Fetal fluid and protein dynamics

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Fetal Fluid

and

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Dynamics

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Fetal fluid and protein dynamics

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Fetal fluid and protein dynamics

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Suzanne Pasman

geboren te Nieuwegein in 1977

Aan elke foetus die ooit foetale therapie nodig zal hebben

Voor iedereen die mij dierbaar is en ooit een foetus was

Contents

1

General introduction

introduction
background
outline of the thesis

Introduction

Fetal medicine is a relatively young and fast growing field of medicine. The first successful fetal treatment was the intrauterine blood transfusion for Rhesus hemolytic disease in 1963 (by Dr. A.W. Liley). Since then, both the number of indications as the number of fetal treatment options have expanded rapidly. With the introduction of ultrasound, prenatal diagnosis of a wide range of fetal diseases became possible. In addition, ultrasound guided techniques enabled the development of minimally invasive prenatal treatments. Therefore, ultrasound and fetal medicine are inextricably linked. Sonographic observations provided a growing insight in the physiology and the pathophysiology of human fetuses. However, as a young and developing field of medicine, many questions still have to be answered at a fundamental level.

Physiology is the basis for our understanding of both health and disease state. Research in this area in human adults has resulted in the development of many diagnostic tools, medicines and other treatment modalities. Furthermore, experimental animal studies have provided many answers on questions that were impossible to investigate in humans. However, distinct differences can make it difficult to translate findings from animal experimental models to the human situation. Especially the human placenta is a unique organ, not comparable to that of any other mammal. Further, lessons can be learned from studies in premature neonates of the same gestational age as a fetus. However, radical changes take place after birth, especially in the cardiovascular system, making direct comparisons often impossible.

As a tertiary fetal therapy center, the LUMC has an obligation, besides high quality patient care and performance of clinical trials, to develop scientific projects on basic research level. A unique possibility is provided to study human fetal (patho-) physiology, by the access to the fetal circulation and amniotic fluid during treatment of several fetal diseases. Both the etiology of prenatal diseases and the reaction of fetuses on prenatal treatment can be investigated. Potentially treatable fetal diseases include fetal anemia, twin related problems, primary cardiac failure, primary hydrothorax and other causes of non-immune hydrops fetalis. All of these diseases can lead to abnormal amniotic fluid volumes, i.e. oligo- or polyhydramnios, which can result in a premature delivery of a neonate in a critical condition. Moreover, all of these diseases inevitably lead to the development of hydrops fetalis, eventually leading to intra uterine fetal demise. To the surviving fetuses, impaired neurological outcome poses a serious threat.

The development of abnormal amniotic fluid volumes and hydrops fetalis form the final common pathway of all these fetal diseases and are in fact shifts in fluid and protein in the different fetal compartments. These shifts take place between the amniotic fluid and the intravascular and the interstitial compartment in both the fetus and the placenta. The mechanisms involved in these **fetal fluid and protein dynamics** will be investigated in the studies described in this thesis. With increasing insight in the pathophysiological processes, improvements can be made in the diagnosis of disease stages, in timing of fetal treatment, in new treatment modalities and even in preventive strategies for these high risk prenatal conditions.

From clinical practice, several questions arose. First of all, diagnosis of fetal anemia and the timing of intrauterine blood transfusion has been a subject of interest at our department since several decades. One of the oldest diagnostic tools, measuring bilirubin content in amniotic fluid, developed by Bevis in 1956 [1] and introduced into clinical practice by Liley in 1961 [2], has been a clinically useful tool for many years. Surprisingly, detailed studies on the background of this test are lacking. Secondly, treatment of fetuses with intrauterine blood transfusion still carries a substantial risk of complications or fetal loss. Treatment methods were developed empirically, and are practically unchanged since the late 1980s. More basic knowledge on fetal condition during developing anemia and on fetal reaction to blood transfusion could potentially lead to adaptation and refinement of management protocols, with increased safety of this procedure.

Finally, the studies described in this thesis are aimed to bring forth an increased understanding of fetal physiology in general. This can contribute to the development of fetal therapy for a wider range of obstetric complications as oligo- and polyhydramnios and intrauterine growth restriction.

Background

The studies in this thesis were performed in fetuses with hemolytic alloimmune anemia. Yearly, around 90 intrauterine blood transfusions are performed in the LUMC to treat this disease.

Fetal hemolytic alloimmune anemia
Hemolytic alloimmune anemia used to be the main cause of hydrops fetalis [3] and one of the most important causes of perinatal death before 1960 [4]. It was commonly referred to as erythroblastosis fetalis. In red cell alloimmunization a woman's immune system is sensitized to foreign red blood cell surface antigens, stimulating the production of IgG antibodies. The most common routes of maternal sensitization are via blood transfusion or after feto-maternal hemorrhage. Feto-maternal hemorrhage can occur for example during spontaneous or induced abortion, ectopic pregnancy, trauma, invasive obstetric procedures, and delivery, espacialy traumatic parturition, with consequences for a subsequent pregnancy. The antibodies can cross the placenta and, if the fetus is positive for the red blood cell surface antigens, lead to hemolysis of fetal red blood cells and fetal anemia. Of the more than 50 different antigens causing hemolytic disease in the fetus and newborn, the D antigen of the Rhesus blood group system (Rh D) causes the most cases of prenatal severe hemolytic disease in the fetus and newborn [5;6]. With the introduction of intrauterine blood transfusion, the possibility of prenatal detection of anemia, improved neonatal care and last but not least the preventive administration of anti-D immunoglobulins, an enormous decrease has taken place of alloimmune anemia and immune hydrops fetalis, in the last 40 years [7;8].

Diagnosis of fetal hemolytic anemia
The diagnosis of fetal hemolytic anemia can be established by fetal blood sampling. However, the invasive nature of this procedure introduces a risk to the pregnancy and to further boostering of alloimmunization. Signs of fetal anemia can be observed with ultrasound i.e. cardiomegaly, hepato- and splenomegaly and signs of hydrops. Furthermore, fetal anemia can be predicted by Doppler blood flow measurements or by amniotic fluid analysis. Both of these diagnostic tools are quite accurate in prediction of fetal anemia [9;10], however, the measurement of the peak systolic velocity in the middle cerebral artery can predict severe anemia with higher accuracy than bilirubin determinination in amniotic fluid [9]. Moreover, the great advantage of sonographic measurements is the fact that it is not harmfull for the pregnancy. However, below 18 weeks and above 36 weeks of gestation, the measurement of the peak systolic velocity in the middle cerebral artery appears less accurate or more

difficult to obtain. Also, maternal obesity, abnormal position of the fetus or concomitant pathology can make prediction of fetal anemia with ultrasound difficult. Then measurement of bilirubin content (usually delta OD450 measurement) by amniocentesis may help the clinician in timing of the more invasive cordocentesis and a first transfusion. The so-called Liley or Queenan charts show the cut-off values for bilirubin content in amniotic fluid that indicate the risk of fetal anemia. The diagnosis of fetal anemia is finally confirmed by the sampling of fetal blood.

Intrauterine transfusion

Intrauterine transfusion is an ultrasound guided procedure. Puncture of the umbilical vein is performed either at the cord insertion, through the anterior placenta, or in the intra-abdominal hepatic portion of the umbilical vein. Fetal blood is sampled for analysis and an intravascular blood transfusion can be performed. Another option is an intraperitoneal transfusion of donor blood. Red cells will then be absorbed from the peritoneal cavity, through lymphatic drainage, towards the intravascular compartment. Although this method was replaced by the intravascular method in the 1980s, renewed interest has brought it back to use recently, often in combination with intravascular transfusion to prolong the transfusion-interval. At the LUMC, intrauterine transfusions are performed as early as 16 weeks of gestation and repeat transfusions are given every 2-5 weeks up to 35 weeks [11]. After birth, aimed between 36 and 38 weeks of gestation, phototherapy, transfusions and/or exchange transfusions may be necessary to treat recurrent anemia and hyperbilirubinemia. The procedure related risk of fetal loss is 1.6% for every intrauterine transfusion [12]. Risk factors are low gestational age and severe hydrops. Improvement of the most commonly used fetal therapy is therefore still an important subject of investigation.

Hydrops fetalis

Hydrops fetalis is the condition where a fetus retains an abundant amount of fluid. It is defined as the presence of an abnormal fluid collection in two or more fetal compartments. It can be recognized on ultrasound as fetal ascites, pericardial effusion, hydrothorax, (generalized) subcutaneous tissue edema, placental edema or polyhydramnios. Hydrops fetalis can be classified as immune or non-immune hydrops, based on whether or not alloimmunization underlies the etiology. This classification was traditionally used, since non-immune hydrops usually was not treatable and implied a poor prognosis. Recently, classification as anemic or non-anemic hydrops has been proposed [13]. Nowadays, this is a useful classification, since many hydropic fetuses can benefit from an intrauterine blood transfusion. Besides alloimmune hemolytic anemia, this includes cases of Parvo B19 viral infection or feto-maternal

hemorrhage. Other groups of causes can be identified that can benefit from fetal therapy [14]. One of these groups are twin related problems, such as twin reversed arterial perfusion sequence or twin-to-twin transfusion syndrome, that can be treated with laser ablation of intertwin connecting blood vessels. Other causes include primary hydrothorax that can benefit from thoraco-amniotic shunt placement or fetal arrhythmia that can benefit from transplacental drug treatment. Chromosomal, genetic, or metabolic disorders or congenital infections as CMV should be excluded since these are generally non-curable causes of hydrops fetalis.

The similarity between both anemic and non-anemic hydrops fetalis is the occurrence of cardiovascular changes, either as a primary or a secondary effect. Understanding of the cardiovascular pathophysiology in fetal hemolytic anemia and immune hydrops can therefore be helpful in the understanding of many other fetal diseases and might improve different types of fetal therapy.

Outline of the thesis

The studies described in this thesis explore fetal pathophysiology in hemolytic anemia and immune hydrops fetalis. Measurements performed in fetal blood as well as in amniotic fluid, before or during intrauterine transfusion, where used for our research.

The studies in this thesis can be summarized as follows:

The mechanism behind the curve of the so-called Liley chart has never been fully understood. In **chapter two**, we investigated the relation between bilirubin concentration in fetal blood and that in amniotic fluid. We hypothesized on the most plausible pathway for bilirubin to enter and leave the amniotic fluid.

In **chapter three**, we tested the hypothesis that the concentration of bilirubin is determined by the binding to albumin. Thereby we tried to explain the relation between fetal anemia and the Liley chart. This led to the next question: how does albumin enter and leave the amniotic fluid?

In **chapter four**, we reviewed the available evidence on the origin of albumin in amniotic fluid and the transport mechanisms that determine amniotic fluid composition. We speculate on the function of albumin in amniotic fluid and propose directions for future research and development of new fetal therapy strategies.

In **chapter five**, we investigated whether low albumin concentration was a causative or secondary effect in the development of hydrops fetalis. Concentration of albumin in fetal blood was analyzed to assess the relation with severity of anemia and severity of hydrops.

Fetal cardiovascular physiology may be distinct from adults and even from neonates. In **chapter six**, the maintenance of blood volume was investigated. The effect of severity of anemia and the presence of hydrops on total fetoplacental blood volume were analyzed.

In **chapter seven**, we investigated the extravascular fluid shift that takes place from the fetal circulation during intrauterine transfusion. The effect of volume and speed of transfusion, and the severity of anemia and presence of hydrops were analyzed.

Purpose of this thesis was to gain insight in human fetal (patho-)physiology. In **chapter eight**, old, current en acquired knowledge of fetal fluid and protein dynamics is described. Furthermore, implications for current practice that followed from our studies are discussed and implications for future research are proposed.

Finally, **chapter nine** summarizes the results of the presented studies.

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2

On the Origin of Amniotic Fluid Bilirubin

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Humphrey H. H. Kanhai, Frank P. H. A. Vandenbussche

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 [1]. Bilirubin concentration is about four times higher in fetal than in maternal blood [2,3]. As a result of this concentration gradient, the unconjugated (liposoluble) bilirubin diffuses through trophoblastic layers from fetal to maternal blood [4]. It is unclear whether active or passive carrier-mediated transport mechanisms play an additional role in placental transfer [5]. Glucuronyl transferase activity in the fetal liver is minimal, less than 1 per cent of its activity in neonatal and later life, and only a minor fraction of fetal bilirubin is conjugated [3,6]. 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 [7]. Unconjugated (hydrophobic) bilirubin in fetal and maternal blood is linked to albumin almost completely, and only a minute fraction is free [8].

