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General discussion

insights in fetal (patho-)physiology
implications for current practice
implications for future research
Around the year 1650, the renowned professor Sylvius taught his medical students at the University of Leiden about the fetal circulation [1]. He described a continuous circulation of blood that flowed from the mother, through the placenta where it was somehow transformed, to the fetus and vice versa. The beating of the fetal heart had no function other than *ad solam propriam vitam conservadam*, or “just to be alive”. This of course is the fetal physiological equivalent of a flat world model. We have since learned that the fetus has its own circulation, pumping its blood through its body, through the umbilical cord and the placenta and back. A layer of chorionic cells surrounds the entire fetal compartment: the syncytiotrophoblast is the fetal border of the placenta; the chorionic membrane encloses the amniotic fluid compartment in which the fetus moves and develops. All exchange of substances between mother and fetus takes place through this chorionic layer of cells. The type of cells and the specific architecture of the placenta are unique to the human species. Therefore, human fetal physiology differs from that of other mammals.

Also, the fetus is not merely an unborn baby. The fetus and neonate differ in several aspects. First, a substantial part of the fetal circulation is extracorporal (in the umbilical cord and placenta). Further, since breathing is not yet required to obtain oxygen from the air, the lung circulation is only a fraction of what it will be after birth due to a much higher pulmonary vascular resistance [2]. Before birth two shortcuts exist to bypass the lung circulation [3]. A great part of the blood that enters the right half of the heart, immediately flows to the left side of the heart or to the aorta and thus to the head and body instead of the lungs. The first shortcut is the *foramen ovale* between the atria; the second shortcut is the *ductus arteriosus* between the pulmonary artery and the aorta. Finally, there is an intricate system, the *ductus venosus*, which regulates the amount of flow coming from the placenta to either go directly to the right atrium, or first to flow through the liver [3]. This system divides the oxygen and nutrient rich blood from the placenta over the fetal brain and the fetal liver. Physiology research in fetal lambs has shown that fetuses are different from neonates in other aspects as well. The compliance of the fetal circulation seems higher than that of neonates [4]. The compliance of the interstitial space is also higher and is therefore more prone to expansion [5]. The lymphatic return is much more vulnerable to a rise in venous pressure [6] and the capillary permeability is higher in fetuses compared to neonates [7]. It is unknown whether all these effects gradually change dependent on gestational age or if this is largely changed directly after birth.
General discussion

In this thesis we studied fetal pathophysiology in hemolytic anemia and hydrops, with a focus on fluid and protein dynamics. Our studies have led to acquired insights in the following aspects:

First, we studied bilirubin clearance in the fetus. Where hemolytic anemia in neonates is frequently accompanied by hyperbilirubinemia, potentially leading to kernicterus, fetuses are not known to develop this problem. (Free) bilirubin concentrations in fetuses never reach the dangerous levels as they occur in neonates with hemolytic anemia. This is due to an (in part active) transport of unconjugated bilirubin over the placenta towards the mother [8]. Before bilirubin is transferred over the placenta though, it seems to exchange between the fetal blood and the amniotic fluid (chapter 2 and 3). Unconjugated bilirubin is almost completely bound to albumin in extracellular fluids [9]. The bilirubin content in the entire fetal compartment is thus determined not only by formation and clearance of bilirubin from the fetal blood, but also by the total binding capacity of albumin in the different fetal compartments. Literature review showed that amniotic fluid albumin is likely to be of maternal or membrane origin instead of fetal origin (chapter 4), thus delineating the hypothesis that bilirubin exchanges over the intramembraneous pathway, releasing and binding to albumin on either side of the membrane.

