

ORIGINAL ARTICLE

Doppler Ultrasonography versus Amniocentesis to Predict Fetal Anemia

Dick Oepkes, M.D., P. Gareth Seaward, M.B., B.Ch.,
Frank P.H.A. Vandebussche, M.D., Rory Windrim, M.B., John Kingdom, M.D.,
Joseph Beyene, Ph.D., Humphrey H.H. Kanhai, M.D., Arne Ohlsson, M.D.,
and Greg Ryan, M.B., for the DIAMOND Study Group*

ABSTRACT

BACKGROUND

From the Department of Obstetrics (D.O., F.P.H.A.V., H.H.H.K.), Leiden University Medical Center, Leiden, the Netherlands; and the Fetal Medicine Unit, Department of Obstetrics and Gynaecology (P.G.S., R.W., J.K., G.R.), and the Department of Paediatrics (A.O.), Mount Sinai Hospital; and the Department of Public Health Sciences, University of Toronto (J.B.) — all in Toronto.

*Other members of the Diagnostic Amniocentesis or Noninvasive Doppler (DIAMOND) Study Group are listed in the Appendix.

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Pregnancies complicated by Rh alloimmunization have been evaluated with the use of serial invasive amniocentesis to determine bilirubin levels by measuring in the amniotic fluid the change in optical density at a wavelength of 450 nm (ΔOD_{450}); however, this procedure carries risks. Noninvasive Doppler ultrasonographic measurement of the peak velocity of systolic blood flow in the middle cerebral artery also predicts severe fetal anemia, but this test has not been rigorously evaluated in comparison with amniotic-fluid ΔOD_{450} .

METHODS

We performed a prospective, international, multicenter study including women with RhD-, Rhc-, RhE-, or Fy^a-alloimmunized pregnancies with indirect antiglobulin titers of at least 1:64 and antigen-positive fetuses to assess whether Doppler ultrasonographic measurement of the peak systolic velocity of blood flow in the middle cerebral artery was at least as sensitive and accurate as measurement of amniotic-fluid ΔOD_{450} for diagnosing severe fetal anemia. The results of the two tests were compared with the incidence of fetal anemia, as determined by measurement of hemoglobin levels in fetal blood.

RESULTS

Of 165 fetuses, 74 had severe anemia. For the detection of severe fetal anemia, Doppler ultrasonography of the middle cerebral artery had a sensitivity of 88 percent (95 percent confidence interval, 78 to 93 percent), a specificity of 82 percent (95 percent confidence interval, 73 to 89 percent), and an accuracy of 85 percent (95 percent confidence interval, 79 to 90 percent). Amniotic-fluid ΔOD_{450} had a sensitivity of 76 percent (95 percent confidence interval, 65 to 84 percent), a specificity of 77 percent (95 percent confidence interval, 67 to 84 percent), and an accuracy of 76 percent (95 percent confidence interval, 69 to 82 percent). Doppler ultrasonography was more sensitive, by 12 percentage points (95 percent confidence interval, 0.3 to 24.0), and more accurate, by 9 percentage points (95 percent confidence interval, 1.1 to 15.9), than measurement of amniotic-fluid ΔOD_{450} .

CONCLUSIONS

Doppler measurement of the peak velocity of systolic blood flow in the middle cerebral artery can safely replace invasive testing in the management of Rh-alloimmunized pregnancies. (ClinicalTrials.gov number, NCT00295516.)

IN PREGNANCIES COMPLICATED BY RED-cell alloimmunization, progressive hemolytic anemia develops in the fetus. Fetal anemia may lead to hydrops and death as early as 17 weeks of gestation. Survival rates can exceed 90 percent if anemia is diagnosed and treated with intrauterine blood transfusions in a timely manner.¹ Fetuses at risk for severe anemia are identified on the basis of the mother's obstetrical history and serum antibody levels and are followed closely at specialized referral centers. The standard test to evaluate the need for fetal transfusion is serial amniocentesis for the determination of bilirubin levels in amniotic fluid. Hemolysis leads to the accumulation of bilirubin in amniotic fluid, and the amniotic-fluid bilirubin level correlates with the severity of hemolysis.² The bilirubin level is quantified by spectrophotometry and expressed as the change in optical density at a wavelength of 450 nm (ΔOD_{450}); the ΔOD_{450} values are then plotted on a chart devised by Liley² to estimate the severity of anemia. Although this test is accurate,² it requires amniocentesis, which carries risks of membrane rupture, infection, worsening of sensitization, and fetal loss.³⁻⁵

