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## Fetal fluid and protein dynamics

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## Bilirubin/Albumin Ratios in Fetal Blood and in Amniotic Fluid in Rhesus Immunization

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## Abstract

### **Objective**

To test the hypothesis that unconjugated bilirubin is equally distributed over the albumin molecules present in fetal blood and amniotic fluid in Rhesus (Rh) immunization.

### **Methods**

Molar concentrations of unconjugated bilirubin and albumin were measured in fetal blood and amniotic fluid samples, obtained before the first intrauterine transfusion in 30 nonhydropic, anti-D-alloimmunized fetuses, with gestational ages ranging from 20 to 35 weeks.

### **Results**

Bilirubin concentration in amniotic fluid was best predicted by a combination of bilirubin concentration in fetal blood ( $p < .001$ ), albumin concentration in fetal blood ( $p = .008$ ), and albumin concentration in amniotic fluid ( $p < .001$ ) (adjusted  $R^2 = 0.91$ ). The bilirubin/albumin ratios in fetal blood were linearly correlated with the bilirubin/albumin ratios in amniotic fluid ( $R^2 = 0.75$ ,  $p < .001$ ). However, the bilirubin/albumin ratios in fetal blood were always higher than the bilirubin/albumin ratios in amniotic fluid (regression coefficient 1.4, 95% confidence interval 1.1–1.7). In our population, a bilirubin/albumin ratio in amniotic fluid of 0.10 or greater had a better sensitivity and specificity to predict severe anemia (Z-hemoglobin  $-5$  standard deviations or less) than the Queenan 4 or the Liley 2c line.

### **Conclusion**

The relation between fetal hemolysis and amniotic fluid bilirubin concentration is based on the linear correlation between bilirubin/albumin ratios in fetal blood and in amniotic fluid. The slope in Queenan's and Liley's chart follows that of the albumin concentration in amniotic fluid during gestation.

## Introduction

Bilirubin is the degradation product of hemoglobin. Its main configuration in the fetus is unconjugated [1]. Unconjugated bilirubin is hydrophobic and tightly but reversibly bound to albumin in extracellular fluids [2]. In fetal blood the albumin concentration increases between 20 and 35 weeks of gestation [3]. In amniotic fluid the albumin concentration initially increases between 20 and 24 weeks, but then decreases between 25 and 35 weeks [4,5]. Unconjugated bilirubin is cleared from fetal blood over the placenta to maternal blood [6]. Conjugation of bilirubin and excretion through the gall bladder or the kidneys are usually not triggered until a few days after birth [7,8]. The small amount of conjugated bilirubin that is formed prenatally is probably converted to unconjugated bilirubin in the fetal intestines and reabsorbed in the fetal circulation [9]. Thus, most of the bilirubin in the fetus and in amniotic fluid is unconjugated and bound to albumin.

Since the early 1960s, measurement of the concentration of bilirubin in amniotic fluid has been used to predict the severity of fetal hemolytic anemia and to decide on the necessity of intrauterine red cell transfusion [10,11]. Recently, noninvasive Doppler studies have been introduced to predict fetal anemia [12]. Nevertheless, the Queenan chart or the Liley chart still are important diagnostic tools in determining the timing of the first intrauterine red cell transfusion because these tests have a high sensitivity in this respect [13]. The mechanisms behind these diagnostic tools, however, have not been completely unraveled. Yet, understanding the pathways that bilirubin takes to distribute to the fetal compartments could lead to a better comprehension of the pathophysiology of fetal hemolytic disease, which may further improve our management of fetal anemia and neonatal hyperbilirubinemia caused by alloimmunization.

We hypothesized that unconjugated bilirubin is equally distributed over the albumin molecules that are present in all fetal compartments, including amniotic fluid, before it is transported across the placenta toward the maternal blood. Therefore, we measured the molar concentration ratios of bilirubin to albumin in fetal blood and in amniotic fluid and investigated their correlation. We expected that, if our hypothesis were true, there would be a significant linear relation between the two ratios. Furthermore, assuming that there would be no difference in binding capacity of albumin for bilirubin in the different compartments, the regression coefficient of this relation would be 1.

