Diagnostic procedures for assessing the severity of alloimmune fetal anemia

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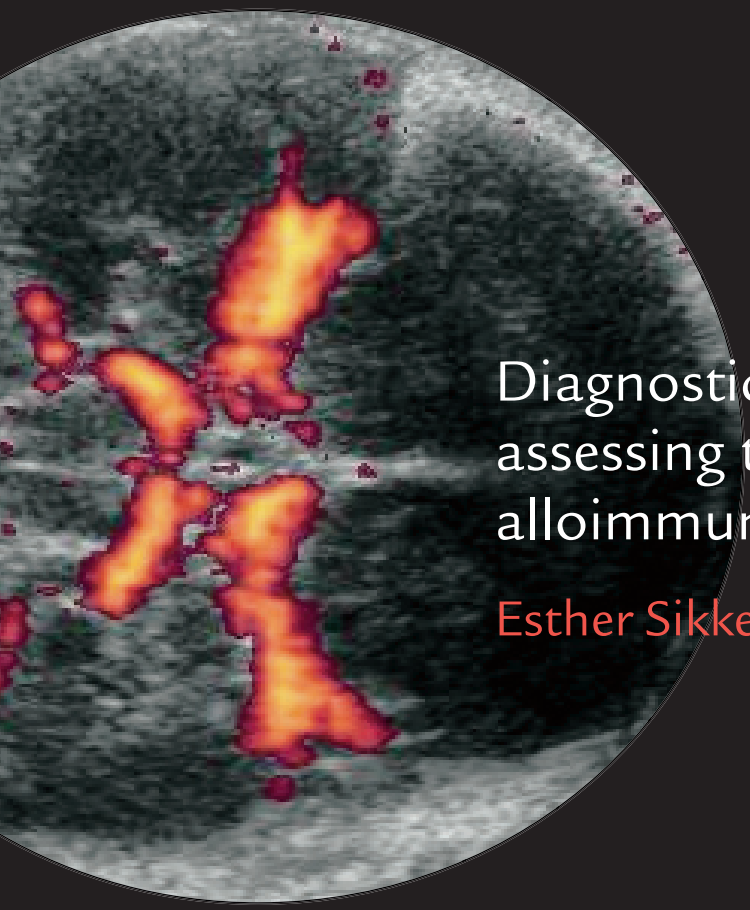

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Esther Sikkel

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For the ones I love

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List of abbreviations

ACOG	American College of Obstetricians and Gynecologists
ADCC	Antibody-dependent cellular cytotoxicity
ANOVA	Analysis of variance
CIs	Confidence intervals
ΔOD 450	Deviation of optical density at 450 nm
EDD	End-diastolic dimension
ESD	End-systolic dimension
FBV	Fetoplacental blood volume
FHR	Fetal heart rate
Hb	Hemoglobin concentration
Ht	Hematocrit
IUT	Intrauterine transfusion
LUMC	Leiden University Medical Center
LVSF	Left ventricular shortening fraction
MCA	Middle cerebral artery
MCA peak	Middle cerebral artery peak velocity
MoM	Multiples of median
MoM-MCA	Standardized MCA peak velocity
RVSF	Right ventricular shortening fraction
SD	Standard deviation
SEM	Standard error of the mean
UV	Umbilical vein
UV max	Umbilical venous maximum velocity
z_Ht	Standardized fetal hematocrit
z_UV	Standardized UV max velocity

General introduction

1

Alloimmune hemolytic anemia of the fetus and newborn

A woman, negative for a red cell antigen, may produce antibodies when she comes into contact with erythrocytes positive for the offending antigen either by blood transfusion, an organ transplant, or as a consequence of a fetomaternal transfusion. In a pregnant woman, IgG antibodies will cross the placenta leading to hemolysis in the fetus and more or less severe fetal anemia. This process is often called erythroblastosis fetalis and consequences include intrauterine demise and neonatal hyperbilirubinaemiae, eventually leading to kernicterus with its concomitant morbidity and mortality.

In 1940, Landsteiner and Weiner discovered the clinically most important red cell antigen, the "rhesus factor" through their research with Macacus rhesus, a monkey species.[1] In the following decades, doctors learned to understand the pathogenesis of alloimmune hemolytic anemia, found ways to diagnose and treat the condition and even discovered how to effectively prevent the disease. It was a success story in medicine and at the same time it was the start of a new subspecialty: fetal medicine.

Prevention

Hemolytic disease of the newborn was once a major contributor to perinatal morbidity and mortality. During the past 4 decades, there has been a remarkable improve of fetal outcome, due to the improved neonatal care, the introduction of intrauterine transfusion, and the introduction of prevention with anti-D. Administration of anti-D to a rhesus-negative mother after the birth of a rhesus-positive child leads to decreased immunization rates. However, in some cases, rhesus immunization will already have occurred (during pregnancy) or the amount of anti-D may not suffice in cases of large fetomaternal transfusions. Blood transfusions and organ (kidney, bone) transplant are other possible causes of alloimmunization. Finally, maternal non-rhesus D red blood cell antibodies may also lead to fetal alloimmune anemia.

Screening

In the Netherlands, there is a nationwide screening policy since 1998. All pregnant women are tested in the first trimester of pregnancy for the presence of red cell antibodies. Screen-positive women are categorized as no-risk, low-risk or high-risk for fetal anemia according to the type of antigen and the concentration of antibodies. In case of high antibody titers, the Antibody Dependent Cell Cytotoxicity (ADCC) test is performed.[2] Pregnancies at high risk for fetal anemia, for example in case of anti-D antibodies with an indirect Coombs titer of >1/32 and an ADCC test of >50% are referred to Leiden University Medical Center (LUMC).

Diagnosis

When a woman is referred to LUMC the obstetrical history is taken into account. Further, ADCC tests and antibody titers are followed. The women are instructed to pay special attention to fetal movements. Ultrasound examinations are performed: depending on the severity of the immunization, weekly or bi-weekly. The ultrasound examination is focussed on fetal movements, early signs of hydrops, fetal liver and spleen enlargement, cor-thorax ratio, umbilical vein maximum flow and middle cerebral artery peak flow measurement. In case of a severe immunization and inconclusive ultrasound findings, amniocentesis and bilirubin concentration measurement is performed. Bilirubin is the main degradation product of hemoglobin and therefore a measure of its destruction. Bilirubin concentration in amniotic fluid is measured colorimetrically by performing a spectral absorption curve. The bilirubin absorption, expressed as Δ OD 450 is calculated as the difference between the measured light absorption at 450 nm and the background absorption at 450 nm.[3] If then there is suspicion of severe anemia, fetal blood examination and an intrauterine transfusion is performed.

Therapy

In case of a less advanced pregnancy the therapy of choice of fetal anemia is intrauterine blood transfusion. At LUMC intrauterine transfusions are performed as early as 16 weeks of gestation and repeat transfusions are given every 2-4 weeks up to 35 weeks,[4;5] aiming that women give

birth between 36 and 38 weeks of gestation. After birth, phototherapy, transfusions and/or exchange transfusions may be necessary.

Despite its undoubted merits, an intrauterine transfusion is not without risk. Procedure-related fetal loss rates are in the range of 1-3% per procedure.[4;5] Furthermore, every intrauterine transfusion carries the risk of fetomaternal hemorrhage, which may increase the severity of alloimmunization. A significant increase in antibody concentration occurs in 50% of cases after intrauterine transfusion.[6]

National strategy in the Netherlands
In the Netherlands all pregnant women have a blood test taken for irregular antibodies in the first trimester of pregnancy. In case of a rhesus negative mother without irregular antibodies the blood test will be repeated at 30 weeks and the mother will receive anti-D. In the Netherlands we have approximately 200 pregnancies yearly with new cases of potentially dangerous red cell antibodies. (170 anti-D, approximately 30 anti-K and anti-c). Of these, approximately 100 pregnant women a year will have high antibody titers. In the Netherlands, care for fetal alloimmunization is centralised and all severely immunized pregnancies are referred to the LUMC. In the LUMC, pregnant women with high antibody titers are followed with ultrasound examinations and, if deemed necessary, amniocentesis, until, in approximately 1/3 of them, suspicion of severe fetal anemia leads to the performance of an intrauterine transfusion. Yearly, around 100 intrauterine transfusions in approximately 30 fetuses are performed.

Outline of the thesis

This thesis is about the physiological basis and the diagnostic accuracy of two methods to asses the severity of fetal hemolytic anemia: measurement of bilirubin concentration in amniotic fluid on the one hand and fetal ultrasound on the other hand.

Part 1: Chemical approach

Since the 1960s, Δ OD 450 measurements have been the main diagnostic procedure for prediction of fetal anemia and since the 1990s, the Doppler technique is upcoming. We performed a Medline search *(chapter 2)* to compare those two diagnostic tools.

The Δ OD 450 technique has been shown to have good diagnostic qualities from 27 to 36 weeks of pregnancy. In *chapter 3,* we assessed the diagnostic accuracy of amniotic fluid Δ OD 450 in our patient population from 18 weeks onwards.

It is still unknown how bilirubin enters the amniotic fluid. We studied *(chapter 4)* the relationship between bilirubin concentration in amniotic fluid and fetal blood in rhesus D alloimmunized anemic fetuses in order to speculate on the possible pathway of bilirubin from fetal blood to amniotic fluid.

Part 2: Ultrasonographic approach

We have been performing intrauterine transfusions with a large volume load since many years. We investigated *(chapter 5)* the effects of acute large increases of hematocrit on fetal blood flow velocities. This therapeutic experiment may help to explain the relationship between hematocrit and blood flow velocities in the fetus.

Theoretically, fetal anemia may lead to a reactive increase in cardiac contractility and, consequently, to an increased cardiac wall thickness and an increased cardiac size. In everyday practice, sonographers often mention their impression that the heart is increased in size in a fetus with anemia. Therefore, to test this hypothesis and to explore potential applications, we assessed *(chapter 6)* the diagnostic value of cardiac size measurements in the prediction of severe alloimmune anemia.

Another impression of sonographers during intra-uterine transfusions is that the contractility of the fetal heart is decreased after intrauterine transfusion. Therefore, we assessed the effect of fetal anemia and intrauterine transfusion on ventricular shortening fraction *(chapter 7)*.

It is hoped that the studies in this thesis will add to a better understanding of the physiologic changes and to a more accurate diagnosis and hence to the timely therapeutic intervention in fetal anemia.

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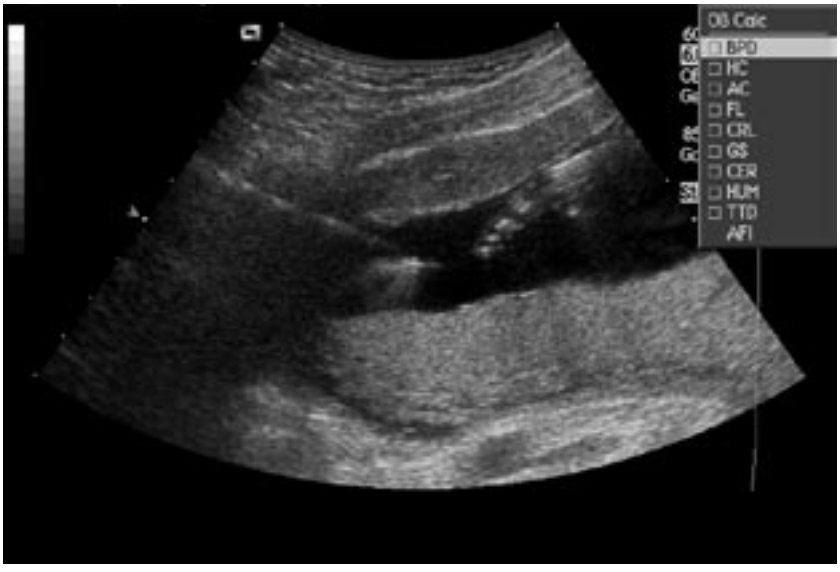
Part 1: Chemical approach



Diagnostic accuracy of Δ OD 450 measurements and middle cerebral artery peak systolic velocity in the prediction of severe fetal alloimmune anemia: a literature review

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2

Introduction

The severity of fetal alloimmune anemia can be diagnosed biochemically or sonographically. The biochemical method is based on the fact that hemolysis results in increased bilirubin concentrations in fetal blood and in amniotic fluid.[1] Already in 1956, Bevis found that bilirubin concentrations in amniotic fluid are indicative of the severity of the hemolytic process in fetuses of alloimmunized mothers.[2] 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.[3]
The American College of Obstetricians and Gynecologists (ACOG) still recommends serial amniocentesis in pregnancies at risk, followed by intrauterine transfusion (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.[4] Amniotic fluid Δ OD 450 can also be plotted in other charts (Queenan, extended Liley) or be used as Ovenstone factor, or transmutance ratio.[5-8]

The invasive nature of amniocentesis remains a disadvantage, however. With each procedure, there is a risk of iatrogenic rupture of the fetal membranes or infection, both of which can lead to fetal loss. There is also the risk of increasing severity of sensitization by either boosting of antibody titer or formation of additional antibodies.[9] Since the introduction of non-invasive methods to diagnose fetal anemia, the evaluation of the diagnostic performance of invasive Δ OD 450 measurement is now warranted.[10;11]

Sonographic prediction of severe anemia is easy when the fetus is hydropic. However, treatment results are definitely worse in hydropic than in non-hydropic fetuses.[12] Therefore, severe fetal anemia should preferably be diagnosed and treated before hydrops develops. During the last decade, different methods for this purpose have been proposed: sonographic liver[13;14] and spleen[15] measurements, Doppler measurements of the middle cerebral artery[10], intrahepatic umbilical vein[16;17], descending aorta[18], splenic artery[19] or combined measurements.[11;20] Of these methods, measurement of Middle cerebral artery (MCA) peak systolic velocity is the most widely used. An increased peak systolic velocity in the MCA as

predictor of severe fetal anemia was first described by Mari et al.[21] It is thought that this increase in systolic velocity is caused by a hyperdynamic circulation with increased contractility of the heart and decreased viscosity of the blood.[18] In a prospective series, Mari et al., established the normal median for MCA peak systolic velocity throughout gestation and drew the demarcation line between moderate and severe anemia around 1.5 MoM.[10]

We aimed to compare the accuracy of amniotic fluid Δ OD 450 with the accuracy of the more recent non-invasive Doppler measurement of MCA peak systolic velocity. Therefore we performed a literature review on the accuracy of, first, Δ OD 450 and, second, MCA peak systolic velocity. We calculated the sensitivities and specificities for the different cut-offs used in each study.

Methods

Δ OD 450

English language journals indexed in Medline between 1961 and 2003 were searched for articles addressing amniotic fluid bilirubin levels in the management of red cell alloimmunization. Search terms included "rhesus", "Liley", "Queenan", "OD 450", "amniotic bilirubin", and "amniotic optical density". Selected abstracts were reviewed for relevant information on the test characteristics of amniotic fluid Δ OD 450 to predict fetal anemia. The references of retrieved articles were reviewed for additional articles not identified through the database search. Data on hydropic fetuses were excluded. Two groups were recognized. The first group describes test characteristics of amniotic fluid Δ OD 450 in the prediction of fetal anemia at fetal blood sampling. The second group describes test characteristics of amniotic fluid Δ OD 450 in the prediction of fetal anemia at birth. Sensitivity, specificity, and overall accuracy (combined rate of true-positive and true-negative results) were calculated for different Δ OD 450 cut-offs in the prediction of anemia by two of the authors (ES and FV).

MCA peak systolic velocity

We also searched English language journals indexed in Medline between 1995 and 2005 addressing MCA peak systolic velocity in predicting fetal anemia. The following search term was used: "middle cerebral artery and fetal anemia". Selected abstracts were reviewed for relevant information on the test characteristics of MCA peak systolic velocity to predict fetal anemia. The references of retrieved articles were reviewed for additional articles not identified through the database search. Sensitivity and specificity were calculated for different MCA cut-offs in the prediction of anemia by two of the authors (ES and FV).

Simultaneous Δ OD 450 and MCA peak velocity

In addition, the search consisted of English language journals indexed in Medline between 1995 and 2005 addressing both Δ OD 450 and MCA peak systolic velocity in predicting fetal anemia. The following search term was used: "amniocentesis and middle cerebral artery". Selected abstracts were reviewed for relevant information on the test characteristics of both Δ OD 450 and MCA peak systolic velocity to predict fetal anemia in the same patient population. The references of retrieved articles were reviewed for additional articles not identified through the database search. Sensitivity, specificity, and overall accuracy (combined rate of true-positive and true-negative results) were calculated by two of the authors (ES and FV).

Results

Studies with test characteristics of Δ OD 450

The literature search resulted in 73 abstracts. In 28 papers, test characteristics were mentioned and these papers were read in detail. Twelve additional papers were found by checking the references of these papers. Finally, five papers compared Δ OD 450 with hemoglobin concentration obtained by fetal blood sampling and gave sufficient data to calculate test characteristics.[22-26] All patients in these studies were rhesus-D immunized. These papers are listed in Table I. Sensitivities of Liley's zone III and Queenan's zone 4 in the prediction of severe anemia (not uniformaly

Table 1 - Test characteristics of amniotic fluid Δ OD 450 in the prediction of fetal anti-rhesus D alloimmune anemia at fetal blood sampling (non-hydropic fetuses).

First author, year	*Number of patients*	*Number of amnio centeses*	*Range of gestational age (weeks)*	*Test*	*Test cut-off*	*Definition of*	*Sensitivity (%)*	*Specificity (%)*	*Accuracy (%)*
Nicolaides,[23] 1986	45	45	< 26	Extrapolated Liley	Zone III	Hb < 6 g/dl	47	82	69
					Zone IIB		94	43	62
					Zone III	Hb < 9.7 g/dl	38	92	53
					Zone IIB		84	62	78
MacKenzie,[22] 1988	36	63	17 - 35	Extrapolated Liley	Zone III	Ht < 25 (17-25 weeks) Ht < 30 (25-35 weeks)	--	--	79
Rahman,[24] 1998	43	43	< 27	Queenan	Zone 4	Ht < 15 %	33	36	35
					Zone 3		80	7	33
					Zone 4	Ht < 30 %	44	22	40
					Zone 3		88	11	72
Scott,[25] 1998	35	72	16 - 38	Queenan	Zone 4	Hb-deficit > 7 g/dl	100	79	81
					Zone 3		100	38	42
					Zone 4	Hb-deficit > 2 g/dl	88	95	93
					Zone 3		100	47	60
	--	36	27 - 38	Liley	Zone III	Hb-deficit > 7 g/dl	--	92	92
					Zone III	Hb-deficit > 2 g/dl	100	97	97
Sikkel,[26] 2002	79	79	20 - 35	Extrapolated Liley	Zone III	Hb-deficit > 5 g/dl	79	50	75
					Zone IIc		97	25	86
	24	24	< 27		Zone III		74	100	79
					Zone IIc		95	60	88
	55	55	≥ 27	Liley	Zone III		81	14	73
					Zone IIc		98	0	85

Hb: Hemoglobin concentration, Ht: Hematocrit, --: not given

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

defined) ranged from 33% to 100%. Sensitivities of the upper half of Liley's zone II (IIB) or Queenan's zone 3 ranged from 80% to 100%. Table 2 lists another 12 studies, where Δ OD 450 was compared with the severity of clinically defined fetal anemia or hemoglobin concentration at birth.[3;5-8;27-33] Although the majority of patients in these studies were rhesus-D immunized, other antibodies (including anti-Kell) may have played a role in some of the patients. In case of anti-Kell antibodies, anemia may be partially caused by erythroid precursor damage and not merely by hemolysis. Consequently, the haemolytic-induced rise in amniotic fluid bilirubin may be less pronounced in case of anti-Kell antibodies and severe anemia may remain undetected.[34-36]

Studies with test characteristics of MCA peak systolic velocity

This literature search resulted in 75 abstracts. In 32 papers, test characteristics were mentioned and these papers were read in detail. There were no additional papers found by checking the references of these papers. Finally, 14 papers compared MCA peak systolic velocity with fetal hemoglobin concentration at fetal blood sampling or at birth and gave sufficient data to calculate test characteristics.[10;20;37-48] These papers are listed in Table 3. Sensitivities of MCA peak systolic velocity in the prediction of severe anemia (according to different definitions) ranged from 31% to 100%.

Studies with test characteristics of both Δ OD 450 and MCA peak systolic velocity in the same fetuses

Our search resulted in 12 abstracts. In 3 papers, test characteristics were mentioned and these papers were read in detail. One additional paper was found by checking the references of these papers. Finally, three papers compared Δ OD 450 and MCA peak systolic velocity with hemoglobin concentration and gave sufficient data to calculate test characteristics.[49-51] These papers are listed in Table 4. Sensitivities of Δ OD 450 in the prediction of severe anemia (according to different definitions) ranged from 53% to 86%. Sensitivities of MCA peak systolic velocity in the prediction of severe anemia (according to different definitions) ranged from 64% to 100%.

Table 2 - Test characteristics of amniotic fluid Δ OD 450 in the prediction of neonatal anemia at birth.