Noninvasive testing for fetal anemia can be performed by Doppler ultrasonography. Ultrasonographic measurements of the fetal liver and spleen have been suggested as alternatives to invasive testing but have been shown to be less predictive than Doppler measurements of fetal blood-flow velocities.⁶ Fetuses with anemia have a high cardiac output and decreased blood viscosity, resulting in high blood-flow velocities. In 2000, Mari and colleagues reported that fetal anemia could be detected reliably by Doppler measurement of blood-flow velocity in the middle cerebral artery.⁷ We tested the hypothesis that Doppler ultrasonography of the middle cerebral artery is not inferior to measurement of amniotic-fluid ΔOD_{450} for the prediction of fetal anemia.

METHODS

STUDY SUBJECTS

The study was conducted at tertiary perinatal centers in Toronto, Ottawa, London, and Hamilton, all in Ontario; Halifax, Nova Scotia; Winnipeg, Manitoba; and Vancouver, British Columbia — all in Canada; Dublin, Ireland; San Diego, California; and Leiden, the Netherlands. We included all pregnant women with Rh alloimmunization

with clinically relevant antibodies (D, E, c, or Fy^a) and a maternal serum antiglobulin titer of at least 1:64, as measured by the indirect Coombs' test.^{8,9} The study was approved by the institutional review board at each center. All of the women gave oral or written informed consent. Fetuses were excluded from the study if they had Kell antibodies, hydrops, or major congenital anomalies. Kell-related anemia is caused mainly by destruction of erythroid precursors, resulting in decreased production of red cells rather than hemolysis; assessment of bilirubin by amniotic-fluid ΔOD_{450} levels is unreliable for detecting anemia when this condition is present.¹⁰ Fetuses negative for the maternal alloantigen were also excluded. Women with a partner heterozygous for the antigen concerned were offered fetal genotype testing. The test was performed by analysis of maternal-plasma-free fetal DNA,¹¹ if it was available, or of DNA from amniocytes obtained by amniotic-fluid sampling at approximately 16 weeks of gestation (without ΔOD_{450} measurement). If the results of fetal DNA typing were inconclusive or unavailable, or if the patient declined amniocentesis for this indication, fetal genotyping was performed at the first amniocentesis, at the first fetal blood sampling, or at birth.

Because to our knowledge there were no previous studies comparing Doppler ultrasonography with measurement of amniotic-fluid ΔOD_{450} , our assumptions for sample-size calculations were based on personal experience and unpublished data from the Leiden University Medical Center database. Using a method for paired proportions described by Connor,¹² with an estimated difference in sensitivity between Doppler ultrasonography and measurement of amniotic-fluid ΔOD_{450} of 11 percent and a discordant proportion of 31 percent, we estimated that a sample size of 157 would be needed to show that the sensitivity of Doppler ultrasonography is not inferior to the sensitivity of amniotic-fluid ΔOD_{450} with a type I error of 5 percent and a statistical power of 80 percent.

DOPPLER STUDIES

The peak velocity of systolic blood flow in the middle cerebral artery was measured according to previously described methods.⁷ The measurements were made in the absence of fetal breathing movements, with the woman in a semirecumbent position. An axial section through the fetal

