Diagnostic procedures for assessing the severity of alloimmune fetal anemia

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Amniotic fluid Δ OD 450 values accurately predict severe fetal anemia in D-alloimmunization

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Abstract

Objective: To assess the diagnostic accuracy of the extrapolated Liley curve.
Methods: We searched our database for singleton D-alloimmunized pregnancies with non-hydropic fetuses, where amniocentesis was performed within 4 days of first fetal blood sampling. Amniotic fluid Δ OD 450 values were plotted on an extrapolated Liley chart. Sensitivity and specificity were calculated for two commonly used cut-off levels, Liley's zone 3 and the upper third of Liley's zone 2. Severe fetal anemia was defined as a hemoglobin concentration of 5 standard deviations below the normal mean for corresponding gestational age.
Results: Seventy-nine pregnancies met our inclusion criteria. Overall accuracy of the extrapolated Liley curve in predicting severe fetal anemia was 75% (95%CI: 64-84) for zone 3 and 86% (95%CI: 77-93) when the upper third of zone 2 was included. Sensitivity of Δ OD 450 values in Liley's zone 3 or the upper third of Liley's zone 2 was 95% (95%CI: 74-100) before and 98% (95%CI: 89-100) after 27 weeks.
Conclusion: Liley's extrapolated curve predicts severe fetal anemia with reasonable accuracy and high sensitivity.

Introduction

In 1961, Liley proposed amniotic fluid sampling to measure deviation of optical density at 450 nm (Δ OD 450) to predict life-threatening fetal anemia in the third trimester.[1] After intrauterine intravascular transfusion (IUT) became a relatively safe procedure as early as 18 weeks, the original Liley chart was extrapolated to the second trimester to also predict severe anemia there. This was done by linear extension of the two lines that divide Liley's three zones.[2-4] The American College of Obstetrians and Gynecologists (ACOG) recommends serial amniocentesis in pregnancies at risk, followed by IUT or early delivery when Δ OD 450 values are in Liley zone 3 or in the upper third of Liley zone 2 and rising.[5] Several authors have proposed management schemes based on different cut-off values for Δ OD 450.[6-12] Among these, the Queenan chart is the most popular.[10] In 1986, Nicolaides et al. concluded that Δ OD 450 values were unreliable as predictors of severe anemia in second trimester pregnancies.[13] Others also questioned the value of Δ OD 450 during the third trimester.[8,1 4-16] These doubts applied both to Liley's original chart and to modified versions.[14] However, these studies included amniotic fluid samples that were taken more than a week before the 'gold standard' blood sample, and included cases with Kell antibodies where anemia is partially due to erythroid precursor damage and not merely the results of hemolysis.[17, 18] These studies also included cases of hydrops fetalis, where Δ OD 450 is not only unreliable but also superfluous.[19,20]

A critical evaluation of the diagnostic performance of Δ OD 450 measurement is warranted because new non-invasive methods are being introduced to replace amniocentesis.[21,22] These methods are based on the fact that blood viscosity, which declines along with hematocrit, is inversely related to maximum blood flow velocities in fetal vessels. The proponents of these Doppler methods claim great accuracy in the prediction of fetal anemia.[22-25] As a first step in comparing amniocentesis and non-invasive Doppler, we evaluated the ability of Δ OD 450 values to predict severe fetal anemia.