## Materials and Methods

Leiden University Medical Centre is the national referral center for the treatment of fetal anemia in the Netherlands. Our methods for diagnosis and treatment of severe fetal alloimmune anemia have been described previously [14]. From January 2001 to December 2004, we simultaneously sampled blood and amniotic fluid of singleton, nonhydropic, not previously transfused fetuses suffering from severe Rhesus D alloimmunization with gestational ages ranging from 20 to 35 weeks gestation. None of the fetuses had chromosomal or congenital abnormalities. Bilirubin and albumin concentrations were measured in fetal blood samples taken before the first intrauterine transfusion and in amniotic fluid samples taken within two days before commencing intrauterine transfusion. No additional amniocenteses or cordocentesis were performed to collect the data. This study was an addition to the “Diagnostic amniocentesis or non-invasive Doppler for the diagnosis of severe fetal anemia” study [12], that was approved by the medical ethics committee of the Leiden University Medical Center, and for which all woman gave oral or written informed consent.

Fetal blood samples were sent to our diagnostic laboratories. In our routine clinical chemistry laboratory measurements were made of total bilirubin, conjugated bilirubin and albumin on Oya Hitachi p800 modular autoanalyzer (Roche, Mannheim, Germany). Also, hemoglobin was measured in our routine hematology laboratory on Sysmex XE 2100 (Sysmex, Kobe, Japan). In fetal blood, conjugated bilirubin was subtracted from total bilirubin to calculate the concentration of unconjugated bilirubin. These measurements are reported in micromolars per liter. Albumin was converted from grams per liter to micromolars per liter by multiplying by a factor of 14.4 [15]. Amniotic fluid was stored light protected, and delta OD450 was measured within 1 hour after sampling, as published before [13]. It has been shown that this method measures merely unconjugated bilirubin [16]. The concentration of bilirubin in micromolars per liter was established by multiplying the delta OD450 value by a factor of 27.1 [17]. The concentration of albumin in amniotic fluid was measured by using a turbidimetric method on a Cobas Integra 800 autoanalyzer (Roche, Mannheim, Germany). This analysis took place at the section of liquor cerebri analysis in the Department of Clinical Chemistry.

Standardized Z scores of hemoglobin (Z-hemoglobin) were defined as the number of standard deviations (SDs) that an actual value deviated from the normal mean for gestational age. Reference values for hemoglobin were derived from the literature

[18]. Pearson correlation coefficients were calculated to study relations between different variables, since data were normally distributed. Normality was tested by the Kolmogorov-Smirnov test. We considered  $p < .05$  to be significant. The statistical software program SPSS 12.0.1 (SPSS Inc., Chicago, IL) was used. The form of the relation between the concentration of albumin in amniotic fluid and gestation was studied with polynomial regression. The ratio of bilirubin concentration to albumin concentration was expressed as a fraction (mol/mol). The relation between the bilirubin/albumin ratio in fetal blood and severity of anemia and gestational age was studied with linear regression. The same was done for the bilirubin/albumin ratio in amniotic fluid. After that, linear regression was performed to study the relation between the bilirubin/albumin ratio in fetal blood and the bilirubin/albumin ratio in amniotic fluid. Because in clinical practice the bilirubin concentration in amniotic fluid is used as a predictor for the amount of hemolysis in the fetal blood, the bilirubin/albumin ratio in fetal blood was chosen as the dependent variable and the bilirubin/albumin ratio in amniotic fluid as the independent variable. To study the additional influence of gestational age and severity of anemia, a multivariable linear regression analysis was performed with the bilirubin/albumin ratio in fetal blood as dependent variable and the bilirubin/albumin ratio in amniotic fluid, gestational age, and Z-hemoglobin as independent variables. Because bilirubin originates in fetal blood and subsequently enters the amniotic fluid, we also performed a multivariable linear regression with bilirubin concentration in amniotic fluid as dependent variable and bilirubin concentration in fetal blood, albumin concentration in amniotic fluid, and albumin concentration in fetal blood as independent variables. Finally, a receiver operating characteristic curve was made to determine the optimal cutoff value of the bilirubin/albumin ratio in amniotic fluid to predict severe anemia. Fetuses were considered severely anemic at Z-hemoglobin of  $-5$  SD or less. The cutoff was considered optimal when the sum of the sensitivity and specificity was maximal. The sensitivity and specificity of the chosen cutoff value was then compared with the sensitivity and specificity of the cutoff line 4 in the Queenan chart and the cutoff line 2c in the extended Liley chart [12].