Some of the fetal bilirubin is excreted into the amniotic fluid compartment, and less than 10 per cent of this amniotic fluid bilirubin is conjugated [9]. Each day, the fetus swallows about 75 per cent of the amniotic fluid volume [10]. Amniotic fluid bilirubin concentration is an important diagnostic tool in the management of blood group alloimmunization [11]. 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 per cent of amniotic fluid [10]. 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 [12]. 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 keratinization [13–15]. 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 [16]. A fifth possible pathway is the intramembranous pathway [17]. The fetal surface of the placenta is well vascularized and probably plays an important role in the volume regulation and composition of amniotic fluid [18]. 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 [7,17].

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 centre for the treatment of fetal anemia in the Netherlands. Our methods for diagnosis and treatment of severe fetal alloimmune anemia have been described previously [19]. 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, nonhydropic, and not previously transfused fetuses. Amniotic fluid samples had to have been taken less than 4 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 1000 g for 10 min 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 delta OD450, 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 [11].

Normal total bilirubin concentrations in fetal blood increase during gestation. We used the reference values proposed by Nava et al. [3], which were derived from a large number of normal fetuses undergoing percutaneous umbilical blood sampling between 18 and 39 weeks [3]. Normal bilirubin concentrations in amniotic fluid decrease during gestation. We used the reference values proposed by Nicolaides et al. [20]; these were derived from a large number of amniocenteses in normal pregnancies, equally distributed between 16 and 37 weeks [20]. A factor of 1.585 was used to convert all delta OD450 values to bilirubin concentrations (mg/dl) [21,22].

Normal concentrations of albumin in amniotic fluid and fetal blood were based on the literature [23,24].

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 SD below the normal mean) and 60 were severely anemic (hemoglobin concentration more than 5 SD 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 per cent 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 [11,25].

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 [3,20,23,24], and showed a similar pattern of decrease as pregnancy progressed.

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 SD) [37]*. The lower line separates moderate (between -2 and -5 SD) from severe (less than -5 SD) fetal anemia.

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 SD) total bilirubin concentration in fetal blood [3]*.

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 SD) bilirubin in amniotic fluid [20]*.

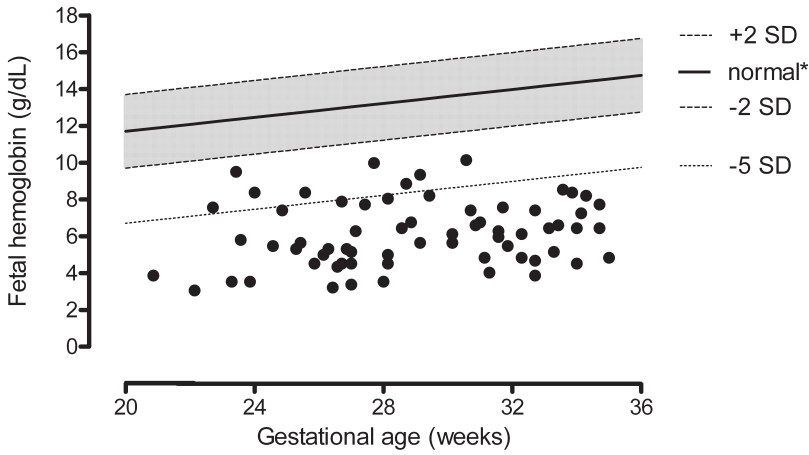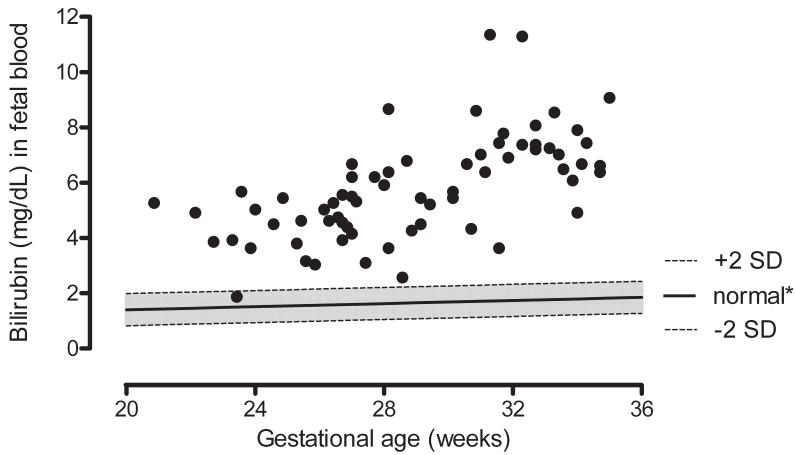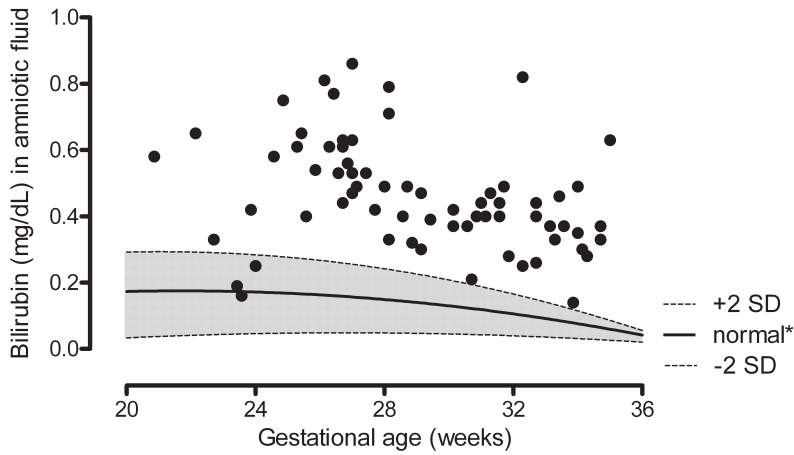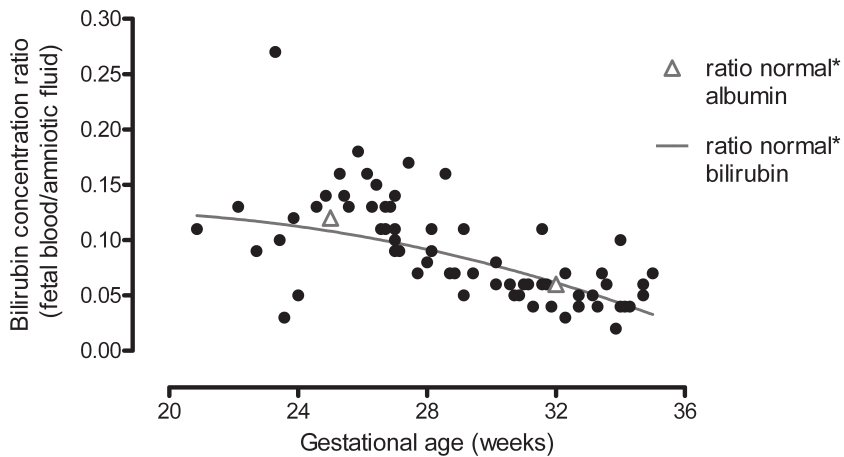**Figure 4** Ratio between amniotic fluid and fetal blood concentrations of total bilirubin in 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey line marks the ratio between normal bilirubin concentrations in amniotic fluid and fetal blood [3,20]*. The grey open triangles mark the ratio between normal albumin concentrations in amniotic fluid and fetal blood [23,24]*.

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 [26]. 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 [27,28]. 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 [3,20]. 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 [1]. Unconjugated bilirubin is hydrophobic and in aqueous solutions linked to albumin almost completely [1]. Transfer of bilirubin between body compartments, however, is due to diffusion of albumin-free unconjugated bilirubin [4]. 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 [4]. As early as 1970, Cherry et al. proposed a strong experimental argument for this theory, measuring delta OD450 before and 12 h after the injection of albumin in the amniotic fluid compartment in 3 alloimmunized pregnancies [28]. They found a highly significant linear relationship between delta OD450 and albumin concentration. In 1967, Cherry and Rosenfield had already suggested that bilirubin/

protein ratios in amniotic fluid could replace plotting delta OD450 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 [29]. 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 [30,31]. 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 [30,32–34]. The protein concentration in amniotic fluid, on the other hand, is only 10 to 20 times lower than in fetal plasma [23,24,27,31]. 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 keratinization begins at approximately 17 weeks and a complete stratum corneum is present by approximately 25 weeks [35]. 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 [15]. The fetal membranes, on the other hand, retain a high permeability

until term [36]. 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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Addendum

Hydropic cases

The study results as presented in chapter 2, are shown here with an addition of 21 hydropic fetuses. We present 15 mildly hydropic fetuses (semi open dots) and 6 severely hydropic fetuses (open dots). Fetuses were classified as mildly hydropic in case a distinct rim of ascites and/or pericardial effusion is observed. Fetuses were classified as severely hydropic in case an abundant amount of fluid collection or skin edema is observed.

In figure 1 it is shown that the bilirubin concentration in fetal blood usually is increased in anemic fetuses. However, the severely hydropic fetuses have a relatively low concentration of bilirubin in fetal blood, in some cases within or even below the normal range. Figure 2 shows the increase in bilirubin concentration in amniotic fluid in anemic fetuses. There is no difference observed between nonhydropic and hydropic fetuses. Figure 3 shows the ratio of the bilirubin concentration in fetal blood to that in amniotic fluid. A large increase is observed in most severely hydropic fetuses, compared to non- and mildly hydropic fetuses.

The low concentration of bilirubin in fetal blood in severely hydropic fetuses could be explained either by a diminished hematopoiesis or by a diminished concentration and/or binding capacity of albumin in fetal blood. It is intriguing that mildly hydropic fetuses seemed not to differ from nonhydropic fetuses, though severely hydropic fetuses show distinct differences.