brain was obtained just caudal to the plane for measurement of the biparietal diameter. The middle cerebral artery was identified by color or power Doppler ultrasonography. The angle of insonation was kept as close as possible to 0 degrees and never exceeded 30 degrees. When an angle between 0 and 10 degrees could not be obtained, on-screen angle correction was applied. The sample volume was placed close to the internal carotid artery. After at least five consecutive uniform waveforms had been recorded, a caliper was placed on the peak of the Doppler waveform. Before the study, all centers were visited by one of the investigators to ensure that all sonographers used the method described here. All centers used state-of-the-art ultrasound equipment approved for obstetrical use, with a spatial peak temporal average intensity of less than 100 mW per square centimeter. Reproducibility studies were not performed by the participating centers. Other studies have shown that Doppler measurements of the peak velocity of systolic blood flow in the middle cerebral artery are highly reproducible, with intraclass correlation coefficients for interobserver variation ranging from 0.98 to 0.99.¹³ Doppler studies were performed before amniocentesis, and therefore, the sonographers were unaware of the ΔOD_{450} results.

AMNIOTIC-FLUID ΔOD_{450} MEASUREMENT

Amniocentesis was performed with a 22-gauge needle under continuous ultrasonographic guidance; 10 ml of fluid was obtained, protected from light, and sent to the local laboratory for immediate processing. After centrifugation, the supernatant was aspirated for spectrophotometry. The absorbance (A) of the supernatant was measured at 360, 410, 450, and 550 nm and then plotted logarithmically against wavelength. A straight baseline was drawn from 360 nm to 550 nm. The baseline absorbance at 410 and 450 nm was subtracted from the absorbance readings at 410 and 450 nm to obtain the ΔA_{410} and uncorrected ΔA_{450} , respectively; then 5 percent of the ΔA_{410} was subtracted from the uncorrected ΔA_{450} to compensate for minor amounts of oxyhemoglobin contamination, yielding the corrected ΔA_{450} . Traditionally, in the spectrophotometric estimation of the bilirubin level in amniotic fluid, absorbance has been described as optical density (OD). The ΔOD_{450} value was manually plotted on a copy of the modified Liley chart.

STUDY PROTOCOL

Patients who fulfilled the inclusion criteria visited a center every one or two weeks. The patients received treatment according to existing local protocols and national guidelines, and no Doppler studies were performed before the decision was made to perform amniocentesis. The first amniocentesis for the determination of amniotic-fluid ΔOD_{450} was performed when the clinician decided that it was indicated on the basis of the mother's obstetrical history, an antibody titer of at least 1:64, and ultrasonographic evaluation of the fetus. Since we aimed only to compare the diagnostic accuracy of Doppler ultrasonography of the middle cerebral artery with amniocentesis, our protocol allowed each center to follow its standard management procedures. Doppler measurements were performed within 24 hours before the amniocentesis.

To avoid the possibility that the clinician might decide not to proceed with amniocentesis on the basis of the Doppler results, the results were revealed to the clinician only after the amniocentesis had been performed. When one or both tests suggested severe fetal anemia, fetal blood sampling or delivery with cord-blood sampling was performed within 72 hours. In patients for whom the Doppler and amniotic-fluid ΔOD_{450} results were both within the normal range, both tests were repeated one to three weeks later. If the next test result predicted severe anemia, fetal blood sampling was performed and the test results obtained just before the fetal blood sampling were used for the outcome analysis. Only the results of the first fetal blood sampling were used to determine the accuracy of both tests. Further management was performed according to the center's standard protocol. If the results of both tests continued to be normal, cord-blood hemoglobin values measured at birth were used to evaluate the tests. The results of the last pair of measurements obtained no more than two weeks before delivery were then used in the analyses.

OUTCOME MEASURES

The primary outcome variables were the sensitivity, specificity, and accuracy of Doppler ultrasonography of the middle cerebral artery, with the use of Mari's chart to interpret the values,⁷ and measurement of amniotic-fluid ΔOD_{450} , with the use of Liley's chart,² for the prediction of fetal anemia requiring transfusion. The reliability of the Liley chart before 27 weeks of gestation has been

questioned.^{14,15} We therefore performed a subgroup analysis of the accuracy of both tests before 27 completed weeks of gestation. In addition, the Doppler ultrasonographic results were compared with amniotic-fluid ΔOD_{450} values plotted on the chart developed by Queenan et al. in 1993, which is widely used in the United States.^{15,16}