Second, we studied the available evidence in the literature on the origin and function of albumin in amniotic fluid. We considered all transport mechanisms from the amniotic cavity to and from the fetus and the mother. Based on the literature, we think that it is very likely that the transport of nutrients not only takes place through the placenta, but also in part through the fetal membranes (chapter 4). The amniotic fluid might therefore be considered the first enteral nourishment. Fetuses and neonates are in this aspect not that different, having a combined enteral intake of water and nutrients. A fetus, however, probably drinks much larger amounts daily to maintain the recycling process of water in the amniotic cavity [10]. Another option is to consider the amniotic cavity to be an extracorporal part of the extravasal compartment of the fetus. There seems to be an extensive exchange between the amniotic fluid and the fetal blood through the intramembraneous pathway. In other words, this compartment is comparable to the interstitial space inside the body of the fetus. The difference is that it is not only in contact with the fetal blood, but it is also in contact with the mother. The fetal membranes therefore seem to play a crucial role as border patrol in maintaining not only the volume but also the composition of amniotic fluid.
Third, we studied the difference between hydropic and nonhydropic fetuses. We found a different bilirubin to albumin ratio in hydropic fetuses (addenda chapter 2 and 3), suggesting a difference in albumin binding capacity both in fetal blood and in amniotic fluid. This might be explained by competitive binding of an unknown ligand, by a change in characteristics for example of pH, or by posttranslational changes in albumin, for example an increase in ischemia-modified albumin. Further, we found hypoalbuminemia to be more often present in hydropic or severely anemic fetuses compared to mildly anemic fetuses (chapter 5). This might be the effect of a decrease in albumin production due to excessive hematopoiesis in the fetal liver. The hypoalbuminemia could also be the effect of an increase in plasma volume, as we found in anemic fetuses (chapter 6). With increasing severity of anemia, thus a reduced number of red blood cells, the compensating amount of plasma could cause a dilution of the amount of albumin present in the circulation. An overcompensating plasma volume in hydropic fetuses (chapter 6) would be in agreement with a further decrease in albumin concentration. Finally, hypoalbuminemia could be the effect of capillary leakage, caused by hypoxia-related endothelial damage. This might explain the rise in protein concentration in amniotic fluid that has been reported in severely anemic and hydropic fetuses, possibly due to leakage through the intramembraneous pathway. However, when studying the shift of fluid out of the fetal circulation during an intrauterine blood transfusion, we did not find any difference between hydropic and nonhydropic fetuses (chapter 7). We expected that an altered capillary permeability would have influenced the amount of fluid shift that takes place. Multiple contradicting effects might on the other hand be present in severe anemia and hydrops, thus explaining the indistinct effect on fluid shift.

Generalized hydrops is a phenomenon rarely seen in adults. An interesting similarity of fetal hydrops exists with the, evenly poorly understood symptoms of pre-eclampsia. Generalized hydrops is also rarely seen in children or neonates. Part of the explanation probably lies in the aforementioned differences in physiology between fetuses and neonates. In contrast to neonates and adults, fetuses have greater endurance in withstanding the deleterious effects of the causes leading to generalized hydrops, without intensive care. As long as fetuses are supplied with a certain amount of oxygen, water and nutrients through the placenta and the amniotic fluid, the womb can actually be considered to be a “prenatal intensive care unit”.

Fourth, we studied fetoplacental blood volumes. The average fetoplacental blood volume was about 120 ml/kg (chapter 6). The average neonatal blood volume is about 90 ml/kg [11]. This implies that about a quarter of the fetoplacental blood volume is extracorporal. The extracorporal volume of blood circulating in the placenta
might vary, especially during blood transfusion. It has been suggested that fetuses can tolerate higher volumes and speed of transfusion compared to neonates because the placenta can function as an expanding reservoir. Further, we found that in severe chronic anemia, fetuses maintain their total blood volume, thus compensating the loss of red cells with an increase in plasma volume (chapter 6). This seems to be similar to neonates with hemolytic anemia [12;13]. In adults with chronic anemia, total blood volume has also been reported to be maintained or to be somewhat decreased [14]. In adults, the kidneys function as regulator of both red cell volume and total blood volume and thus of hematocrit [15]. Our study suggests this regulatory function to be active already from an early gestational age onward.