First author, year	*Number of patients*	*Number of amnio centeses*	*Range of gestational age (weeks)*	*Test*	*Test cut-off*	*Definition of*	*Sensitivity (%)*	*Specificity (%)*	*Accuracy (%)*
Liley,[3] 1961	47	47	27 - 38	Liley	Zone III	Hb < 11 g/dl	76	89	79
					Zone IIc		87	67	83
Pridmore,[7] 1972	716	> 716	20 - 39	Transmittance ratio	> 1.06	Hb < 7.5 g/dl or death	--	--	89
Bosch,[27] 1974	312	312	≥ 26	Liley	Zone III	Hb < 11 g/dl	80	98	91
Bowman,[5] 1975	928	2615	21 - 37	Extrapolated Liley	Zone III	hydrops fetalis or need for treatment	91	99	97
MacDougall,[30] 1975	173	173	--	Liley	Zone III	Hb < 10 g%	33	100	88
Fairweather,[29] 1976	141	468	21 - 39	Δ OD 450	<30 wks: >0.25 >30 wks: >0.15	Hb < 7.5 g/dl	72	91	85
Robertson,[31] 1976	288	920	28 - 35	Bilrubin ratio or stillbirth	> 1.1	Hb < 7.4 g/dl	69	86	82
Moore,[6] 1977	46	78	24 - 40	Liley	Zone III	death or multiple exchange transfusions	50	100	85
					Zone IIb		71	88	83
				Ovenstone factor	> 30		36	100	80
					> 20		64	100	89
Weiner,[33] 1981	56	158	--	Liley	Zone B''	fetal demise or need for neonatal transfusion	67	90	79
Skjaeraasen,[32] 1983	71	72	26 - 32	Δ OD 450	>0.3	intrauterine or neonatal death	86	71	79
Ananth,[28] 1989	32	41	16 - 20	Δ OD450	>0.15	fetal death or IUT or exchange transfusion	59	95	76
Queenan,[8] 1993	74	163	16 - 36	Queenan	Zone 4	"Potentially fatal"	100	100	100

Hb: Hemoglobin concentration, --: not given. IUT: Intrauterine transfusion

Table 3 - Test characteristics of MCA peak systolic velocity in the prediction of fetal anemia at fetal blood sampling or at birth.

First author year	*Number of measurements*	*Number of anemic fetuses*	*Number of hydropic fetuses*	*Test cut-off*	*Definition of anemia*	*Sensitivity (%)*	*Specificity (%)*	*Study design*
Mari,[10] 2000	111	35	12	1.5 MoM	Hb < 0.65 MoM	100	88	retrospectively, cut-off drawn at 100 % sensitivity
Teixera,[46] 2000	26	13	1	> 2 SD above the mean	z_Ht < -4	67	90	prospective
Delle Chiaie,[39] 2001	140	108	1	1.29 MoM	Hb < 0.84 MoM	73	82	--
Detti,[41] 2001	64	11	4	1.69 MoM	Hb < 0.55 MoM	100	94	cut-off drawn at 100 % sensitivity
Sikkel,[20] 2001	42	38	0	1.5 MoM	Hb ≤ -5 SD	71	50	prospective
Deren,[40] 2002	103	53	0	1.35 MoM	Hb < 0.6 MoM	100	82	prospective
Zimmerman,[48] 2002	125	15	3	1.5 MoM	Hb < 0.65 MoM	88	87	prospective, < 35 weeks
Alshimmiri,[38] 2003	66	29	27	1.5 MoM	Hb < 0.65 MoM	31	97	prospective
Duckler,[42] 2003	16	6	0	1.5 MoM	Hb deficit > 5 SD	100	100	prospective
Sikkel,[45] 2003	60	46	12	1.5 MoM	z_Ht ≤ -5 SD	54	57	prospective
Mc Lean,[43] 2004	42	3	0	1.5 MoM	Hb < 0.65 MoM	100	90	retrospective
Scheier,[44] 2004	58	23	9	1.5 MoM	Hb deficit > 6 SD	96	86	cross-sectional
Ahmed,[37] 2005	65	4	0	1.5 MoM	--	50	97	prospective
v Dongen,[47] 2005	27	18	10	1.5 MoM	Hb deficit > 5 SD	89	89	prospective

Hb: Hemoglobin concentration, Ht: Hematocrit, MCA: Middle cerebral artery, MoM: Multiples of the median value for gestational age in normal fetuses, SD: Standard deviation, --: not given

Table 4 - Test characteristics of Δ OD 450 and MCA peak systolic velocity in the prediction of fetal anemia in the same patients.

First author year	*Number of fetuses*	*Number of severely anemic fetuses*	*Definition of anemia*	*Cut-off MCA*	*MCA*			*Cut-off Δ OD 450*	*Δ OD 450*			*Study design*
					sensitivity (%)	*specificity (%)*	*accuracy (%)*		*sensitivity (%)*	*specificity (%)*	*accuracy (%)*	
Nishie,[51] 2003	28	7	Hb deficit > -5 SD	> 1.5 MoM	100	65	73	Bowman's curve zone 3	86	100	96	prospective
Pereira,[50] 2003	28	4	Hb < 0.55 MoM	> 1.5 MoM	100	88	89	Liley high zone 2 or zone 3	75	75	75	retrospective
Bullock,[49] 2005	38	22	Hb < 5th percentile	> 1.5 MoM	64	81	71	Liley curve "over the action line"	53	71	59	cross-sectional

Hb: Hemoglobin concentration, MCA: Middle cerebral artery, MoM: Multiples of the median value for gestational age in normal fetuses, SD: Standard deviation.

Discussion

This study shows that sensitivities to predict severe anemia at fetal blood sampling (Table1) were between 80 % and 100% for Δ OD 450 in the upper half of Liley's zone II (IIB) or Queenan's zone 3. These results are excellent, because the procedure-related risk of amniocentesis is low compared to the procedure-related risk of fetal blood sampling. The sensitivities of Δ OD 450 in the prediction of neonatal anemia at birth (Table 2) were much more variable. This is readily explained by the commonly longer time period between amniocentesis and birth. Also, it should be noted that different inclusion criteria and different definitions of severe anemia were used in the different studies.

The ACOG recommends diagnostic amniocentesis for red cell 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.[4] The results of our previous study support this guideline: a 95 % sensitivity for severe fetal anemia was found.[26] However, a specificity of 50% and the risk associated with repeated amniocentesis remain the major drawbacks of this approach. In addition, fetal and perinatal procedure-related loss rates are reported to be 0.25 to 1% per amniocentesis.[52;53] Further, false positive results of amniocentesis can lead to unnecessary IUTs with procedure-related fetal loss rates of 1 to 3%.[54] Finally, another drawback of amniocentesis or fetal blood sampling is the risk of feto-maternal hemorrhage that may increase the severity of alloimmunization. Feto-maternal hemorrhage occurs in 2.3% of cases after amniocentesis.[9] A significant increase in antibody titers and induction of additional antibodies occurs in respectively 50% and 26% of cases after IUT.[9;55;56] 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.[10;46;57;58] These studies report that Doppler measurements, when performed by experienced operators, have sensitivities between 67% and 100% and specificities between 70% and 100% in the prediction of severe fetal anemia.[10;46;57;58] However, there is a

tendency to be overly optimistic about early results with new techniques. In the present study, we also performed a literature review on the accuracy of Doppler measurements of MCA peak systolic velocity in the prediction of severe fetal anemia. The selected studies showed sensitivities and specificities that were comparable to those reported in the Δ OD 450 studies.

In three small studies, each with less than 40 patients, Doppler and Δ OD 450 were compared.[46-48] Two of these studies were retrospective, only one was prospective. In these studies, the accuracy of MCA peak systolic velocity was better than that of Δ OD 450.

From our literature review, we conclude that Δ OD 450 measurement predicts severe anemia with sensitivities ranging between 80 and 100 % in most studies. In recently published series on MCA Doppler velocimetry, sensitivities for the prediction of severe fetal anemia range between 54 and 100 %. It is still unknown which test, the traditional minimally invasive amniocentesis with Δ OD 450 measurements, or the more recent non-invasive MCA Doppler measurements, is the more accurate. Only a prospective trial, comparing the characteristics of the two tests (Δ OD 450 and MCA peak systolic velocity) simultaneously measured in the same patients, with the gold standard test (fetal hemoglobin concentration) can provide the answer. We have been engaged in such a trial, called the DIAMOND ("diagnostic amniocentesis or non-invasive Doppler") study and the results of this trial will become available soon.[59]

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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).

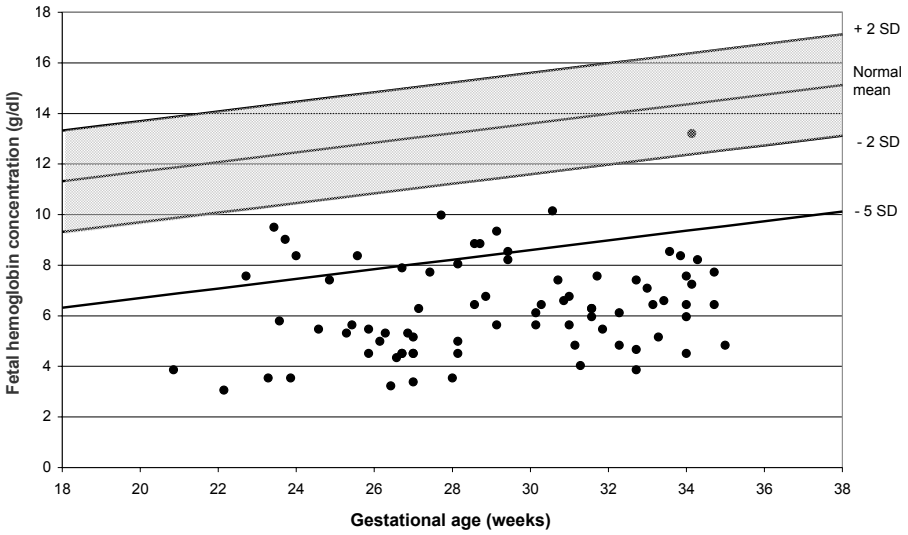

Figure 1 - Hemoglobin values of 79 non-hydropic Rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey zone between 3 upper ascending lines marks the limit of normal (mean ± 2 SD) fetal hemoglobin concentrations. The lower line seperates moderate (between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

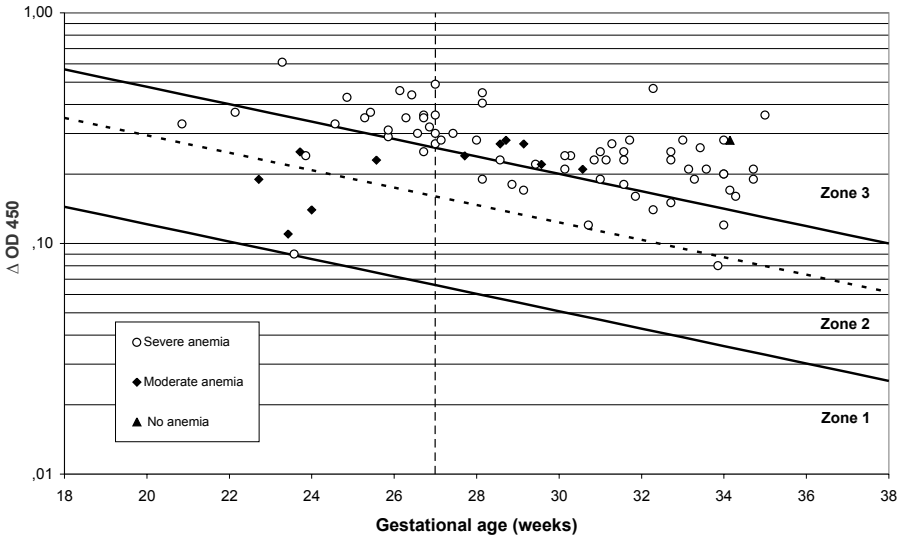

Figure 2 - Amniotic fluid Δ OD 450 values of 79 non-hydropic Rhesus D-alloimmunized fetuses at the time of first blood sampling, plotted in the extrapolated Liley curve. No anemia (▲) corresponds with hemoglobin concentrations within the normal range (mean ± 2 SD) for gestational age; moderate anemia (◆) 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,

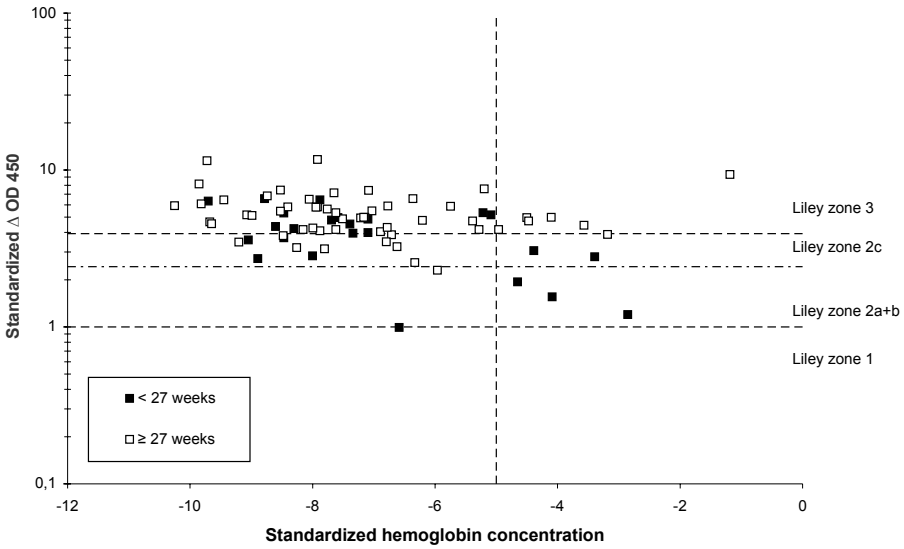

Figure 3 - Relationship between standardized Δ OD 450 and standardized hemoglobin concentrations in 79 non-hydropic Rhesus D-alloimmunized fetuses at the time of first blood sampling. Amniotic fluid Δ OD 450 were standardized by dividing the actual value by the value on the line between zone 1 and 2 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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On the origin of human amniotic fluid bilirubin

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Abstract

We studied the relationship between bilirubin concentrations in amniotic fluid and fetal blood in 68 non-hydropic rhesus D-alloimmunized anemic fetuses at first blood sampling. In these alloimmunized fetuses, the amniotic fluid / fetal blood ratio for bilirubin decreased from 0.09 at 28 weeks to 0.05 at 33 weeks. In normal fetuses, amniotic fluid / fetal blood ratios for bilirubin, and for albumin, are in the same range and show a similar decrease during gestation. We conclude that amniotic fluid bilirubin concentration is determined, firstly, by fetal blood bilirubin concentration and, secondly, by the amniotic fluid / fetal blood ratio of albumin. Among five possible pathways bilirubin could take to build up a concentration in amniotic fluid (fetal kidneys, lungs, skin, bowel, membranes), the intramembranous pathway is the only one that is compatible with the amniotic fluid / fetal blood ratios for bilirubin that we found and must therefore be the most important.

Introduction

Bilirubin is formed during the degradation of haem-containing compounds, mainly hemoglobin (Rosenthal, 1992). Bilirubin concentration is about four times higher in fetal than in maternal blood (Girling, Dow and Smith, 1997; Nava *et al.*, 1996) As a result of this concentration gradient, the unconjugated (liposoluble) bilirubin diffuses through trophoblastic layers from fetal to maternal blood (Odell, 1959). It is unclear whether active or passive carrier-mediated transport mechanisms play an additional role in placental transfer (Serrano *et al.*, 2002). Glucuronyl transferase activity in the fetal liver is minimal, less than 1% of its activity in neonatal and later life, and only a minor fraction of fetal bilirubin is conjugated (Kawade and Onishi, 1981; Nava *et al.*, 1996). In the fetal situation, this low glucuronyl transferase activity is probably beneficial because the clearance of conjugated (hydrophilic) bilirubin through the placental barrier is very slow (Bashore, Smith and Schenker, 1969). Unconjugated (hydrophobic) bilirubin in fetal and maternal blood is linked to albumin almost completely, and only a minute fraction is free (Brodersen, 1980).

Some of the fetal bilirubin is excreted into the amniotic fluid compartment, and less than 10% of this amniotic fluid bilirubin is conjugated (Halitsky and Krumholz, 1970). Each day, the fetus swallows about 75% of the amniotic fluid volume (Brace, 1999). Amniotic fluid bilirubin concentration is an important diagnostic tool in the management of blood group alloimmunization (Liley, 1961). Little is known, however, about how bilirubin reaches the amniotic fluid. Theoretically, there are five major possible pathways bilirubin can take to leave the fetal circulation and enter the amniotic fluid: via fetal kidneys, lungs, skin, bowel, or via placenta and membranes, which is called the intramembranous pathway. A first possible pathway would be via the kidneys. Fetal urine is, after all, the major constituent of amniotic fluid after 16-weeks´ gestation. A second pathway would be via the lungs. Fetal lung fluid contributes to approximately 10% of amniotic fluid (Brace, 1999). Many clinicians and investigators believe that the fetal lung pathway explains the clinically useful relation between amniotic fluid bilirubin concentration

and the degree of fetal anemia (American College of Obstetricians and Gynecologists, 1996). A third possible pathway, excretion of liposoluble substances through the fetal skin along a concentration gradient, probably occurs early in pregnancy, but is hampered during the second half of human gestation due to increasing keratinisation (Evans and Rutter, 1986; Parkin, Lind and Cheyne, 1969; Parmley and Seeds, 1970). Passage of meconium is a fourth possible pathway for bilirubin to enter the amniotic fluid. Fetuses regularly pass meconium into the amniotic fluid and small lumps of meconium have regularly been seen during fetoscopy (Hakguder *et al.*, 2002). A fifth possible pathway is the intramembranous pathway (Gilbert and Brace, 1989). The fetal surface of the placenta is well vascularized and probably plays an important role in the volume regulation and composition of amniotic fluid (Gilbert, Eby-Wilkens, and Tarantal, 1997). Under normal conditions, diffusion of fluid and solutes between amniotic fluid and fetal blood along this pathway is a fairly rapid process, one that has been shown to occur in both directions (Bashore *et al.*, 1969; Gilbert and Brace, 1989).

We wanted to study bilirubin concentrations in human amniotic fluid and fetal blood in cases with highly increased hemoglobin degradation, in order to gain more insight into the enigmatic relation between these concentrations and to possibly draw some conclusions regarding the origin of amniotic fluid bilirubin.

Methods

Leiden University Medical Center 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 (Kanhai *et al.*, 1990). We searched our database from January 1988 to October 2000 for contemporaneous amniotic fluid and fetal blood samples that were taken from singleton, Rhesus D-alloimmunized, non-hydropic, and not previously transfused fetuses. Amniotic fluid samples had to have been taken less than four days before fetal blood sampling.

Fetal blood samples were sent to our central laboratory for bilirubin and hematological measurements. Values were automatically entered into our database and checked by a specialized nurse. 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 spectrophotometer (Amersham Pharmacia Biotech, 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, derived from the logarithmic function of the absorptions between 365 and 550 nm (Liley, 1961).

Normal total bilirubin concentrations in fetal blood increase during gestation. We used the reference values proposed by Nava *et al.*, 1996, which were derived from a large number of normal fetuses undergoing percutaneous umbilical blood sampling between 18 and 39 weeks (Nava *et al.*, 1996). Normal bilirubin concentrations in amniotic fluid decrease during gestation. We used the reference values proposed by Nicolaides *et al.*, 1986; these were derived from a large number of amniocenteses in normal pregnancies, equally distributed between 16 and 37 weeks (Nicolaides *et al.*, 1986). A factor of 1.585 was used to convert all Δ OD 450 values to bilirubin concentrations (mg/dl) (Egberts *et al.*, 2002, Egberts *et al.*, 2003). Normal concentrations of albumin in amniotic fluid and fetal blood were based on the literature (Legras *et al.*, 1978; Takagi *et al.*, 1989).