Doppler ultrasonography and amniotic-fluid studies were considered to indicate fetal anemia requiring transfusion when the results exceeded the following cutoff levels: for Doppler ultrasonography, values above the line indicating 1.5 multiples of the median in Mari's chart; and for amniotic-fluid ΔOD_{450} , values above the line between the middle and the upper third of zone 2 in the modified Liley chart.¹⁶ Liley's original chart was confined to the third trimester and was divided into three zones. Linear extension of the zones to the second trimester has been shown to be clinically useful.¹⁷ Most obstetrical guidelines advise fetal-blood sampling when the ΔOD_{450} level reaches the upper part of zone 2.¹⁶ Queenan's chart has four zones; transfusion is advised when the value reaches the "intrauterine death risk" zone 4.¹⁵ The measured ΔOD_{450} values were manually plotted on Queenan's chart, with the line marking zone 4 used as a cutoff to predict fetal anemia.

The reference test for the diagnosis of fetal anemia was measurement of the hemoglobin level in umbilical-cord blood with the use of the reference range published by Nicolaides et al.¹⁸ In normal fetuses, the hemoglobin level increases with gestational age, and therefore, a fixed hemoglobin cutoff point cannot be used to define fetal anemia. Severe fetal anemia was defined as a hemoglobin level 5 SD or more below the mean for gestational age. Below this level, a fetus requires a blood transfusion to prevent cardiac decompensation and hydrops, which typically occur when the hemoglobin level is 7 SD or more below the mean for gestational age.^{18,19}

STATISTICAL ANALYSIS

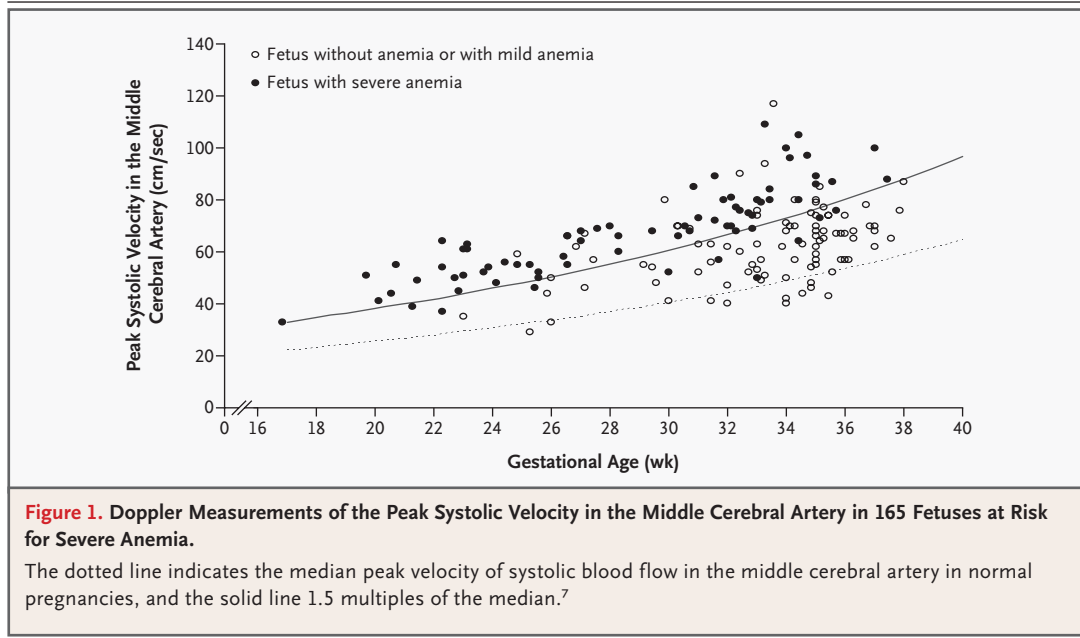
Sensitivity, specificity, and positive and negative predictive values were calculated by standard formulas for a binominal proportion, and 95 percent confidence intervals were calculated by the Wilson interval method.^{20,21} The accuracy of the test was determined by dividing the sum of the true positive and true negative results by the total number of subjects, and 95 percent confidence inter-

vals were calculated by Wilson's method. Comparisons of the test characteristics of Doppler ultrasonography and amniotic-fluid analysis were based on the paired-sample design. McNemar's test for paired proportions was applied, and confidence intervals for differences in sensitivities and specificities were calculated by the methods for paired proportions.²² Two-sided P values less than 0.05 were considered to indicate statistical significance.