## Results

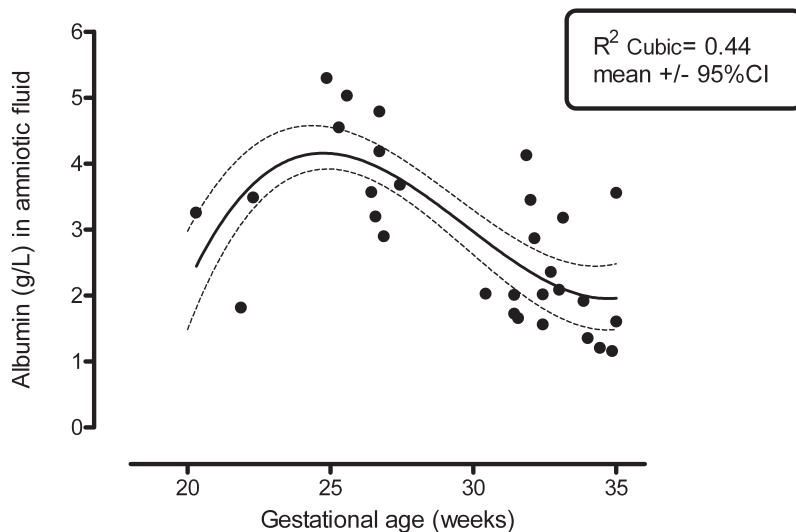
In the study period, 89 Rhesus D-immunized fetuses received their first intrauterine transfusion. Simultaneous sampling of amniotic fluid and fetal blood was performed in 30 singleton, nonhydropic fetuses. Maternal and fetal characteristics are shown in Table 1.

**Table 1** Maternal and fetal characteristics

Characteristics	Mean (Range)
Maternal age (years)	31.5 (20 – 41)
Gravidity	3.3 (1 – 8)
Parity	1.6 (0 – 4)
Last determined antibody titer	1:64 – 1:8000
Last determined ADCC* (%) [19]	55 to more than 80
Gestational age at transfusion (weeks)	29.8 (20 – 35)
Hematocrit at transfusion	0.23 (0.09 – 0.32)
Hemoglobin at transfusion (g/dL)	7.3 (2.6 – 10.8)

\* ADCC: Antibody-Dependent Cell-mediated Cytotoxicity assay.

Figure 1 shows the albumin concentration (grams per liter) in amniotic fluid during gestation. A cubic regression line fitted the data best (adjusted  $R^2$  linear 0.29, adjusted  $R^2$  quadratic 0.39, adjusted  $R^2$  cubic 0.44). An increase in albumin concentration between 20 and 24 weeks of gestation and a decrease between 25 and 35 weeks of gestation was observed. However, the interindividual variance was large.