In conclusion, even though the concentration of bilirubin is relatively low in fetal blood in most severely hydropic cases, the bilirubin extinction plotted in Queenan's or Liley's chart still would predict the presence of severe anemia. A shift in albumin concentration (low in fetal blood and high in amniotic fluid) or a change in albumin binding capacity could explain the increase in the ratio of bilirubin in fetal blood to that in amniotic fluid, in severely hydropic fetuses.
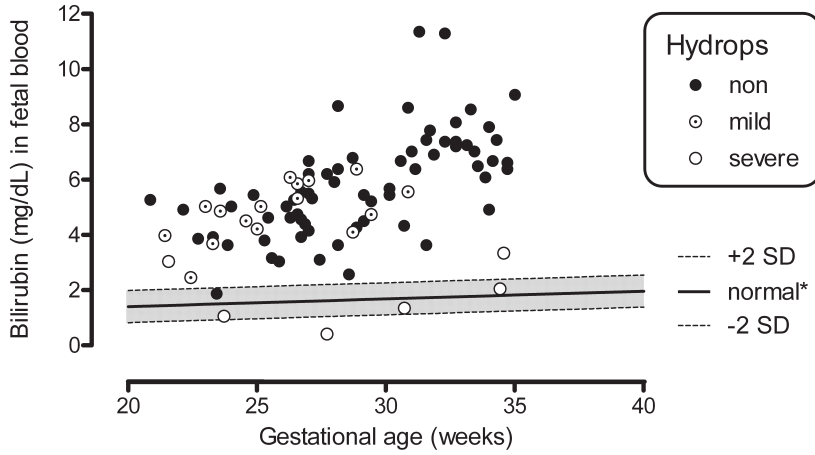
Figure 1 Bilirubin concentration in fetal blood as a function of gestational age. Normal values are shown [1]*. Non-, mildly and severely hydropic fetuses are depicted.
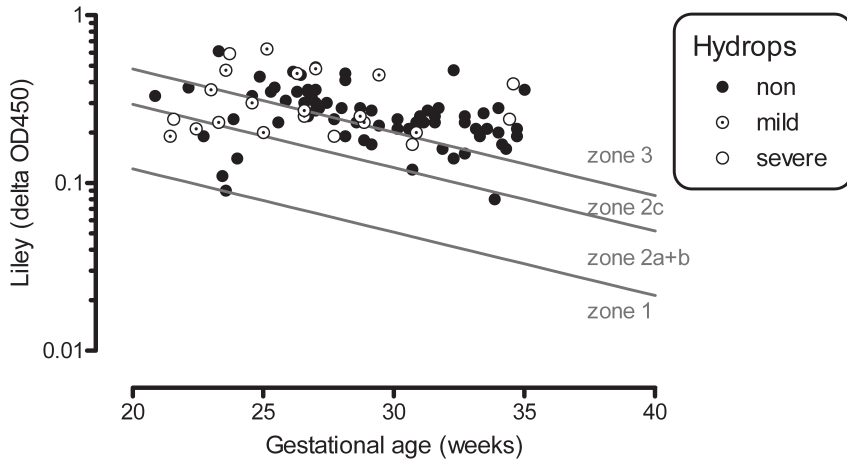
Figure 2 Amniotic fluid ΔOD450 (bilirubin extinction) as a function of gestational age. Cut-off values of the (linearly extended) Liley chart are shown. Non-, mildly and severely hydropic fetuses are depicted.
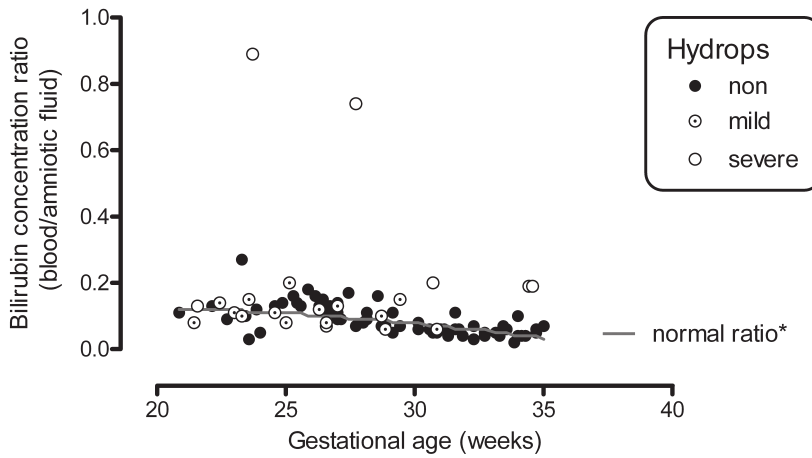
Figure 3 The ratio of the bilirubin concentration in fetal blood to that in amniotic fluid as a function of gestational age. The grey line shows the normal ratio* based on reference values of bilirubin in fetal blood [1] and in amniotic fluid [2]. Non-, mildly and severely hydropic fetuses are depicted.

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3

Bilirubin/Albumin Ratios in Fetal Blood and in Amniotic Fluid in Rhesus Immunization

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Abstract

Objective
To test the hypothesis that unconjugated bilirubin is equally distributed over the albumin molecules present in fetal blood and amniotic fluid in Rhesus (Rh) immunization.

Methods
Molar concentrations of unconjugated bilirubin and albumin were measured in fetal blood and amniotic fluid samples, obtained before the first intrauterine transfusion in 30 nonhydropic, anti-D–alloimmunized fetuses, with gestational ages ranging from 20 to 35 weeks.

Results
Bilirubin concentration in amniotic fluid was best predicted by a combination of bilirubin concentration in fetal blood (p<.001), albumin concentration in fetal blood (p=.008), and albumin concentration in amniotic fluid (p<.001) (adjusted R^2=0.91). The bilirubin/albumin ratios in fetal blood were linearly correlated with the bilirubin/albumin ratios in amniotic fluid (R^2=0.75, p<.001). However, the bilirubin/albumin ratios in fetal blood were always higher than the bilirubin/albumin ratios in amniotic fluid (regression coefficient 1.4, 95% confidence interval 1.1–1.7). In our population, a bilirubin/albumin ratio in amniotic fluid of 0.10 or greater had a better sensitivity and specificity to predict severe anemia (Z-hemoglobin –5 standard deviations or less) than the Queenan 4 or the Liley 2c line.

Conclusion
The relation between fetal hemolysis and amniotic fluid bilirubin concentration is based on the linear correlation between bilirubin/albumin ratios in fetal blood and in amniotic fluid. The slope in Queenan's and Liley's chart follows that of the albumin concentration in amniotic fluid during gestation.

Introduction

Bilirubin is the degradation product of hemoglobin. Its main configuration in the fetus is unconjugated [1]. Unconjugated bilirubin is hydrophobic and tightly but reversibly bound to albumin in extracellular fluids [2]. In fetal blood the albumin concentration increases between 20 and 35 weeks of gestation [3]. In amniotic fluid the albumin concentration initially increases between 20 and 24 weeks, but then decreases between 25 and 35 weeks [4,5]. Unconjugated bilirubin is cleared from fetal blood over the placenta to maternal blood [6]. Conjugation of bilirubin and excretion through the gall bladder or the kidneys are usually not triggered until a few days after birth [7,8]. The small amount of conjugated bilirubin that is formed prenatally is probably converted to unconjugated bilirubin in the fetal intestines and reabsorbed in the fetal circulation [9]. Thus, most of the bilirubin in the fetus and in amniotic fluid is unconjugated and bound to albumin.

Since the early 1960s, measurement of the concentration of bilirubin in amniotic fluid has been used to predict the severity of fetal hemolytic anemia and to decide on the necessity of intrauterine red cell transfusion [10,11]. Recently, noninvasive Doppler studies have been introduced to predict fetal anemia [12]. Nevertheless, the Queenan chart or the Liley chart still are important diagnostic tools in determining the timing of the first intrauterine red cell transfusion because these tests have a high sensitivity in this respect [13]. The mechanisms behind these diagnostic tools, however, have not been completely unraveled. Yet, understanding the pathways that bilirubin takes to distribute to the fetal compartments could lead to a better comprehension of the pathophysiology of fetal hemolytic disease, which may further improve our management of fetal anemia and neonatal hyperbilirubinemia caused by alloimmunization.

We hypothesized that unconjugated bilirubin is equally distributed over the albumin molecules that are present in all fetal compartments, including amniotic fluid, before it is transported across the placenta toward the maternal blood. Therefore, we measured the molar concentration ratios of bilirubin to albumin in fetal blood and in amniotic fluid and investigated their correlation. We expected that, if our hypothesis were true, there would be a significant linear relation between the two ratios. Furthermore, assuming that there would be no difference in binding capacity of albumin for bilirubin in the different compartments, the regression coefficient of this relation would be 1.

3

Materials and Methods

Leiden University Medical Centre 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 [14]. From January 2001 to December 2004, we simultaneously sampled blood and amniotic fluid of singleton, nonhydropic, not previously transfused fetuses suffering from severe Rhesus D alloimmunization with gestational ages ranging from 20 to 35 weeks gestation. None of the fetuses had chromosomal or congenital abnormalities. Bilirubin and albumin concentrations were measured in fetal blood samples taken before the first intrauterine transfusion and in amniotic fluid samples taken within two days before commencing intrauterine transfusion. No additional amniocenteses or cordocentesis were performed to collect the data. This study was an addition to the "Diagnostic amniocentesis or non-invasive Doppler for the diagnosis of severe fetal anemia" study [12], that was approved by the medical ethics committee of the Leiden University Medical Center, and for which all woman gave oral or written informed consent.

Fetal blood samples were sent to our diagnostic laboratories. In our routine clinical chemistry laboratory measurements were made of total bilirubin, conjugated bilirubin and albumin on Oya Hitachi p800 modular autoanalyzer (Roche, Mannheim, Germany). Also, hemoglobin was measured in our routine hematology laboratory on Sysmex XE 2100 (Sysmex, Kobe, Japan). In fetal blood, conjugated bilirubin was subtracted from total bilirubin to calculate the concentration of unconjugated bilirubin. These measurements are reported in micromolars per liter. Albumin was converted from grams per liter to micromolars per liter by multiplying by a factor of 14.4 [15]. Amniotic fluid was stored light protected, and delta OD450 was measured within 1 hour after sampling, as published before [13]. It has been shown that this method measures merely unconjugated bilirubin [16]. The concentration of bilirubin in micromolars per liter was established by multiplying the delta OD450 value by a factor of 27.1 [17]. The concentration of albumin in amniotic fluid was measured by using a turbidimetric method on a Cobas Integra 800 autoanalyzer (Roche, Mannheim, Germany). This analysis took place at the section of liquor cerebri analysis in the Department of Clinical Chemistry.

Standardized Z scores of hemoglobin (Z-hemoglobin) were defined as the number of standard deviations (SDs) that an actual value deviated from the normal mean for gestational age. Reference values for hemoglobin were derived from the literature

[18]. Pearson correlation coefficients were calculated to study relations between different variables, since data were normally distributed. Normality was tested by the Kolmogorov-Smirnov test. We considered p<.05 to be significant. The statistical software program SPSS 12.0.1 (SPSS Inc., Chicago, IL) was used. The form of the relation between the concentration of albumin in amniotic fluid and gestation was studied with polynomial regression. The ratio of bilirubin concentration to albumin concentration was expressed as a fraction (mol/mol). The relation between the bilirubin/albumin ratio in fetal blood and severity of anemia and gestational age was studied with linear regression. The same was done for the bilirubin/albumin ratio in amniotic fluid. After that, linear regression was performed to study the relation between the bilirubin/albumin ratio in fetal blood and the bilirubin/albumin ratio in amniotic fluid. Because in clinical practice the bilirubin concentration in amniotic fluid is used as a predictor for the amount of hemolysis in the fetal blood, the bilirubin/albumin ratio in fetal blood was chosen as the dependent variable and the bilirubin/albumin ratio in amniotic fluid as the independent variable. To study the additional influence of gestational age and severity of anemia, a multivariable linear regression analysis was performed with the bilirubin/albumin ratio in fetal blood as dependent variable and the bilirubin/albumin ratio in amniotic fluid, gestational age, and Z-hemoglobin as independent variables. Because bilirubin originates in fetal blood and subsequently enters the amniotic fluid, we also performed a multivariable linear regression with bilirubin concentration in amniotic fluid as dependent variable and bilirubin concentration in fetal blood, albumin concentration in amniotic fluid, and albumin concentration in fetal blood as independent variables. Finally, a receiver operating characteristic curve was made to determine the optimal cutoff value of the bilirubin/albumin ratio in amniotic fluid to predict severe anemia. Fetuses were considered severely anemic at Z-hemoglobin of –5 SD or less. The cutoff was considered optimal when the sum of the sensitivity and specificity was maximal. The sensitivity and specificity of the chosen cutoff value was then compared with the sensitivity and specificity of the cutoff line 4 in the Queenan chart and the cutoff line 2c in the extended Liley chart [12].

Results

In the study period, 89 Rhesus D–immunized fetuses received their first intrauterine transfusion. Simultaneous sampling of amniotic fluid and fetal blood was performed in 30 singleton, nonhydropic fetuses. Maternal and fetal characteristics are shown in Table 1.

Table 1 Maternal and fetal characteristics

Characteristics	Mean (Range)
Maternal age (years)	31.5 (20 – 41)
Gravidity	3.3 (1 – 8)
Parity	1.6 (0 – 4)
Last determined antibody titer	1:64 – 1:8000
Last determined ADCC* (%) [19]	55 to more than 80
Gestational age at transfusion (weeks)	29.8 (20 – 35)
Hematocrit at transfusion	0.23 (0.09 – 0.32)
Hemoglobin at transfusion (g/dL)	7.3 (2.6 – 10.8)

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity assay.