Because our study was designed to show that Doppler ultrasonography is no worse than the "gold-standard" test (amniocentesis), we used a one-sided hypothesis.²³ We tested the hypothesis that Doppler ultrasonography is not inferior to measurement of amniotic-fluid ΔOD_{450} , using Liley's chart to compare the differences between the sensitivities and accuracies of the two tests with a predetermined clinically meaningful limit of 5 percent. Confidence intervals for the differences were calculated by the sample-based method of Liu et al.²³

RESULTS

From October 2000 through April 2004, 178 pregnant women were enrolled in the study. Data from 14 pregnancies had to be excluded from the analysis: 8 because of incomplete data (mainly missing cord-blood hemoglobin levels at birth) and 6 because of withdrawal. A total of 164 pregnancies with 165 fetuses (one set of twins) remained for analysis. RhD antibodies, with or without RhC antibodies, were present in 147 pregnancies; 13 women had anti-c antibodies; and 2 each had anti-E and anti-Fy^a antibodies. Fetal blood was sampled for hemoglobin analysis in 83 pregnancies. In the other 81 pregnancies, both the Doppler and the amniotic-fluid results remained normal throughout pregnancy, and the hemoglobin level was determined in cord blood obtained at birth. Seventy-four fetuses had severe anemia (hemoglobin deficit at least 5 SD below the mean for gestational age), 25 had mild anemia (hemoglobin deficit, 2 to 5 SD below the mean for gestational age), and the remaining 66 fetuses had normal hemoglobin levels. One mother delivered spontaneously at 25 weeks of gestation, before amniocentesis had been performed. The child did not survive the neonatal period, and the mother and child were excluded from the analysis. One other neonatal death occurred when a child



with growth restriction was delivered by cesarean section at 30 weeks of gestation because of maternal preeclampsia. Overall, 177 of the 179 fetuses (99 percent) survived.

The results of Doppler ultrasonography are shown in Figure 1. The distribution of the results of Doppler ultrasonography and amniotic-fluid ΔOD_{450} is shown in Table 1. The positive and negative predictive values for Doppler ultrasonography were 80 percent and 89 percent, respectively. Amniotic-fluid ΔOD_{450} had a positive predictive value of 73 percent and a negative predictive value of 80 percent. Table 2 shows the results of Doppler ultrasonography and amniotic-fluid ΔOD_{450} , as plotted on Liley's chart, for fetuses without anemia or with mild anemia and for fetuses with severe anemia. The amniotic-fluid ΔOD_{450} results plotted on Liley's and Queenan's charts are shown in Figure 2.

Table 3 compares the test characteristics of Doppler ultrasonography and amniotic-fluid analysis with the results assessed by both Liley's and Queenan's methods. For Doppler ultrasonography, the overall accuracy was 85 percent (95 percent confidence interval, 79 to 90 percent) and the sensitivity was 88 percent (95 percent confidence interval, 78 to 93 percent), as compared with 76 percent (95 percent confidence interval, 69 to 82 percent) and 76 percent (95 percent confidence interval, 65 to 84 percent), respectively, for amniotic-fluid ΔOD_{450} according to Liley's

method. The difference in accuracy between the two methods was 9 percentage points (95 percent confidence interval, 1.1 to 15.9), and the difference in sensitivity was 12 percentage points (95 percent confidence interval, 0.3 to 24.0); these intervals exclude zero, indicating that accuracy and sensitivity are significantly better for Doppler ultrasonography than for amniotic-fluid ΔOD_{450} .