Results

We found 68 contemporaneous amniotic fluid and blood samples from untransfused non-hydropic D-alloimmunized fetuses. Mean gestational age was 29 weeks (range 21-35). Mean fetal hemoglobin concentration was 6.1 g/dl (range 3.1-10.1). Figure 1 shows the individual hemoglobin concentrations of fetuses in our study plotted against their gestational age. Eight fetuses were moderately anemic (hemoglobin concentration 2 to 5 standard deviations below the normal mean) and 60 were severely

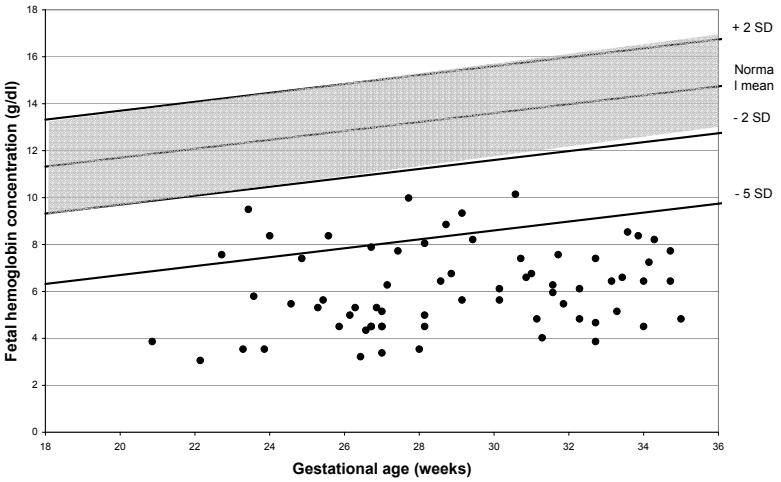

Figure 1 - Hemoglobin values of 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey zone between the three upper ascending lines marks the limits of normal fetal hemoglobin concentrations (mean ± 2 standard deviations) (Nicolaides *et al.*, 1988). The lower line separates moderate (between –2 and –5 standard deviations) from severe (less than –5 standard deviations) fetal anemia.

anemic (hemoglobin concentration more than 5 standard deviations below the normal mean) at the time of first blood sampling. Mean total bilirubin concentration in fetal blood was 5.8 mg/dl (range 1.9-11.4). In all but three cases, the conjugated bilirubin concentration was less than 10% of the total bilirubin concentration. Figure 2 plots the concentrations of total bilirubin in fetal blood against gestational age. Values were above normal in all but one fetus. Figure 3 shows the amniotic fluid bilirubin concentrations against gestational age. Values were above normal in all but three fetuses. In our study, 50 amniotic fluid bilirubin values were in Liley's zone 3, 13 in the upper third of zone 2 and the remaining 5 in the lower two thirds of zone 2 (Liley, 1961; Sikkel *et al.*, 2002). Figure 4 shows the ratios between bilirubin concentrations in amniotic fluid and in blood of the fetuses in our study, plotted against their gestational age. Roughly, these ratios decreased from around 0.09 at 28 weeks to around 0.05 at 33 weeks. Thus, in our alloimmunized fetuses, these ratios were in the same range as bilirubin and albumin ratios in non-immunized fetuses (Nava *et al.*, 1996; Nicolaides *et al.*, 1986; Legras *et al.*, 1978; Takagi *et al.*, 1989), and showed a similar pattern of decrease as pregnancy progressed.

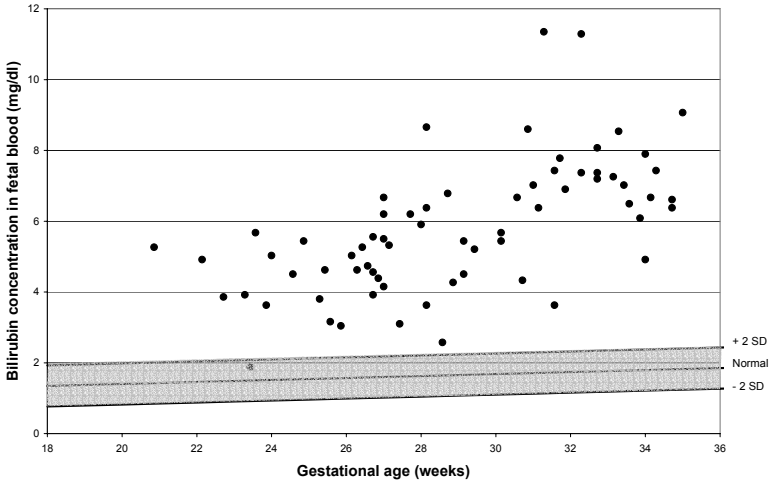

Figure 2 - Total bilirubin values in blood of 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey zone between the three lines marks the limits of normal (mean ± 2 standard deviations) total bilirubin concentration in fetal blood (Nava *et al.*, 1996).

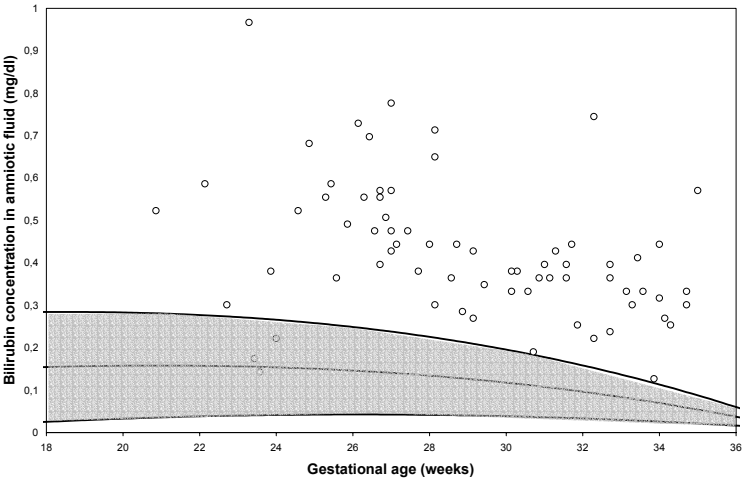

Figure 3 - Amniotic fluid bilirubin values of 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey zone between the three lines marks the limits of normal (mean ± 2 standard deviations) bilirubin in amniotic fluid (Nicolaides *et al.*, 1986).

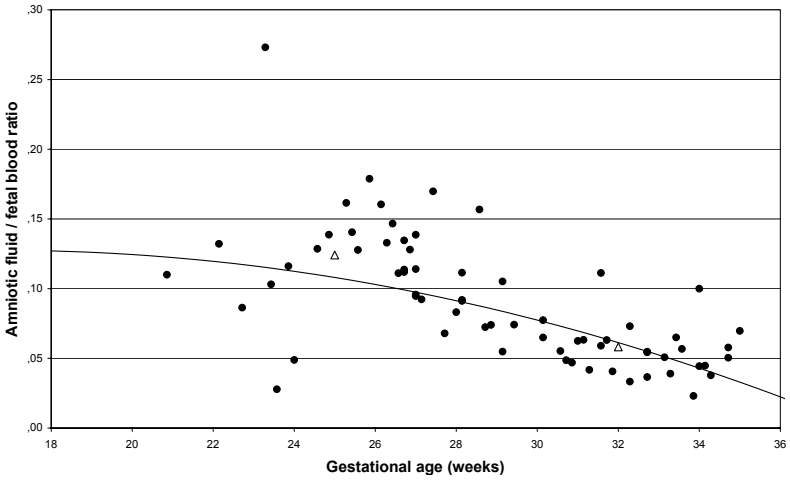

Figure 4 - Ratio between amniotic fluid and fetal blood concentrations of total bilirubin in 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The black line marks the ratio between normal bilirubin concentrations in amniotic fluid and fetal blood (Nava *et al.*, 1996; Nicolaides *et al.*, 1986). The grey open triangles mark the ratio between normal albumin concentrations in amniotic fluid and fetal blood (Legras *et al.*, 1978; Takagi *et al.*, 1989).

Discussion

We studied bilirubin concentrations in amniotic fluid and blood in 68 alloimmunized fetuses and found that bilirubin values in blood were on average three times as high as in non-anemic fetuses. All values were, however, well below the threshold associated with a kernicterus risk (Poland, 2002). Amniotic fluid bilirubin values were also elevated, and most values were in Liley's zone 3, which warrants immediate treatment. We then calculated ratios of bilirubin in amniotic fluid to that in blood for these anemic fetuses and found these ratios to be very similar to ratios in normal fetuses. These ratios were also very similar to ratios of albumin in amniotic fluid to that in blood in normal fetuses. These ratios decreased with gestational age from around 0.09 at 28 weeks to 0.05 at 33 weeks.

The strength of the present study is that we measured bilirubin in a relatively large number of D-alloimmunized anemic fetuses. None of these fetuses were hydropic and this may be important because hydrops

is associated with an increase in the amniotic fluid / fetal blood ratio of albumin: it has been shown that in hydropic fetuses, the blood concentration of albumin decreases and the amniotic fluid concentration of albumin increases (Nicolaides, Warenski and Rodeck, 1985; Cherry, Rosenfield and Kochwa, 1970). A weakness of our study is that amniotic fluid samples were taken up to three days before fetal blood sampling (we called this contemporaneous) whereas one would prefer completely simultaneous samples. Prehydropic changes in some of our severely anemic fetuses may also have influenced our results. Finally, we did not measure bilirubin in non-anemic fetuses, and therefore we had to use normal mean values of bilirubin in amniotic fluid and in blood found in the literature (Nava *et al.*, 1996; Nicolaides *et al.*, 1986). Still, we think our results suggest rather convincingly that amniotic fluid / fetal blood bilirubin ratios in anemic and non-anemic fetuses are very similar.

Albumin contains one high affinity binding site for bilirubin and one or two secondary sites of lower affinity (Rosenthal, 1992). Unconjugated bilirubin is hydrophobic and in aqueous solutions linked to albumin almost completely (Rosenthal, 1992). Transfer of bilirubin between body compartments, however, is due to diffusion of albumin-free unconjugated bilirubin (Odell, 1959). The bilirubin gradient between compartments is a function of the concentration of albumin-free bilirubin and thus of the ratio between bilirubin and albumin in both compartments (Odell, 1959). As early as 1970, Cherry *et al.* proposed a strong experimental argument for this theory, measuring Δ OD 450 before and 12 hours after the injection of albumin in the amniotic fluid compartment in 3 alloimmunized pregnancies (Cherry, Rosenfield and Kochwa, 1970). They found a highly significant linear relationship between Δ OD 450 and albumin concentration. In 1967, Cherry and Rosenfield had already suggested that bilirubin / protein ratios in amniotic fluid could replace plotting Δ OD 450 in Liley's curve and suggested a bilirubin / protein ratio of 0.55 as the cut-off. In 1974, Bosch *et al*. found that this "Cherry-ratio" led to slightly more accurate predictions than the Liley chart (Bosch, Robinson and Fisher, 1974). Our study suggests the existence of a fixed amniotic fluid / fetal blood ratio for bilirubin. This ratio decreases between 26 and 34 weeks, probably concurrent with the decrease of the

amniotic fluid / fetal blood ratio for albumin. It is still unclear which factors contribute to the albumin concentration in amniotic fluid. In animal experiments, it has been shown that amniotic fluid albumin is, to a large extent, of maternal origin and that clearance occurs through fetal swallowing and digestion, as well as through absorption through fetal membranes (Gitlin *et al.*, 1972; Faber and Anderson, 2002). It seems clear that the origins and pathways of amniotic fluid albumin are distinct from those of bilirubin, but they are, at present, even more puzzling.

We conclude that the bilirubin concentration in amniotic fluid reflects the bilirubin concentration in fetal blood. This finding provides a logical explanation for the longstanding good performance of Liley's method in the diagnosis of severe fetal alloimmune hemolytic anemia. Further, we found that the amniotic fluid / fetal blood ratio for bilirubin mimicked that of albumin. Therefore, we suggest that the ratio between bilirubin and albumin in amniotic fluid equals the ratio between bilirubin and albumin in blood. The existence of a fixed ratio would shed some light on the origin of human amniotic fluid bilirubin: of the five possible pathways bilirubin could take, only one would agree with such a fixed ratio. To our knowledge, urinary or alveolar fluid concentrations of bilirubin have not been measured in the human fetus. It is very improbable, however, that urine or alveolar fluid contribute substantially to the bilirubin concentration in amniotic fluid because the protein concentrations in both fetal urine and alveolar fluid are 100 to 200 times lower than in fetal plasma (Awad *et al.*, 2002; Boston *et al.*, 1968; Muller *et al.*, 1996; Gitlin *et al.*, 1972). The protein concentration in amniotic fluid, on the other hand, is only 10 to 20 times lower than in fetal plasma (Legras *et al.*, 1978; Takagi *et al.*, 1989; Faber and Anderson, 2002; Nicolaides, Warenski and Rodeck, 1985). Because of the very low albumin concentrations in urine and alveolar fluid, these fluids act as a barrier for unconjugated bilirubin leaving the plasma and entering the amniotic fluid compartment. A meconial origin of amniotic fluid bilirubin is inconsistent with a clinically relevant correlation between amniotic fluid and fetal blood bilirubin concentration. The fetal skin probably serves as a major pathway for solute and water exchange between amniotic fluid and fetus in early gestation. Fetal skin keratinisation begins at approximately 17 weeks

and a complete stratum corneum is present by approximately 25 weeks (Hashimoto *et al.*, 1966). At 14 to 18 weeks, the skin has been shown to have similar permeability as chorion laeve and amnion. However, in fetuses of 24 weeks and older, the skin has become quite impermeable (Parmley and Seeds, 1970). The fetal membranes, on the other hand, retain a high permeability until term (Lloyd *et al.*, 1969). Therefore, bilirubin exchange between fetal blood and amniotic fluid most probably occurs through the intramembranous pathway, where both excretion and reabsorption of bilirubin take place throughout gestation.

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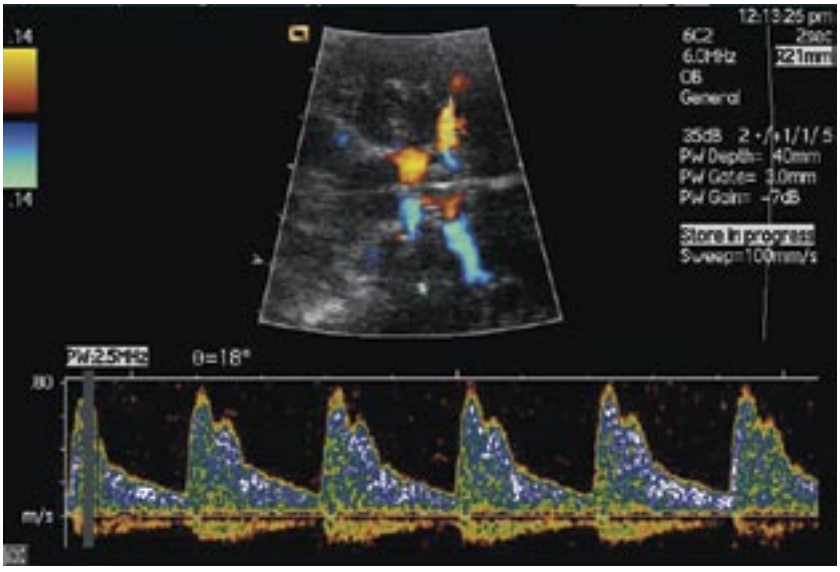
Part 2: Ultrasonographic approach



Effect of an increase of the hematocrit on middle cerebral artery peak and umbilical vein maximum velocities in anemic fetuses

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5

Abstract

ObjectIve: To measure the effects of acute large increases of the hematocrit on fetal peak arterial and maximum venous blood flow velocities.
Methods: Middle cerebral artery peak flow velocities and umbilical vein maximum flow velocities were measured before, immediately after, and 12-24 h after intrauterine transfusions. All measurements were standardized for gestational age.
Results: Complete measurements were obtained at 60 intrauterine transfusions. The mean hematocrit before intrauterine transfusion was 0.19 l/l and after 0.40 l/l. The middle cerebral artery peak flow velocity decreased immediately after transfusion in 59 of the 60 cases. There was a rise in umbilical vein maximum flow velocity immediately after intrauterine transfusion in 37 of the 60 cases. The sensitivity of middle cerebral artery peak flow velocity for severe anemia before intrauterine transfusion was 54% and the specificity 57%. The sensitivity of umbilical vein maximum flow velocity for severe anemia before intrauterine transfusion was 67% and the specificity 57%.
Conclusions: An acute large increase of the fetal hematocrit significantly decreases middle cerebral artery peak flow velocity. The effect on umbilical vein maximum velocity is, however, unpredictable.

Introduction

Middle cerebral artery peak velocity (MCA peak) and umbilical venous maximum velocity (UV max) increase in fetuses when anemia develops.[1–7] Measurements of arterial and venous flow velocities have even been described as sensitive indicators of the degree of fetal anemia in red cell alloimmunization.[3-7] As such, these measurements have recently been proposed as an alternative diagnostic method to invasive procedures like amniocentesis and cordocentesis.[3-7] A lowered blood viscosity due to a decrease in hematocrit is an obvious explanation for raised blood velocities in anemia. However, flow velocities are influenced by many other factors, and normal values change during gestation: in uncomplicated pregnancies, the MCA peak increases from 23 cm/s at 18 weeks to 58 cm/s at 38 weeks[5], and the UV max increases from 15 cm/s at 18 weeks to 24 cm/s at 38 week.[3,8] Arterial flow velocities depend on blood viscosity, cardiac output, vessel diameter, and peripheral resistance.[9,10] Venous flow velocities depend on blood viscosity, vessel diameter, right atrial pressure, and ductus venosus function.

We wanted to study the effects of acute changes in hematocrit on fetal maximum blood flow velocities. Therefore, we measured the hematocrit before and after intrauterine transfusion (IUT), and MCA peak and UV max before, immediately after, and the day after transfusion.

Methods

Setting and Patients

The Leiden University Medical Center 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.[11] Between November 2000 and August 2001, Doppler ultrasound was performed before, immediately after, and 1 day after all IUTs. The study was approved by the institutional review board, and all women gave oral informed consent. Fetal blood sampling was performed either at the intrahepatic vein or at the placental cord insertion (at the

discretion of the operator), and packed red cells with a hematocrit of around 80% were given intravenously. Before the procedure, meperidine (75 mg), promethazine (25 mg) and indomethacin (50 mg) were given to the mother. Atracurium (0.4 mg/kg) was given to the fetus immediately after the initial blood sample was taken.

Measurements

MCA peak and UV max measurements were performed before IUT (0-6 h), immediately after (within 30 min) and the day after (12-24 h). All Doppler measurements were performed with the angle between the ultrasound beam and the direction of the blood flow as close to 0° as possible and never exceeding 30°. If the angle was > 0°, an angle correction was applied. Flow velocity waveforms were obtained in the absence of fetal breathing and body movements. Doppler studies were done by one of five experienced operators (E.S., K.T., R.H.M., F.K., F.V.) using an Acuson Sequoia (Acuson, Mountain View, CA) ultrasound machine with a 6.0 MHz probe. The circle of Willis was visualized and the middle cerebral artery was examined close to its origin from the internal carotid artery. In the absence of visible changes in the waveform patterns, we measured for at least five waveforms. The highest point of the flow velocity waveform (peak systolic velocity) was measured. The UV max was measured in the straight intrahepatic portion of the umbilical vein. The maximum velocity was measured when a uniform Doppler signal of at least 3 seconds was obtained. The hematocrit was measured in the initial fetal blood sample and at the end of the transfusion.

Normal and standardized values

Normal hematological and blood flow velocity values change during gestation, and we therefore standardized all measurements (hematocrit, MCA peak, and UV max) for gestational age. For fetal hematocrit, we used the reference values proposed by Forestier et al.[12,13] Standardized fetal hematocrit (z_Ht) was defined as the number of SDs that the actual value deviated from the normal mean for gestational age. Severe fetal anemia was defined as z_Ht ≤ -5, moderate fetal anemia as -2 ≥ z_Ht > -5. For MCA peak, we used the reference values proposed by Mari et al.[5], and the

standardized MCA peak velocity was expressed as multiples of median (MoM_MCA) for gestational age. An abnormally increased MCA peak was defined as a MoM_MCA ≥ 1.5. For UV max, we used the reference values proposed by Oepkes et al.[3,8] The standardized UV max velocity (z_UV) was defined as the number of SDs the measured value deviated from the normal mean for gestational age. An abnormally increased UV max was defined as z_UV ≥ 2. Hydrops was classified as mild when a distinct rim of ascites was present with or without pericardial effusion.[14] Hydrops was classified as severe when ascites was abundant (free-floating intra-abdominal organs) with or without pericardial effusion, skin edema, and pleural effusion.[14]

Statistics

To compare the changes in Doppler velocities (MoM_MCA and z_UV) between the three different time points and changes in z_Ht between the two different time points, repeated measures analysis of variance was used. A value of $p < 0.05$ was considered significant. Comparison between subgroups at each time point was performed using t-test (puncture site, type of immunization) and ANOVA (presence of hydrops). Comparison of the changes between the three different time points in the subgroups (according to puncture site, type of immunization and presence of hydrops) was done using repeated measures analysis of variance. Because of multiple testing, a Bonferoni correction was used for comparison of subgroups, and only p-values < 0.01 were considered significant. We thereby assumed that z_Ht on the day after transfusion was identical to the value obtained immediately after IUT [15]. Sensitivity and specificity of MoM_MCA and z_UV before IUT in the prediction of severe fetal anemia were calculated using 2x2 tables. Comparison between sensitivities and specificities of the subgroups (puncture site, type of immunization, presence of hydrops, order of IUT) before transfusion was performed using χ^2 tests. Statistical analysis was performed uing SPSS version 10.0 (SPSS, Chiago, IL.).

Results

During the study period, 80 IUTs were performed in 30 pregnancies. Twenty procedures were excluded from analysis because of incomplete measurements due to fetal breathing, body movements, or fetal position in utero (n=16), or because the final fetal blood sample could not be obtained (n=3). In 1 case, there was persistent bleeding from the puncture site (after the final fetal blood sample) and the fetus was found to be severely anemic again 7 days later (n=1). Complete measurements were obtained from 60 IUTs in 30 fetuses in 29 women (1 with a twin pregnancy). Characteristics of these 60 IUTs are shown in Table 1.