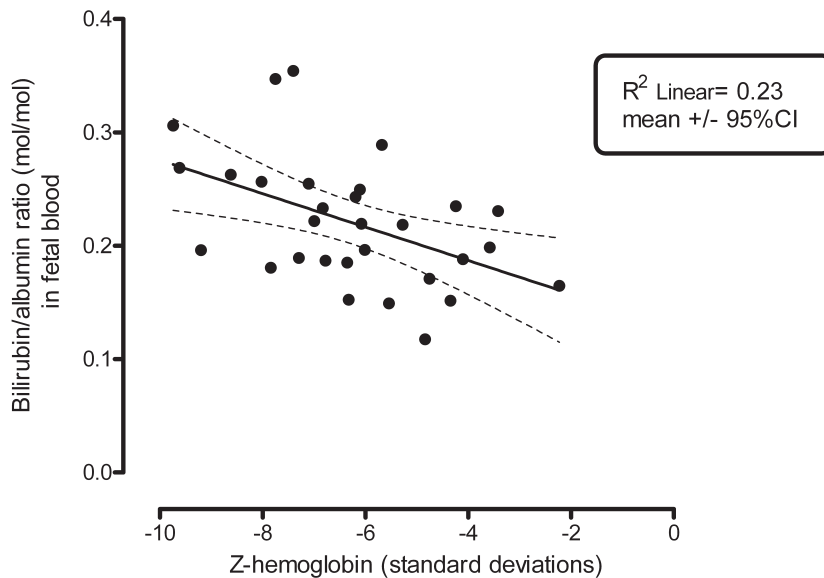


**Figure 1** Concentration of albumin (g/L) in amniotic fluid as a function of gestational age (weeks). Mean and its 95%CI are plotted.

In fetal blood, the bilirubin/albumin ratio ranged from 0.12 to 0.35. Figure 2 shows the bilirubin/albumin ratio in fetal blood plotted against standardized hemoglobin concentrations (Z scores). There was a significant correlation of the bilirubin/albumin ratio in fetal blood with the severity of anemia ( $R^2$  0.23,  $p=.007$ ). There was no relation between the bilirubin/albumin ratio in fetal blood and gestational age ( $R^2$  0.00,  $p=.92$ ).

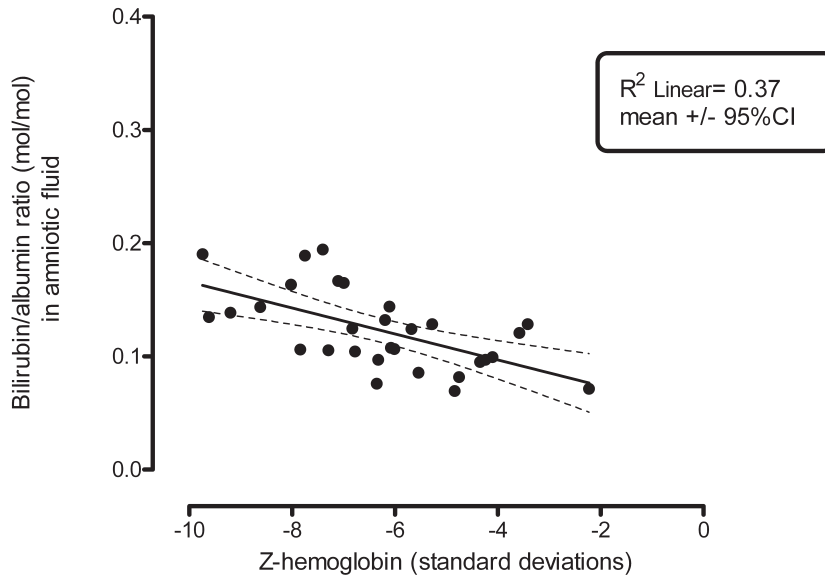
In amniotic fluid, the bilirubin/albumin ratio ranged from 0.07 to 0.19. Figure 3 shows the bilirubin/albumin ratio in amniotic fluid plotted against standardized hemoglobin concentrations (Z scores). There was a significant correlation of the bilirubin/albumin ratio in amniotic fluid with the severity of anemia ( $R^2$  0.37,  $p<.001$ ). There was no relation between the bilirubin/albumin ratio in amniotic fluid and gestational age ( $R^2$  0.004,  $p=.74$ ).

Figure 4 shows the linear relation between the bilirubin/albumin ratio in fetal blood and the bilirubin/albumin ratio in amniotic fluid ( $R^2$  0.75,  $p<.001$ ). Notably, the bilirubin/albumin ratios in fetal blood were always higher than the bilirubin/albumin ratios in amniotic fluid.