In a post hoc subgroup analysis of data from 41 fetuses up to a gestational age of 27 completed weeks, Doppler ultrasonography had a sensitivity of 90 percent (95 percent confidence interval, 75 to 97 percent) and a specificity of 60 percent (95 percent confidence interval, 31 to 83 percent). The sensitivity of amniotic-fluid ΔOD_{450} assessed by Liley's method was 84 percent (95 percent confidence interval, 67 to 93 percent), and the specificity was 40 percent (95 percent confidence interval, 17 to 69 percent), whereas amniotic-fluid ΔOD_{450} assessed by Queenan's method had a sensitivity of 94 percent (95 percent confidence interval, 79 to 98 percent) and a specificity of 40 percent (95 percent confidence interval, 17 to 69 percent). There were no significant differences among the test characteristics of the three methods in the second trimester.

DISCUSSION

We designed the present study to assess whether the results of Doppler measurement of the peak

Table 1. Peak Systolic Velocity of Blood Flow in the Middle Cerebral Artery Measured by Doppler Ultrasonography and Amniotic-Fluid ΔOD_{450} , Classified According to Liley Zones as Predictors of Anemia.*

Measurement	Severe Fetal Anemia†	No Anemia or Mild Anemia	Total
	<i>number of pregnancies</i>		
MCA blood flow			
>1.5 MoM	65	16	81
≤1.5 MoM	9	75	84
Total	74	91	165
Amniotic-fluid ΔOD_{450}			
Liley zone 2c or 3	56	21	77
Liley zone 1 or 2	18	70	88
Total	74	91	165

* MCA denotes middle cerebral artery, and MoM multiples of the median. Liley's method is described in Liley.²

† Severe fetal anemia is defined as a hemoglobin level at least 5 SD below the mean for gestational age.

Table 2. Relation between Peak Systolic Velocity of Blood Flow in the Middle Cerebral Artery Measured by Doppler Ultrasonography in Fetuses with and Those without Severe Anemia and Amniotic-Fluid ΔOD_{450} , Classified According to Liley Zones.*

MCA Blood Flow	Amniotic-Fluid ΔOD_{450}		Total
	Liley Zone 2c or 3	Liley Zone 1 or 2	
<i>number of pregnancies</i>			
No anemia or mild anemia			
>1.5 MoM	9	7	16
≤1.5 MoM	12	63	74
Total	21	70	91
Severe fetal anemia†			
>1.5 MoM	50	15	65
≤1.5 MoM	6	3	9
Total	56	18	74

* MCA denotes middle cerebral artery, and MoM multiples of the median. Liley's method is described in Liley.²