Methods

Leiden University Medical Center is the national referral center for the treatment of alloimmune fetal anemia in The Netherlands. Our methods for diagnosing and treating severe fetal anemia have been described previously.[26] Briefly, patients with high antibody titers are followed with weekly ultrasound examinations for signs of incipient hydrops or fetal anemia. These signs include hepatosplenomegaly, cardiomegaly, placental thickening, decreased fetal movements, and increased maximum flow velocities in the descending aorta and intrahepatic umbilical vein.[21] When severe anemia is suspected at or after 27 weeks, amniocentesis for Δ OD 450 is performed to avoid unnecessary fetal blood sampling. For the data in this study, we followed our center's established procedure, performing the first IUT when Δ OD 450 was in zone 3, or in upper third of zone 2 and rising.[5] In some fetuses after 27 weeks and in most before 27 weeks, the decision to perform the first IUT was based on ultrasound findings alone. In these cases, when a transamniotic approach to the fetal umbilical vein was necessary, amniotic fluid was collected and Δ OD 450 measured for the purpose of this study. This procedure was approved of by the hospital's ethical committee, and in each case oral informed consent of the mother was obtained. The data of all patients were stored in our database (*Paradox 9.0*, Corel Corporation, Ottawa, Canada). We searched this database for the period January 1988 to October 2000 for contemporaneous amniotic fluid and fetal blood samples that met the following criteria: they were taken from fetuses that were 1) Rhesus D-alloimunized, 2) non-hydropic, 3) not previously transfused, 4) singleton; and 5) amniotic fluid samples were taken less than four days before fetal blood sampling.

Amniotic fluid samples (5-10 ml), protected from light during transport, were centrifuged at 1000g for 10 minutes to remove vernix and erythrocytes. The absorption of the supernatant was measured at the wavelengths 365, 450 and 550 nm with an UltrospecPlus spectrofotometer (Amersham Pharmacia Biotech, Little Chalfont, UK). The bilirubin absorption, expressed as Δ OD 450, was calculated as the difference between the measured absorption at 450 nm and the background

absorption at 450 nm. The latter was derived, as described by Liley, from the logarithmic function of the absorptions between 365 and 550 nm.[1] Each Δ OD 450 was measured and entered into our database within an hour after amniocentesis. Only values at or after 27 weeks were used clinically. At IUT, a small portion of the initial fetal blood sample was used for on the spot measurement of hemoglobin concentration and mean red cell volume. Fetal hematocrit was used to calculate the volume of intravascular red cell transfusion.[27] The remaining fetal blood of the initial sample was sent to our central laboratory for hematological and other measurements. These latter values were automatically entered into our database and checked by a specialized nurse. Statistical analysis was performed using *SPSS 10.0* (SPSS Inc., Chicago, USA).

We copied Liley's original chart,[1] and found that the upper line that defined zone 2 crossed the vertical lines corresponding with 27 and 41 weeks at Δ OD 450 of 0.260 and 0.077 respectively; the (parallel) lower line defining zone 2 crossed the vertical line corresponding with 27 weeks at 0.066. We then drew a third parallel line through the Δ OD 450 of 0.160 at 27 weeks, as the delineation of the upper third of zone 2 (2c). All three lines were extrapolated backwards in a linear fashion from 27 to 18 weeks. Standardized amniotic fluid Δ OD 450 was calculated by dividing the Δ OD 450 measurement by the value on the line between zones 1 and 2 for the corresponding gestational age. For example, a Δ OD 450 of 0.260 nm at 27 weeks and a Δ OD 450 of 0.141 at 34 weeks are both on the border between zone 2 and 3. Both correspond with a standardized Δ OD 450 of 3.94. The latter value is found by dividing the Δ OD 450 measurements (0.260 and 0.141) by the cut-off values on the line between zone 1 and zone 2 (0.066 and 0.036 respectively) for the corresponding gestational ages. In this way, the standardized Δ OD 450 is independent of gestational age and indicates how much the measured value was higher than the corresponding boundary value between zone 1 and 2. Standardized values above 3.94 correspond to Liley values in zone 3.

Normal fetal hemoglobin values increase during gestation. We used the reference values proposed by Nicolaides et al. in 1988.[28] These reference values were obtained from 210 fetuses, ranging from 17 to 40 weeks, and

they have a constant standard deviation (SD) of 1 g/dl.[28] For the purpose of this study, we defined severe anemia as hemoglobin concentrations > 5 SD below the normal mean for gestational age. This cut-off was chosen because a higher cut-off would include fetuses in whom the need of treatment is not warranted, whereas a lower cut-off would include too many cases of hydrops fetalis, which would not only render the use of diagnostic amniocentesis redundant, but would also worsen the prognosis significantly.[29] Moderate anemia was defined as a hemoglobin concentration > 2 SD but ≤ 5 SD below the normal mean for gestational age. Standardized fetal hemoglobin scores were defined as the number of SDs that the actual value deviated from the normal mean for gestational age.

