood volume. Then the average fetal total blood volume per kg body weight was determined in nonhydropic, mildly hydropic and severely hydropic fetuses. To study the additional influence of the severity of anemia and presence and severity of hydrops on fetal blood volume, multivariate regression analysis was performed, with fetal blood volume per kg body weight as dependent and severity of anemia and hydrops (as an ordinal variable) as independent variables. The null hypothesis was that there was no significant correlation between fetal total blood volume per kg body weight and severity of anemia or hydrops. We considered \( p < 0.05 \) to be significant. The statistical software SPSS 14.0.1 and Graphpad Prism 5.0 were used.

### Results

From January 2002 to April 2006, we performed 112 first intrauterine transfusions in alloimmunized anemic fetuses. We included 86 transfusions with complete data. Alloimmunization, at the time of transfusion, was caused by anti-D or anti-D+C (\( n = 69 \)), anti-Kell (\( n = 10 \)), anti-c (\( n = 4 \)), anti-Jka (\( n = 1 \)), anti-Kpa (\( n = 1 \)) and anti-Verdegaal (\( n = 1 \)). Patient baseline characteristics are shown in table 1. Mean hemoglobin deficit was 6.8 standard deviations below the normal mean, with a range of \(-2.1\) to \(-11.7\) standard deviations.

The mean MCHC before transfusion was 20.4 (\( n = 85, \text{SD} = 1.2 \)), the mean MCHC of the donor blood was 20.9 (\( n = 19, \text{SD} = 0.5 \)) and the mean MCHC after transfusion was 21.1 (\( n = 81, \text{SD} = 0.8 \)). There was a mean increase of 0.7 in MCHC after transfusion, and this was a significant, albeit small, difference (\( n = 80, \text{SD} = 1.02, p < 0.01 \)).
In 9 nonhydropic cases an interim blood sample was taken. In these cases the measurements of the blood volumes, calculated with the interim and the final blood samples, were compared in a Bland-Altman plot. The bias between the 2 methods was small: the interim measurement was on average 1.1 ml higher. The SD of the differences, however, was 15.8 ml (95% limits of agreement= –32.1 to 29.9 ml; percent SD= 10.2%, with 95% limits of agreement= –21.7% to 18.4%). Figure 1 shows that the average fetal total blood volume had no influence on the extent of the difference between the 2 methods.

Figure 2 shows that there is a strong relation between fetal total blood volume and estimated fetal weight, both in nonhydropic and hydropic fetuses. Variance of fetal total blood volume increased with increasing estimated fetal weight. Under 1 kg, the SD was 30 ml; between 1 and 2 kg, the SD was 53 ml; above 2 kg the SD was 61 ml. Since estimated fetal weight and fetal blood volume increase exponentially during gestation, a log transformation was performed. There was a significant correlation between log(estimated fetal weight) and log(total blood volume) (p< 0.001, Pearson correlation coefficient= 0.96).

Figure 3 shows the relation between severity of anemia and fetal blood volume per kg body weight. The mildly hydropic fetuses had a Z-hemoglobin of less than –6.2 SD and the severely hydropic cases of less than –9.8 SD. The average total blood volume per kg body weight in nonhydropic fetuses was 123 ml/kg (SD= 23 ml/kg). However, in hydropic fetuses the average total blood volume per kg body weight was higher: 144 ml/kg (SD= 29 ml/kg) (average in mildly hydropic fetuses 144 ml/kg, and in severely hydropic fetuses 142 ml/kg). Multivariate regression analysis showed that there was a significant relation between fetal total blood volume per kg body weight and the presence of hydrops (p= 0.035, r= 14.6, 95%CI= 1.1–28.1); however, there was no relation with severity of anemia (Z-hemoglobin; p= 0.94, r= 0.12, 95%CI= –3.1 to 3.3).
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Table 1 Patient baseline characteristics.

<table>
<thead>
<tr>
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<th>first intrauterine transfusion, initial values (n=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>27.5 (17.1 – 35.4) *</td>
</tr>
<tr>
<td>Estimated fetal weight (g)</td>
<td>1272 (167 – 3033) *</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.19 (0.05 - 0.36) *</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>6.3 (1.8 – 11.8) *</td>
</tr>
<tr>
<td>Fetal hemoglobin (%)</td>
<td>91 (81 - 96) *</td>
</tr>
<tr>
<td>Presence of hydrops</td>
<td>74 no hydrops,</td>
</tr>
<tr>
<td></td>
<td>9 mild hydrops,</td>
</tr>
<tr>
<td></td>
<td>3 severe hydrops</td>
</tr>
</tbody>
</table>

* mean (range)

Figure 1 The differences between the interim and final blood volumes are compared with their average in a Bland-Altman plot. The bias and 95% limits of agreement are depicted.
Figure 2  Relation between calculated fetal total blood volume (ml) and estimated fetal weight (g). Nonhydropic, mildly hydropic and severely hydropic cases are depicted.

Figure 3  Relation between fetal total blood volume per kg body weight (ml/kg) and severity of anemia (Z-hemoglobin). Z-values (in standard deviations) were calculated with normal values adapted from Nicolaides et al. [23]. Nonhydropic, mildly hydropic and severely hydropic cases are depicted.
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Discussion

In this study, we found that the calculated total blood volume was strongly correlated with the estimated fetal weight. We found no relation between total blood volume and severity of anemia. Therefore, we conclude that, with decreasing red cell volume, there is a compensating increase in plasma volume. However, there was on average a higher fetal total blood volume per kg body weight in hydropic fetuses.