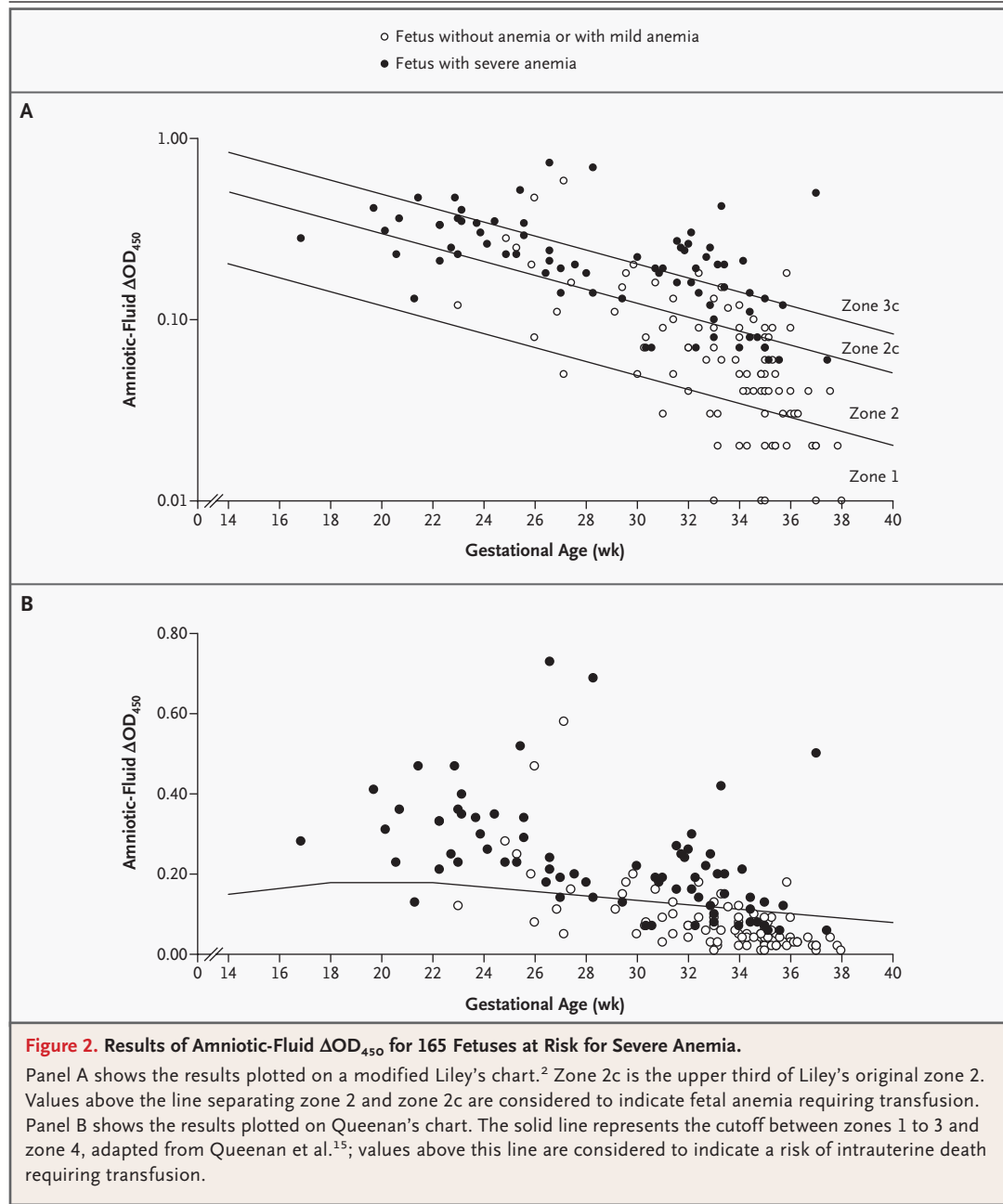
† Severe fetal anemia is defined as a hemoglobin level at least 5 SD below the mean for gestational age.

systolic velocity in the middle cerebral artery were at least equal to those of the traditional amniotic-fluid ΔOD_{450} measurement. The results showed that Doppler measurement was significantly more accurate and sensitive than amniotic-fluid ΔOD_{450} measurement. Given the invasive nature of amniocentesis and the associated risks, these data support the use of the noninvasive Doppler test in the evaluation of Rh-alloimmunized pregnancies.

Our findings regarding Doppler ultrasonography of the middle cerebral artery are consistent with those of a previous prospective study of 125

women with Rh alloimmunization, which reported a sensitivity of 88 percent and a specificity of 87 percent for the prediction of fetal anemia.²⁴ Our data also confirm and extend the findings of two smaller prospective studies (with 28 and 38 patients) comparing Doppler ultrasonography of the middle cerebral artery with amniotic-fluid analysis in pregnant women with Rh alloimmunization.^{25,26} Both studies found nonsignificantly higher sensitivities of Doppler ultrasonography for the prediction of fetal anemia.

Despite the observation of Nicolaides et al. in 1986 that Liley charts had limited reliability be-



fore 27 weeks of gestation,¹⁴ serial amniocentesis has remained the cornerstone for the management of Rh-alloimmunized pregnancies, since reliable and safe alternative diagnostic tools have not been available. Serial diagnostic fetal-blood sampling has been proposed as an alternative,²⁷ but it is considered too hazardous by most clinicians.^{15,28} Our study shows that noninvasive Doppler ultrasonography of the middle cerebral artery

is at least as reliable as measurement of amniotic-fluid ΔOD_{450} assessed by either Liley's or Queenan's method. Two previous studies have compared these two methods. One favored Liley's method,²⁹ and the other Queenan's.³⁰ Our study, which enrolled more patients than the previous studies, suggests a slightly higher diagnostic accuracy for Queenan's method.