Sensitivity, specificity, and overall accuracy (combined rate of true-positive and true-negative results) were calculated for different Δ OD 450 cut-offs (Liley 3 and 2c) in the prediction of severe anemia, together with their exact 95% CI. Separate analyses were done for gestational ages above and below 27 completed weeks. Pearson R^2 were calculated between standardized Δ OD 450 and standardized hemoglobin. To study if this relation differed before and after 27 weeks, linear regression was performed with standardized hemoglobin as outcome variable and standardized Δ OD 450 as independent variable. The slopes of the regression lines before and after 27 weeks were compared by adding a dummy variable in the regression model, indicating whether the pregnancy was more than 27 weeks, and testing the significance of the interaction term between standardized Δ OD 450 and the dummy variable.

Results

In the 13 year study period, 249 fetuses were treated for alloimmune anemia with one or more IUT's; 139 of these were anti D-alloimmunized and non-hydropic, and 79 of them fulfilled our inclusion criteria of singletons with contemporaneous sampling of amniotic fluid and fetal blood at their first IUT. Mean gestational age of these 79 fetuses at the time of first IUT was 29 completed weeks (range 20-35) and mean

hemoglobin concentration was 6.3 g/dl (range 3.1- 13.2). Mean age of the 79 women was 32 years (range 23-44), and parity was 3.3 (range 2-14). There was one fetal and one neonatal death among these 79 cases. The fetal death occurred at 25 weeks due to an intrauterine infection following the 3rd IUT. In this case, the first IUT (the data from which were used in this study) took place at 21 weeks. The neonatal death occurred after an emergency cesarean section, 4 hours after the first IUT. This IUT took place at 35 weeks and was complicated by continuing leakage of blood from the cord at the puncture site.

Figure 1 plots hemoglobin values of these 79 fetuses against their gestational age, compared to the normal range (mean ± 2 SD) of fetal hemoglobin concentration as established by Nicolaides et al.,[28] as well as the –5 SD line that we used as the cut-off for severe anemia. Of the 79 fetuses included in this study, only one had a hemoglobin concentration in the normal range, 11 showed moderate anemia, and 67 had severe anemia at the time of first fetal blood sampling. Amniotic fluid Δ OD 450 values of the 79 fetuses are shown on the extrapolated Liley chart (Figure 2).

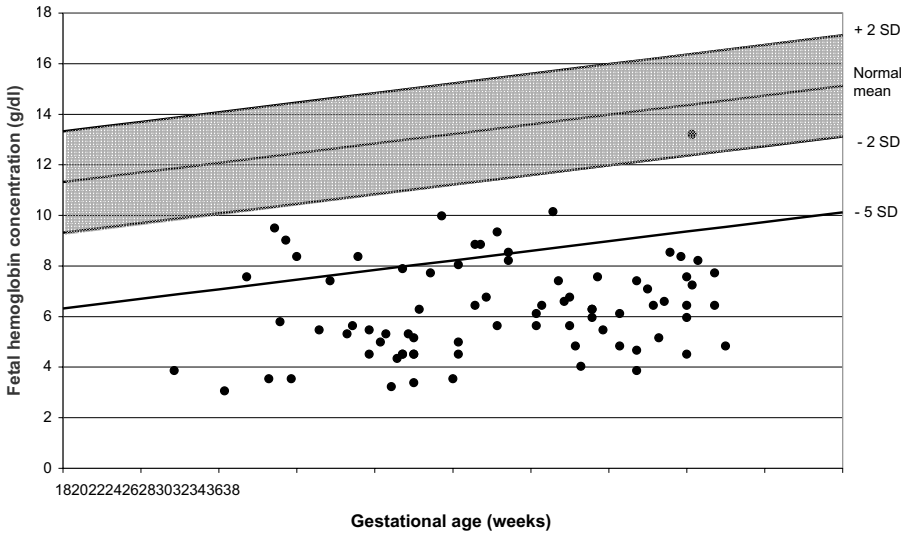

Figure 1 - Hemoglobin values of 79 non-hydropic Rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey zone between 3 upper ascending lines marks the limit of normal (mean ± 2 SD) fetal hemoglobin concentrations. The lower line seperates moderate (between –2 and –5 SD) from severe (< –5 SD) fetal anemia.