The acquired insights on the diagnosis, the pathophysiology and the reaction to treatment in severe anemic or hydropic fetuses led to several implications for current practice that will hereafter be discussed. Furthermore, implications for future research are described.
Chapter 8

Implications for current practice

We investigated some of the pathophysiological processes that occur in hemolytic anemia and immune hydrops fetalis. This resulted in the following recommendations for clinical practice:

1) Bilirubin concentration in amniotic fluid is determined by both the formation of bilirubin in fetal blood, and the amount and binding capacity of albumin in the fetus and in the amniotic fluid (chapter 2 and 3). The formation of bilirubin is increased during hemolysis. The clearance of bilirubin that is formed in the fetus depends on the transport over the placenta towards the mother. For many years, prediction of fetal hemolytic anemia was performed by measuring the amount of bilirubin in amniotic fluid. The Queenan and Liley chart show the cut-off values for the concentration of bilirubin in amniotic fluid that indicate the presence of severe hemolytic anemia [16]. While working on the studies presented in this thesis, we realized that, since most of the bilirubin is bound to albumin, the slope in both the original non-extended Liley chart and in the Queenan chart follows that of the average albumin concentration in amniotic fluid during gestation. This is illustrated in figure 1 and 2.

Figure 1  Queenan and Liley curve for amniotic fluid ΔOD450 (bilirubin) values
We advise to use the Queenan chart instead of the linearly extended Liley chart, for prediction of fetal anemia below 24 weeks' gestation, since the Queenan chart better corresponds with the average albumin concentration in this time period.

2) There are several conditions in which amniotic fluid albumin concentration can deviate from the normal average, for example intrauterine growth restriction, polyhydramnios or renal abnormalities. In these cases, it therefore must be anticipated that bilirubin concentration in amniotic fluid will change accordingly. Also, hydrops is associated with a shift in albumin concentration, both in fetal blood (chapter 5), and in amniotic fluid [17]. Bilirubin concentration in fetal blood is lower in hydropic fetuses compared to nonhydropic severely anemic fetuses (chapter 2). But, because of the relatively increased binding capacity in amniotic fluid (chapter 3), the concentration of bilirubin in amniotic fluid will still be high in hydropic fetuses. This actually increases the clinical usefulness of the Queenan and Liley chart, although in the last decades, the use of ultrasound to detect fetal hydrops reduced the need for amniocentesis and bilirubin measurement in the severely affected group.

We advise to beware of differences in the amniotic fluid albumin concentration or its binding capacity for bilirubin, that might occur in several pathological situations. This influences the bilirubin concentration in amniotic fluid, and thus its predictive value for fetal anemia.

Figure 2 Albumin concentration in amniotic fluid during gestation

\[ R^2 \text{ Cubic} = 0.44 \]

mean +/- 95% CI
3) Fetal bilirubin spreads through all fetal compartments including the amniotic fluid (chapter 3), before it is transported towards the maternal blood. Bilirubin most likely enters the amniotic fluid through the intramembranous pathway, then binding to the albumin that is present there. Albumin in amniotic fluid seems to be mostly of maternal origin (chapter 4). Thus, the bilirubin-albumin complex detaches and the free bilirubin crosses the membrane, after which it attaches again to form a new bilirubin-albumin complex. This process takes place from fetal blood towards the amniotic fluid, towards the maternal blood and most likely also towards the extravascular compartment in the fetus. After birth, bilirubin is being cleared from the neonatal blood after the commencing of the conjugation and excretion process and after intestinal re-uptake is lowered [18;19]. Then the bilirubin content that is built up in the interstitium is recruited into the blood stream. Thus, forementioned process reverses, from the extravascular compartment towards the fetal blood. Clinicians need to be aware this phenomenon can occur, also after an exchange transfusion, in neonates that have had a potentially large accumulation of bilirubin before birth. We advise to take the possible prenatal accumulation of bilirubin in the extravascular compartment into account, that can cause subsequent hyperbilirubinemia after birth.

4) In the past, two different assumptions have been made for the calculation of the fetoplacental blood volume. First assumption was that the blood volume before transfusion equals that after transfusion [20]. Second assumption was that the blood volume after transfusion increases by the amount of donor blood volume that is administered during transfusion [21]. Both assumptions do not seem to represent reality. During transfusion, part of the plasma immediately leaves the circulation, on average about a third of the transfused volume (chapter 7). Further, we found that the fetoplacental blood volume, measured before transfusion, was maintained in nonhydropic fetuses. Thus, the decrease in red cell volume, due to hemolysis, is compensated by an increase in plasma volume. In hydropic fetuses there even seems to be an overcompensation of plasma volume (chapter 6). These findings make it very likely that after transfusion the fetoplacental blood volume will also normalize rapidly. Thus, the other two thirds of the transfused volume probably leave the circulation after transfusion, i.e. further reducing the amount of plasma volume. Therefore, an increase in hematocrit should be expected to still take place shortly after an intrauterine blood transfusion.