Table 1 - Characteristics of the 60 IUTs

Gestational age (completed weeks), median (range)	29 (19-35)
Type of alloimmunization:	
D	45 (of which 6 mildly hydropic)
Kell	14 (of which 3 mildly and 3 severely hydropic)
c	1
Order of IUTs:	
first	22
second	15
third	11
fourth	7
fifth	4
sixth	1
Degree of anemia:	
moderate (-2 SD ≥ Ht > -5 SD)	14
severe (Ht ≤ - 5 SD)	46
Puncture site:	
placental cord insertion	44
intrahepatic vein	16

IUT: intra uterine transfusion; Ht: Hematocrit

There were no complications following IUT in our study population. The MCA peak decreased immediately after transfusion in 59 of the 60 cases. There was a rise in UV max immediately after the procedure in 37 of the 60 cases. Table 2 shows the mean values (range) of MCA peak, UV max

and Ht and their standardized values before, immediately after and 1 day after IUT. MCA peak and MoM_MCA as well as UV max and z_UV differed significantly between the three time points ($p<0.0001$). Ht and z_Ht differed significantly between the two time points ($p<0.0001$). One day after IUT, z_UV was significantly ($p < 0.003$) higher in severely hydropic fetuses than in mildly hydropic or non-hydropic fetuses. There were no other significant differences at any of the time points in z_Ht, MoM_MCA, and z_UV between the subgroups (according to puncture site, presence of hydrops, type of immunization). In addition, there were no significant difference in the change that occurred after IUT in the subgroups.

Table 2 - Actual and standardized measurements (mean (range)) before, immediately after and 1 day after 60 IUTs

	before IUT	*after IUT*	*1 day after IUT*	*repeated measures ANOVA between time points*
Actual values				
MCA peak (cm/s)	61.87 (26-109)	32.68 (10-60)	44.40 (18-73)	p < 0.0001
UV max (cm/s)	29.72 (15-58)	36.32 (14-101)	22.75 (10-35)	p < 0.0001
Ht (l/l)	0.19 (0.04-0.28)	0.40 (0.22-0.47)		p < 0.0001
Standardized values				
MoM_MCA	1.59 (0.94-2.68)	0.83 (0.26-1.75)	1.13 (0.67-1.62)	p < 0.0001
z_UV	3.09 (-1.38-11.35)	5.19 (-2.6-25.53)	0.87 (-2.92-5.08)	p < 0.0001
z_Ht	5.79 (3.13-8.76)	0.43 (-1.79-3.93)		p < 0.0001

IUT: intrauterine transfusion; MCA: Middle cerebral artery; UV: Umbilical vein; Ht: Hematocrit; MoM_MCA: Multiples of median of MCA for gestational age; z_UV: Number of SDs the actual value of UV deviated from normal mean for gestational age; z_Ht: Number of SDs the actual value of Ht deviated from normal mean for gestational age

Figure 1 plots hematocrit values of 30 fetuses at 60 IUTs against their gestational age. Figure 2 shows the relation between MoM_MCA and z_Ht before, immediately after, and 1 day after IUT. A horizontal line is drawn at MoM_MCA of 1.5 to separate test-positives (above) from test-negatives (below). A vertical line is drawn at the threshold of severe anemia: cases on the left of the vertical line were severely anemic, and cases on the right were moderately anemic. Figure 3 shows the relation between z_UV and z_Ht before, immediately after, and 1 day after IUT. A horizontal

line is drawn at z_UV of 2.0 to separate test-positives (above) from test-negatives (below). A vertical line is again drawn at the threshold of severe anemia: cases on the left of the vertical line were severely anemic and cases on the right were moderately anemic. As such, Figure 2 and 3 can be read as two-by-two tables. Table 3 lists sensitivities and specificities of MoM_MCA and z_UV before IUT in the prediction of severe fetal anemia in the study population. There were no significant differences in sensitivity and specificity before IUT between subgroups (according to puncture site, presence of hydrops, type of immunization, order of IUT).

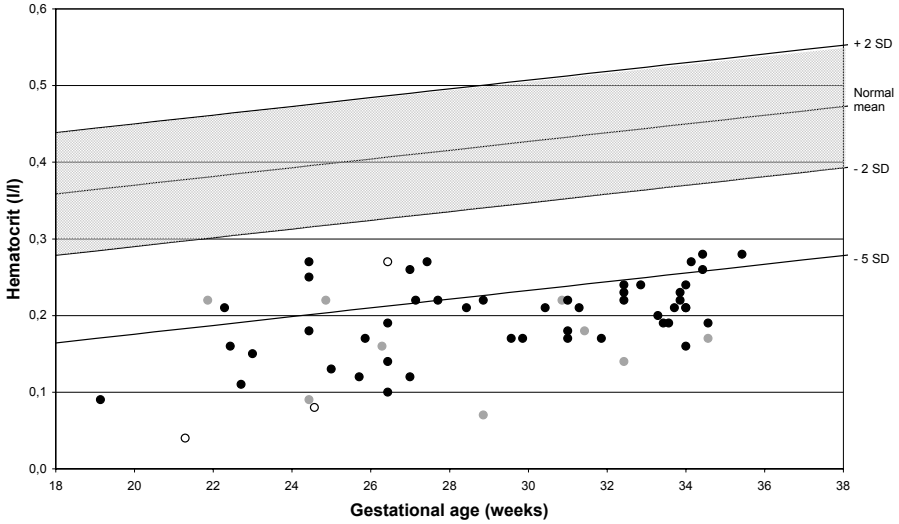

Figure 1 - Hematocrit values before 60 intrauterine transfusions (in 30 fetuses), plotted against gestational age at blood sampling. The grey zone marks the limits of normal (mean ± 2 SD) fetal hematocrit concentrations [13]. The lower line separates moderate (between –2 and -5 SD) from severe (≤ –5 SD) fetal anemia. Dark circles represent non-hydropic fetuses, grey circles represent mildly hydropic fetuses, and open circles represent severely hydropic fetuses.

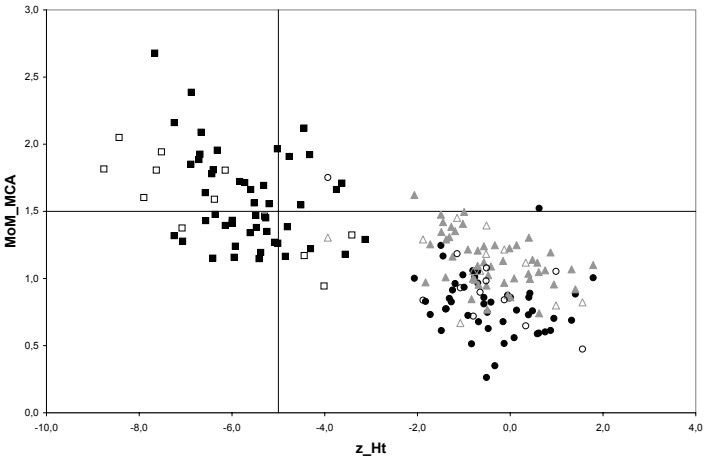

Figure 2 - Relation between standardized middle cerebral artery peak velocity (MoM_MCA) and standardized hematocrit (z_Ht) before (squares), immediately after (circles), and 12-24 h after (triangles) 60 intrauterine transfusions. Closed symbols represent non-hydropic fetuses, and open symbols represent hydropic fetuses. z_Ht was defined as the number of SDs that the actual value deviated from the normal mean for gestational age. MoM_MCA was expressed as multiples of median for gestational age. The horizontal line drawn at 1.5 MoM_MCA seperates test-positives (above) from test-negatives (below). The vertical line drawn at -5 z_Ht is the threshold for severe anemia. Cases on the left of the vertical line were severely anemic, cases on the right were non-anemic or only moderately anemic.

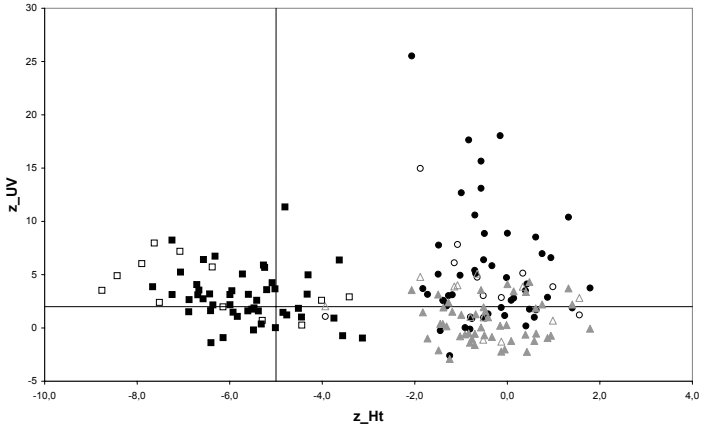

Figure 3 - Relation between standardized umbilical vein maximum velocity (z_UV) and standardized hematocrit (z_Ht) before (squares), immediately after (circles), and 12-24 h after (triangles) 60 intrauterine transfusions. Closed symbols represent non-hydropic fetuses, and open symbols represent hydropic fetuses. z_Ht was defined as the number of SDs that the actual value deviated from the normal mean for gestational age. z_UV was defined as the number of SDs the measured value deviated from the normal mean for gestational age. The horizontal line drawn at 2 z_UV seperates test-positives (above) from test-negatives (below). The vertical line drawn at –5 z_Ht is the threshold for severe anemia. Cases on the left of the vertical line were severely anemic, cases on the right were non-anemic or only moderately anemic.

Table 3 - Sensitivity and specificity of MoM_MCA and z_UV before IUT in the prediction of severe (Ht ≥ -5 SD) fetal anemia

	total study population (n = 60)		*untransfused fetuses (n = 22)*		*previously transfused fetuses (n = 38)*	
	MoM_MCA	z_UV	MoM_MCA	z_UV	MoM_MCA	z_UV
Sensitivity % (95% CI)	54 (39-69)	67 (52-80)	58 (33-80)	74 (49-91)	52 (32-71)	63 (42-81)
Specificity % (95% CI)	57 (29-82)	57 (29-82)	0 (0-71)	33 (1-91)	73 (39-94)	64 (31-89)

MoM_MCA: Multiples of median of MCA for gestational age; z_UV: Number of SDs the actual value of UV deviated from normal mean for gestational age; IUT: Intrauterine transfusion; Ht: Hematocrit; SD: Standard deviation; 95% CI: 95% Confidence Interval.

Discussion

As expected, we found a substantial decrease in MCA peak immediately after IUT. Quite unexpectedly, however, we found a steep, though temporary, increase in UV max immediately after IUT in 37 of our 60 cases. The latter finding challenges the view that the fetal hematocrit is the main determinant of the blood flow velocity. Furthermore, sensitivities of MCA peak and UV max in the prediction of severe fetal anemia were disappointing in our study. There were no significant differences in these sensitivities between untransfused and previously transfused fetuses.

We think that the strength of our study lies in the fact that it resembles a laboratory setting: we measured fetal blood flow velocities in the same fetus before and after substantially changing its hematocrit. There are, however, several shortcomings too. First, we not only changed hematocrit during IUT, but also infused a relatively large volume into the fetal vascular space. It is known from animal experiments, though, that through loss of plasma from the fetal circulation after packed red cell transfusion, the fetal blood volume increases by only half of the transfused volume.[15] It is also known from these experiments, that the fetal blood volume after IUT remains at the same level for 24 h.[15] Second, our standard medication before IUT was another factor that, in addition to the changes in hematocrit and vascular volume, may have influenced the fetal response to IUT. Third, in individual cases, small hematomas or edemas at the

intrahepatic course of the umbilical vein may have influenced UV max near the puncture site. However, we did not find a significant difference in post IUT standardized UV max between procedures according to puncture site.

Mari et al. measured MCA peak before and immediately after IUT in 17 procedures.[10] These authors used no tocolytic agents and did not puncture intrahepatically. They found a significant decrease in MCA peak following IUT. Delle Chiaie et al. measured MCA peak before and after 39 IUTs.[2] They found a significant reduction of post transfusion MCA peak values. Oepkes measured UV max before and 12 -20 h after the first IUT in 36 fetuses.[16] This author found a significant decrease in UV max the day after IUT. Recently, Stefos et al. measured MCA peak before and immediately after 54 procedures.[17] They found a decrease in MCA peak in all but 1 fetus. Our findings on the effect of IUT on MCA peak and UV max are consistent with those of these four studies. The rise in UV max that we found immediately after IUT, however, has not been reported previously.

In an often cited study, Mari et al. have proposed a cut-off value for MoM_MCA of 1.5 for moderate anemia, and one of 1.55 for severe anemia, as these cutoff values resulted in 100% sensitivity, based on their retrospective analysis of 111 fetuses.[5] Recently, Detti et al. proposed a cutoff value for MoM_MCA of 1.69 for severe anemia in previously transfused fetuses, again based on 100% sensitivity in their retrospective series of 64 fetuses.[18] Delle Chiaie et al. found 73% sensitivity of MCA peak before 108 fetal blood samples by using a MCA peak threshold value of 1.29 multiples of median in the prediction of anemia (Hb ≤ 0.84 MoM).[2] Texeira et al. prospectively studied 26 alloimmunized fetuses and found a sensitivity of 67% of an MCA peak of > 2 SD above the mean in predicting severe anemia (z_Ht < -4).[7] In the present study, we found a sensitivity of 54% for MCA peak (MoM_MCA ≥ 1.5) in the prediction of severe anemia (z_Ht ≤ -5). This may seem disappointing. However, it is well known that sensitivities are always lower in prospective series than in the retrospective series from which the cut-off values have been derived.

Our study probably raises more questions than that it gives answers. First, there was the unexpected rise in UV max immediately after IUT.

From this finding, it seems obvious that the fetal hematocrit is not the only and probably not the most important factor determining maximum umbilical venous blood flow velocities. The venous pressure may be far more important in this matter. Second, the wide range in pre-transfusion MoM_MCA for fetuses with identical z_Ht points to the fact that, besides hematocrit, other factors such as cardiac output and peripheral resistance must play an important role in determining MCA peak. Finally, the protein concentration is also a major determinant of blood viscosity.[19]

In conclusion, an increasing fetal hematocrit has a clear effect on maximum arterial and venous flow velocities. This effect is partly the result of the changes in blood viscosity, but other factors are certainly involved. Given the potential consequences of a false negative result, the sensitivities of MCA peak and UV max for severe fetal anemia were disappointing in our study. Therefore, monitoring of pregnancies at risk of fetal anemia should not rely solely on measuring blood flow velocities.

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Cardiac ventricular wall thickness and cardio-thoracic ratio in fetuses with severe alloimmune anemia

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6

Abstract

Objective: To test the diagnostic accuracy of cardiac ventricular wall thickness and cardio-thoracic ratio in the prediction of severe fetal alloimmune anemia.
Methods: The thickness of cardiac wall of the left and the right ventricles and the inter-ventricular septum were measured in diastole using M-mode ultrasound. The cardio-thoracic circumference ratio was measured on the B-screen. Reference ranges were obtained in 24 non-immunized control pregnancies. The measurements were then obtained in alloimmunized fetuses and two by two tables were constructed to compare the frequency of abnormal cardiac ultrasound measurements in severe and non-severe fetal alloimmune anemia. Ultrasound measurements were considered abnormal in case values were > 2 SD above the normal mean for gestational age. Severe anemia was defined as a hemoglobin concentration of > 5 SD below the normal mean for gestational age.
Results: Complete measurements were obtained in 15 alloimunized fetuses with severe anemia and in 16 alloimmunized fetuses without severe anemia. Sensitivities of cardiac ultrasound ranged between 0 en 47% and specificities between 77 and 97%.
Conclusion: Diagnostic accuracy of ventricular wall thickness and cardio-thoracic ratio in the prediction of severe fetal alloimmune anemia was disappointing. More than 50% of measurements in severely anemic fetuses were within the normal reference ranges.

Introduction

Fetal adaptation to chronic anemia includes changes, such as liver and spleen enlargement,[1-3] and a hyperdynamic circulation.[4] One of the signs of a hyperdynamic circulation is an increase in the peak systolic velocity of the middle cerebral artery. It is widely used as a diagnostic test, because peak systolic velocity of the middle cerebral artery is easy to measure and quite accurate in predicting severe anemia.[5] The hyperdynamic circulation is probably caused by a decrease in blood viscosity and by an increase in cardiac output. Several authors, did indeed find an increased cardiac output with the development of anemia in dogs, in fetal lambs and in human fetuses.[6-9]

We hypothesized that an increase in cardiac output would be accompanied by cardiac changes that are visible on ultrasound. In addition, our sonographers had the impression that severe anemia was accompanied by enlargement of the fetal heart, thickening of the ventricular walls and the interventricular septum, and hyperdensity of the myocard. Ouzounian et al. measured the biventicular outer dimension in alloimmunized patients.[10] They found a 50% sensitivity of the biventricular outer dimension / biparietal dimension ratio in predicting the necessity of neonatal transfusion. Oberhoffer et al. measured cardiac wall thickness in fetuses with anemia.[11] They found a significant increase in cardiac wall thickness in anemic fetuses compared to their own reference ranges.

We wanted to test the diagnostic value of cardiac ultrasound in fetal alloimmune anemia, and therefore measured cardiac ventricular wall thickness and cardio-thoracic ratio in non-anemic, moderately and severely anemic alloimmunized fetuses and in non-anemic controls. Thus, we hoped to expand our diagnostic possibilities for determining the need for fetal transfusion in red cell alloimmunized pregnancies.

Methods

Setting and Patients

Leiden University Medical Center is the national referral center for the management of fetal hematocytopenias in the Netherlands. Our methods for diagnosis and treatment of severe fetal alloimmune anemia have been described previously.[12] Briefly, patients with high antibody titres (> 1/16) and ADCC test > 50 % were followed with weekly ultrasound examinations for signs of fetal anemia.[13] These signs include hepatosplenomegaly, placental thickening, decreased fetal movements, increased maximum flow velocities in intrahepatic umbilical vein, increased mean velocity in the descending thoracic aorta, increased peak systolic velocity in the middle cerebral artery, or incipient hydrops. When severe anemia was suspected at or after 27 weeks, (repeated) amniocentesis for Δ OD 450 measurement was performed and fetal blood sampling was performed when Δ OD 450 was in zone 3 or the upper third of zone 2 and rising.[14] In pregnancies before 27 weeks, the decision to perform the first IUT was based on ultrasound findings alone.

Between March 2001 and May 2002, red cell alloimmunized women visiting our center were also followed with detailed fetal cardiac ultrasound. Exclusion criteria were: single consultation, and factors prohibiting cardiac ultrasound such as abundant fetal breathing or body movements, unfavorable fetal position, lack of time to perform the measurements, maternal obesity. Additional exclusion criteria were: fetuses negative for the offending antigen, hydrops fetalis, no intravascular acces and thus hemoglobin concentration unknown at IUT, or no cardiac measurements at the time severe anemia was diagnosed. Furthermore, 24 normal controls were followed with 4-weekly detailed cardiac ultrasound. The institutional review board approved this prospective study, and all women gave oral informed consent.

Measurements

Ultrasound measurements were performed at least every two weeks and 0-6 hours before IUT. M-mode measurements of fetal ventricular wall thickness and B-mode cardio-thoracic ratio were obtained in diastole. Ultrasound

measurements were both obtained in the absence of fetal breathing and body movements by one of two experienced operators (ES, KT) using an Acuson Sequoia (Acuson, Mountain View, CA) ultrasound machine with a 6.0 MHz probe. Sonographers were blinded for fetal hemoglobin measurements but not for Doppler measurements. The M-mode cursor was placed in the four-chamber view of the fetal heart perpendicular to the interventricular septum, just below the tips of the atrioventricular valves. The ventricular wall thickness of the left and the right ventricles and of the interventricular septum were then obtained (Figure 1). These cardiac wall measurements were only performed when there was no doubt about the definition of endocardial surfaces.

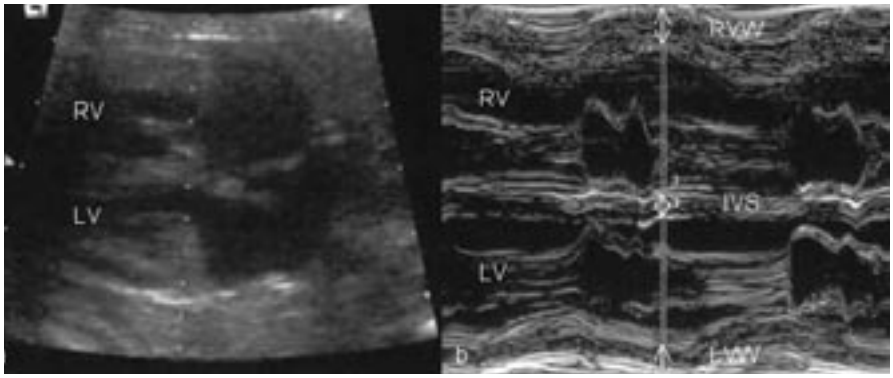

Figure 1 - Sonographic image of the fetal heart (a) and the orientation of the M-mode cursor, placed perpendicular to the interventricular septum, just below the tips of the atrioventricular valves. M-Mode measurement (b) in diastole of the left and the right ventricular walls and the septum.
LV = left ventricle, RV = right ventricle, RVW = right ventricular wall, LVW = left ventricular wall, IVS = inter-ventricular septum

Cardio-thoracic ratios were calculated after measuring the cardiac and thoracic circumferences at the level of the four-chamber view of the fetal heart (Figure 2). The cardiac circumference was measured on the outmost pericard of the heart and the thoracic circumference was measured on the outmost of the ribs of the fetus. This technique is slightly different from the one described by Paladini et al., who measured thoracic circumference at the level of the fetal skin.[15] In the alloimmunized patients, fetal hemoglobin was measured at the time of fetal blood sampling or in cord blood after birth.