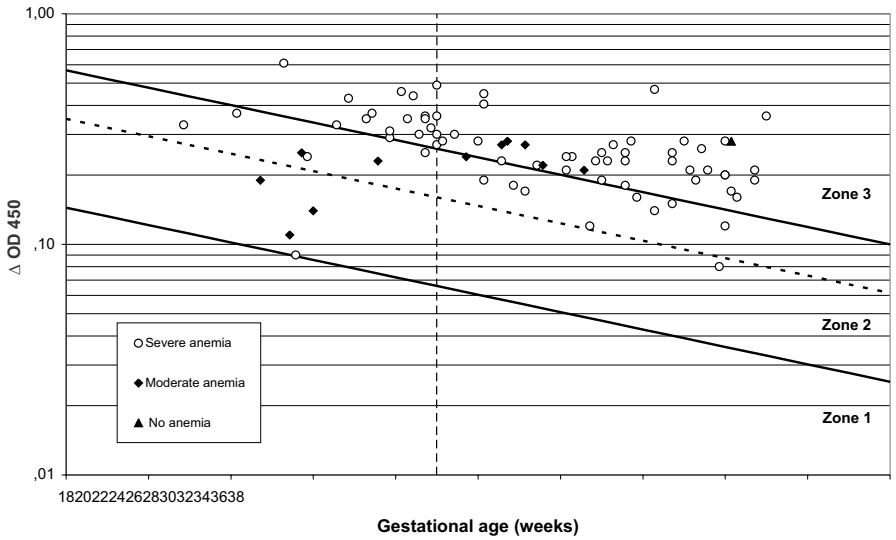

Figure 2 - Amniotic fluid Δ OD 450 values of 79 non-hydropic Rhesus D-alloimmunized fetuses at the time of first blood sampling, plotted in the extrapolated Liley curve. No anemia (▲) corresponds with hemoglobin concentrations within the normal range (mean ± 2 SD) for gestational age; moderate anemia (◆) corresponds with hemoglobin concentrations between 2 and 5 SD below the normal mean; and severe anemia (○) corresponds with fetal hemoglobin concentrations > 5 SD below the normal mean. The vertical axis (Δ OD 450) has a logarithmic scale and the horizontal axis (gestational age) has a linear scale. The vertical broken line is drawn at 27 weeks to divide Liley's original chart from the extrapolated part. The descending broken line divides zone 2 in an upper third (2c) and two lower thirds. Note that this upper third is on a visual and not on a logaritmic scale.

Figure 3 shows the relationship between standardized hemoglobin values at first IUT and contemporaneous standardized Δ OD 450 values of the 79 fetuses in our study. The linear correlation between standardized Δ OD 450 on a logarithmic scale and standardized hemoglobin was low (R^2 = 0.096). Pearson R^2 between Δ OD 450 and hemoglobin values was 0.315 for the samples taken before 27 weeks (n=24) and 0.018 when taken at or after 27 weeks (n=55). However, the slopes of the regression lines, with standardized hemoglobin as outcome variable and standardized Δ OD 450 as independent variable, did not differ significantly (p=0.21) before and after 27 weeks pregnancy. In addition, in Figure 3, horizontal lines were drawn at the thresholds of Liley zones 1, 2c and 3 and a vertical line at the threshold of severe anemia. As such, Figure 3 can be read as a two-by-two table. Cases on the left of the vertical line were severely anemic,