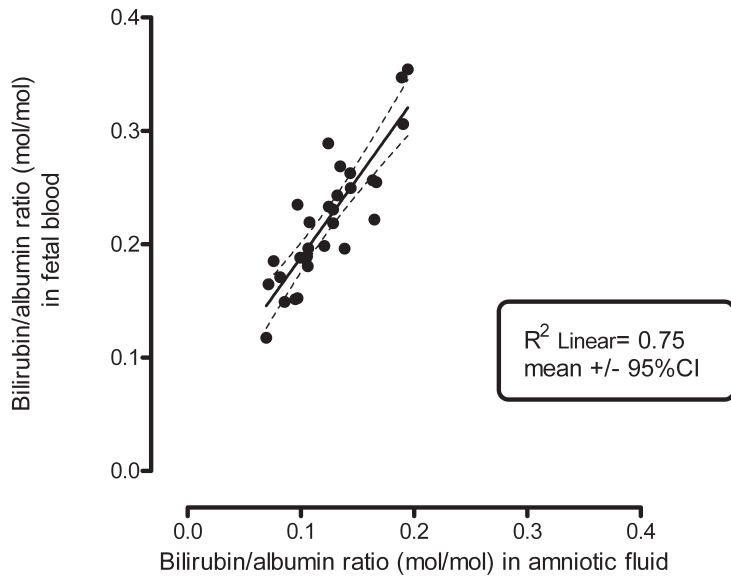


**Figure 2** Bilirubin/albumin molar concentration ratio in fetal blood against standardized hemoglobin concentration (Z scores). Mean and its 95%CI are plotted.





**Figure 3** Bilirubin/albumin molar concentration ratio in amniotic fluid against standardized hemoglobin concentration (Z scores). Mean and its 95%CI are plotted.



**Figure 4** Bilirubin/albumin molar concentration ratio in fetal blood against bilirubin/albumin molar concentration ratio in amniotic fluid. Mean and its 95%CI are plotted.

The formula of the regression line shown in figure 4 was as follows: mean bilirubin/albumin ratio in fetal blood =  $0.05 + 1.4 \times$  mean bilirubin/albumin ratio in amniotic fluid (95% confidence interval of the constant is 0.01–0.09; 95% confidence interval of the regression coefficient is 1.1–1.7). To study the additional influence of gestational age and severity of anemia, a multivariable linear regression analysis was performed. This showed that the bilirubin/albumin ratio in amniotic fluid was still significantly related to the bilirubin/albumin ratio in fetal blood (regression coefficient = 1.5,  $p < .001$ ), while there was no significant influence of gestational age (regression coefficient = 0.00,  $p = .72$ ) and severity of anemia (Z-hemoglobin) (regression coefficient = 0.002,  $p = .59$ ).

Because bilirubin originates in fetal blood and subsequently enters the amniotic fluid, we also performed a multivariable linear regression with bilirubin concentration in amniotic fluid as the dependent variable. This showed that bilirubin concentration in fetal blood ( $p < .001$ ), albumin concentration in fetal blood ( $p = .008$ ), and albumin concentration in amniotic fluid ( $p < .001$ ) were all independently related to the bilirubin concentration in amniotic fluid. The adjusted  $R^2$  of this model was 0.91.

In the receiver operating characteristic curve (ROC curve, not shown), we found that 0.10 was the optimal cutoff value for the bilirubin/albumin ratio in amniotic fluid to predict severe anemia. Table 2 shows the comparison between sensitivities and specificities in our study population of this chosen cutoff value and commonly used cutoffs in the Queenan and extended Liley charts.

**Table 2** Test characteristics of the bilirubin/albumin ratio and Queenan and extended Liley charts to diagnose severe anemia\*

	<b>Cutoff</b>	<b>Sensitivity (%)</b>	<b>Specificity (%)</b>
Bilirubin/ albumin molar ratio	0.10 or greater	86	75
Queenan chart	4 line or greater	82	25
Extended Liley chart	2c line or greater	72	25

\* Severe anemia is defined as Z-hemoglobin of -5 standard deviations or less

## Discussion

In this study, we found a strong linear correlation between the bilirubin/albumin molar concentration ratio in fetal blood and this same ratio in amniotic fluid. This is a strong argument in favor of our hypothesis that bilirubin is distributed over the available albumin in fetal blood and amniotic fluid. However, in contrast with our hypothesis, bilirubin is not distributed in an equal manner over the albumin in these fetal compartments because the bilirubin/albumin ratio in fetal blood was always higher than the bilirubin/albumin ratio in amniotic fluid.