Figure 1 shows the albumin concentration (grams per liter) in amniotic fluid during gestation. A cubic regression line fitted the data best (adjusted R^2 linear 0.29, adjusted R^2 quadratic 0.39, adjusted R^2 cubic 0.44). An increase in albumin concentration between 20 and 24 weeks of gestation and a decrease between 25 and 35 weeks of gestation was observed. However, the interindividual variance was large.
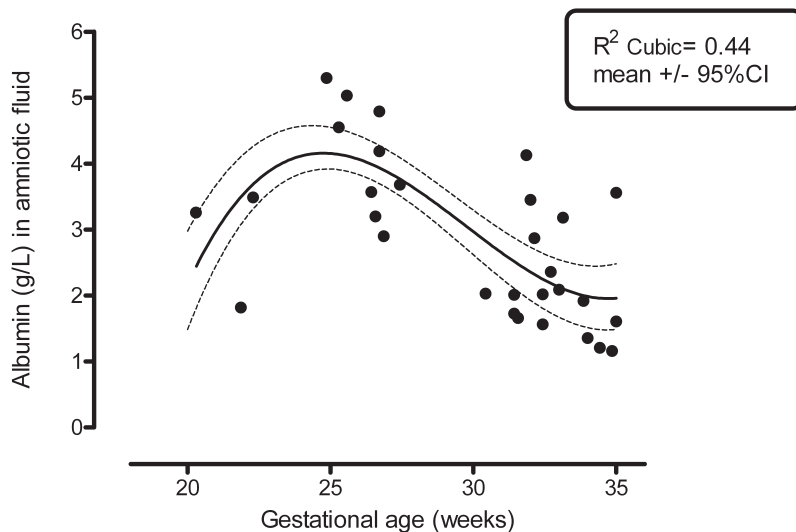
Figure 1 Concentration of albumin (g/L) in amniotic fluid as a function of gestational age (weeks). Mean and its 95%CI are plotted.

In fetal blood, the bilirubin/albumin ratio ranged from 0.12 to 0.35. Figure 2 shows the bilirubin/albumin ratio in fetal blood plotted against standardized hemoglobin concentrations (Z scores). There was a significant correlation of the bilirubin/albumin ratio in fetal blood with the severity of anemia (R^2 0.23, p=.007). There was no relation between the bilirubin/albumin ratio in fetal blood and gestational age (R^2 0.00, p=.92).

In amniotic fluid, the bilirubin/albumin ratio ranged from 0.07 to 0.19. Figure 3 shows the bilirubin/albumin ratio in amniotic fluid plotted against standardized hemoglobin concentrations (Z scores). There was a significant correlation of the bilirubin/albumin ratio in amniotic fluid with the severity of anemia (R^2 0.37, p<.001). There was no relation between the bilirubin/albumin ratio in amniotic fluid and gestational age (R^2 0.004, p=.74).

Figure 4 shows the linear relation between the bilirubin/albumin ratio in fetal blood and the bilirubin/albumin ratio in amniotic fluid (R^2 0.75, p<.001). Notably, the bilirubin/albumin ratios in fetal blood were always higher than the bilirubin/albumin ratios in amniotic fluid.

Figure 2 Bilirubin/albumin molar concentration ratio in fetal blood against standardized hemoglobin concentration (Z scores). Mean and its 95%CI are plotted.
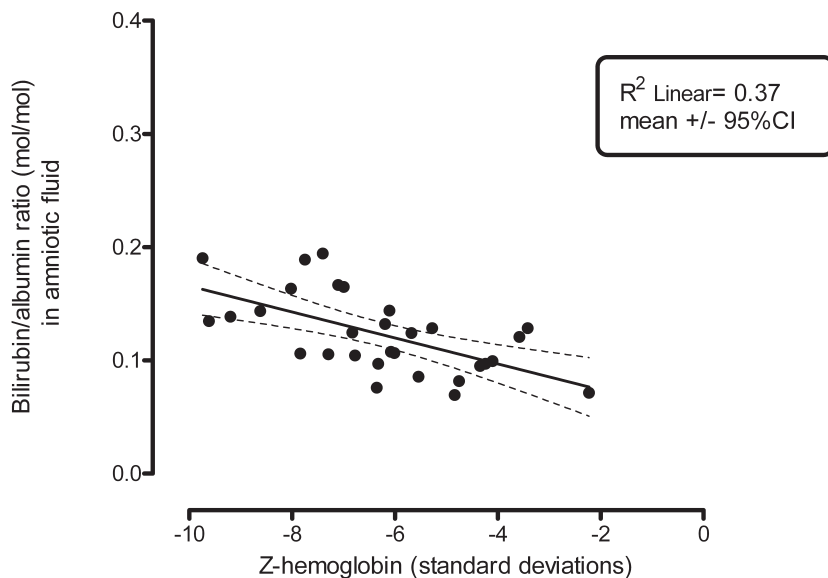
Figure 3 Bilirubin/albumin molar concentration ratio in amniotic fluid against standardized hemoglobin concentration (Z scores). Mean and its 95%CI are plotted.
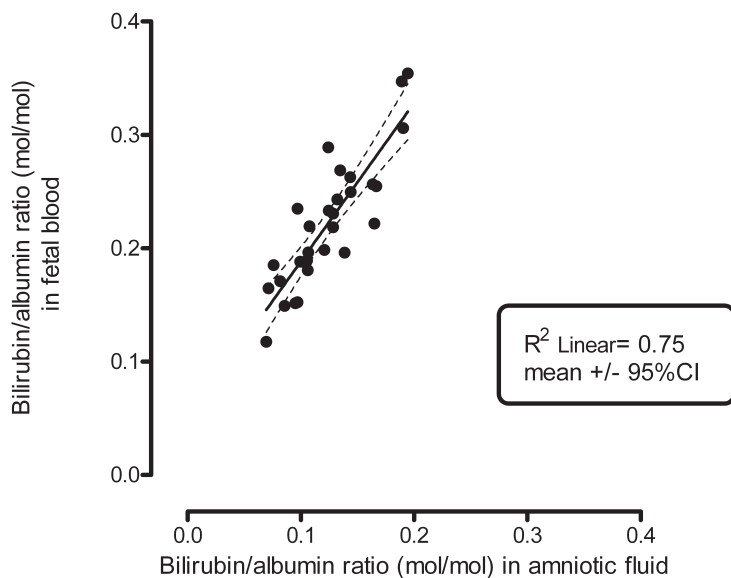
Figure 4 Bilirubin/albumin molar concentration ratio in fetal blood against bilirubin/albumin molar concentration ratio in amniotic fluid. Mean and its 95%CI are plotted.

The formula of the regression line shown in figure 4 was as follows: mean bilirubin/albumin ratio in fetal blood=0.05+1.4×mean bilirubin/albumin ratio in amniotic fluid (95% confidence interval of the constant is 0.01–0.09; 95% confidence interval of the regression coefficient is 1.1–1.7). To study the additional influence of gestational age and severity of anemia, a multivariable linear regression analysis was performed. This s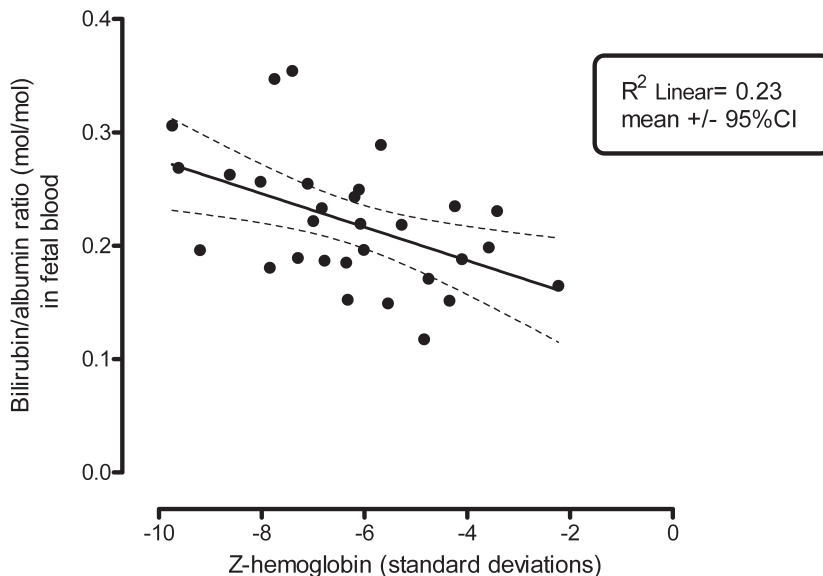howed that the bilirubin/albumin ratio in amniotic fluid was still significantly related to the bilirubin/albumin ratio in fetal blood (regression coefficient=1.5, p<.001), while there was no significant influence of gestational age (regression coefficient=0.00, p=.72) and severity of anemia (Z-hemoglobin) (regression coefficient=0.002, p=.59).

Because bilirubin originates in fetal blood and subsequently enters the amniotic fluid, we also performed a multivariable linear regression with bilirubin concentration in amniotic fluid as the dependent variable. This showed that bilirubin concentration in fetal blood (p<.001), albumin concentration in fetal blood (p=.008), and albumin concentration in amniotic fluid (p<.001) were all independently related to the bilirubin concentration in amniotic fluid. The adjusted R^2 of this model was 0.91.

In the receiver operating characteristic curve (ROC curve, not shown), we found that 0.10 was the optimal cutoff value for the bilirubin/albumin ratio in amniotic fluid to predict severe anemia. Table 2 shows the comparison between sensitivities and specificities in our study population of this chosen cutoff value and commonly used cutoffs in the Queenan and extended Liley charts.

Table 2 Test characteristics of the bilirubin/albumin ratio and Queenan and extended Liley charts to diagnose severe anemia*

	Cutoff	**Sensitivity (%)**	**Specificity (%)**
Bilirubin/ albumin molar ratio	0.10 or greater	86	75
Queenan chart	4 line or greater	82	25
Extended Liley chart	2c line or greater	72	25

* Severe anemia is defined as Z-hemoglobin of -5 standard deviations or less

Discussion

In this study, we found a strong linear correlation between the bilirubin/albumin molar concentration ratio in fetal blood and this same ratio in amniotic fluid. This is a strong argument in favor of our hypothesis that bilirubin is distributed over the available albumin in fetal blood and amniotic fluid. However, in contrast with our hypothesis, bilirubin is not distributed in an equal manner over the albumin in these fetal compartments because the bilirubin/albumin ratio in fetal blood was always higher than the bilirubin/albumin ratio in amniotic fluid.

Our curve of the mean amniotic fluid albumin concentration during pregnancy confirms previously published data [4,5]. It is also very similar to the cutoff lines of amniotic fluid bilirubin concentration during gestation in the Queenan and Liley charts [10,11]. This similarity is readily explained by the fact that, in amniotic fluid, bilirubin is bound to albumin. Our data are in support of findings by Queenan et al. [11] that linearly extending the Liley graph below 22 weeks is not to be advised.

The range of the bilirubin/albumin ratio of 0.12–0.35 that we observed in fetal blood was similar to findings of Ritter et al. [20]. In neonates, approximately 30% binding of bilirubin to albumin in blood was found [20]. We observed no correlation between the bilirubin/albumin ratios and gestational age. Robertson et al. [21] studied albumin reserve binding capacity for bilirubin in umbilical cord serum and also found no difference between 18 to 42 weeks of gestation.

It is known that there is one strong binding site on albumin for bilirubin and several weaker binding sites [2]. Our observed regression coefficient of 1.4 of the regression line between the bilirubin/albumin ratios in fetal blood and amniotic fluid may be explained by a difference in biochemical qualities between blood and amniotic fluid, which influence the binding force of one or more binding sites on albumin. Another possible cause for the fact that the bilirubin/albumin ratio in fetal blood was always higher than the bilirubin/albumin ratio in amniotic fluid could be a difference in competitive binding. Either way, the linear relation between the ratios seems to be caused by a constant difference in property between fetal blood and amniotic fluid. We speculate that a difference in pH could explain the observed regression coefficient of 1.4 between the ratios in fetal blood and amniotic fluid. In vitro experiments have shown that 1 mol albumin in serum binds 1.9 mol bilirubin at a pH of 7.4. With a decline in pH, albumin will bind less bilirubin [22]. The difference in pH between fetal blood and amniotic fluid (respective means of 7.3 and 7.1 in alloimmunized fetuses

[23]) could thus be the cause of the observed difference in binding capacity of albumin for bilirubin.

Our results contain strong arguments in favor of the theory that amniotic fluid bilirubin concentration is determined both by the bilirubin concentration in fetal blood and by the albumin concentrations in fetal blood and amniotic fluid. Although urine is the principal source of amniotic fluid, it is unlikely that the fetal kidneys are the pathway over which bilirubin can enter the amniotic fluid because the concentration of protein is 100 times lower in fetal urine than in amniotic fluid. The most likely pathway over which bilirubin can constantly be balanced out over the available albumin, therefore, seems to be the intramembraneous pathway. The intramembraneous pathway is the combined permeable surface that is adjacent to the amniotic fluid. Initially, the fetal skin and mucous membranes are an important component of this pathway, and after keratinization of the skin, which occurs between 17 and 25 weeks of gestation, the main component that remains is the fetal side of the placenta [24]. Knowledge on the origin of albumin in amniotic fluid could complete our understanding of this fetal physiological mechanism.

