Diagnostic procedures for assessing the severity of alloimmune fetal anemia

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General Discussion

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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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