Our curve of the mean amniotic fluid albumin concentration during pregnancy confirms previously published data [4,5]. It is also very similar to the cutoff lines of amniotic fluid bilirubin concentration during gestation in the Queenan and Liley charts [10,11]. This similarity is readily explained by the fact that, in amniotic fluid, bilirubin is bound to albumin. Our data are in support of findings by Queenan et al. [11] that linearly extending the Liley graph below 22 weeks is not to be advised.

The range of the bilirubin/albumin ratio of 0.12–0.35 that we observed in fetal blood was similar to findings of Ritter et al. [20]. In neonates, approximately 30% binding of bilirubin to albumin in blood was found [20]. We observed no correlation between the bilirubin/albumin ratios and gestational age. Robertson et al. [21] studied albumin reserve binding capacity for bilirubin in umbilical cord serum and also found no difference between 18 to 42 weeks of gestation.

It is known that there is one strong binding site on albumin for bilirubin and several weaker binding sites [2]. Our observed regression coefficient of 1.4 of the regression line between the bilirubin/albumin ratios in fetal blood and amniotic fluid may be explained by a difference in biochemical qualities between blood and amniotic fluid, which influence the binding force of one or more binding sites on albumin. Another possible cause for the fact that the bilirubin/albumin ratio in fetal blood was always higher than the bilirubin/albumin ratio in amniotic fluid could be a difference in competitive binding. Either way, the linear relation between the ratios seems to be caused by a constant difference in property between fetal blood and amniotic fluid. We speculate that a difference in pH could explain the observed regression coefficient of 1.4 between the ratios in fetal blood and amniotic fluid. In vitro experiments have shown that 1 mol albumin in serum binds 1.9 mol bilirubin at a pH of 7.4. With a decline in pH, albumin will bind less bilirubin [22]. The difference in pH between fetal blood and amniotic fluid (respective means of 7.3 and 7.1 in alloimmunized fetuses

[23]) could thus be the cause of the observed difference in binding capacity of albumin for bilirubin.

Our results contain strong arguments in favor of the theory that amniotic fluid bilirubin concentration is determined both by the bilirubin concentration in fetal blood and by the albumin concentrations in fetal blood and amniotic fluid. Although urine is the principal source of amniotic fluid, it is unlikely that the fetal kidneys are the pathway over which bilirubin can enter the amniotic fluid because the concentration of protein is 100 times lower in fetal urine than in amniotic fluid. The most likely pathway over which bilirubin can constantly be balanced out over the available albumin, therefore, seems to be the intramembraneous pathway. The intramembraneous pathway is the combined permeable surface that is adjacent to the amniotic fluid. Initially, the fetal skin and mucous membranes are an important component of this pathway, and after keratinization of the skin, which occurs between 17 and 25 weeks of gestation, the main component that remains is the fetal side of the placenta [24]. Knowledge on the origin of albumin in amniotic fluid could complete our understanding of this fetal physiological mechanism.

Already in 1965, Cherry et al. [25] investigated the correlation of the bilirubin/protein ratio in amniotic fluid with the severity of anemia. Sensitivities to predict anemia with this ratio were, however, variable [26-28]. The diversity of the methods of measurements may explain some of these variable results. Furthermore, false-negative prediction was reported in fetuses that turned out to be hydropic [29]. Nowadays, delta OD450 will not be used clinically, in an alloimmunized patient, when hydrops is identified sonographically.

Our findings do have clinical implications. First, understanding the background of a diagnostic test gives one the opportunity to understand exceptional cases. In an anemic fetus with an abnormal concentration of albumin in the amniotic fluid—for example, due to kidney disease, hydramnios, hydrops, or growth restriction—an unexpected result in the Queenan or Liley charts may be found. In an anemic fetus with a low bilirubin concentration in fetal blood—for example, in Kell immunization—bilirubin concentration in amniotic fluid may also be lower than expected [30]. Second, we observed a significant correlation of the bilirubin/albumin ratio, both in fetal blood and amniotic fluid, with severity of anemia. Theoretically, the reliability of the Queenan or Liley charts in predicting the degree of hemolysis should be impaired by the wide interindividual variation of amniotic fluid albumin concentration. Using the bilirubin/albumin ratio in amniotic fluid may, therefore, improve our ability to predict the severity

of fetal anemia. Also, the bilirubin/albumin ratio does not change during gestation, making the test easy to interpret. In our study population, the bilirubin/albumin ratio was a more accurate test for diagnosing severe anemia than the Queenan and Liley charts. However, the sensitivities and specificities of the Queenan and Liley charts in our study were lower than in other studies [12,13]. The suggested cutoff value of the bilirubin/albumin ratio of 0.10 should be validated in an independent data set, preferably a prospective cohort.