Already in 1965, Cherry et al. [25] investigated the correlation of the bilirubin/protein ratio in amniotic fluid with the severity of anemia. Sensitivities to predict anemia with this ratio were, however, variable [26-28]. The diversity of the methods of measurements may explain some of these variable results. Furthermore, false-negative prediction was reported in fetuses that turned out to be hydropic [29]. Nowadays, delta OD450 will not be used clinically, in an alloimmunized patient, when hydrops is identified sonographically.

Our findings do have clinical implications. First, understanding the background of a diagnostic test gives one the opportunity to understand exceptional cases. In an anemic fetus with an abnormal concentration of albumin in the amniotic fluid—for example, due to kidney disease, hydramnios, hydrops, or growth restriction—an unexpected result in the Queenan or Liley charts may be found. In an anemic fetus with a low bilirubin concentration in fetal blood—for example, in Kell immunization—bilirubin concentration in amniotic fluid may also be lower then expected [30]. Second, we observed a significant correlation of the bilirubin/albumin ratio, both in fetal blood and amniotic fluid, with severity of anemia. Theoretically, the reliability of the Queenan or Liley charts in predicting the degree of hemolysis should be impaired by the wide interindividual variation of amniotic fluid albumin concentration. Using the bilirubin/albumin ratio in amniotic fluid may, therefore, improve our ability to predict the severity

of fetal anemia. Also, the bilirubin/albumin ratio does not change during gestation, making the test easy to interpret. In our study population, the bilirubin/albumin ratio was a more accurate test for diagnosing severe anemia than the Queenan and Liley charts. However, the sensitivities and specificities of the Queenan and Liley charts in our study were lower than in other studies [12,13]. The suggested cutoff value of the bilirubin/albumin ratio of 0.10 should be validated in an independent data set, preferably a prospective cohort.

In conclusion, amniotic fluid bilirubin concentration is determined by both the bilirubin concentration in fetal blood and by the albumin concentrations in fetal blood and in amniotic fluid. The relation between fetal hemolysis and amniotic fluid bilirubin concentration is based on the linear correlation between bilirubin/albumin ratios in fetal blood and in amniotic fluid. The slope in Queenan's and Liley's charts follows that of the albumin concentration in amniotic fluid during gestation.

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3

Addendum

Hydropic cases

The study results as presented in chapter 3, are shown here with an addition of 13 hydropic fetuses. We present 10 mildly hydropic fetuses (semi filled dots) and 3 severely hydropic fetuses (open dots). Fetuses were classified as mildly hydropic in case a distinct rim of ascites and/or pericardial effusion is observed. Fetuses were classified as severely hydropic in case an abundant amount of fluid collection with skin edema is observed.

In figure 1 it is shown that the bilirubin to albumin ratio (BAR) in fetal blood increases with increasing severity of anemia. However, the severely hydropic fetuses have a relatively low BAR in fetal blood. Figure 2 shows the increase in BAR in the amniotic fluid with severity of anemia. There is no difference observed between nonhydropic and hydropic fetuses. Figure 3 shows the relation of the BAR in fetal blood to the BAR in amniotic fluid. In nonhydropic and mildly hydropic fetuses, the BAR in fetal blood is always higher compared to that in amniotic fluid. Interestingly though, in the severely hydropic fetuses the BAR in fetal blood was almost the same as the BAR in amniotic fluid.

The low BAR in fetal blood in severely hydropic cases could be explained by either a diminished hematopoiesis or a dimished binding capacity of albumin in fetal blood. Again (as in the addendum of chapter 2), the mildly hydropic fetuses seemed not to differ from the nonhydropic fetuses, though severely hydropic fetuses show distinct differences.

In conclusion, it is possible that there is a decrease in binding capacity of albumin for bilirubin in fetal blood in severely hydropic fetuses. This might be the result of a change of fetal blood pH, a change in albumin posttranslational modifications or a change in competitive binding of other ligands to albumin. In addition, it is possible that there is an increase in albumin binding capacity in amniotic fluid. It is intriguing to consider the rise of protein in amniotic fluid, which has been reported in hydropic cases, to be the result of a capillary leakage of fetal albumin into the amniotic fluid.
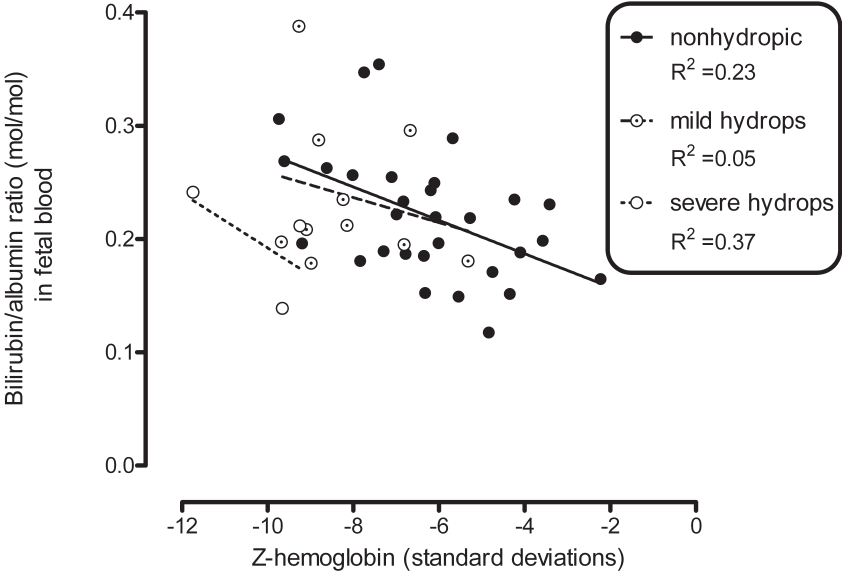
Figure 1 Bilirubin to albumin ratio (mol/mol) in fetal blood in relation to severity of anemia (Z-hemoglobin). Non-, mildly and severely hydropic fetuses are depicted.
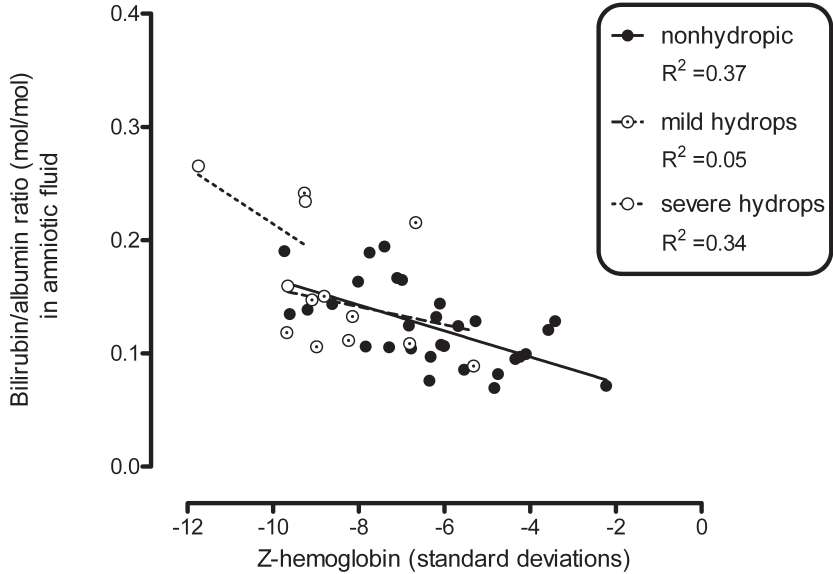
Figure 2 Bilirubin to albumin ratio (mol/mol) in amniotic fluid in relation to severity of anemia (Z-hemoglobin). Non-, mildly and severely hydropic fetuses are depicted.
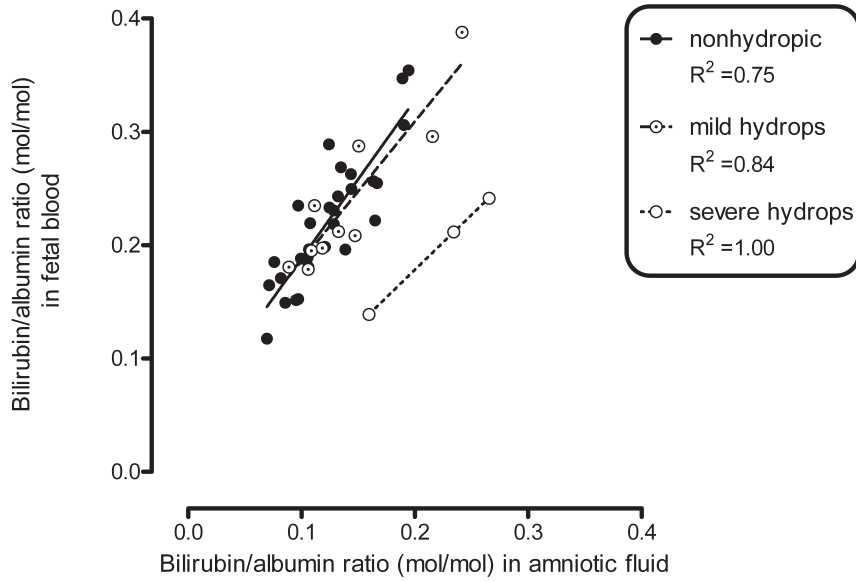
Figure 3 Bilirubin to albumin ratio (mol/mol) in fetal blood in relation to that in amniotic fluid. Non-, mildly and severely hydropic fetuses are depicted.

4

Origin and function of amniotic fluid albumin: a review of the available evidence

Suzanne A. Pasman, Emile de Heer, Frank F.P.H. Vandenbussche

Abstract

Little is known about the origin of albumin in amniotic fluid. A fetal origin is questionable, because of the low concentration of protein in fetal urine and lung fluid. A maternal origin of proteins in amniotic fluid was already proposed in the 1970s. This review focuses on the possible sources of albumin in amniotic fluid and on the pathways for albumin to enter and leave the amniotic fluid in the second and third trimester. Based on the available evidence, it is unlikely that the fetus makes a large contribution to amniotic fluid albumin. Maternal albumin probably reaches the amniotic fluid through the fetal membranes, though the amniotic membrane itself also produces albumin. The functions of albumin in amniotic fluid are even less understood. Albumin may influence the homeostasis of amniotic fluid volume. Furthermore, intake of albumin, and the fatty acids which albumin carries, could be a substantial part of fetal nourishment. More knowledge on the mechanisms that determine amniotic fluid composition and on the function of albumin in amniotic fluid could provide the basis for new fetal therapy strategies.

Introduction

Although albumin is the most prevalent protein in amniotic fluid [18;25;26], its origin in amniotic fluid remains unclear. Most clinicians in the field of fetal and maternal medicine assume that albumin in the amniotic fluid is of fetal origin, though this assumption has never been established by research. While the amniotic fluid is mainly formed of fetal urine and lung fluid [27], the concentration of protein in fetal urine and lung fluid is many times lower than that in amniotic fluid (Table 1). This suggests that the albumin in amniotic fluid has a different origin, most likely a maternal source. It was already proposed in the 1970s that albumin in amniotic fluid is transported from maternal blood to amniotic fluid without passing through the fetal blood [28]. Using polymorphisms, multiple larger proteins that are found in the amniotic fluid, such as transferrin and alpha1-antitrypsin, have been shown to have a maternal origin [29;30]. Finally, the placenta and even the membranes cannot be ignored, and should be considered as possible sources of albumin in amniotic fluid.

The functions served by albumin in amniotic fluid are even more elusive. Albumin is a protein that hardly shows any differences between species, underlining its importance to sustain life. It surrounds embryo's in abundance, either when they are hatching from an egg or growing inside a uterus. It is the most prevalent serum protein with a crucial role in maintaining intravascular osmotic pressure and functioning as a carrier protein of numerous substances [31;32]. One might expect albumin to have a similar role in amniotic fluid as it has in the blood. This could imply it has an important role in amniotic fluid volume and pressure regulation. Albumin may also provide an important contribution to fetal nourishment. When considering the potential non-fetal origin of albumin, the amniotic fluid can be seen as a high-energy substance, providing prenatal feeding through the gastrointestinal tract. In other words, it could provide a kind of fetal "breast" feeding.