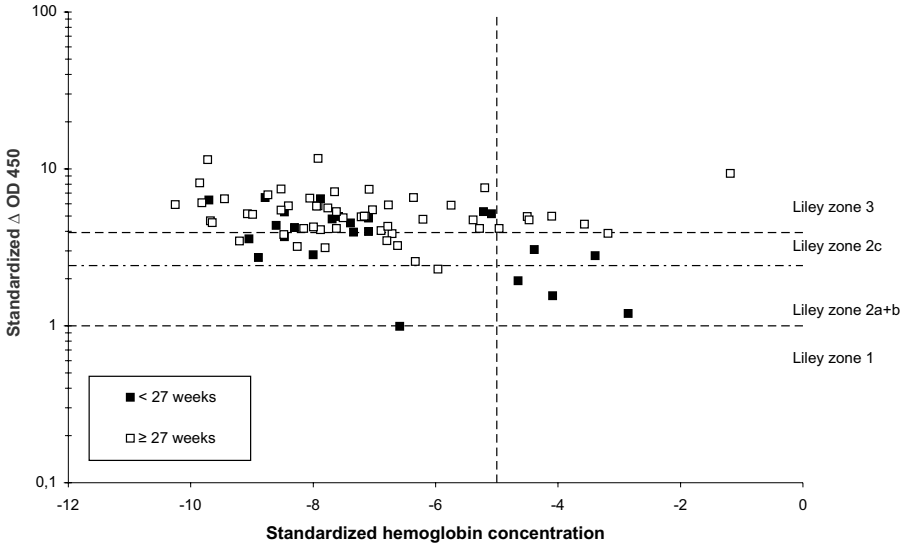

Figure 3 - Relationship between standardized Δ OD 450 and standardized hemoglobin concentrations in 79 non-hydropic Rhesus D-alloimmunized fetuses at the time of first blood sampling. Amniotic fluid Δ OD 450 were standardized by dividing the actual value by the value on the line between zone 1 and 2 for corresponding gestational age. Standardized hemoglobin concentrations were defined as the number of standard deviations that the actual value deviated from the normal mean for gestational age. The vertical broken line is drawn at the threshold of severe anemia (–5 SD). Thus, this figure can be read as a two-by-two table: cases on the left of the vertical line were severely anemic, and those above the chosen horizontal cut-off line (e.g., zone 3 or zone 2c) were true positives and those below were false negatives. Cases on the right of the vertical line were non-anemic or only moderately anemic, and those above the chosen cut-off were false positives, while those below were true negatives.

and those above the chosen horizontal cut-off line (e.g., zone 3 or zone 2c) were true positives and those below were false negatives. Cases on the right of the vertical line were non-anemic or only moderately anemic and those above the chosen cut-off were false positives, while those below were true negatives. Table 1 lists the two-by-two tables and test characteristics of amniotic fluid Δ OD 450 in the prediction of severe anemia. Accuracy of the extrapolated Liley curve in predicting severe fetal anemia was 75% (95%CI: 64-84) for zone 2 and 86% (95%CI: 77-93) when the upper third of zone 2 was included. Sensitivity of Liley's zone 3 was 74% (95%CI: 49-91) before and 81% (95%CI: 67-91) after 27 weeks. Sensitivity of Liley's zone 3 including zone 2c was 95% (95%CI: 74-100) before and 98% (95%CI: 89-100) after 27 weeks.

Table 1 - Two-by-two tables and test characteristics of Δ OD 450 in the prediction of severe anemia

Number of patients	*Range of gestational age (weeks)*	*Test cut-off*	*HB-deficit > 5 g/dl*	*Hb-deficit ≤ 5 g/dl*	*Sensitivity (%)*	*Specificity (%)*	*Accuracy (%)*
79	20 - 35	≥ Zone 3	53	6	79	50	75
		< Zone 3	14	6			
		≥ Zone 2c	65	9	97	25	86
		< Zone 2c	2	3			
24	< 27	≥ Zone 3	14	0	74	100	79
		< Zone 3	5	5			
		≥ Zone 2c	18	2	95	60	88
		< Zone 2c	1	3			
55	≥ 27	≥ Zone 3	39	6	81	14	73
		< Zone 3	9	1			
		≥ Zone 2c	47	7	98	0	85
		< Zone 2c	1	0			