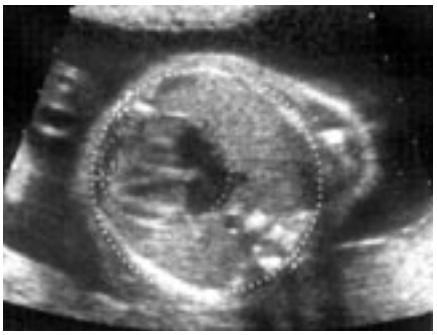

Figure 2 - Sonographic image of the fetal thorax at the level of the four-chamber view of the heart during diastole and the ellipses for measuring cardiac and thoracic circumferences. The cardiac circumference was measured on the outmost pericard of the heart and the thoracic circumference was measured on the outmost of the ribs of the fetus.

Normal values

M-mode ventricular wall thickness and cardio-thoracic ratios were also measured in 24 uncomplicated pregnancies with normal outcome. Each patient was measured five times with an interval of 4 weeks, between 18 - 36 weeks by one of two experienced operators (ES, MS). Interobserver variability (SD of differences/mean of measurements) for wall thickness was 4.2% for the left ventricle, 6.1% for the right ventricle and 8.2% for the interventricular septum. Inter-observer limits of agreement (95 % confidence interval of differences) were +0.45 to -0.30 mm for ventricular wall thickness of the left ventricle and +0.28 to -0.23 mm of the right ventricle and +0.36 to –0.31 mm of the interventricular septum.[16]

Statistics

Statistical analyses were performed using SPSS 10.0 (SPSS, Chicago, IL) and SAS proc mixed (SAS, Cary, NC). To construct normal references ranges, linear mixed models were fitted to the data of the 24 uncomplicated pregnancies, assuming a linear relation between the fetal cardiac measurements and gestational age with a per person random intercept and slope. This yielded an estimate for the mean and standard deviation as function of gestational age. Values of > 2 SD above normal mean for gestational age were considered as abnormal (positive test result). Severe fetal anemia was defined as hemoglobin > 5 SD below the normal mean.[17] Four two by two tables were then created to assess the diagnostic accuracy of thickening of the left ventricle, right ventricle, interventricular septum, and the cardio-thoracic ratio in the prediction of severe fetal anemia. When there was a first fetal blood sampling that showed severe fetal anemia, the cardiac measurements from immediately before the IUT were used. If there was no need for an IUT all the cardiac measurements were used in the analyses.

Results

The left, right and interventricular septum ventricular wall thickness in the 24 normal control fetuses (120 measurements) increased between 17 and 37 weeks from 1.1 to 4.0, 1.4 to 4.1 and 1.0 to 3.7 mm respectively. The cardio-thoracic ratio in these 24 normal fetuses (120 measurements) increased between 17 and 37 weeks from 0.52 to 0.55.
During the study period, 81 alloimmunized women where followed in our center of which 31 did receive at least one IUT. After exclusion, 15 severely anemic and 16 moderate or non-anemic fetuses with complete measurements remained (Figure 3). Study population characteristics are shown in Table 1.

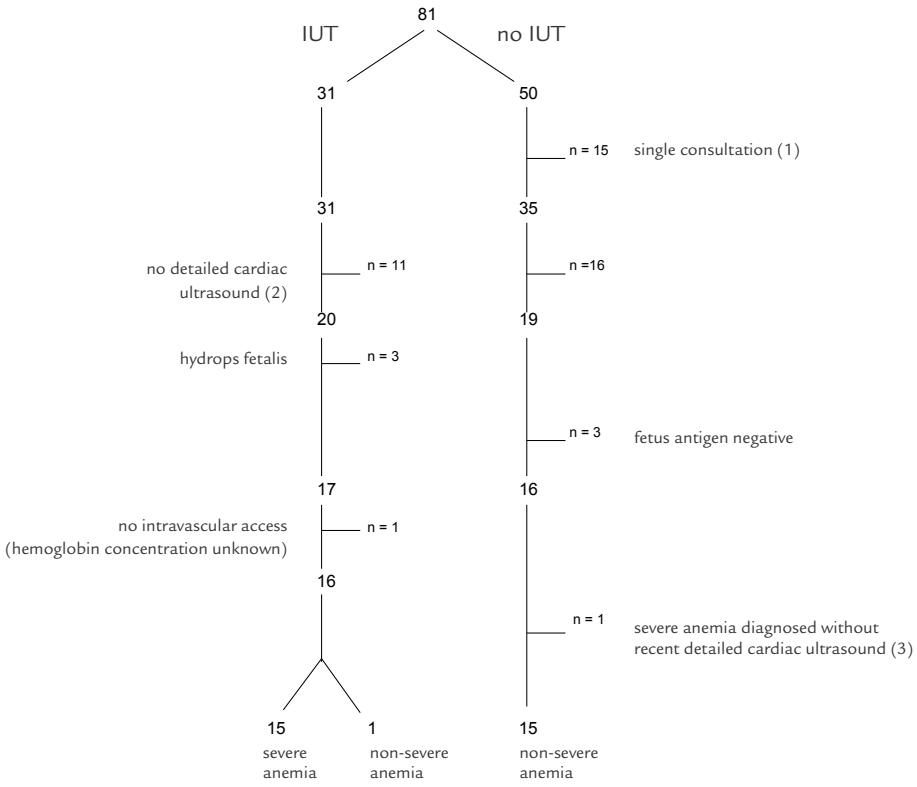

Figure 3 - Flow-chart of study population with exclusion criteria. After exclusion, 31 fetuses remained of which 15 were severely anemic and 16 were moderate or non-anemic.

(1) Because of antigen-negative fetus, low suspicion of anemia, or late referral
(2) Because of lack of time, maternal obesity, fetal breathing or position or body movements
(3) At the time of birth by cesarean section (36 weeks) severe anemia (hemoglobin concentration 7,5 g/dl) was diagnosed, whereas the last detailed prenatal cardiac ultrasound had taken place 5 weeks earlier (31 weeks).

Table 1 - Patient characteristics

	Contols (n = 24)	*Alloimmunized pregnancies without severe fetal anemia*	*Alloimmunized pregnancies with severe fetal anemia*
Maternal age			
(completed years), median (range)	33 (26 - 40)	31 (25 - 40)	31 (25 - 39)
Gestational age			
(completed weeks), median (range)			
at first visit	19 (17 - 21)	20 (13 - 28)	27 (14 - 33)
at birth	40 (36 - 42)	37 (33 - 38)	36 (35 - 37)
at first intrauterine transfusion	---	---	31 (17 - 34)
Type of alloimmunization			
D	---	14	12
c	---	1	3
E	---	1	---
Hemoglobin concentration			
before IUT (g/dl), median (range)	---	---	6.0 (3.7 - 9.3)
at birth (g/dl), median (range)	---	12.4 (10.0 - 21.6)	---
Number of detailed cardiac sonographic measurements included in analysis			
median (range)	5 (4-5)	4 (1 - 12)	1

Figure 4 shows reference ranges for left and right cardiac ventricular wall and interventricular septum thickness. Figure 5 shows reference ranges for cardio-thoracic ratio. Ventricular wall thickness and cardio-thoracic ratio of fetuses with and without severe anemia are plotted in these curves. Most measurements in alloimmunized pregnancies were within the normal reference ranges.

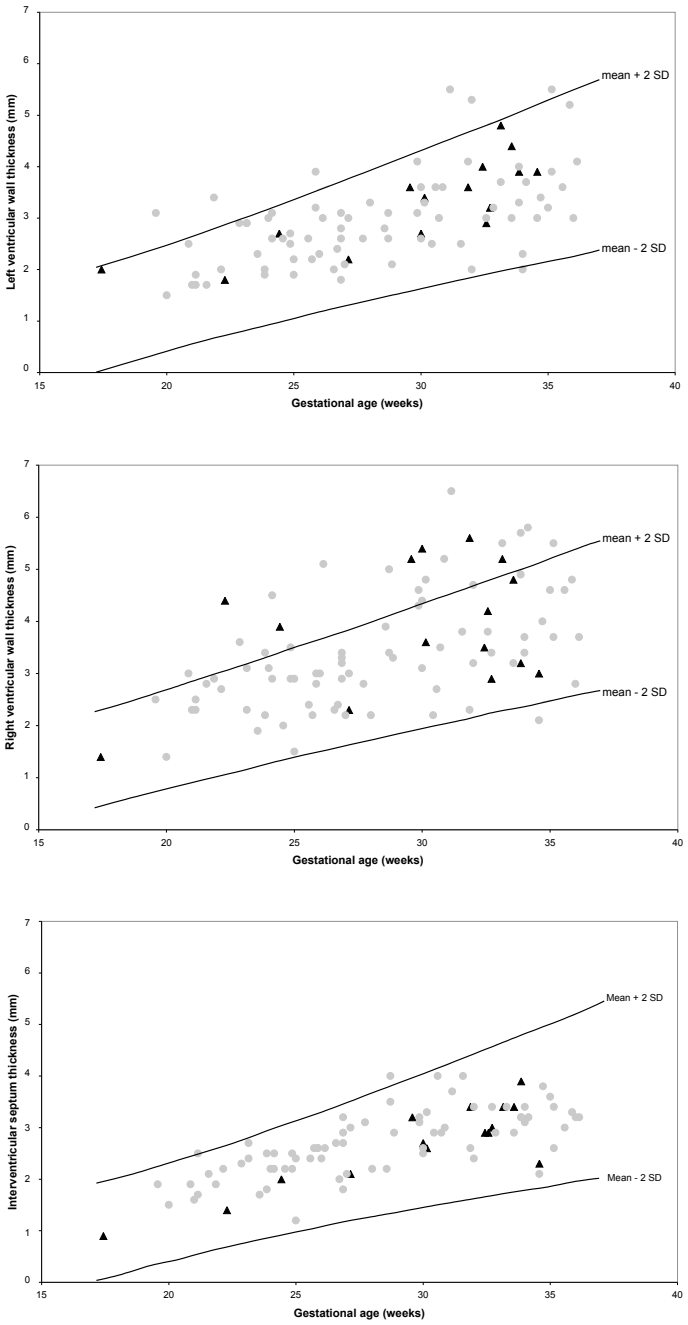

Figure 4 - Reference ranges for left and right cardiac ventricular wall and interventricular septum thickness (mean ± 2 SD). Cardiac ventricular wall thicknesses of fetuses with (black triangles) and without (grey circles) severe anemia were plotted in the curves.

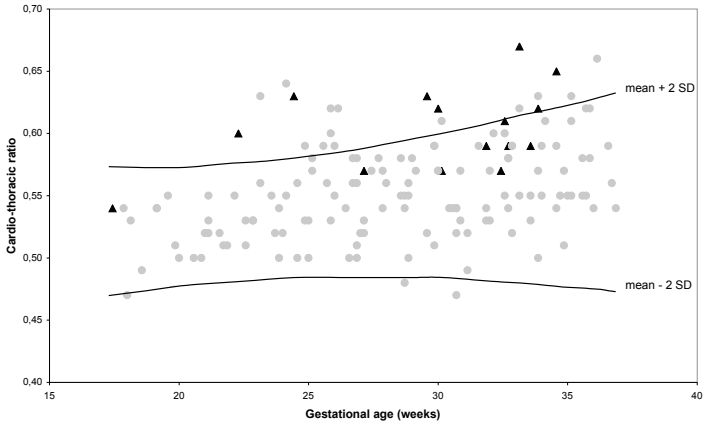

Figure 5 - Reference ranges for cardio-thoracic ratio (mean ± 2 SD). Cardio-thoracic ratios of fetuses with (black triangles) and without (grey circles) severe anemia were plotted in the curve.

Table 2 lists the sensitivities and specificities of the different cardiac ultrasound measurements in the prediction of severe fetal anemia. All sensitivities were below 50%.

Table 2 - Diagnostic accuracy of cardiac wall thickness and cardio-thoracic ratio in the prediction of severe fetal anemia in alloimmunized pregnancies.

	Sensitivity	Specificity
Cardiac wall thickness of:		
- left ventricle	0	92.0 %
- septum	0	97.3 %
- right ventricle	40.0 %	77.3 %
Cardio-thoracic ratio	46.7 %	90.4 %

Discussion

We measured the cardiac ventricular wall and interventricular septum thickness and the cardio-thoracic ratio in alloimmunized pregnancies with and without severe anemia and in non-anemic controls. We found that most of these measurements in alloimmunized pregnancies were within normal ranges. Their diagnostic accuracy is therefore too low to recommend them as a diagnostic tool in predicting severe fetal anemia.

Other authors have sonographically measured cardiac wall thickness. Alan et al. measured cardiac wall thickness in 200 normal fetuses using M-mode ultrasound.[18] They found an increase in left ventricular wall thickness from 1.3 to 4.1 mm between 16 and 39 weeks. Sutton et al. measured cardiac wall thickness in 16 normal fetuses using M-mode ultrasound.[19] They found a linear increase in left cardiac wall thickness from 2.0 to 3.5 mm and in right cardiac wall thickness from 2.0 to 3.0 mm between 20 to 40 weeks. Veille et al. measured cardiac wall thickness in 80 normal fetuses with M-mode ultrasound.[20] They found an increase in left ventricular wall thickness from 1.5 to 3.4 and right ventricular wall thickness from 1.6 to 3.3 between 17 and 41 weeks. Tan et al. measured cardiac wall thickness in 100 normal fetuses.[21] They used calipers on B-screen image for their measurements. They found an increase in left cardiac wall thickness from 1.4 to 3.3 mm and in right cardiac wall thickness from 1.3 to 3.3 mm between 17 and 37 weeks. Oberhoffer et al. measured cardiac wall thickness in 200 normal fetuses using M-mode ultrasound.[22] They found that the left ventricular wall thickness increased from 1.6 to 3.3 mm, the right ventricular wall thickness from 1.6 to 3.7 and the interventricular wall thickness from 1.6 to 3.7 mm between 19 and 40 weeks. Our normal reference ranges obtained in non-anemic controls are in agreement with the results of the five cited studies. Oberhoffer et al. also measured cardiac wall thickness in 30 anemic fetuses and found a symmetrical myocardial hypertrophy of the ventricular walls.[11] In arround 1/3 of their measurements, the interventricular septum thickness was above their normal range. In our study, however, the interventricular septum thicknesses in anemic fetuses were all within the normal range.

Other authors have sonographically measured cardio-thoracic ratio. Paladini et al. measured cardio-thoracic circumference ratio in 410 normal fetuses with gestational ages between 17 weeks and term.[15] They found a fairly constant circumference ratio throughout pregnancy, with a slight increase from 0.45 at 17 weeks to 0.50 at term. Respondec et al. measured cardio-thoracic area ratio in 99 normal fetuses and found this area ratio to remain relatively constant from 20 to 38 weeks.[23] Further, these authors measured the heart/chest antero posterior diameter and this measurement was also relatively stable throughout pregnancy. Oberhoffer et al. measured cardio-thoracic area ratios in 30 fetuses with anemia but they found that an increased cardio-thoracic ratio was not a consistent finding in this population.[11] Ouzounian et al. measured the biventricular outer dimension in diastole with M-mode in 63 alloimmunized patients without IUT.[10] They stated that this measurement is not ideal as a screening method or as a method of surveillance in the management of alloimmunized pregnancies. In our study, the slight increase in cardio-thoracic ratio in normal fetuses throughout pregnancy was similar to that in the cited studies. However, we found that the cardio-thoracic ratio was within the normal range in 8 of 15 severely anemic fetuses.

The strength of the present study is that we longitudinally studied cardiac wall thickness on both sides of the heart and cardio-thoracic ratio in a relatively large number of non-hydropic anemic fetuses. A weakness of our study is that the reproducability of M-mode measurements in the fetus has been described as poor.[24] However, Oberhoffer et al., found an intraobserver variability of cardiac wall thickness of ≤ 5.4%.[11] Tan et al. found an interobserver variability of 7%.[21] Veille et al. found an interobserver variability of ≤ 4.5%.[20] In our study, we found an interobserver variability of ≤ 8.2%. Another weakness is that we have not measured hemoglobin concentration during the pregnancy of fetuses with moderate anemia at birth. However, we think that it is very unlikely that a neonate with moderate anemia at birth has had periods of severe anemia due to alloimmunisation earlier in pregnancy.

In conclusion, we hypothesised that an increase in cardiac output in severe anemia would be accompanied by cardiac changes that are visible on ultrasound. These subjective signs included cardiac wall thickness, enlargement of the fetal heart and hyperdensity of the cardiac walls. However, we found no clear effect of severe fetal anemia on either M-mode measured cardiac ventricular wall thickness or B-mode measured cardio-thoracic ratio. Therefore, we think these measurements are not helpful in the diagnosis of severe fetal alloimmune anemia.

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Fetal cardiac contractility before and after intrauterine transfusion

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7

Abstract

Objective: To evaluate the effect of fetal anemia and intrauterine transfusion on ventricular shortening fraction.
Methods: Intrauterine transfusion was performed at a median gestational age of 31 weeks (range 19-35). Median hemoglobin concentration before and after intrauterine transfusion was 7.9 g/dl (range 2.7-13.7) and 14.3 g/dl (range 12.7-16.1) respectively. The end-diastolic and end-systolic transverse dimensions of the left and right ventricles were obtained using M-mode ultrasound. The shortening fractions of both ventricles were calculated at three time points: before, immediately after and one day after intrauterine transfusion. The blood volume given at intrauterine transfusion was expressed as a percentage of estimated fetoplacental blood volume.
Results: Complete measurements were obtained from 49 transfusions in 23 fetuses. Both, left and right, ventricular shortening fractions differed significantly between the three time points. Left ventricular shortening fraction decreased immediately after transfusion in 43 (88 %) of the 49 fetuses. Right ventricular shortening fraction decreased immediately after transfusion in 42 (86 %) of the 49 fetuses. At the first intrauterine transfusion, there was only a weak correlation between the decrease in shortening fraction of both ventricles and the transfused volume (left: $R^2 = 0.15$; $p = 0.20$ / right: $R^2 = 0.005$; $p = 0.81$).
Conclusion: Transfusion significantly decreases the shortening fraction of both ventricles of the fetal heart. There is, however, little correlation between the decrease in shortening fraction and the volume of red cells given at intra-uterine transfusion.

Introduction

A variety of sonographic measurements can be used to describe fetal cardiac function. These include morphological measurements, such as the cardio thoracic ratio[1], dynamic measurements, such as ventricular shortening fraction measured in the four-chamber view as transverse ventricular diameter[2-4] ventricular length[5], or area[6], Doppler measurements, such as isovolumetric contraction time [7], and combined measurements such as stroke volume or cardiac output.[2] Fetal anemia, as well as intrauterine transfusion (IUT), is supposed to have an effect on fetal cardiac contractility.

Previous studies have focused on functional[8-11], morphological[12], Doppler[13-17] or combined measurements[9; 10; 18; 19] In this study, we have chosen to evaluate ventricular shortening fraction in severely anemic fetuses before and after IUT. M-mode measured four-chamber view transverse shortening fraction probably corresponds best with the visual impression of cardiac contractility on a B-mode image. A further advantage of ventricular shortening fraction is that it has been shown to be fairly constant throughout normal pregnancy.[2-4]

We wanted to investigate the possible use of ventricular shortening fraction as a diagnostic tool in the diagnosis of fetal anemia and in the assessment of cardiac overload during IUT. Our hypothesis for this study was that fetal anemia increases and that intra-uterine transusion decreases the fetal cardiac contractility. To test this hypothesis, we measured the left and right ventricular shortening fractionsin a group of alloimmune anemic fetuses before intra-uterine transfuion, immediately after (within 30 minutes) and a day after IUT.

Methods

Setting and Patients

Leiden University Medical Center 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.[20] Between March 2001 and May 2002, M-mode ultrasound of the fetal heart was performed before, immediately after, and one day after all IUTs. The institutional review board gave approval for this prospective study, and all women gave oral informed consent. Severe fetal anemia was defined as hemoglobin-deficit ≥ 5 SD, moderate fetal anemia as 2 SD ≤ hemoglobin-deficit < 5 SD, and absence of anemia as hemoglobin-deficit < 2 SD of the normal mean.[21] Hydrops was classified as mild when a distinct rim of ascites was present with or without pericardial effusion.[22] Hydrops was classified as severe when ascites was abundant (free-floating intra-abdominal organs) with or without pericardial effusion, skin edema, or pleural effusion.[22]

Procedures

Fetal blood sampling was performed, and packed red cells with a hematocrit of 76-84% were given intravenously. The volume given at IUT depended on gestational age and on the severity of anemia. Hemoglobin was measured in the initial fetal blood sample of the transfusion. The hemoglobin concentration target after IUT was 14 g/dl. The transfused volume was expressed as a percentage of the estimated fetoplacental blood volume (FBV): (transfused volume at IUT / estimated FBV) *100. To calculate estimated FBV, we used estimated fetal weight determined by ultrasound, together with the assumption of a FBV of 100 ml/kg.[23] Before the procedure, meperidine (75 mg), promethazine (25 mg) and indomethacin (50 mg) were given to the mother. Atracurium (0.4 mg/kg) was given to the fetus immediately after the initial blood sample was taken.