This review considers the current evidence on the origin of albumin in amniotic fluid in the second and third trimester of gestation. It will discuss the possible pathways from a fetal, a maternal and a placental origin, based on present literature. Due to the relative scarcity of human data, results from animal studies will be described where human evidence is absent. We will then speculate on the function of albumin in amniotic fluid, and will finally propose potential directions for future research.

4

Fetal blood as a possible source of amniotic fluid albumin

In the embryonic period, production of albumin starts in the yolk sac [33]. By the second and third trimester, albumin is probably mainly formed in the fetal liver [34-36]. There are a number of possible routes by which fetal serum albumin could be transferred to the amniotic fluid (see Figure 1).

Table 1 Normal values of total protein, albumin and AFP concentration in different compartments during 2nd and 3rd trimesters of human pregnancy.

	Total protein (g/L)[‡]	Albumin (g/L)[‡]	Changes during gestation of total protein and albumin:	AFP (g/L)[‡]	Changes during gestation of AFP:
Maternal serum	**65** [1;2]	**30** [1;3]	lower than in non-pregnant women	**0.00005 - 0.0001** [4-6]	increases until 32 weeks and then decreases
Fetal serum	**25 - 55** [7-10]	**20 - 35** [7-10]	increases between 20 and 35 weeks	**0.05 - 2** [4-6]	rapidly decreases from beginning of 2nd trimester
Fetal lung fluid	**[A] [0.006 - 0.4]** [11-13]	?	?	?	?
Fetal urine	**[B] [0.04 - 0.1]** [14;15]	**[C] [0.002 - 0.06]** [16-18]	?	?	?
Amniotic fluid	**3 - 6** [19-22]	**1.5 - 4** [21-24]	increases until 25 weeks, decreases until 35 weeks; can increase again after 38 weeks	**0.0001 - 0.03** [4-6]	rapidly decreases from beginning of 2nd trimester

[‡] Values are given as means. Two values indicate minimum and maximum means at different gestational ages. Two values within [ ] indicate the range found in different publications.
[A] tracheal fluid of fetal sheep
[B] fetal bladder punctions in obstructive uropathy
[C] first voided neonatal urine after birth

Urine

Urine is the main contributor of amniotic fluid, and as such it is the most obvious candidate for the source of amniotic fluid albumin. However, the concentration of total protein and albumin in fetal urine is 25 to 750 times lower than the concentration in amniotic fluid (Table 1). Table 1 shows the range of reported values for (mean) albumin concentration, taken from studies that measured albumin in first voided postnatal urine of premature and term neonates. The range of values reported for (mean) total protein concentration is also shown, obtained from human fetal bladder punctions in obstructive uropathy, in cases with good outcome after birth. Although these urine measurements were not obtained in healthy fetuses, it is more likely that they overestimate the actual values than underestimate them. The low concentration of protein in urine compared to that in amniotic fluid implies that either the amniotic fluid is highly concentrated, with water extracted on a daily basis, or that the albumin in the amniotic fluid has another source.
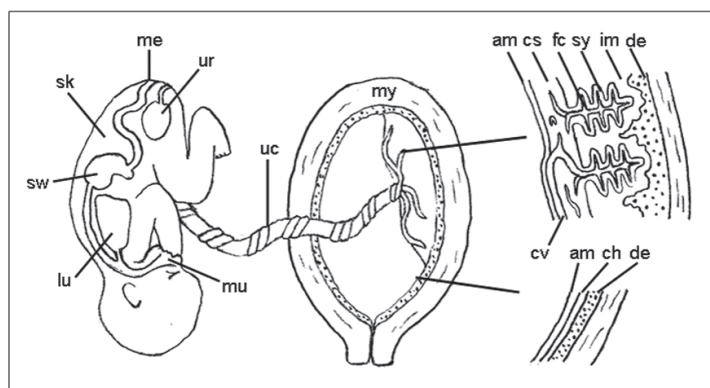
Figure 1 Possible pathways to and from human amniotic fluid in 2nd and 3rd trimester. Uterus is depicted, with fetus, placenta and fetal membranes in detail.

Fetus	**Uterus**	**Placenta**	**Fetal membranes**
lu= lung fluid	de= decidua	cs= chorion stroma	am= amniotic
me= meconium	my= myometrium	cv= chorial vessels	membrane
mu= mucous		fc= fetal capillaries	ch= chorionic
membranes		im= intervillous	membrane
sk= ski		maternal blood	
sw= swallowing		sy= syncytio-	
uc= umbilical cord		trophoblast	
ur= urine			

Intra-membraneous pathway: sk,mu,uc,cs/cv↔am
Trans-membraneous pathway: de↔ch↔am

Note that all pathways are bidirectional except fetal urine and meconium.

During the 3rd trimester of pregnancy, the amniotic fluid concentrations of several proteins decrease [18;22;23]. There are several explanations for this. In the case of proteins such as beta2-microglobulin (12 kD), which have a molecular weight lower than that of albumin (67 kD), it has been shown that the concentration in first voided urine after birth is similar to that in corresponding amniotic fluid [18]. The concentration of these proteins in amniotic fluid decreases during pregnancy [18;23]. This suggests that tubular resorption increases strongly from 24 weeks gestation onward [18;23]. The change in the concentration of low molecular weight proteins in amniotic fluid during gestation can thus be explained by renal development. In the case of albumin and proteins with higher molecular weight, it has been shown that albumin concentration in first voided urine after birth is much lower than in the corresponding amniotic fluid [18]. Burghard et al. found that the concentration of postnatal urinary albumin and high molecular weight proteins was only 5% and 15% of that in amniotic fluid [18]. Gitlin et al. estimated that amniotic fluid albumin was less than 5% of urinary origin at term [17]. They calculated that a term fetus would have to produce 8 litres of urine daily to explain the amount of albumin present in amniotic fluid. Urine production and clearance of water (and low molecular weight proteins) are clearly not in this order of magnitude [27;37]. A urinary origin of albumin combined with a strong daily extraction of water thus seems an implausible explanation for the concentration of amniotic fluid albumin. Furthermore, bulk amniotic fluid turnover increases strongly during gestation due to an increase in urine production during pregnancy [37], as well as an increase in the amount of fluid that is swallowed by the fetus [27]. This has led to the conclusion that the decrease of albumin concentration in amniotic fluid in the 3rd trimester is a result of a rise in bulk fluid turnover, which exceeds the inflow of albumin from a separate source [18;28]. This would indeed explain the decrease of amniotic fluid albumin between 25 and 35 weeks of gestation and the low concentration of albumin in urine compared to amniotic fluid. It seems that the observed changes in amniotic fluid albumin concentration during gestation can only be explained by a non-urinary origin of amniotic fluid albumin.

Another indicator that fetal urine is not the main source of amniotic fluid albumin is the ratio between AFP and albumin concentrations. AFP is a fetal protein with a molecular weight similar to that of albumin (AFP, 70 kD; albumin, 67 kD). In normal pregnancies, the fetal urine to fetal blood concentration ratio is roughly similar for AFP and albumin [38]. However, at term, as can be derived from Table 1, the amniotic fluid to fetal blood concentration ratio for AFP is 30 times smaller than that of albumin. This relatively high concentration of albumin, implies a mainly non-urinary source of amniotic fluid albumin. In pregnancies complicated by congenital nephrosis,

fetal proteinuria leads to markedly raised amniotic fluid AFP [39]. Amniotic fluid albumin, however, is only slightly elevated or even normal [40]. Thus, although it is probable that AFP in amniotic fluid originates mainly from fetal urine, it is likely that the albumin in amniotic fluid has another source.

Lung fluid

Lung fluid is the second largest contributor to amniotic fluid. It is estimated that around 15% of the daily production of amniotic fluid is attributable to lung fluid [27]. Although more lung fluid is produced, about half of it is immediately swallowed [41]. However, total protein concentration in fetal lung fluid is many times lower than in the amniotic fluid (Table 1). A number of experiments have been conducted using ovine fetuses to study fetal lung fluid production. When radioactively labelled albumin was injected, hardly any was found to pass from fetal blood to lung fluid [13]. Furthermore, it was shown that chloride is actively secreted by fetal lungs and, along with it, a fluid with a protein concentration much lower than that in fetal lung interstitial fluid [42;43]. Of course, care should be taken when interpreting these above mentioned findings, as they were all derived from studies in ovine fetuses. However, current knowledge of human fetal lung physiology shows similar results to those in ovine studies. For example, using pulmonary Doppler ultrasonography, human fetal lung fluid outflow was shown to increase until 36 weeks of gestation [44], similar to measurements in ovine fetuses [45]. To our knowledge, no data exists on the concentration of albumin in human fetal lung fluid. Unfortunately, neonatal bronchoalveolar lavage measurements can not be compared with the fetal situation, since lung fluid production is strongly decreased after birth mainly due to a strong decrease in active chloride secretion [43]. To conclude, though little is known on human fetal lung fluid composition, based on ovine experimental studies, it is unlikely that lung fluid is a significant contributor to amniotic fluid albumin.

Intestines

Fetal intestines are another possible source of amniotic fluid albumin. It is often assumed that meconium is voided into the amniotic fluid only just before or during birth. However, during fetoscopy, small amounts of meconium have been observed as early as the early second trimester of gestation. In fact, intestinal enzymes are present in amniotic fluid throughout pregnancy [46]. However, fetal continence for meconium seems to increase markedly after 18 weeks of gestation [47]. It has also been shown that after 18 weeks, intestinal enzymes in amniotic fluid decrease significantly [48-50]. Furthermore, it has been shown between 35 and 42 weeks gestation that there is no significant difference in albumin concentration between

amniotic fluids that contain meconium and those that do not [21]. Finally, in an ovine experimental study, iatrogenic jejuno-ileal atresia did not alter albumin concentration in amniotic fluid [51]. In conclusion, although there may be an intestinal contribution to amniotic fluid albumin before 18 weeks, it is very unlikely that there is a significant contribution in the late 2nd trimester or the 3rd trimester.

Skin

The skin is another possible pathway through which albumin could permeate. In 1969, Lind et al. described microvilli on human fetal skin at 14 weeks gestation [52]. Such microvilli are also present on amniotic membrane [53;54] and indicate an important transport function. However, a marked change takes place shortly after 14 weeks. From 25 weeks gestation, human fetal skin consists of multiple layers and is keratinised, making it much less permeable for water and solutes [52;55-57]. In conclusion, it is unlikely that fetal skin is a significant contributor to amniotic fluid albumin in the second half of pregnancy.

Intramembraneous pathway

Finally, the so-called intramembraneous pathway could contribute to the albumin in the amniotic fluid. This is a pathway between the fetal blood and the amniotic fluid, which is thought to explain important fluid shifts in ovine fetuses, and which probably also exists in humans (see Figure 1) [58]. Brace and Gilbert introduced this possible pathway, pointing out that not all uptake of amniotic fluid was due to swallowing, but that part of the reabsorption must take place through the membranes. Therefore, they hypothesised a kind of lymphatic drainage, between the amnion and chorion leave, draining amniotic fluid towards the fetal blood vessels on the fetal surface of the placenta. In ovine pregnancy, an anatomic substrate for fluid drainage is present in the form of micro blood vessels between the amnion and chorion leave. Brace and Gilbert named this the intramembraneous pathway. In humans, though, there are no micro blood vessels between the amniotic and chorionic membranes, but there is a large surface area of fetal blood vessels present in the umbilical cord and at the fetal surface of the placenta adjacent to the amniotic fluid. Using a mathematical model, Mann et al. calculated that human intramembraneous flow of water must be around 390 ml daily [59]. The term intramembraneous pathway has subsequently been proposed for the combination of fetal skin, the mucous membranes (mainly oro-nasal saliva), the umbilical cord surface, and the fetal side of the placenta [27].