For the calculation of the donor volume that has to be given at an intrauterine transfusion, the Rodeck formula can be used [20]. Currently, the aim is to reach a
desired hematocrit of 50% at the end of the transfusion. Thus, a final sample is taken to check if the desired level is achieved and blood is transfused until this is corrected. The Rodeck formula, however, does not take a fluid shift during transfusion into account. Moreover, it should be realized that the hematocrit at the end of transfusion is probably not the final hematocrit, because fluid will continue to shift in the hours or days after transfusion. It is possible that hazardous levels of hematocrit are reached with potential adverse effects. In a recent (possibly pre-selected) cohort of neonates that had received an intrauterine transfusion, 24% showed moderate to severe abnormalities on cranial ultrasound (personal communication G. van Wezel-Meijler), which may be due to hypoxia but also to polycythemia mediated damage. Because the Rodeck formula does not take a change in blood volume into account, it actually is a useful formula, assuming that blood volume returns to normal shortly after transfusion. It might be acceptable to aim for a hematocrit of 55% in nonhydropic fetuses [22], when striving for a maximal prolongation of time until delivery or next transfusion. However, it should not be expected to be reached at the end of transfusion and the calculated amount of donor blood should not be exceeded. When choosing the desired level of hematocrit, the risk of brain damage mediated by polycytemia has to be weighed against the risk of an additional intrauterine intervention [23]. Finally, estimated blood volumes as reported in chapter 6 can be used for the calculation of the donor volume.

We advise to use the Rodeck formula to calculate the volume of donor blood that has to be given during transfusion. The hematocrit is expected to still increase after transfusion and should thus not be expected to already be at the desired level at the end of transfusion.

5) A relative high transfusion speed probably lowers the amount of fluid shift during intrauterine blood transfusion (chapter 7). This would imply that in case of high transfusion speed, the measured final hematocrit is even less predictive since the hematocrit will increase even more after transfusion. In our study period, we found that in younger fetuses, relative volume and speed of transfusion was many times higher than in older fetuses. Since fetoplacental blood volume increases exponentially during gestation (figure 3), volume and pressure burden will be many times higher if transfusion policy is not adjusted at lower gestational ages. Advisable is, since donor blood is administered at 5 ml/min in 30 to 35 weeks old fetuses, transfusion speed should be lowered to 4 ml/min in 25 to 30 weeks, to 3 ml/min at 20 to 25 weeks, and to 2 ml/min or less below 20 weeks gestation (table 1). In addition, desired hematocrit or desired hemoglobin level should be adjusted to normal values for gestational age [24].
We advise to adjust the transfusion speed and the donor volume administered during intrauterine transfusion, according to the gestational age or estimated fetal weight of the fetus.

6) In case of hydrops, extra care should be taken to avoid volume or pressure overload, thus even lower speed of transfusion is advisable. Even though no obvious relation was present between fluid shift and severity of anemia or presence of hydrops (chapter 7), based on the literature (and clinical experience) it seems

<table>
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<th>EFW*</th>
<th>FPV**</th>
<th>ml/min</th>
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<th>ml/min</th>
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<td>3,1%</td>
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* EFW: fetal weight in g. [25], ** FPV 123 ml/kg (chapter 6)
hazardous to achieve a large difference in hematocrit in a short period of time [26;27]. Thus, in hydropic or severely anemic fetuses we prefer to aim for a lower final hematocrit e.g. 30%. Exchange transfusion or intraperitoneal addition of transfusion are other options to avoid direct volume and pressure overload. Finally, the relative increase in total blood volume in hydropic fetuses (chapter 6) may normalize with improvement of fetal condition, thus still somewhat increasing hematocrit. **We advise that severely anemic and hydropic fetuses should only be burdened with relatively low volume and pressure during intrauterine blood transfusion.**
Chapter 8

Implications for future research

The results from the studies performed in this thesis provide further insight in the pathophysiology of alloimmune anemia, bringing forth new questions to be researched. These questions can also be extended to the field of other fetal diseases and in general to fetal physiology. These questions can be translated to the following propositions for future research.