Measurements

M-mode measurements were performed before IUT (0-6 hours), immediately after (within ½ hour), and one day after (12-24 hours). In the

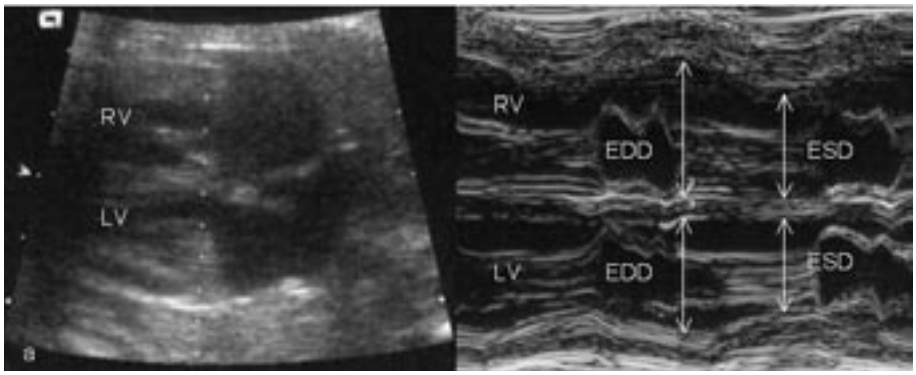

Figure 1 - Fetal heart (a) and the orientation of the M-mode cursor, placed perpendicular to the interventricular septum, just below the tips of the atrioventricular valves.
M-Mode measurement (b) of the systolic and diastolic transverse ventricular diameters.
LV = left ventricle, RV = right ventricle, EDD = end-diastolic dimension, ESD = end-systolic dimension

four-chamber view of the fetal heart, the cursor was placed perpendicular to the interventricular septum, just below the tips of the atrioventricular valves. The end-diastolic dimension (EDD) and the end-systolic dimension (ESD) of the left and the right ventricles were then obtained (Figure 1). M-mode measurements were obtained in the absence of fetal breathing and body movements. M-mode studies were done by one of two experienced operators (ES, KT) using an Acuson Sequoia (Acuson, Mountain View, CA) ultrasound machine with a 6.0 MHz probe. The left ventricular shortening fraction (LVSF) and the right ventricular shortening fraction (RVSF) were calculated using the following formula: shortening fraction = (EDD – ESD) / EDD. The percentage decrease between shortening fraction before and that after transfusion was calculated as (1 – (shortening fraction $_{\text{after IUT}}$ / shortening fraction $_{\text{before IUT}}$)) * 100. The percentage decrease in shortening fraction was correlated with the transfused volume as a percentage of estimated FBV.

Normal values

LVSF and RVSF were measured in 13 uncomplicated pregnancies with normal outcome. Each patient was measured five times with an interval of 4 weeks, between 18 - 36 weeks gestation. Inter-observer limits of agreement (95 % confidence interval of differences) for LVSF and RVSF were -0.014 to +0.024 and -0.023 to +0.023 respectively.[24]

Statistics

To compare the changes in shortening fraction between the three time points in patients with the first IUT and in patients with the following IUT, a linear mixed model with random effect was used.[25] This model was also used to compare means between controls and patients, to correct for the fact that several measurements per person were performed. Pearson R^2 values were calculated between percentage decrease in shortening fraction at first IUT and transfused volume as a percentage of estimated FBV. Statistical analysis was performed using SPSS 10.0 (SPSS, Chicago, IL) and SAS proc mixed (SAS, Cary, NC). A value of $p < 0.05$ was considered significant.

Results

During the study period, 85 IUTs were performed in 30 pregnancies. Thirty-six procedures were excluded from analysis, because of incomplete measurements due to fetal breathing, body movements or fetal position in utero (n=23), lack of time to perform the measurements (n=8), maternal obesity (n=4), or because intravascular access was not obtained and intraperitoneal transfusion was performed (n=1). Complete measurements were obtained from 49 IUTs in 23 fetuses in 23 women. Study population characteristics are shown in Table 1. There were no major complications following IUT in our study population. Minor complications occurred in 4 IUTs: fetal bradycardia for less than 1 minute (n=2), bleeding from the puncture site for less than 3 minutes (n=2).

Figure 2 shows mean (± 2 SEM) LVSF and RVSF in normal (control) fetuses (these measurements were longitudinally obtained) as well as in anemic fetuses before the first IUT, immediately after the first IUT, and one day after the first IUT. Ventricular shortening fraction was higher in anemic (first IUT) than in normal fetuses but his difference was not statistically significant (LVSF p=0.280, RVSF p=0.075). Mean (± 2 SEM) LVSF was 0.27 (0.24-0.29) in normal (control) fetuses. For the 13 fetuses that received their first transfusion, mean LVSF was 0.30 (0.24-0.35) before IUT, 0.19 (0.16-0.23) immediately after IUT, and 0.30 (0.26-0.34) one day after IUT

Table 1 - Characteristics of the 49 IUTs.

Maternal age (completed years), median (range)	32 (19-43)
Gestational age (completed weeks), median (range)	31 (19-35)
Type of alloimmunization	
D	41
Kell	3
c	5
Hydrops	
none	43
mild	3 (3 D alloimmunizations)
severe	3 (1 D and 2 Kell alloimmunizations)
Order of IUTs	
first	13
second	15
third	11
fourth	5
fifth	4
sixth	1
Infused volume	
infused volume (ml), median (range)	71 (14-114)
infused volume/estimated FBV (%), median (range)	44 (14-85)
Hemoglobin	
hemoglobin before IUT (g/dl), median (range)	7.9 (2.7 – 13.7)
hemoglobin after IUT (g/dl), median (range), (n = 47)	14.3 (12.7 – 16.1)
Degree of anemia	
none (Hb > -2 SD)	1
moderate (-2 SD ≥ Hb > -5 SD)	11
severe (Hb ≤ - 5 SD)	37

IUT = intra-uterine transfusion, FBV = fetoplacental blood volume, Hb = Hemoglobin, SD = standard deviation

(Figure 2). Mean (± 2 SEM) RVSF was 0.21 (0.18-0.25) in normal (control) fetuses. For the 13 fetuses that received their first transfusion, mean RVSF was 0.27 (0.23-0.32) before IUT, 0.16 (0.11-0.22) immediately after IUT, and 0.23 (0.18-0.27) one day after IUT (Figure 2). In previously transfused fetuses, similar changes were observed: LVSF was 0.28 (0.26-0.31) before IUT, 0.22 (0.18-0.25) immediately after IUT, and 0.33 (0.30-0.35) one day after IUT. RVSF was 0.31 (0.28-0.35) before IUT, 0.18 (0.14-0.23) immediately after IUT, and 0.23 (0.20-0.26) one day after IUT. Mean fetal heart rate (± 2 SEM) was 138 (136-140) in normal fetuses, 139 (133-144)

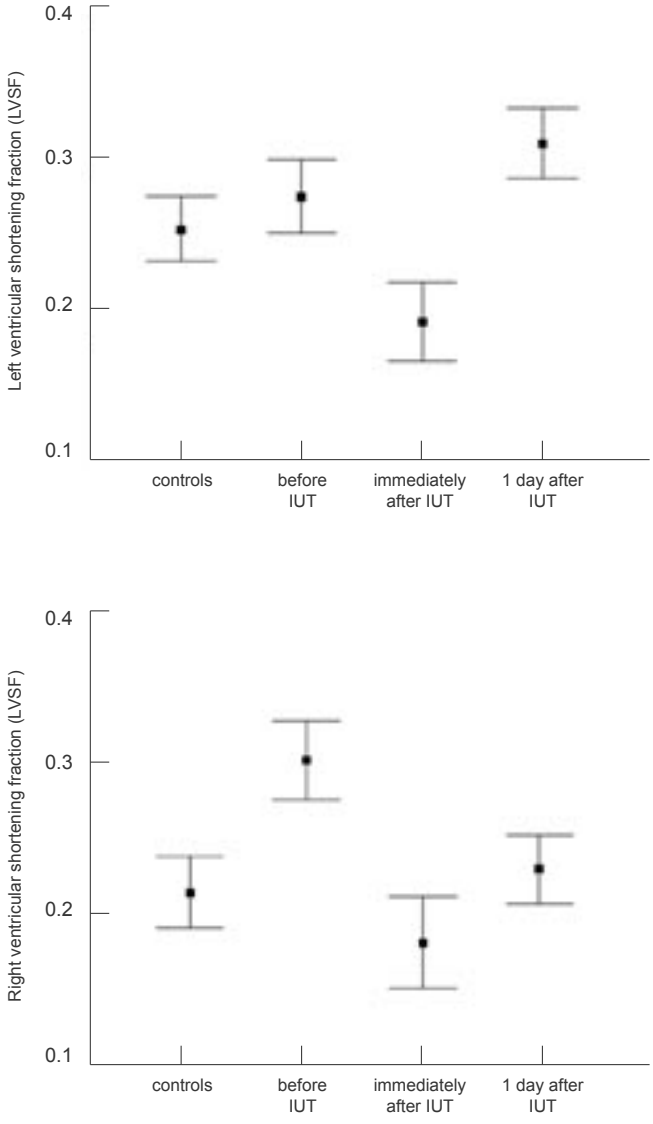

Figure 2 - Shortening fraction (mean ± 2 SEM) of the left (LVSF) and the right (RVSF) ventricle in 13 normal controls (taken in account that the measurements are longitudinally obtained with according change in SEM), and in 13 anemic fetuses before, immediately after, and one day after the first IUT. Ventricular shortening fraction was higher in anemic than in normal fetuses but this difference was not statistically significant. In anemic fetuses, LVSF and RVSF differed significantly between the three time points ($p<0.001$).

in anemic fetuses before the first IUT, 133 (126-139) immediately after the first IUT, and 141 (138-145) one day after the first IUT. In anemic fetuses LVSF and RVSF, as well as fetal heart rate, differed significantly between the three time points. This applied to fetuses receiving the first transfusion as well as for previously transfused fetuses ($p<0.004$).

Figure 3 shows the relation between ventricular shortening fraction before and immediately after IUT. The 45-degree line divides the fetuses with a decrease in shortening fraction from the fetuses with an increase. LVSF decreased in 43/49 (88 %) of IUTs, RVSF decreased in 42/49 (86 %) of IUTs. Hydropic fetuses showed a similar pattern as non-hydropic fetuses, although there was a tendency towards higher shortening fractions in hydropic fetuses.

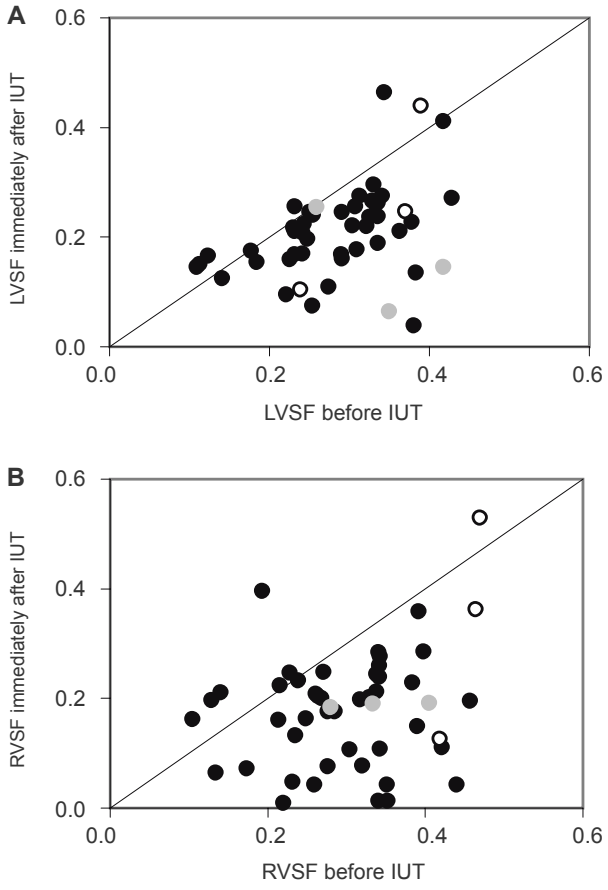

Figure 3 - Relation between ventricular shortening fractions before and immediately after IUT (49 IUTs in 23 fetuses). Each dot represents the two ventricular shortening fractions concerning one IUT, the value before IUT is on the x-axis and the post-transfusion value is on the y-axis. (A) Diagram for the left ventricular shortening fraction (LVSF) and (B) for right ventricular shortening fraction (RVSF).
The 45º line divides the fetuses with a decrease in shortening fraction (right of the line) from the fetuses with an increase in shortening fraction (left of the line). Black circles represent non-hydropic fetuses, grey circles represent mildly hydropic fetuses, and open circles represent severely hydropic fetuses.

Figure 4 shows the relation between percentage decrease in shortening fraction during transfusion and the transfused volume as a percentage of estimated FBV in 49 IUTs. We found only weak correlations for this relation at the first IUT (left: R^2 = 0.15; p= 0.20 / right: R^2 =0.005; P = 0.1.

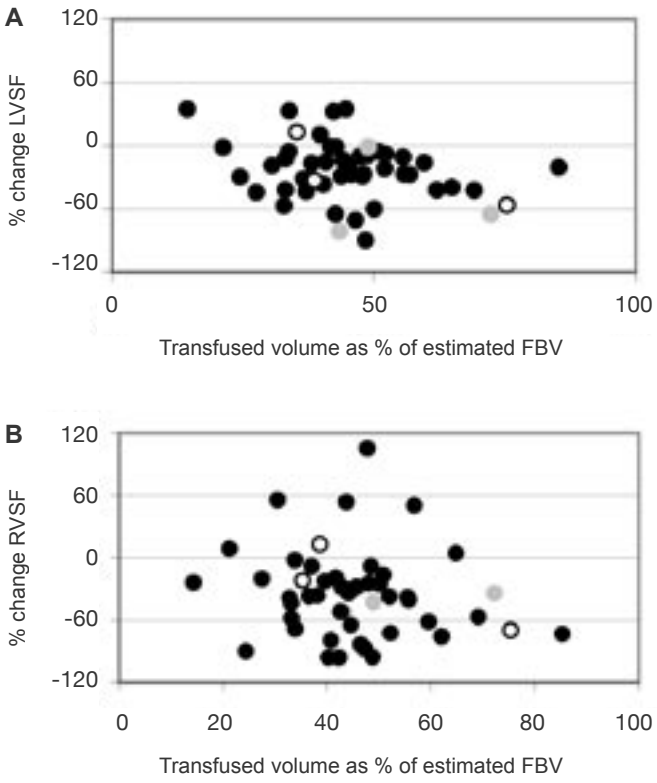

Figure 4 - Relation between change in shortening fraction and transfused volume in 49 IUTs. The change in shortening fractions is expressed as a percentage of the pre-transfusion value (minus value for decrease and plus value for increase). The percentage decrease in shortening fraction during transfusion was calculated as (1 – (shortening fraction after IUT / shortening fraction before IUT)) * 100. The transfused volume is expressed as a percentage of estimated fetoplacental blood volume (FBV). Transfused volume was calculated as: (transfused volume at IUT / estimated FBV) *100. Black circles represent non-hydropic fetuses, grey circles represent mildly hydropic fetuses, and open circles represent severely hydropic fetuses.
(A) Diagram for effect on left ventricular shortening fraction (LVSF) and (B) for right ventricular shortening fraction (RVSF).

Discussion

We measured LVSF and RVSF in normal fetuses and in anemic fetuses before and after IUT. We found that both LVSF and RVSF were higher in anemic than in normal fetuses, though this was not statistically significant. Fetal heart rate significantly decreased immediately after IUT. Furthermore, we found a substantial and statistically significant decrease in both LVSF and RVSF immediately after IUT. One would expect shortening fraction to decrease more after the administration of relatively large volumes of packed red cells. However, we found only a very weak correlation between the decrease in shortening fraction and the transfused volume (as a percentage of estimated FBV).

A review of normal LVSF and RVSF values as measured by different researchers is presented in Table 2.[2-4; 26; 27] Some researchers found a small decrease in ventricular shortening fraction with advancing gestational age; most researchers, however, found no significant effect of gestational age. As shown in Table 2, mean LVSF and RVSF are, in different research papers, close to 33%, ranging between 0.26 to 0.48 for the left ventricle and 0.21 to 0.42 for the right ventricle. We do not have an explanation

Table 2 - Literature review of reference values for ventricular shortening fraction measured by M-mode in normal fetuses.

First author, year	*Range of gestational age (weeks)*	*Number of fetuses*	*Number of measurements*	*Mean LVSF (SD)*	*Mean RVSF (SD)*
Wladimiroff et al,[27] (1981)	27-33	27	27	0.29 (0.07)	0.29 (0.06)
	34-40	26	26	0.26 (0.06)	0.26 (0.06)
De Vore et al,[3] (1984)	18-41	82	82	0.33 (0.04)	0.32 (0.04)
Koyanagi et al,[26] (1990)	18-41	104	104	0.26 (0.02)	0.29 (0.02)
Agata et al,[2] (1991)	37-40	34	34	0.34 (0.06)	---
Hsieh et al,[4] (2000)	10-40	42	241	0.48 (0.13)	0.42 (0.11)
Present study	18-36	13	65	0.27 (0.08)	0.21 (0.10)
Weighted mean of above mentioned studies	---	328	---	0.32 (0.06)	0.31 (0.06)

LVSF = left ventricular shortening fraction, RVSF = right ventricular shortening fraction, SD = standard deviation

for the differing range of normal values in previous studies, other than the difference between imaging techniques and the position within the ventricle from where the measurements are taken.[3] In our study mean LVSF was 0.27 and RVSF 0.21. These normal values are rather low in comparison to most of those in the studies listed in Table 2. Therefore, we suggest that sonographers should create their own reference ranges for ventricular shortening fraction.

We think that the strength of our study lies in the fact that we measured the shortening fraction on both sides of the heart in the same fetus before and after changing its FBV. This change was substantial in most cases. Further, we measured a relatively large number of fetuses with severe anemia. There are also some weaknesses in our study. First, the repeatability of M-mode measurements in the fetus has been described as poor.[28] However, in 1990, Veille et al. assessed the error of the cursor placement and found that there was no statistical difference between measurements of the right and the left ventricle at two different levels (at the tips of the atrioventricular valves and at the insertions of the valves) either during diastole or during systole.[29] Therefore the placement of the M-mode cursor at the atrioventricular valves in ventricular shortening fraction measurements is less critical than would be expected. Further, inter-observer variability in M-mode ventricular shortening fraction was measured by two groups of researchers.[27;29] They found a measuring error of 5%. Inter-observer limits of agreement in our study were –0.014 to +0.024 for LVSF and –0.023 to +0.023 for RVSF. Second, our standard medication before IUT included indomethacin. In some fetuses indomethacin causes transient constriction of the ductus arteriosus, even after short-term use.[30] This may have influenced LVSF and RVSF after IUT. Indeed, in 1997, Harada et al. showed that administration of indomethacin decreased the right ventricular area shortening fraction, but not the left ventricular area shortening fraction.[31]

Other authors have reported on cardiac function before and after intrauterine transfusion. Their findings are summarized in Table 3. In short, cardiac output decreased immediately after IUT whereas measures of afterload increased immediately after IUT.[8-11; 14; 16; 17; 19] Twelve hours

Table 3 - Summary of results of previous studies on cardiac function before and after intrauterine transfusion.

Authors, year	*No. of fetuses*	*No. of IUTs*	*Measure of cardiac function*	*Immediate effect of IUT*	*Effect of IUT after > 12 hours*
Copel et al,[13] 1988	24	64	Umbilical artery, pulsatility index Descending aorta, pulsatility index		no change in the pulsatility index for any of the vessels
Copel et al,[18] 1989	11	11	Left ventricular output Right ventricular output		no significant changes no significant changes
Weiner et al,[11] 1989	8	20	Umbilical venous pressure	increase with 4.2 mmHg	
Mari et al,[15] 1990	16	16	Middle cerebral artery, pulsatile index Internal carotid artery, pulsatility index Anterior cerebral artery, pulsatility index Thoracic aorta, pulsatility index Abdominal aorta, pulsatility index Renal artery, pulsatility index Femoral artery, pulsatility index Umbilical artery, pulsatility index Heartrate		no significant difference in the pulsatility index for any of the vessels no change in heartrate
Mari et al,[16] 1990	13	13	Middle cerebral artery, pulsatilel index Internal carotid artery, Anterior cerebral artery, pulsatility index Umbilical artery, pulsatility index Heartrate	decrease with 0.70 decrease with 0.57 decrease with 0.69 decrease with 0.29 significant increase in heartrate	decrease with 0.01 decrease with 0.10 decrease with 0.08 decrease with 0.05 no significant changes
Moise et al,[10] 1990	21	38	Umbilical venous pressure Left ventricular output Right ventricular output Heartrate	increase with 1.7 mm Hg decrease with 19% decrease with 22% no change in heartrate	
Rizzo et al,[19] 1990	12	12	Left ventricular output Right ventricular output Heartrate	decrease with 63.84 ml/min/kg decrease with 52.35 ml/min/kg no significant changes	
Oepkes et al,[17] 1993	21	21	Ductus venosus peak velocity no significant changes	increase with 0.26 m/s decrease with 0.09 m/s	decrease with 0.09 m/s
d' Ancona et al,[14] 1997	14	14	Portal vein	Increase with 0.7 of the H/L ratio	
Goodrum et al,[8] 1997	21	27	Mean umbilical arterial pressure Heartrate	increase with 4.6 mm Hg bradycardia in 5/16 IUTs	
Kilby et al,[9] 1998	6 fetal lambs	6	Left ventricular afterload End-diastolic pressure End-diastolic vollume Left ventricular output Heartrate	increase with 7.2 mm Hg/ml increase with 6.5 mm Hg increase with 0.9 ml/kg decrease with 28 ml/kg/min no significant changes	
This study	23	49	Left ventricular shortening fraction Right ventricular shortening fraction Heartrate	decrease from 0.30 to 0.19 decrease from 0.27 to 0.16 significant decrease	no significant decrease no significant decrease no significant decrease

H/L ratio, ratio between high (peak) and low (nadir) velocities in the portal vein

after IUT, however, these changes were no longer demonstrable.[13; 15;18] Our findings, that VSF decreased immediately after IUT and normalized within 12 hours are, thus, in accordance with the findings of these previous studies. Further, we have related the change in VSF with the relative increase in blood volume during IUT. However, we found only a weak correlation.