The components of the intramembraneous pathway could all contribute to the build-up of albumin concentration in amniotic fluid. The contribution of human fetal skin

has already been discussed. The contribution of mucous membranes in the human situation is unknown. In 3 kg ovine fetuses, fluid secretions from the head amounted to 25 ml daily [27]. The contribution of the umbilical cord is also unknown, but depends on the transport characteristics of Wharton's jelly. Since Wharton's jelly contains mucopolysaccharides, this may facilitate the transport of some amniotic fluid solutes like bilirubin [28]. In fact, albumin has been shown to pass through ovine umbilical cords in vitro [28]. Finally, the main contributor of the intramembraneous pathway is expected to be the fetal surface of the placenta. Both the chorionic tissue and the chorial blood vessels could transport fetal serum albumin to the amniotic fluid. However, animal research has not yet demonstrated albumin transport from the fetus to amniotic fluid through this pathway, but has instead shown an albumin transport in the opposite direction [60;61]. In view of this, it is unlikely that the intramembraneous pathway is a significant contributor to amniotic fluid albumin.

4

Several investigators have studied the dynamics of transport between amniotic fluid and fetal blood through the intramembraneous pathway. Passive diffusion of solutes seems to occur in both directions over the intramembraneous pathway, limited only by solute size [62]. However, passive diffusion only accounted for a minor part of the flow over the intramembraneous pathway. The majority of intramembraneous flow seems to be due to bulk flow of water and solutes, which is only explainable by unidirectional transport, in the form of active vesicular transport [62-64]. This hypothesis was developed in part due to several studies that used albumin tracers to investigate intramembraneous flow. In 2002, in an ovine study by Faber et al., ^{125}I labelled albumin was injected into the amniotic fluid [60]. All urine and lung fluid was diverted and fetuses were made unable to swallow. They found that ^{125}I-albumin was cleared from amniotic fluid at a rate of 25-30 ml/h bulk flow. In addition, after ^{125}I labelled albumin was injected in fetal serum, hardly any ^{125}I-albumin appeared in the amniotic fluid after 30 hours. Previously, Mann et al. had performed a similar study and obtained comparable results [61]. Besides radioactively-labelled albumin, they also injected radioactively-labelled creatinin into the amniotic fluid. They found that creatinin clearance from amniotic fluid was greater than albumin clearance, 5 hours after injection. Notably, they found a higher radioactivity in fetal blood compared to maternal blood. However, caution should be made in extrapolating data from ovine studies. Osmolality is higher in ovine than in human amniotic fluid, and differences in osmolality and composition suggest that human and ovine amniotic fluid have different regulating mechanisms [65]. Nonetheless, vacuolation was recognised in human amnion with electron microscopy [54;66], which might support the hypothesis of active vesicular transport. It has also been proposed that aquaporins play an

important role in intramembraneous flow [67]. These are small transcytotic channels that transport water and in some cases small molecules such as 0.1 kD creatinin. However, aquaporins are unable to transfer larger 67 kD albumin. Human studies on the intramembraneous pathway are scarce. In 1972, Gitlin et al. injected radioactively-labelled protein into human amniotic fluid in 2 cases after the occurrence of intrauterine fetal death [17]. Birth took place 2 days after administration. As would be expected, no ^{125}I-albumin was retrieved in the stomach. Nonetheless, 10% of the injected ^{125}I-albumin was still cleared from the amniotic fluid. This was believed to have taken place through the fetal membranes, since the highest radioactivity was registered in the membranes. After birth, some radioactivity could also be measured in the cord blood and some in the maternal blood. In conclusion, it does not appear that albumin is transported from fetal blood to amniotic fluid through the intramembraneous pathway in significant amounts. It is, however, plausible that the intramembraneous pathway is in part responsible for the transport of amniotic fluid albumin to the fetal blood.

Summary

Based on the available evidence from the literature, it is unlikely that the fetus contributes significantly to the amniotic fluid albumin. Data from human and animal experiments seem to rule out fetal urine, lung liquid, skin or intestines as a large source of albumin. Finally, studies on the intramembraneous pathway have shown uptake of albumin rather than excretion into the amniotic fluid.

Maternal blood as a possible source of amniotic fluid albumin

Most clinicians think the transfer of molecules between mother and fetus takes place exclusively from maternal blood to fetal blood over the trophoblast. However, there are other possible pathways that albumin could take to leave the maternal blood and build up a concentration in the amniotic fluid.

Via fetal blood

First of all, the possibility should be considered that albumin is transferred via the trophoblast from maternal to fetal blood, after which it enters the amniotic fluid. In 1997, Malek et al. performed a study in a dually-infused human placental lobe [68]. ^{14}C-labelled albumin and IgG were injected into the maternal side of the placental lobe. Only small traces of albumin were retrieved at the fetal side of the placenta.

After 1 hour, this low concentration of albumin remained constant for the study period of 4 hours. In contrast, after an internalization phase of 2 hours, the concentration of IgG kept increasing markedly in the fetal blood. Although albumin and IgG are both bound by the so-called neonatal Fc receptor (FcRn), this phenomenon could be explained by the observation that a specific Fc receptor (FcgammaRIIb) is present in fetal capillary endothelium, which can transfer IgG into the fetal blood, but not albumin [69]. In the previous chapter, we already saw the unlikelihood that fetal serum accounts for significant amounts of the albumin in the amniotic fluid. In conclusion, it is unlikely that maternally derived albumin would be transported via fetal blood and then contribute significantly to amniotic fluid albumin.

There are, however, other possible pathways that albumin could take to move from the maternal compartment to the amniotic fluid, without first entering the fetal blood.

4

Transmembraneous pathway

The transfer of albumin from maternal blood to amniotic fluid and vice versa can theoretically take place through the amnion and chorion leave. The exchange of fluid and solutes through the amniotic and chorionic membrane towards the decidua parietalis and the maternal uterus is frequently referred to as the transmembraneous pathway (see Figure 1). In ovine experiments, the transmembraneous flow of water, in second and third trimester, seems to be directed from amniotic fluid towards the decidua. It is estimated that at term, about 1% of all outward flow from the amniotic fluid takes place via the transmembraneous pathway [70]. This is in contrast to early pregnancy, when the chorion is the major pathway for inward flow of water and solutes into the coelomic fluid [71]. In 20 and 42 day old human embryos, albumin has been histochemically shown to be present in the yolk sac, chorion stroma and amnion [72]. In 1982, Wang et al. identified breaks in the basement membrane in fresh full term human amnion with electron microscopy. Based on this finding, and the fact that intercellular occluding junctions are absent in the amnion, they proposed the possibility of a transmembraneous transfer of proteins into the amniotic fluid [53]. In 1983, Wang and Bartels supported this hypothesis with the finding that the intercellular channels in human chorion also contain very few occluding junctions, making a paracellular route for proteins plausible [73;74]. In conclusion, the transmembraneous pathway is a possible route for the build-up of amniotic fluid albumin concentration.

Via placental tissue

Finally, though it is seldom considered, the placental tissue could serve as a direct pathway. It is theoretically possible that a direct exchange takes place from the

maternal blood in the placenta, through the chorion tissue, through the amniotic membrane on the fetal side of the placenta, to the amniotic fluid (see Figure 1). Passive diffusion of molecules through the placenta can only take place up to 0.6 kD [75]. Active transport to the amniotic fluid, however, may be possible, through this part of the intramembraneous pathway. For example, an ovine experimental model suggested that there is an active transport of glucose and lactate through the intramembraneous pathway into the amniotic fluid [63]. It is therefore conceivable that there is also an active transport of albumin through the fetal side of the placenta. Different receptors, for example FcRn, could be involved in binding albumin [76]. Binding receptors can theoretically either concentrate albumin on the cell surface, induce formation of endocytose vesicles, or they can save albumin from degradation in the acid environment of the endosomes, thus recycling albumin back to the apical cell surface or facilitating transcytotic transport. Though several receptors that can bind albumin have been shown to be present in the human placenta [77-79], it is not known to what extent these receptors contribute to the transport of albumin through the placenta. In 2006, Lambot at al. showed that in the term human placenta, uptake of maternal albumin takes place in the trophoblast, via clathrin-mediated vesicular transport [78]. In their study, however, a quarter of the albumin was rapidly recycled towards the maternal side of the trophoblast. Most of the internalized maternal albumin was degraded, and it did not seem to reach the villous stroma or cross the endothelium of fetal capillaries. However, this may not rule out a pathway for albumin directly through the chorion stroma in the placenta to the amniotic fluid. Lambot et al. suggested that 150 grams of albumin enters the trophoblast layer daily [78]. Even if most of this is recycled to the maternal blood or degraded, a significant amount could still remain, which could be transported through the placenta to the amniotic fluid. It is worth noting that a study of adult mice showed that FcRn saved as much albumin from degradation in the endosomes as the liver produced daily [80]. It is imaginable that such a recycling mechanism also takes place on the fetal side of the placenta. Thus, after uptake of amniotic fluid albumin via vesicular transport in the intramembraneous pathway, albumin may be released back into the amniotic fluid, thus increasing its concentration there. In conclusion, we can only speculate on the contribution of maternal albumin transferred via placenta tissue to the amniotic fluid. Recycling of amniotic fluid albumin on the fetal side of the placenta should also be considered.

Studies indicating a maternal origin
The first indication for the maternal origin of amniotic fluid albumin came from several studies using radioactively-labelled albumin. In an experiment by Gitlin et al. in 1964,

[131]I labelled albumin was injected into human maternal blood at term [81]. After 3 to 9 hours, radioactivity and albumin concentration were measured in maternal blood, fetal blood and amniotic fluid. A 3 to 5 times higher specific activity per mg albumin was found in amniotic fluid than in fetal blood. In contrast, radioactively-labelled IgG, injected into maternal serum, showed a 2.5 times lower specific activity in amniotic fluid than in fetal blood after 4 hours. A month after the injection of the ^{131}I labelled albumin (25 and 32 days), there was no protein-bound radioactivity left in the amniotic fluid, although there was still radioactivity present in fetal and maternal blood. In a comparable study, performed by Dancis et al. in 1961, radioactively-labelled albumin was injected into human maternal blood at three months gestation [82]. After 24 hours, radioactivity in amniotic fluid was lower than that in fetal blood. However, since radioactivity was only measured per ml and not per mg albumin in this study, specific activity per mg albumin may have been higher in amniotic fluid, as was found by Gitlin et al. Theoretically, measurement of radioactivity per mg is a preferable measure, since it reflects the proportion of maternally derived albumin compared to fetally produced albumin, ignoring the difference of the fluid volume in which the tracer is dissolved. In 1986, Tomoda et al. published a study where ^{125}I-labelled albumin was injected into the amniotic fluid of ovine fetuses [83]. It was shown that as the fetus digested the albumin, most radioactive iodine was spliced from the albumin and cleared via the placenta to the mother, and was subsequently retrieved in maternal urine. Fetal urine was measured for 9 days, using a catheter, before recycling it to the amniotic fluid. Hardly any ^{125}I re-entered the amniotic fluid through fetal urine. Based on Gitlin et al.'s findings, in 1975, Sutcliffe argued that if the albumin in the amniotic fluid had been of fetal origin, its specific activity would not have exceeded that in the fetal serum. Thus, he concluded, most of the albumin in amniotic fluid at term must be of maternal origin [28]. Dancis et al. came to the opposite conclusion, but it is not possible to say whether this is because the transfer mechanism is different at different gestational ages (Dancis' study was conducted at three months gestation, whereas Gitlin's study was conducted at term), or whether it is due to the difference in measurement method (per ml instead of per mg). Finally, Tomoda et al. pointed out that the results of studies with radioactively-labelled albumin should be interpreted with caution since ^{125}I-albumin can be spliced when digested by the fetus. Dancis et al. also addressed this problem, pointing out that the possibility that radioactive iodine is spliced in the placenta can not be ruled out. This may explain why hardly any protein-bound radioactivity was measured in the amniotic fluid after 25 or more days. In conclusion, radioactive labelling studies at term endorse the idea that maternal albumin is transported directly from maternal blood to the amniotic fluid without passing through fetal blood.

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The second indication for the maternal origin of amniotic fluid albumin is the fact that proteins with a molecular weight of over 260 kD are not detected in amniotic fluid [25;28]. This suggests that proteins enter the amniotic fluid by ultrafiltration and that they are only able to permeate the fetal membranes when they are below a certain molecular weight. One striking example is haptoglobin 1-1, with a molecular weight of 85 kD, which is found in amniotic fluid, whereas the other forms 2-1 and 2-2, with a molecular weight of over 260 kD, are not. Thanks to the difference in the isoforms of haptoglobin, it has been possible to prove the maternal origin of haptoglobin 1-1 in amniotic fluid in some cases [84]. Since albumin has a molecular weight of 67 kD, it should be able to permeate the membranes. In 1960, Abbas et al. found that at term, the electrophoretic protein pattern of amniotic fluid was very similar to that obtained by dialysing maternal serum through the fetal membranes [85]. In conclusion, based on its molecular weight, it is likely that albumin enters the amniotic fluid by passive diffusion through the membranes.