A strength of our study is that the participation

Table 3. Comparison of Test Characteristics for Peak Systolic Velocity of Blood Flow in the Middle Cerebral Artery Measured by Doppler Ultrasonography and Amniotic-Fluid ΔOD_{450} , Assessed According to Liley's and Queenan's Methods.

Test	Sensitivity	Specificity	Accuracy
MCA blood flow	88 (78.4 to 93.5)	82 (73.3 to 88.9)	85 (78.6 to 89.5)
Amniotic-fluid ΔOD_{450}			
Liley's method	76 (64.8 to 84.0)	77 (67.3 to 84.0)	76 (69.3 to 82.2)
Queenan's method	81 (70.7 to 88.4)	81 (72.0 to 88.0)	81 (74.6 to 86.4)
Differences			
MCA blood flow vs. amniotic-fluid ΔOD_{450} by Liley's method	12 (0.3 to 24.0)	6 (-3.8 to 14.8)	9 (1.1 to 15.9)
MCA blood flow vs. amniotic-fluid ΔOD_{450} by Queenan's method	7 (-4.1 to 17.6)	1 (-7.8 to 10.0)	4 (-3.3 to 10.5)
Amniotic-fluid ΔOD_{450} : Queenan's method vs. Liley's method	5 (0.3 to 10.6)	4 (0.2 to 8.6)	5 (1.6 to 8.1)

* MCA denotes middle cerebral artery. The methods used to interpret ΔOD_{450} measurements are described by Liley² and by Queenan et al.¹⁵

of clinicians in 10 centers in Europe and North America, all using their own standard protocols for the management of Rh-alloimmunized pregnancies except for the addition of Doppler ultrasonography, makes the results generalizable. The 45 percent prevalence of severe fetal anemia in our study confirms that we selected appropriately high-risk pregnancies. Our study was not aimed at pregnancies with mild Rh alloimmunization.

The high accuracy of Doppler ultrasonography of the middle cerebral artery obviates the need for invasive testing, even in many high-risk pregnancies involving antibody titers of 1:64 or more. If we had relied on Doppler ultrasonography alone, 51 percent of the patients in our study would not have undergone any invasive procedure. Although Doppler ultrasonography can replace amniocentesis for the measurement of ΔOD_{450} , it is important to note that the sensitivity of Doppler ultrasonography is not 100 percent. All studies that have found Doppler ultrasonography of the middle cerebral artery to be highly accurate for the prediction of fetal anemia, including ours, were conducted in specialized re-

ferral centers and involved patients whose fetuses were at high risk for anemia. The participating sonographers and clinicians had extensive experience both in performing fetal Doppler studies and in managing Rh-alloimmunized pregnancies. We agree with other authors^{24,31} that pregnant women with severe Rh alloimmunization should be cared for in centralized facilities and should undergo serial complete fetal ultrasonographic examinations and Doppler evaluation by experts in fetal medicine.

On the basis of smaller studies that reported that Doppler ultrasonography of the middle cerebral artery was highly accurate in the prediction of fetal anemia, most centers have gradually implemented a less invasive strategy for the management of Rh-alloimmunized pregnancies.³² The results of our large prospective study confirm that such a strategy is justified.

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APPENDIX

In addition to the authors, the following investigators participated in the DIAMOND study: **Canada** — *McMaster University Hospital, Hamilton, Ont.*: B. Brennan, B. DeFrance; *St. Joseph's Health Centre, London, Ont.*: R. Gratton, R. Gagnon; *Ottawa Hospital, Ottawa*: K. Fung-Kee-Fung, M. Walker; *Women's Hospital, Winnipeg, Man.*: C. Schneider; *IWK Grace Health Centre, Halifax, N.S.*: M. Van Den Hof, V. Allen; *British Columbia Women's Hospital, Vancouver, B.C.*: A. Gagnon; **United States** — *University of California, San Diego, School of Medicine, San Diego*: A. Hull; **Ireland** — *National Maternity Hospital, Dublin*: P. McParland.

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