In 1999 Ulm et al. described two pregnancies in the same woman where they performed 11 and 13 transfusions respectively.[32] In their case report, they suggest that continuation of intravascular therapy until term may represent a reasonable alternative to selective premature delivery even in cases with highly aggressive maternal rhesus alloimmunization. Our study suggests that a smaller number of IUTs but with a larger volume do not endanger the condition of the fetal heart. Massive IUT (a mean of 45% of FPV in 15 minutes) was well tolerated in our study. Although we perform IUT from 16 weeks' gestation onwards and aim at term delivery, the maximum number of IUT per pregnancy in our clinic has been 7 with low overall mortality and morbidity.[22]

In anemic fetuses middle cerebral artery peak velocity is increased.[33] After IUT this peak velocity of the middle cerebral artery decreases.[34] It has been suggested that fetal hematocrit as well as left ventricular contractility are the main determining factors for these changes in middle cerebral artery peak velocity.[35; 36] Our finding of a decreased LVSF after IUT supports the suggestion that decreased cardiac contractility contributes to the decrease in middle cerebral artery peak velocity after IUT.

In conclusion, fetal anemia had only a minor effect on M-mode measured shortening fraction of the left and right ventricles of the heart. Intrauterine transfusion, on the other hand, had a clear effect, with both LVSF and RVSF decreasing significantly. This corresponds well with the visual impression of decreased contractility on B-mode ultrasound during and immediately after IUT. This effect on contractility showed, however, little correlation with the transfused volume given at IUT.

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General Discussion

8

The studies *(chapters 2 to 7)* in this thesis concern diagnostic methods that are used to differentiate between moderate and severe anemia in red cell alloimmunization. In this thesis, severe fetal anemia is defined as ≥ 5 SD below the normal mean for gestation, because then anemia will become life-threatening and, thus, in need of therapy. Moderate fetal anemia is defined as > 2 SD but < 5 SD below the normal mean for gestation. Only a small percentage of pregnancies with red-cell antagonism is at risk for severe anemia. In the Netherlands, there are approximately 200,000 pregnancies >16 weeks yearly, around 4000 of these mothers have irregular red-cell antibodies, but only around 100 pregnancies are at risk for severe anemia. It is of great importance that the diagnosis of severe fetal anemia is made not too late because of the risk of fetal demise but also not too early, because of the risk and disadvantages of unnecessary cordocenteses and intrauterine transfusions.

In the Netherlands, pregnant women have a screening test for antibodies against red blood cells in the first trimester. In pregnant women with irregular antibodies, there will be a risk selection on the basis of obstetric history and antibody titers.[1] Pregnancies at risk for moderate to severe fetal anemia will be referred to the national center for intrauterine treatment, the Leiden University Medical Center (LUMC). At each visit, distinction between moderate (leading to continued close monitoring) and severe (leading to immediately intrauterine treatment) fetal anemia has to be made. This distinction will be preferably made by non-invasive methods: maternal perception of fetal movements and different ultrasonographic measurements (B-mode, M-mode and Doppler). Invasive diagnostics methods (amniocentesis, cordocentesis) will only be used when non-invasive methods indicate severe anemia.

The research questions in this thesis were:

1. What is the basis of the relation between decreased concentration of red cells in fetal blood and increased bilirubin concentration in amniotic fluid?
2. What is the basis of the relation between decreased concentration of red cells in fetal blood and increased peak systolic velocity in the middle cerebral artery?

3. Which one of these two measurements (bilirubin concentration in amniotic fluid or middle cerebral artery peak systolic velocity) provides the better differentiation between moderate and severe anemia?
4. Are certain ultrasonographic measurements of the fetal heart useful in the diagnosis of severe fetal anemia?

As a result of the studies in this thesis, the following answers can now be formulated and discussed:

1. Increased destruction of red blood cells caused by alloimmunization leads to an increased concentration of unconjugated bilirubin in fetal blood. This unconjugated bilirubin is liposoluble and crosses the placenta through the *trans*membranous pathway along the concentration gradient between fetal and maternal blood. In normal pregnancies, mean maternal serum bilirubin concentration is 0.35 mg/dl[2], and mean fetal serum bilirubin concentration is 1.5 mg/dl.[3] In case of red cell destruction in alloimmunization, fetal serum bilirubin concentration is in the range of 2 to 12 mg/dl.[4] In addition to this clearance of unconjugated bilirubin from fetal blood into maternal blood, there must also be a pathway through which, during periods with high concentration of bilirubin in fetal blood, small amounts of bilirubin are "leaking" into the amniotic fluid.

By measuring the amniotic fluid/fetal blood ratio of bilirubin at different gestational ages and comparing these with the amniotic fluid/fetal blood ratio of albumin as found in the literature, we found, in a speculative study, indirect evidence *(chapter 4)* for the following hypothesis. Unconjugated bilirubin is almost completely bound to albumin in fetal blood as well as in amniotic fluid. Through a small fraction of unbound unconjugated bilirubin that crosses liposoluble membranes, an equilibrium is established whereby bilirubin will evenly spread over the available albumin. On the basis of our measurements, we provide indirect evidence for this assumption. Following our hypothesis, the bilirubin concentration in amniotic fluid is, thus, defined by the bilirubin concentration in fetal blood (which is increased in case of fetal anemia) and by the amniotic fluid /fetal blood ratio of albumin (which is decreasing with increasing gestational age). In a more recent (yet unpublished) study we have measured bilirubin and albumin

concentrations simultaneously in fetal blood and in amniotic fluid. The findings in this unpublished study are consistent with our hypothesis.

What does this hypothesis mean for the physiology of fetal bilirubin? Which pathway does bilirubin take to enter the amniotic fluid? Among five possible pathways bilirubin could take to build up a concentration in amniotic fluid (fetal kidneys, lungs, skin, bowel, membranes) the consequence of our findings is that the membranes seem the only remaining pathway. Our argumentation is the following. Both in fetal blood and amniotic fluid the bilirubin is, to a large, extent unconjugated. Unconjugated bilirubin is for around 99% tightly bound to albumin. It is highly improbable that urine or alveolar fluid contributes substantially to the bilirubin concentration in amniotic fluid. While protein concentrations in these bodily fluids are 100 to 200 times lower than those in fetal plasma, whereas protein concentration in amniotic fluid is only 10 to 20 times lower than in fetal plasma. Because of the very low albumin concentrations in urine and alveolar fluid, these fluids must act as a barrier for unconjugated bilirubin leaving the plasma and entering the amniotic fluid compartment. A meconial origin of amniotic fluid bilirubin is inconsistent with a clinically relevant correlation between amniotic fluid and fetal blood bilirubin concentration. The fetal skin probably does serve as a major pathway for solute and water exchange, including unconjugated bilirubin, between fetus and amniotic fluid, but only until 16 weeks of gestation. However, in fetuses of 25 weeks, the fetal skin is already completely keratinized. The fetal membranes, on the other hand, retain a high permeability until term. Therefore, bilirubin exchange between fetal blood and amniotic fluid most probably occur through this pathway, called *intra*-membranous pathway.

An intriguing question, raised after the previous discussion on how bilirubin enters the amniotic fluid, is: how does albumin enter the amniotic fluid. And is this albumin of maternal or of fetal origin? This question is the subject of a new study protocol. Characterization of albumin is, however, difficult. On the basis of studies performed in the seventies, there are reasons to believe that the albumin in amniotic fluid is of maternal origin. It is supposed that maternal albumin passes from maternal blood

to amniotic fluid through the transmembraneous pathway. Among others, this would mean that a fetus, drinking large amounts of amniotic fluid every day, digests maternal proteins already before birth. As a consequence, lactation starts, so to speak, already in utero.

The more we learn about fetal physiology, the more we discover that the fetus is already very well prepared for extra uterine life. The changes that occur at birth are somewhat less radical than previously believed. In the beginning of the 19th century, Kergaradec[5] demonstrated the fetal heartbeat. In the first half of the 20th century, Ahlfeld discovered that the fetus was breathing in utero[6]. With these breathing movements, probably triggered by pCO_2 increase in fetal brain stem cells, the fetus is aspirating oxygen-containing blood from the placenta through the umbilical cord vein, ductus venosus and left ventricle of the heart. The extra amount of oxygen-rich blood thus reaches the fetal brain within seconds after the start of "breathing". In the second half of the 20th century, it was proved that the fetal blood pH is identical to that in adults.[7] In the beginning of the 21st century, it may now be shown that a fetus receives motherly albumin as nutritional supplement. During the 3rd trimester, drinking of amniotic fluid contributes for approximately 10% of the daily necessary calorie and amino-acid intake, as has been shown in fetuses with obstructions of the gastrointestinal tract.[8;9]

2. Theoretically, the systolic blood flow velocity in the middle cerebral artery may be increased because of a
a. decrease in hematocrit, resulting in a lowered viscosity of fetal blood.
b. increase of fetal heart contractility as an adaptation to anemia
c. decrease of peripheral vascular resistance in the brain vessels because of a decrease of intracellular pO_2.

Our studies *(chapter 5 and 7)* provide arguments for the fact that, in anemia, a decrease of blood viscosity is not the only factor leading to an increase of middle cerebral artery peak systolic velocity. Probably, the contractility of the heart is a second important factor. However, our studies show some weaknesses. First, there is the fact that our Doppler measurements were performed during an intrauterine transfusion

whereby the mother receives medication. Amongst others, she receives the prostaglandin synthesis inhibitor indomethacine that affects the fetal circulation because it leads to a constriction of the ductus arteriosus. These changes in the fetal circulation may have influenced our measurements. Furthermore, during intrauterine treatment, the blood stream in the umbilical vein is affected by the therapeutic administration of blood in this vein. For the purpose of intrauterine transfusion, umbilical vein puncture was performed at the placental umbilical cord insertion or in the pars intrahepatica. A small hematoma in the Warthons gelly, or a contraction of the capsule of Glisson in the liver, will influence local and systemic hemodynamics. This may, again, have influenced our Doppler measurements.

3. Knowledge about the physiological changes in relation to hemolytic anemia can help in the understanding of diagnostic methods. Many clinicians, however, are only interested in knowing which method, amniocentesis with measurement of bilirubin concentration in amniotic fluid, or Doppler with measurement of the middle cerebral artery systolic peak velocity is better in predicting fetal anemia. The studies in *chapters 2 and 3* are addressing this question. In *chapter 2* we have performed a systematic review of publications on sensitivity and specificity of Δ OD 450 and peak systolic velocity of MCA. On the one hand, this study provides us with a reasonable impression of the quality of both tests. But on the other hand, this study cannot be conclusive. First, there is only one (small) prospective study in this review. Second, different definitions of anemia were used in different studies, which makes comparison and metanalysis impossible. Third, almost all the studies on Δ OD 450 were performed in a different time period than the MCA studies. Anyhow, it is evident that the range of sensitivities and specificities of the Δ OD 450 and the middle cerebral artery systolic peak velocity are overlapping. In *chapter 3*, we have measured the accuracy of amniotic fluid Δ OD 450 values in the prediction of severe fetal anemia in D-alloimmunization in a prospective, non-controlled study. We found that in Liley's extrapolated curve, zone 3 plus the upper third of zone 2 had a sensitivity of 97% and an overall accuracy of 86% for severe non-hydropic fetal anemia. This excellent clinical accuracy is, however, rather surprising. First, there is the fact that

Δ OD 450 has obviously no relation with compensatory hematopoiesis. Second, the clinical performance of the Δ OD 450 test is in contrast with the poor correlation between Δ OD 450 and fetal hemoglobin concentration. Our conclusion is that Liley's chart is a rather rough method. It gives clinically useful information regarding the necessity of IUT, but is a poor predictor of the actual hemoglobin concentration.

Between 2000 and 2004 we have been involved in an international multicenter study. In this so called Diamond study, invasive amniocenteses and non-invasive Doppler, two tests for the prediction of fetal anemia were performed simultaneously in the same patients and compared with the gold standard, fetal hemoglobin concentration. The main results of the Diamond study have been presented as an abstract,[10] and the paper has meanwhile been submitted for publication. In the Diamond study, sensitivity of middle cerebral artery systolic peak velocity is around 85% and that of Δ OD 450 measurement is around 75%. This difference in sensitivity was significant. Thus, MCA is certainly not inferior to Δ OD 450. Moreover, its non-invasiveness is an undisputable advantage. Therefore, middle cerebral artery systolic peak velocity measurements probably will become the diagnostic method of choice and Δ OD 450 a second line method, used only in cases where the non-invasive methods are not conclusive.

4. In this thesis, B mode and M-mode *(chapter 6 and 7)* ultrasound measurements of the fetal heart have been shown to be disappointing in predicting fetal anemia. These measurements seem to add little or nothing to the diagnosis of fetal anemia. There is, however, a measurable effect of IUT on cardiac contractility. In *chapter 7*, we describe that cardiac contractility is significantly decreased after IUT. This corroborates the visual impression on ultrasound.
In *chapter 6*, cardiac ventricular wall thickness and cardio-thoracic ratio in relation to fetal anemia are described. Most measurements in fetuses of alloimmunized pregnancies where within normal ranges. Their diagnostic accuracy is therefore too low to recommend them as a tool in predicting severe anemia. A remaining ultrasound item in cases of severe early fetal anemia, is the subjective impression of increased density of the cardiac walls.

We think a few topics deserve attention in future research: the echogenic density of the cardiac walls but also of the fetal bowel and skin (pre-hydropic changes) could be such a topic. In addition, intra uterine transfusion provides an ideal opportunity to study the changes in blood volume and blood constituents in fetal anemia and during intrauterine treatment. Very few centers in the world have sufficient numbers of patients as the LUMC does, to study these changes. Therefore, the LUMC has an obligation to continue to perform studies in this field.

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Summary

Introduction *(chapter 1)*

Before or during pregnancy, the mother, who types negative for a specific red cell antigen, might develop antibodies against the offending antigen. This so-called alloimmunization can occur after a blood transfusion, after organ transplantation or after a fetomaternal transfusion. The maternal antibodies can pass through the placenta into the fetal circulation. In case the fetus is positive for the antigen, the red cells will be destructed and anemia may develop. In serious cases this can lead to massive hydrops and ultimately to intrauterine death. In the Netherlands, there are annually approximately 200 pregnancies in which potentially dangerous maternal antibodies against fetal erythrocytes are diagnosed (mainly anti-D, anti-c and anti-K antibodies). Pregnant women with a high concentration of such antibodies are at high risk for fetal complications. In approximately 30% of these high-risk pregnancies intrauterine transfusion is warranted. Severe fetal anemia can already occur at 16 weeks of gestation.

Diagnostic procedures are focussed on timely detecting severe fetal anemia, before hydrops or death occur. Early recognition and treatment of fetal anemia before hydrops fetalis develops, leads to a better outcome. However, treatment of a fetus in utero that has only moderate anemia carries several risks, such as procedure-related fetal loss rates, boostering of existing antibodies and the development of new antibodies. In differentiating between moderate and severe fetal anemia, several techniques are used. A first diagnostic tool is the history of the woman. The obstetrical history is a part of this anamnesis. Important is also the perception of fetal movements by the mother. A reduction of the fetal movements felt by the mother is an alarming signal and raises the suspicion of severe fetal anemia. The second diagnostic technique is to follow the concentration of maternal antibodies. When these are rising, the fetus may be in danger for anemia. The third diagnostic technique is measurement of bilirubin concentration in amniotic fluid. This method was described by Liley in 1961 and is based on the increase of optical density at a wavelength of 450 nanometres, the Δ OD 450. A fourth diagnostic technique is ultrasonography. On a B-mode image, discrete sign of hydrops can be seen. Fetal organs involved in the production

and destruction of red cells, such as liver and spleen, can be measured. These organs are often enlarged in severe anemia. Placental thickness can also be measured. With M-mode ultrasound, some measurements can be performed more accurately. With Doppler, the blood velocity in the fetal arteries and veins can be measured. A fifth diagnostic technique is cardiotocography. The fetal heart frequency is measured for 30 to 45 minutes. This technique is, however, inappropriate for diagnosing fetal anemia, because, even in very severe anemia, there is most often a normal pattern of the fetal heart frequency. A final diagnostic technique is fetal cordocentesis. At cordocentesis the exact hemoglobin concentration can be measured. However, the risk of severe complications for the fetus at cordocentesis is approximately 2 or 3 %.

In this thesis, the link between severe fetal anemia and on the one hand, the bilirubin concentration and on the other hand, some more recently used ultrasonographic measurements are studied. The treatment of fetal anemia is symptomatic and is aimed at keeping the fetus in a good condition by intrauterine transfusion until term thus avoiding life-threatening fetal anemia and iatrogenic prematurity at the same time.

The aim of the different studies in this thesis was to analyse the physiology of fetal anemia and the evaluation of diagnostic techniques to predict the optimal timing for fetal blood transfusion.
This thesis contains two parts: the chemical approach and the ultrasonographic approach.

Part 1: Chemical approach

The chemical techniques are based on the fact that destruction of fetal hemoglobin will increase the bilirubin concentration of amniotic fluid. Amniotic fluid is obtained by amniocenteses. The degree of yellowness of the amniotic fluid will be measured as described by William Liley in 1961. This yellowness is caused exclusively by bilirubin.

First, a systematic literature review was performed *(chapter 2)* on the diagnostic accuracy of bilirubin measurement in amniotic fluid and of middle cerebral artery peak systolic velocity in the prediction of severe fetal alloimmune anemia. The advantage of Doppler measurements is their non-invasiveness. The conclusion of this review, based on mainly retrospective studies, was that the sensitivity for both techniques is variable but quite similar. In case of equal sensitivity, it seems clear that the non-invasive technique is preferable. A new large multicenter study in which both techniques are prospectively tested in the same patients has meanwhile been performed.

In *chapter 3* we studied how accurately the bilirubin concentration in amniotic fluid predicts severe fetal anemia in the second and third trimester in D-alloimmunized pregnancies. Seventy-nine non-hydropic singleton pregnancies were included where amniocentesis was performed within 4 days of first fetal blood sampling. Amniotic fluid Δ OD 450 values were plotted on a Liley's chart. In 1961 William Liley described a chart with on the x-as gestational age and on the y-as the Δ OD 450. In this chart, 3 zones were defined: zone 1 (non-anemic), zone 2 (moderate anemia) and zone 3 (severe anemia). The original Liley chart is from 27 to 36 weeks, the extrapolated Liley curve extends from 18 to 36 weeks. Accuracy, sensitivity and specificity were calculated for two commonly used cut-off levels on the Liley chart. Sensitivity of Δ OD 450 values in Liley's zone 3 or the upper third of Liley's zone 2 was 95% before and 98% after 27 weeks. We concluded that Liley's extrapolated curve predicts severe fetal anemia with high sensitivity and reasonable specificity.

In *chapter 4* we investigated the pathways of bilirubin from fetal blood to amniotic fluid. Therefore, we studied the relation between bilirubin concentration in blood and in amniotic fluid in 68 non-hydropic rhesus D-alloimmunized anemic fetuses at first intrauterine transfusion. In these alloimmunized fetuses, the amniotic fluid/fetal blood ratio for bilirubin decreased from 0.09 at 28 weeks to 0.05 at 33 weeks. In normal, non-anemic fetussen, amniotic fluid/fetal blood ratios for bilirubin, and for albumin, are in the same range and show a similar decrease during gestation. On the basis of these findings, we hypothesised that amniotic fluid bilirubin concentration is determined, firstly, by fetal blood concentration and, secondly, by the amniotic fluid/fetal blood ratio of albumin.