Human fetal total blood volume has been estimated, in different studies, to lie between 94 and 176 ml/kg. Yao et al. [24] estimated the fetoplacental blood volume, using a radioactively labelled albumin dilution method, to be 105 ml/kg in the term newborn. This volume was determined from the sum of blood volume in the newborn plus the residual volume in the cord and placenta [24]. However, it is known that plasma is lost from the fetal circulation during labor, resulting in an increase of hematocrit and a lowering of fetal blood volume. The study of Yao et al. [24] is the only study that was performed in a non-anemic human population. Several investigators have used the change in hematocrit during transfusion to calculate the blood volume in anemic fetuses. In a first formula, the assumption was made that the transfusion volume was added to the pre-transfusion blood volume and that no volume is lost during transfusion. With this formula, the mean fetal blood volume was underestimated and calculated to be between 94 and 115 ml/kg [13–16]. With a second formula, the assumption was made that the blood volume before and after transfusion remained the same [12]. With this formula, the mean fetal blood volume was overestimated and calculated to be 176 ml/kg [19]. Another method was proposed by Hoogeveen et al. [19] to calculate total blood volume with the use of a dilutional principle (of fetal hemoglobin with adult hemoglobin), so the amount of plasma that is lost during transfusion does not influence calculations. With this method, which we also used in this study, a mean fetal blood volume of 121 ml/kg was found [19].

We believe our method to calculate the fetal blood volume is quite accurate, without using artificial red cell labelling. A few assumptions have to be made, however. First, we assume that the hemoglobin concentration differences are representational of the red cell volume differences. This is only true if there is no free hemoglobin in the circulation and if there are no differences in MCHC between fetal and donor blood. We tested the variation in MCHC before and after transfusion and found it to be small. Second, we neglected the small amount of fetal hemoglobin that can be found in donor blood, because in the study by Hoogeveen et al., fetal hemoglobin in the
donor blood was always lower than 1% [19]. Finally, we have to accept that errors can occur due to measurement errors of hemoglobin, hematocrit and percentage fetal hemoglobin. In 9 cases, an interim blood sample was taken, giving us the opportunity to verify the range of error that occurs, when using our method to determine the total blood volume. The difference between the interim measurement and the final measurement had an SD of 16 ml. When estimating the total blood volume per kg body weight, there is also a possible error in the sonographically estimated fetal weight. Recent literature showed that, when using the formula of Hadlock et al. [21] or closely related formulas, in only 86.5% of the cases, the prediction was within 15% of actual birth weight [25]. In case of hydrops fetalis, this measurement error may be even larger. With increasing fetal weight, the absolute error becomes larger, which might also explain the wider variance of fetal blood volume that we observed with increasing fetal weight.

Our finding that the severity of anemia has no influence on fetal total blood volume is in agreement with studies in adults and neonates [2–4]. Studies in human fetuses have also concluded that there is no evident relation between blood volume and severity of anemia or hydrops [13–16]. However, these studies all used the formula that underestimates blood volume and that does not take into account a plasma shift during transfusion. Our study is the first study that uses the more accurate calculation method to study the influence of anemia on total blood volume in a large number of human fetuses. In our study population, some very severely anemic fetuses were included, with a hemoglobin deficit of up to 12 SD below the normal mean, making it possible to draw conclusions on the entire scope of severity of anemia. We found it remarkable to find that even very young fetuses obviously adequately maintain their total blood volume.

Although there was a significant relation between hydrops and fetal total blood volume per kg body weight, the sample size of the hydropic fetuses was small, which warrants caution in drawing any conclusions. Hydronic fetuses on average had a 17% higher blood volume. This is in accordance with the hypothesis that hydrops is (in part) a consequence of congestive heart failure, or a state of high-output failure, with a rise in venous return. However, the increase in cardiac output that has been reported in anemic fetuses [6–8] does not always coincide with an increase in total blood volume, as nonhydric fetuses apparently maintain their blood volume. This must result in a hyperdynamic circulation, where the same blood volume has a shorter circulation time. The increase in blood flow velocities corresponds to this phenomenon. The cardiomegaly that is usually observed can be explained by the increased stroke
volume, most probably resulting from the decrease in peripheral resistance due to the decreased viscosity of the blood and vasodilatation [2, 7, 26, 27].

It has been shown that the red cell volume is a better measure for the severity of anemia, or oxygen delivery capacity, in sick neonates, than hemoglobin concentration or hematocrit [28, 29]. Since we found that the decrease in red cell volume, on average, is compensated by an increase in plasma volume, the hemoglobin concentration or hematocrit is a representative measure for the severity of anemia, in the alloimmunized fetus. When performing an intrauterine transfusion, the hematocrit of the initial blood sample therefore is the best basic assumption on which the required amount of donor blood can be calculated. The hematocrit of the final blood sample can, however, be lower than what is actually achieved, because in the days after transfusion there probably will be a return to the initial total blood volume and thus a further increase in hematocrit.

In conclusion, we found an average fetal total blood volume of 123 ml/kg in nonhydropic fetuses and 144 ml/kg in hydropic fetuses. In the human nonhydropic fetus, total blood volume is maintained when severe hemolytic anemia develops. Thus, the decrease in red blood cell volume is compensated by an increase in plasma volume. In hydropic fetuses, however, total blood volume seems to be increased. Thus, there is an overcompensating expansion in plasma volume. This is in accordance with the hypothesis that congestive heart failure plays a role in the pathophysiology of hydrops in anemic fetuses.

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