1) The large interindividual variation in albumin concentration in amniotic fluid can hamper the sensitivity of the Queenan or extended Liley chart. The accuracy for the prediction of fetal anemia by measuring the amniotic fluid bilirubin to albumin ratio (or BAR), that avoids this problem, could be investigated prospectively. Maternal obesity, unusual position of the fetus or concomitant pathology can make prediction of fetal anemia with ultrasound difficult. Especially below 18 weeks gestation the measurement of the peak systolic velocity in the middle cerebral artery may become less accurate or more difficult to obtain. The presently used Liley chart is also not accurate in this time period. Furthermore, the risk of intrauterine transfusion is significantly increased below 20 weeks gestation [23]. Thus, performing an amniocentesis to measure the bilirubin to albumin ratio, to aid in the diagnosis of fetal anemia, may be worthwhile especially at low gestational age. Postnataally, the bilirubin to albumin ratio is already being investigated in a multicenter trial (BARtrial), to see whether this ratio is a valuable addition to total serum bilirubin concentration, when used as phototherapy and exchange transfusion cut-off value, for the prevention of neurological sequelae [28]. It would be of interest to also investigate the albumin binding capacity for bilirubin in hydropic and nonhydropic fetuses and to compare prenatal with postnatal binding capacity [28;29]. A lowered reserve binding capacity could imply an increased risk of free bilirubin toxicity [30].

**Studies on pre- as well as post-natal measurements of the bilirubin to albumin ratio may improve the treatment of fetuses and neonates with blood group immunization problems.**

2) Review of the available evidence on the origin of amniotic fluid albumin showed that there are many questions left unanswered since the 1970s (chapter 4). Studies on the origin of albumin and on the transport mechanism through the so-called intra- and transmembraneous pathways are of great interest. Using albumin as a study object, it may become possible to gain more knowledge on the homeostasis of amniotic fluid volume and composition. The intramembraneous pathway is thought to play a crucial role in the development of oligo- or polyhydramnios. These are conditions that accommodate
many fetal diseases and may pose obstetrical problems. Many in vitro studies (as proposed in chapter 4) using human placenta and membranes can be designed. **Studies on the origin of albumin and on the transport mechanism through the fetal membranes will increase our knowledge, among others, of amniotic fluid homeostasis.**

3) Since albumin in amniotic fluid is probably mostly of maternal origin, the possibility of transamniotic fetal nutrition becomes of interest. Especially since albumin is a carrier protein among others for fatty acids and minerals. Intrauterine growth restriction as a result of placental insufficiency is an important obstetric problem. Although the diminished supply of nutrients is not the only problem of placental insufficiency since it may be accompanied by problems in oxygenation, an increase in prenatal nourishment might improve outcome. Studies on transamniotic feeding in animal studies have so far given paradoxical and disappointing results [31-33]. However, understanding of the physiological role of transamniotic feeding might be the key to designing a fetal therapy for intrauterine growth restriction. **Studies on physiological transamniotic feeding can give directions for the research on the development of fetal therapy for intrauterine growth restriction.**