Among five possible pathways bilirubin could take to build up a concentration in amniotic fluid (fetal kidneys, lungs, skin, bowel, membranes), the intramembranous pathway appears to be the only remaining possibility. During fetal life, bilirubin in amniotic fluid is mainly unconjugated. Unconjugated bilirubin is bound to albumin almost completely. It is very improbable, therefore, that urine or alveolar fluid contribute substantially to the bilirubin concentration in amniotic fluid, because the albumin concentration in these body fluids is 100 to 200 times lower than in fetal plasma. The albumin concentration in amniotic fluid on the other hand, is only 10 to 20 times lower than in fetal plasma. Because of the very low albumin concentrations in urine and alveolar fluid, these fluids act as a barrier for unconjugated bilirubin leaving the plasma and entering the amniotic fluid compartment. A meconium origin of amniotic fluid bilirubin is inconsistent with a clinically relevant correlation between amniotic fluid and fetal blood bilirubin concentration. The fetal skin probably serves as a major pathway for solute and water exchange, including unconjugated bilirubin, between amniotic fluid and fetus till 16 weeks of gestation. In fetuses of more than 25 weeks, however, the fetal skin is completely keratinized. The fetal membranes, on the other hand, retain a high permeability until term. Therefore, bilirubin exchange between fetal blood and amniotic fluid most probably occurs through the intramembraneous pathway.

Part 2: Ultrasonographic approach

Since 1995 several publications have shown that middle cerebral artery blood velocities during systole of the fetal heart are increased in case of fetal anemia. It is still unclear if this increased blood flow velocity in anemia is the result of changes in blood viscosity, in contractility of the heart or in peripheral brain resistance.

In order to study the effect of blood viscosity on the systolic blood flow velocities in the middle cerebral artery, blood flow velocities were measured before and after intrauterine transfusion. After all blood viscosity increases during intrauterine transfusion. In *chapter 5* we describe the effect of a large increase of the heamatocrit on middle cerebral artery peak and umbilical vein maximum velocities in anemic fetuses. Therefore, middle cerebral artery peak flow velocities and umbilical vein maximum flow velocities were measured before, immediately after and 12-24 hours after 60 intrauterine transfusion. The middle cerebral artery peak flow velocity decreased immediately after transfusion in 59 of the 60 cases. There was a rise in umbilical vein maximum flow velocity immediately after intrauterine transfusion in 37 of the 60 cases. The conclusion was that an acute large increase of the fetal hematocrit significantly decreases middle cerebral artery peak flow velocity. The effect on umbilical vein maximum velocity was, however, unpredictable. The fact that the umbilical vein maximum flow velocity in several cases increases after intrauterine transfusion is of course in contradiction with the saying that the viscosity of fetal blood is the most important factor determining blood flow velocity. Also the wide range in arterial blood flow velocities in fetuses with the same hematocrit points to the fact that, besides hematocrit, other factors such as cardiac output and peripheral resistance must play an important role.

In *chapter 6* we studied the diagnostic accuracy of cardiac ventricular wall thickness and cardio-thoracic ratio in the prediction of severe fetal anemia. The thickness of cardiac wall of the left and the right ventricle and the inter-ventricular septum were measured in diastole using M-mode

ultrasound. The cardio-thoracic circumference ratio was measured on the B-screen. The measurements were obtained in alloimmunized fetuses. Then, two by two tables were constructed to compare the frequency of abnormal cardiac ultrasound measurements in severe and non-severe fetal alloimmune anemia. Complete measurements were obtained in 15 alloimmunized fetuses with severe anemia and in 16 alloimmunized fetuses without severe anemia. Sensitivities of cardiac ultrasound ranged between 0 and 47% and specificities between 77 and 97%. The conclusion was that diagnostic accuracy of ventricular wall thickness and cardio-thoracic ratio in the prediction of severe fetal alloimmune anemia was disappointing. More than 50% of measurements in severely anemic fetuses were within the normal reference ranges.

In *chapter 7,* we evaluated the effect of fetal anemia and intrauterine transfusion on ventricular shortening fraction. During systole, the ventricles of the heart decrease in size. The procentual decrease in size of the ventricles during systole can be measured with ultrasound and expressed as ventricular shortening fraction. The end-diastolic and end-systolic transverse dimensions of the left and the right ventricles were obtained in 23 fetuses before and after 49 intrauterine transfusions. The blood volume given at intrauterine transfusion was expressed as a percentage of estimated fetoplacental blood volume. Shortening fractions of the left and right ventricles differed significantly between three time points: before, immediately after and one day after intrauterine transfusion. Left ventricular shortening fraction decreased immediately after transfusion in 43 (88%) of the 49 procedures. Right ventricular shortening fraction decreased immediately after transfusion in 42 (86%) of the 49 procedures. At the first intrauterine transfusion, there appeared to be only a weak correlation between the decrease in shortening fraction of both ventricles and the transfused volume. The conclusion was that transfusion significantly decreases the shortening fraction of both ventricles of the fetal heart. There is, however, little correlation between the decrease in shortening fraction and the volume of red cells given at intrauterine transfusion.

We hope that the different studies in this thesis will contribute to improved understanding of the physiologic changes in fetal anemia and thus to a less invasive and timely diagnosis of severe fetal anemia. Finally, the studies described in this thesis leads to an improved insight into the hemodynamic changes, during and after treatment with intrauterine transfusion.

Samenvatting

Inleiding *(hoofdstuk 1)*

Voor of tijdens de zwangerschap kunnen bij de moeder antistoffen ontstaan tegen rhesus D en andere rode bloedcel antigenen. Deze immunisatie kan veroorzaakt worden door bloedtransfusie, door orgaantransplantatie of door foetomaternale transfusie. De antistoffen die de moeder maakt en die gericht zijn tegen de antigenen op de foetale rode bloedcellen kunnen de placenta passeren. Door antigen gemedieerde destructie van rode bloedcellen kan foetale anemie ontstaan. Deze anemie kan in ernstige gevallen leiden tot massale hydrops en uiteindelijk tot het intra-uterien overlijden van de foetus. In Nederland zijn er jaarlijks ongeveer 200 zwangeren met potentiëel gevaarlijke antistoffen. Het gaat hierbij voornamelijk om anti-D, anti-c en anti-K antistoffen. Bij de helft van deze zwangeren is de concentratie aan antistoffen zo hoog dat men spreekt van ernstige immunisatie. Bij ongeveer 30% van de vrouwen met ernstige immunisatie krijgt de foetus een dusdanig ernstige anemie dat een intra-uteriene transfusie noodzakelijk is. Deze ernstige foetale anemie kan al optreden bij een zwangerschapsduur van 16 weken.

De diagnostiek is gericht op het tijdig opsporen van ernstige foetale anemie, dus vóór er hydrops of sterfte ontstaat. Uit onderzoek is gebleken dat tijdige detectie en behandeling van foetale anemie, voor hydrops ontstaat, leidt tot betere uitkomsten voor het kind. Anderzijds is het riskant en minder zinvol om een foetus met slechts matige anemie in de baarmoeder te behandelen. Tijdig opsporen betekent dus niet alleen niet te laat, maar ook niet te vroeg. Bij de differentiatie tussen matige en ernstige foetale anemie wordt gebruik gemaakt van een aantal technieken. Voor tijdige detectie zijn in de loop van jaren diverse diagnostische technieken ontwikkeld. Een eerste diagnostische techniek is de anamnese van de vrouw. Hierbij speelt de obstetrische voorgeschiedenis een rol. Belangrijk is ook het voelen bewegen van het ongeboren kind, het “leven voelen”. Verminderen van leven voelen is een alarmsignaal en geeft hoge verdenking op ernstige foetale anemie. Een tweede diagnostische techniek is het vervolgen van de concentratie aan moederlijke antistoffen. Zodra deze stijgt loopt de foetus meer gevaar. Een derde diagnostische techniek is de meting van de bilirubine concentratie in vruchtwater. Deze methode

is in 1961 door Liley beschreven en wordt ook wel meting van de toename van de optische dichtheid bij een golflengte van 450 nanometer, de Δ OD 450 genoemd. Een vierde diagnostische techniek is echoscopisch onderzoek. Met behulp van echoscopie kunnen de eerste tekenen van hydrops gezien worden. Tevens kunnen foetale organen die met bloed aanmaak of -afbraak te maken hebben, zoals lever en milt, gemeten worden. Deze organen zijn vaak vergroot bij ernstige anemie. Ook kan de placenta dikte gemeten worden. Met de echoscopische M-mode kunnen metingen aan het hart nauwkeurig verricht worden. En met Doppler kan de bloedstroomsnelheid in de foetale arteriën en venen gemeten worden. Een vijfde diagnostische techniek is cardiotocografie. Hierbij wordt de foetale hartfrequentie over 30 tot 45 minuten geregistreerd. Deze techniek is echter van betrekkelijke waarde, aangezien bij ernstige anemie een normaal hartfrequentie patroon kan voorkomen. Een laatste diagnostiche techniek is de navelstrengpunctie. Bij een navelstrengpunctie kan de mate van anemie exact gemeten worden. Het risico op ernstige complicaties voor de foetus bedraagt bij een navelstrengpunctie echter 2 à 3%.

Het verband tussen ernstige foetale anemie en enerzijds de bilirubine concentratie in vruchtwater en anderzijds een aantal meer recent toegepaste echoscopische metingen vormen het onderwerp van dit proefschrift. De behandeling van foetale anemie is symptomatisch en is er op gericht de foetus met bloedtransfusies in goede conditie te houden tot de à terme periode. Vermeden wordt aldus dat een kind geboren wordt met naast zijn anemisch probleem tevens problemen van (iatrogene) vroeggeboorte.

Het doel van de verschillende studies in dit proefschrift was meer inzicht te krijgen in de foetale fysiologie bij anemie en de evaluatie van een aantal diagnostische methoden voor het voorspellen van het optimale moment voor foetale bloedtransfusie. Dit proefschrift bestaat uit twee delen: de chemische benadering en de echoscopische benadering.

Deel 1: Chemische benadering

De chemische techniek voor de diagnostiek van foetale anemie is gebaseerd op het feit dat bij afbraak van het foetale hemoglobine de bilirubine concentratie in het vruchtwater zal toenemen. Vruchtwater wordt verkregen door middel van amniocentese. De mate van geelverkleuring van het vruchtwater wordt vervolgens gemeten zoals in 1961 door William Liley beschreven. Deze geelverkleuring wordt uitsluitend veroorzaakt door bilirubine.

Allereerst werd een overzicht gemaakt *(hoofdstuk 2)* van de literatuur over de waarde van bilirubine metingen in vruchtwater en tevens van Doppler van de arteria cerebri media in de voorspelling van ernstige foetale alloimmune anemie. Het voordeel van de Doppler methode is dat het een non-invasieve manier is om anemie te voorspellen. De conclusie van deze review is dat de sensitiviteit voor beide technieken in onderzoeken, die voor het grootste deel retrospectief zijn, varieert maar grosso modo overeen komt. Bij gelijk blijvende sensitiviteit dient de vraag gesteld te worden of de niet invasieve techniek dan niet vanzelf als de betere gezien moet worden. Een prospectief en groter onderzoek waarbij beide technieken in dezelfde patiënten worden uitgevoerd moet uitsluitsel gaan geven.

In *hoofdstuk 3* werd onderzocht hoe accuraat de bilirubine concentratie in vruchtwater ernstige foetale anemie voorspelt in het tweede en derde trimester van de zwangerschap. Hiervoor werden 79 niet-hydropische eenling zwangerschappen met een D immunisatie geincludeerd waarbij een amniocentese was verricht maximaal 4 dagen voor de eerste intra-uteriene transfusie. De Δ OD 450 waarden uit het vruchtwater werden in een Liley grafiek geplaatst. William Liley beschreef in 1961 een grafiek met op de x-as de zwangerschapsduur en op de y-as de Δ OD 450. In deze grafiek zijn 3 zones getekend: zone 1 (geen anemie), zone 2 (matige anemie) en zone 3 (ernstige anemie). De originele grafiek van Liley gaat vanaf 27 tot 36 weken, bij de geëxtrapoleerde Liley curve is deze curve doorgetrokken van 27 naar 18 weken. Sensitiviteit en specificiteit werden berekend voor de meest gangbare afkappunten op de Liley curve. De sensitiviteit voor de

bovenste 2/3 van Liley zone 2 was 95% vóór 27 weken en 98% na 27 weken. Geconcludeerd werd dat de geëxtrapoleerde Liley curve met zeer goede sensitiviteit en redelijke specificiteit ernstige anemie voorspelt.

In *hoofdstuk 4* wordt gezocht naar de weg waarlangs de verhoogde concentratie aan bilirubine in het foetale bloed leidt tot een verhoogde concentratie aan bilirubine in vruchtwater. Hiervoor werd de relatie onderzocht tussen bilirubine concentratie in foetaal bloed en deze in vruchtwater bij 68 rhesus D allo-geimmuniseerde foetus met anemie zonder hydrops bij de eerste intra-uteriene transfusie. Bij deze anemische foetussen verminderde de vruchtwater/foetale bloed ratio van bilirubine van 0.09 bij 28 weken naar 0.05 bij 33 weken. In normale, niet anemische foetussen, zijn de vruchtwater/foetale bloed ratio voor bilirubine en voor albumine in dezelfde range en laten dezelfde afname zien tijdens de zwangerschap. Op basis van deze bevindingen hebben wij de hypothese geformuleerd dat de bilirubine concentratie in vruchtwater word bepaald door ten eerste de foetale bloed bilirubine concentratie en ten tweede door de vruchtwater/foetale bloed ratio van albumine.

Van de vijf mogelijke wegen die bilirubine kan nemen om vanuit foetaal bloed in het vruchtwater een concentratie op te bouwen (foetale nieren, longen, huid, darmen en vliezen) lijken de vliezen de meest voor de hand liggende. Tijdens het foetale leven is bilirubine in vruchtwater voor het grootste deel ongeconjugeerd. Ongeconjugeerd bilirubine is voor 99% gebonden aan albumine. Het is onwaarschijnlijk dat bilirubine in het vruchtwater komt via foetale urine of longvocht omdat de albumine concentratie in deze lichaamsvochten 100 tot 200 maal lager is dan in foetaal plasma. De albumine concentratie in vruchtwater is, echter, maar 10 tot 20 keer lager dan in foetaal plasma. Wegens de extreem lage albumine concentratie in foetale urine en longvocht werken deze vloeistoffen als een barrière voor het ongeconjugeerde bilirubine die het plasma wil verlaten en het vruchtwater compartiment wil binnengaan. Dat foetale ontlasting, meconium genaamd, het bilirubine tussen foetaal bloed en vruchtwater zou transporteren is niet in overeenstemming te brengen met een klinisch relevante correlatie tussen concentratie van bilirubine in vruchtwater en in foetaal bloed. De dunne foetale huid laat tot een

zwangerschapsduur van 16 weken waarschijnlijk uitwisseling toe tussen bloed en vruchtwater van allerhande stoffen, waaronder ongeconjugeerd bilirubine. Bij een zwangerschapsduur van 25 weken is de foetale huid echter volledig gekeratiniseerd. De foetale vliezen blijven wel permeabel gedurende de gehele zwangerschap. Daarom vindt de uitwisseling van ongeconjugeerd bilirubine tussen foetaal bloed en vruchtwater hoogstwaarschijnlijk plaats via deze weg.

Deel 2: Echoscopische benadering

Sinds 1995 is in meerdere publicaties aangetoond dat de stroomsnelheid van bloed in de arteria cerebri media tijdens de systole van het hart verhoogd is bij foetale anemie. Men kan zich daarbij afvragen of deze toegenomen stroomsnelheid bij anemie het gevolg is van veranderingen in de viscositeit van het bloed, van de contractie kracht van het hart of van de perifere weerstand in de hersenen.

Om de invloed van de viscositeit op de systolische bloedstroomsnelheden in de arteria cerebri media te onderzoeken werd de stroomsnelheid gemeten voor en na een intra-uteriene transfusie. Bij een transfusie neemt de viscositeit van het bloed immers toe. In *hoofdstuk 5* word het effect beschreven van een acute grote toename van het hematocrit op de piek systolische snelheid in de arteria cerebri media en op de maximum snelheid van de vena umbilicalis. Daartoe werden de snelheden in de arteria cerebri media en de vena umbilicalis gemeten vóór, direct na en de dag na 60 intra-uteriene transfusies. De snelheid in de arteria cerebri media verminderde in 59 van de 60 gevallen direct na transfusie. Er was in 37 van de 60 gevallen een toename van de snelheid in de vena umbilicalis. Geconcludeerd werd dat een acute toename van het hematocriet zoals bij een intra-uteriene transfusie significant de snelheid in de arteria cerebri media vermindert. Het effect op de snelheid in de vena umbilicalis daarentegen bleek onvoorspelbaar. Het feit dat de stroomsnelheid in

de vena umbilicalis in vele gevallen toenam na transfusie is natuurlijk in tegenspraak met de stelling dat de viscositeit van bloed de voornaamste determinant is van de bloedstroomsnelheid. Ook de uitgebreide variatie in arteriële bloedstroomsnelheden bij foetussen met hetzelfde hematocrit wijst erop dat, naast hematocrit, andere factoren zoals slagkracht van het hart en perifere weerstand een belangrijke rol moeten spelen.

In *hoofdstuk 6* werd uitgezocht wat de diagnostische accuraatheid is van metingen van de cardiale ventrikel wand dikte en de cor-thorax ratio om ernstige foetale anemie te voorspellen. De dikte van de cardiale ventrikel wand van de linker en de rechter ventrikel werden gemeten met behulp van M-mode echoscopisch onderzoek. De cor-thorax ratio werd gemeten op het B-beeld. De metingen werden verkregen in geïmmuniseerde zwangerschappen. Vervolgens werden 2 bij 2 tabellen gemaakt om de frequentie van abnormale cardiale echoscopische metingen in ernstige en niet-ernstige foetale anemie te vergelijken. De metingen werden verkregen bij 15 foetussen met ernstige anemie en bij 16 foetussen zonder ernstige anemie. De sensitiviteit van de cardiale echoscopische metingen varieerde van 0 tot 47% en de specificiteit van 77 tot 97%. Geconcludeerd werd dat de diagnostische waarde van de ventriculaire wand dikte en de cor-thorax ratio teleurstellend was. Meer dan 50% van de metingen in ernstige anemische foetussen was immers binnen de normale referentie waarden voor normale foetussen.

In *hoofdstuk 7* werd geëvalueerd wat het effect van foetale anemie en intra-uteriene transfusie was op de ventriculaire verkortings fractie. Tijdens de systole worden de ventrikels van het hart kleiner. Hoeveel kleiner de ventrikels tijdens de systole zijn kan met de echo gemeten worden en wordt uitgedrukt als de ventriculaire verkortings fractie. Bij 23 foetussen werd voor en na 49 transfusies de eind diastolische en eind systolische transversale dimensie van de rechter en de linker ventrikel gemeten. Het bloed gegeven bij intra-uteriene transfusie werd weergegeven als een percentage van het geschatte fetoplacentale bloed volume. Verkortings fracties van de rechter en de linker ventrikel verschilden significant voor, direct na en 1 dag na transfusie. De linker shortening fractie verminderde direct na transfusie in 43 van de 49 procedures. De rechter shortening

fractie verminderde direct na transfusie in 42 van de 49 procedures. Bij de eerste transfusie bleek er maar een matige correlatie te zijn tussen het verminderen van de shortening fractie van beide ventrikels en het getransfundeerde volume. Er werd geconcludeerd dat intra-uteriene transfusie de shortening fractie van beide ventrikels vermindert. Er is echter weinig correlatie tussen de vermindering van de shortening fractie en de hoeveelheid bloed gegeven tijdens transfusie.

Hopelijk dragen de verschillende studies in dit proefschrift bij aan de diagnostiek bij verdenking op foetale anemie en aan een beter inzicht in de veranderingen die optreden bij het opheffen van foetale anemie tijdens intra-uteriene transfusies.

Nawoord

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Curriculum vitae

Esther Sikkel werd geboren op 30 september 1970 te Mijdrecht. Na het behalen van een HAVO diploma (1988) en een VWO diploma (1990) aan het Veenlanden College te Mijdrecht werd in 1991 begonnen met een studie geneeskunde aan de Vrije Universiteit te Amsterdam. Er werd een wetenschappelijke stage gedaan over Dengue virus infecties (Demam berdarah dengue) in Yogjakarta, Indonesië voor de vakgroep Kindergeneeskunde (Prof. Veerman) van de Vrije Universiteit te Amsterdam. In oktober 1998 werd het artsexamen behaald.

Vervolgens is zij 2 jaar werkzaam geweest als echoscopist bij de afdeling verloskunde (hoofd Prof. Dr. H.H.H. Kanhai) in het LUMC, Leiden. In deze tijd is haar interesse in de rhesus problematiek gewekt, waarna zij per oktober 2000 startte als AGIKO in hetzelfde ziekenhuis aan het in dit proefschrift beschreven onderzoek.

Per 1 juli 2003 startte zij in het Groene Hart Ziekenhuis, Gouda, met de opleiding Gynaecologie en Verloskunde (opleider Dr. J.C.M. van Huisseling). Per 1 oktober 2004 werd dit vervolgd voor het academisch deel van de opleiding in het LUMC, Leiden (opleiders prof. Dr. H.H.H. Kanhai en prof. Dr. G.G. Kenter). Voor de afronding van haar opleiding tot gynaecoloog zal zij vanaf 1 oktober 2007 werkzaam zijn in het Groene Hart Ziekenhuis te Gouda.