Hb: Hemoglobin concentration; Hb-deficit: Difference between actual Hb and mean Hb for corresponding gestational age

Discussion

We compared Δ OD 450 with contemporaneous hemoglobin concentration in non-hydropic fetuses who were given their first IUT. The correlation between Δ OD 450 and fetal hemoglobin concentration in our study was weak. However, the clinical usefulness of Δ OD 450 was good since Liley's zone 3 and 2c predicted severe anemia with an overall sensitivity of 79% and 97% respectively. These sensitivities were roughly the same at gestational ages of 20 to 27 weeks and 27 to 35 weeks. Compared to previous studies on this subject,[13-15,30] we used very stringent inclusion criteria and collected data on a relatively large number of patients. The data were prospectively collected in our clinical practice, and we adhered to current guidelines.[5] We did not measure hemoglobin concentration in fetuses after 27 weeks with Δ OD 450 in Liley's zone 2 unless repeated measurements showed a rising trend or ultrasound indicated a high risk of fetal anemia.

In 1986, Nicolaides et al. published a paper with the challenging title "Have Liley charts outlived their usefulness?" in which they suggested that second trimester Δ OD 450 values were unreliable in predicting severe anemia and that fetal blood sampling should replace amniocentesis.[13] After excluding hydropic fetuses from that study, it appears that the upper half of Liley's zone 2 had a 94% sensitivity and a 43% specificity in predicting fetal hemoglobin concentration < 6 g/dl.[13] In 1998, Rahman et al. confirmed the results of Nicolaides study and also stated that predictions made on the basis of second trimester Δ OD 450 measurements are inaccurate.[30] They found an 80% sensitivity of Queenan's zone 3 to predict a fetal hematocrit below 15%. Nevertheless, given the difference in procedure-related risk between amniocentesis and fetal blood sampling, we believe that sensitivities between 80% and 100%, as found by using the upper third of Liley's zone 2, are acceptable. Therefore, we argue that Δ OD 450 measurements in the second and third trimester are still useful.

The ACOG recommends diagnostic amniocentesis for alloimmunization with high antibody titers from as early as 20 weeks gestation and therapeutic intervention when Δ OD 450 is in Liley's zone 3 or rising in the upper third of zone 2.[5] The results of our study support this guideline: a 95% sensitivity for detecting severe fetal anemia was found. A specificity of 50% or less and the risk of repeated amniocentesis remain the major drawbacks of this approach. False positive results of amniocentesis can lead to unnecessary IUTs with procedure-related fetal loss rates of 1 to 3%.[31] Fetal and perinatal procedure-related loss rates are reported to be 0.25 to 1% per amniocentesis.[32,33] Another drawback of amniocentesis or fetal blood sampling is the risk of feto-maternal hemorrhage which can increase the severity of alloimmunization. Feto-maternal hemorrhage occurs in 2.3% of cases after amniocentesis, and a significant increase in antibody titers occurs in 50% of cases after IUT.[34,35] Thus, there is still a need for non-invasive tests that can predict fetal anemia with equal or higher accuracy.

Recent studies suggest that arterial and venous Doppler flow velocities in fetal vessels accurately predict anemia.[22-25] These studies report that

Doppler measurements have sensitivities between 63% and 100% and specificities between 70% and 100% in the prediction of severe fetal anemia when performed by experienced operators. [22-25] However, there is a tendency to be overly optimistic about early results with new techniques. We are presently involved in a prospective multicenter trial to compare the diagnostic accuracy between Δ OD 450 measurements and maximum flow velocity in the fetal middle cerebral artery and the intrahepatic umbilical vein. Until the results of such prospective studies are available, we suggest that amniocentesis for Δ OD 450 measurement is still important in the management of severe Rhesus D-alloimmunization.

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