4) Hypoalbuminemia seems to be a secondary effect of hydrops (chapter 5) and may in part be due to an increase in plasma volume (chapter 6). It would be of interest to investigate the albumin concentration in different fetal compartments as ascites and hydrothorax and compare this with values in amniotic fluid and fetal blood, to gain insight in the amount of albumin that builds up in these different compartments in hydrops. Besides, many other questions on fetal pathophysiology are still unanswered. Research developed using animal or computer models will give rise to hypotheses that have to be verified in human studies. In this thesis, the collection of simultaneous fetal blood and amniotic fluid samples was essential to test our hypothesis (chapter 3). We therefore want to advocate the prospective collection of scarce and unique human fetal samples, that are otherwise discarded. It would be most valuable to collect and store samples of different fetal compartments, that have been taken simultaneously. Preferably a perinatal biobanking should be achieved of pre- and postnatally collected samples, for example of ascites or pleural effusion, in combination with fetal (cord) blood and/or maternal blood and/or amniotic fluid. **Collection of samples simultaneously obtained in different fetal compartments will be very valuable for perinatal pathophysiology research.**
5) Cardiovascular changes play a crucial part in hydrops fetalis. The question remains whether there is an actual myocardial failure in anemia induced hydrops, or if the cardiovascular changes are an effect of the hyperdynamic circulation or in other words, represent a high output state. The low concentration of hemoglobin causes a low arterial pressure due to a decrease in viscosity and possibly due the periferal vasodilatation. Vasodilatation may be mediated for example by endothelium-derived relaxing factor, which is regulated by hemoglobin concentration [34]. The kidneys may mediate a neurohumoral cascade that increases plasma volume, as to rise cardiac filling pressure and increase cardiac output, thus maintaining arterial pressure [35]. This cascade eventually might lead to overcompensation of plasma retention and a rise in venous pressure, thus causing the development of hydrops fetalis. It would be of interest to differentiate between actual myocardial failure and a state of high output congestion with a cardiac function that can quickly return to normal when the underlying condition is treated. Actual myocardial failure might be present for example in recipient twins in severe TTS, or in fetuses effected by Parvovirus B19 with a cardiomyopathy. High output cardiac failure may be present for example in twin arterial perfusion syndrome, or in fetuses with sacrococcygeal teratoma or placental chorioangioma. Studies (as proposed in chapter 7) on the difference between hydropic and nonhydropic fetuses could eventually give insight in the pathophysiology of fetal high output cardiac failure.

**Studies on the cardiovascular mechanisms behind high output cardiac failure will improve our knowledge on hydrops fetalis, the final common pathway of many prenatal diseases.**

6) To optimize fetal intrauterine transfusion policy, the use of additional intraperitoneal transfusion should be studied prospectively. Benefit of the combination of intravascular with intraperitoneal transfusion is the avoidance of a direct vascular overload during the transfusion, while obtaining the longest possible interval until delivery or subsequent transfusion. Possible downsides to this method could be prolonging of the intervention time, damage inflicted to the abdominal wall or the intestines, possibly causing fetal pain and intra-abdominal adhesions, or the induction of polycytemia. A new formula for the amount of donor blood that has to be given intravascularly and intraperitonally, has to be established for this prospective study. With the use of the studies in this thesis (chapters 6 and 7) this new formula can be established. A prospective study should be designed with the aim of safety analysis and with comprehensive pre- and postnatal neuro-imaging and (neurological) follow-up.

**A prospective study on the combination of intravascular and intraperitoneal transfusion should be designed to improve intrauterine transfusion safety.**
Final thoughts

We want to advocate the importance of human fetal physiology research. In particular, the intrauterine transfusion is a unique situation where ultrasound measurements can be combined with blood sampling and other invasive diagnostics. Both the initial values as the reaction of the fetus on a therapeutic intervention makes it an ideal situation for physiological research. From the experience with alloimmunized hemolytic anemia and immune hydrops fetalis, we can translate our increased knowledge in fetal (patho-) physiology to other prenatal diseases. Hereby, creating a basis for the development of new or improved fetal therapies for a wide range of prenatal diseases.

Further, the similarities between (premature) neonates and fetuses of the same gestational age and the continuum of disease processes before and after birth, makes an intense cooperation between fetal medicine specialists and neonatologist, a goal that has to be strived for. Also, ongoing collaboration with specialized pediatricians e.g. in the field of cardiology, neurology and urology, and specialist e.g. in the field of radiology, pathology, embryology, hematology, immunology and genetics are essential for the development of fetal medicine. Joint ventures can increase the much needed chances to access funding resources. Research at the beginning of life is poorly funded compared to research at the end of life [36]. The emphasis on the long term consequences should however obviate the importance of perinatal research.

**Fetal physiology research should continue to be performed, preferably in collaboration with other (pediatric) specialists and should emphasize the possibility that it will lead to the improvement of long term health consequences.**

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