In conclusion, amniotic fluid bilirubin concentration is determined by both the bilirubin concentration in fetal blood and by the albumin concentrations in fetal blood and in amniotic fluid. The relation between fetal hemolysis and amniotic fluid bilirubin concentration is based on the linear correlation between bilirubin/albumin ratios in fetal blood and in amniotic fluid. The slope in Queenan's and Liley's charts follows that of the albumin concentration in amniotic fluid during gestation.

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## Addendum

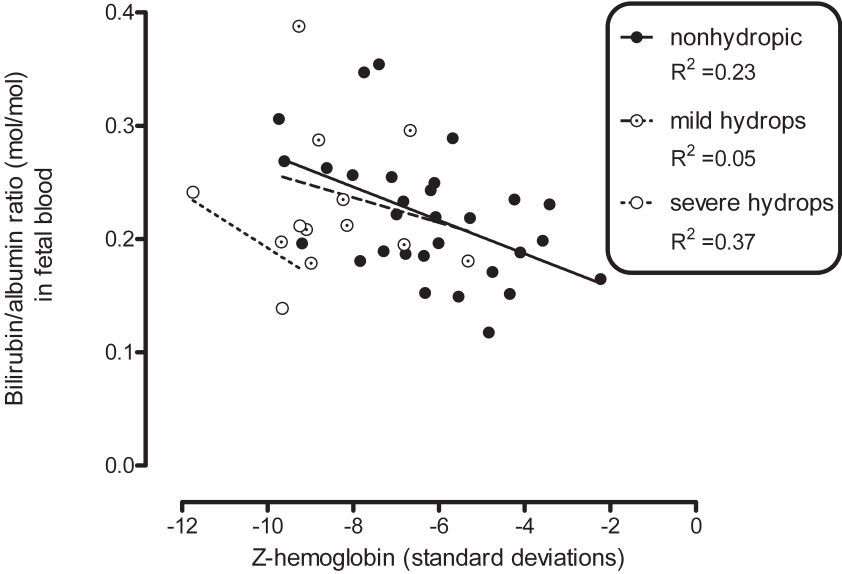
### **Hydropic cases**

The study results as presented in chapter 3, are shown here with an addition of 13 hydropic fetuses. We present 10 mildly hydropic fetuses (semi filled dots) and 3 severely hydropic fetuses (open dots). Fetuses were classified as mildly hydropic in case a distinct rim of ascites and/or pericardial effusion is observed. Fetuses were classified as severely hydropic in case an abundant amount of fluid collection with skin edema is observed.

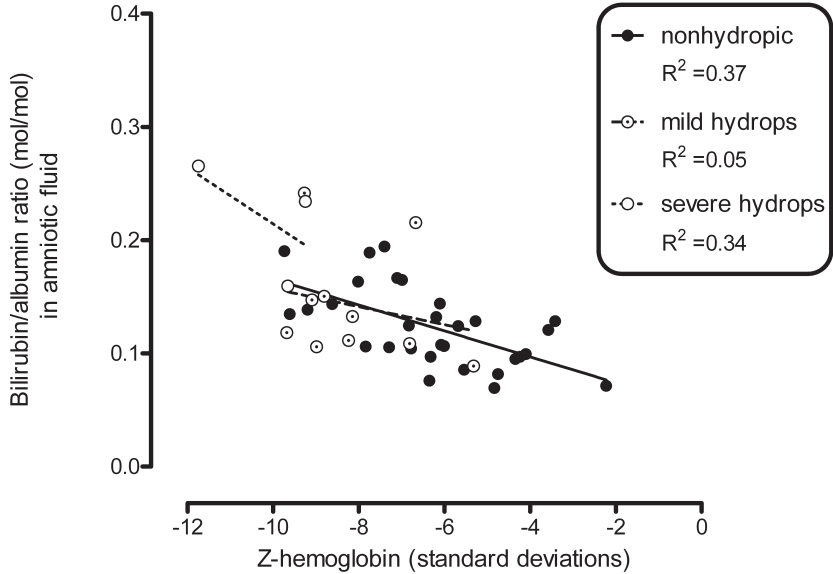
In figure 1 it is shown that the bilirubin to albumin ratio (BAR) in fetal blood increases with increasing severity of anemia. However, the severely hydropic fetuses have a relatively low BAR in fetal blood. Figure 2 shows the increase in BAR in the amniotic fluid with severity of anemia. There is no difference observed between nonhydropic and hydropic fetuses. Figure 3 shows the relation of the BAR in fetal blood to the BAR in amniotic fluid. In nonhydropic and mildly hydropic fetuses, the BAR in fetal blood is always higher compared to that in amniotic fluid. Interestingly though, in the severely hydropic fetuses the BAR in fetal blood was almost the same as the BAR in amniotic fluid.

The low BAR in fetal blood in severely hydropic cases could be explained by either a diminished hematopoiesis or a diminished binding capacity of albumin in fetal blood. Again (as in the addendum of chapter 2), the mildly hydropic fetuses seemed not to differ from the nonhydropic fetuses, though severely hydropic fetuses show distinct differences.

In conclusion, it is possible that there is a decrease in binding capacity of albumin for bilirubin in fetal blood in severely hydropic fetuses. This might be the result of a change of fetal blood pH, a change in albumin posttranslational modifications or a change in competitive binding of other ligands to albumin. In addition, it is possible that there is an increase in albumin binding capacity in amniotic fluid. It is intriguing to consider the rise of protein in amniotic fluid, which has been reported in hydropic cases, to be the result of a capillary leakage of fetal albumin into the amniotic fluid.

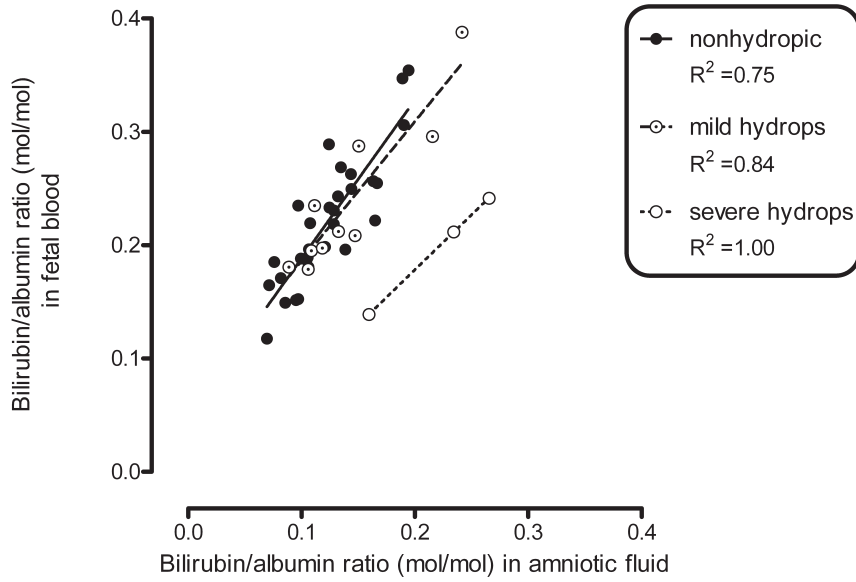


**Figure 1** Bilirubin to albumin ratio (mol/mol) in fetal blood in relation to severity of anemia (Z-hemoglobin). Non-, mildly and severely hydropic fetuses are depicted.



**Figure 2** Bilirubin to albumin ratio (mol/mol) in amniotic fluid in relation to severity of anemia (Z-hemoglobin). Non-, mildly and severely hydropic fetuses are depicted.





**Figure 3** Bilirubin to albumin ratio (mol/mol) in fetal blood in relation to that in amniotic fluid. Non-, mildly and severely hydropic fetuses are depicted.