The third indication for the maternal origin of amniotic fluid albumin comes from measurements of protein concentration ratios. It has been hypothesised that if serum proteins enter the amniotic fluid by ultrafiltration, then proteins of similar molecular weights will diffuse into the amniotic fluid at approximately the same rate. Thus, the relative concentration of proteins would be similar in two compartments. Derrington et al. investigated the ratio of transferrin to albumin and found that, at term, the ratio of transferrin to albumin in amniotic fluid was more similar to that in maternal blood than to that in fetal blood [87]. Transferrin has already been shown to be of maternal origin by comparing polymorphisms of the protein in amniotic fluid and in fetal and maternal blood [29]. Both Sutcliffe et al. and Johnson et al. studied the change in protein concentration during the course of pregnancy, comparing the concentrations of proteins in amniotic fluid to the concentrations in fetal and maternal serum [38;86]. The albumin concentration ratios during pregnancy were similar to the ratios of proteins like orosomucoid and Gc-globulin that are of proven maternal origin, and were very different from the ratios of AFP, which is of proven fetal origin.

Several of the studies discussed above were already mentioned in an extensive review published in 1975 entitled "The nature and origin of the soluble protein in human amniotic fluid," in which R.G. Sutcliffe concluded that albumin in amniotic fluid must be, to a large extent, of maternal origin [28].

Summary
Based on the available evidence, it is likely that amniotic fluid albumin is, to a significant degree, of maternal origin. From in vivo and in vitro experiments, it seems

probable that albumin is transported via the transmembraneous pathway. It is not known to what extent the fetal side of the placenta contributes to maternally derived albumin transport or to recycling of amniotic fluid albumin.

Placenta or fetal membranes as a possible source of amniotic fluid albumin

Another possible explanation for the relatively high concentration of albumin in amniotic fluid compared to fetal urine and lung fluid, which are the main sources of amniotic fluid, is that the placenta or fetal membranes produce the albumin that enters the amniotic fluid.

Placenta
Several studies have investigated the synthesis of albumin in the placenta. In baboon fetuses, no albumin mRNA was detected in the placenta or the amniotic membrane [36]. In the term human placenta, however, McKinnon et al. identified albumin mRNA in the syncytiotrophoblast by RT-PCR and albumin protein by immunohistochemistry [87]. Similarly, AFP synthesis was detected in the trophoblast, though only in the first trimester and not in the term human placenta [88]. In conclusion, albumin does seem to be synthesized in the placenta, but it is not known to what extent this reaches the amniotic fluid.

Fetal membranes
To our knowledge, there are no reports on the synthesis of albumin in the chorionic membrane. However, the amniotic membrane itself is a plausible contributor to amniotic fluid albumin. The amniotic membrane consists of a single layer of epithelial cells on a thicker basement membrane and a collagen spongy layer containing mesenchymal cells. Albumin gene expression has been observed in human amniotic epithelial cells [89]. Moreover, Takashima et al. demonstrated that these cells can synthesize and excrete albumin at term. This synthesis was 30 fold greater in intact amnion compared to a cultivated monolayer of amniotic epithelial cells [90]. Furthermore, albumin mRNA expression was observed in mesenchymal cells from human amniotic membrane. However, production of albumin and AFP only increased significantly when these cells were induced to differentiate to hepatocyte type cells in vitro [91]. In conclusion, it is likely that the amniotic membrane contributes to the albumin in the amniotic fluid, although it is not known to what extent.

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Summary

Based on the available literature, it is not known if the placenta or the chorionic membrane contribute to amniotic fluid albumin. It is, however, likely that amniotic fluid albumin is to a certain extent of amniotic membrane origin.

The function of amniotic fluid albumin

Human serum albumin is a relatively small protein that accounts for around 75% of protein molecules and about half of total protein mass in the plasma of healthy adults [32]. In fetal blood, albumin forms an even larger fraction of total protein (Table1). Because of its relatively large contribution to the plasma protein pool, albumin is responsible for approximately 75% of plasma colloid oncotic pressure in adult serum [32]. Besides this, its main function is to act as a carrier protein: e.g., for fatty acids, steroid and thyroid hormones, calcium, nitric oxide, bilirubin, and numerous types of toxins and drugs [31;92]. Serum albumin has other functions, among them an antioxidant function and an enzymatic function, and it also modulates inflammatory response [32].

Volume regulation

We can only speculate about the function of albumin in amniotic fluid. As in blood, albumin in amniotic fluid is the major component of total protein [18;25;26]. Albumin in amniotic fluid may also have a function in maintaining osmotic pressure, and it could therefore have a regulatory function in the volume and pressure of the fluid. Albumin concentration in maternal blood, has been shown to influence human placental lactogen secretion by the placenta [93;94]. Lactogen receptors in the amniotic membrane, in turn, may be involved in amniotic fluid volume regulation [95]. Albumin may also influence vesicular transport in the intramembraneous pathway, since it has been shown that albumin concentration regulates caveolin-1 production in the human liver [96]. Caveolin-1 is essential in forming caveolae and has been found in fetal endothelium in the human placenta [97].

Carrier protein

Albumin may play an important part in recycling and detoxifying solutes in amniotic fluid. As a carrier protein, it binds numerous solutes that enter the amniotic fluid and it can hold toxins, rendering them harmless [31]. Uptake and degradation can then take place in the fetal gastrointestinal tract or in the fetal side of the placenta.

Nutrition

Considering its non-fetal origin, the albumin in amniotic fluid may have another important function. We know that the fetus at term swallows and digests about 80% of amniotic fluid every 24 hours [27]. In a study by Gitlin et al. in 1972, radioactively-labelled proteins (such as ^{125}I-albumin) were injected into the amniotic fluid, hours or days before birth [17]. In live fetuses, two thirds or more of the amniotic fluid volume was cleared of protein per day and most of this could be retrieved in the stomach after birth. Swallowing prior to birth can thus add significantly to fetal protein intake. Albumin could serve as an extra source of amino-acids and energy, especially considering its carrier function of fatty acids in aqueous solution. Fetuses that lack this extra source because of an oesophageal or intestinal obstruction are more often growth restricted. In a fetal rabbit experiment, oesophagus ligation restricted growth by 10 to 15% [98]. A retrospective study in fetuses with gastro-intestinal malformations showed that 38% were small for gestational age [99]. This growth retardation was found to be most significant in the last weeks of gestation [100]. Since more than 90% of fat deposition in the fetus occurs in the last ten weeks of pregnancy [101], a large amount of fatty acid transfer is required during this period.

Regulation of amino acid uptake from mother to fetus is not yet fully understood [102]. If the albumin in amniotic fluid is indeed of maternal origin, it is clear this would contribute to daily amino- and fatty acid uptake. Also, it is possible that fatty acids or other ligands are brought into the amniotic fluid through the fetal membranes, along with albumin. Maternal diet has already been shown to influence fatty acid composition in the amniotic fluid and fetal intestinal membrane in rats [103]. Further, even if the albumin in amniotic fluid has an amniotic membrane or placental origin, its amino-acids must still come from the mother. Placental insufficiency might then be not just a matter of diminished transport but also of diminished production of albumin. In conclusion, it is important to realize that the fetal membranes could actively participate in the exchange processes between the maternal and fetal compartment.

Summary

Based on the known functions of albumin in blood, it is probable that albumin in amniotic fluid influences the homeostasis of amniotic fluid volume. It is also possible that it functions as a carrier protein, for example for fatty acids. Finally, it is very likely that albumin in amniotic fluid forms a substantial part of fetal nourishment.

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Future research

To date, research has not brought forth solid conclusions regarding the origin of albumin in amniotic fluid. In recent years, the proteins in the amniotic fluid have again been studied extensively, this time in the field of proteomics [25;26;104]. However, the possible maternal origin of these proteins has hardly been addressed. Research on the transport mechanism that regulates albumin concentration in amniotic fluid may have wider implications, ultimately leading to new treatment modalities involving the amniotic fluid.

Albumin polymorphisms

In the case of a maternal or paternal abnormality or polymorphism of albumin, the type of albumin in amniotic fluid could be compared to that in maternal serum to establish evidence for the fetal or maternal origin. There are several polymorphisms known in human albumin that could easily be measured with tandem mass spectrometry [105]. Unfortunately, polymorphisms are rare, with frequencies reported to lie between 1 in 1700 to 1 in 3000 in humans [106;107]. However, in case an amniocentesis would be performed in a mother with a known albumin polymorphism, it would become possible to measure the proportion of maternal and fetal contribution to amniotic fluid albumin at a given gestational age.

Albumin tracing

Labelling studies could be designed without the use of radioactivity or toxins, to study the tracer dynamics of maternally injected albumin into the fetal blood and amniotic fluid. For example, stable isotope tracers could be measured with gas chromatography/ mass spectrometry (GC/MS) or GC-combustion-isotope ratio mass spectrometry (GC-C-IRMS). This method has already been used to study surfactant metabolism in premature neonates [108]. Intrauterine transfusion would be a unique situation where simultaneous samples could be obtained from maternal blood, fetal blood and amniotic fluid at different gestational ages.

Albumin synthesis

The production of albumin in the placenta, and chorionic and amniotic membranes could also be investigated. Real time-PCR of mRNA and immunohistochemistry of albumin and its precursors, can be compared at different gestational ages [87;88]. In addition, mesenchymal stem cells or other free floating cells that are present in the amniotic fluid could be considered as a source of albumin.

Albumin transport

The transport mechanisms through the chorion and amnion can be studied, to gain insight into the pathways that albumin takes and the functioning of the intra- and transmembraneous pathway in humans. When doing this, a bidirectional transfer of albumin must be considered. Passive diffusion and active excretion, as well as active vesicular transport should be considered. Albondin, caveolin, megalin, cubilin or clathrin-mediated vesicle transport and macropinocytosis can be studied in human in vitro placenta and membrane models [78]. Finally, the possibility should also be considered that albumin is recycled after vesicular transport, due for example to binding to FcRn [80].

Poly- and oligohydramnios

Another possibility to gain insight into the intramembraneous pathway, is to investigate the concentration of amniotic fluid albumin in poly- and oligohydramnios, of different types of causes. Since these pathological situations represent a dysfunction of the homeostatic properties of the intramembraneous pathway, comparison between different causes and non-pathological cases may tell us about how this pathway functions.

Transamniotic nutrition

Transamniotic feeding of amino- and fatty acids may be a complement to transplacental feeding and can therefore be considered in investigations on intrauterine growth restriction and fetal therapy for this problem. Since the importance of prenatal nutrition on adult metabolism has become apparent in recent years, transamniotic feeding should also be considered in the research on the developmental origins of health and disease.

Conclusion

From the currently available evidence, it appears that the fetus does not make a large contribution to albumin in the amniotic fluid in the second and third trimesters. It seems that a significant amount of the albumin in the amniotic fluid is of maternal origin, and this maternal albumin probably reaches the amniotic fluid through the fetal membranes. In addition to passive diffusion, it is possible that there is a form of active transport or even recycling of albumin over the intra- or transmembraneous pathway. The amniotic membrane itself also produces albumin. At present, we do not know with certainty what the relative contribution is of each of the possible sources for

amniotic fluid albumin. These relative contributions presumably change during the course of the pregnancy.

The albumin in amniotic fluid has a number of possible functions. These include volume regulation and transport of either toxins or nutrients. Intake of non-fetal albumin, and the fatty acids which albumin carries, could be a substantial part of fetal nourishment.

Different suggestions have been proposed in this review to investigate the origin, the transport mechanisms, and the function of albumin in amniotic fluid. This could lead to the development of new fetal therapy strategies, including relatively simple measures for otherwise hard-to-treat conditions such as poly- and oligohydramnios and intrauterine growth restriction.

Acknowledgements

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Hypoalbuminemia:
A cause of fetal hydrops?

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Abstract

Objective

The pathophysiology of fetal hydrops is still unclear. One factor that is believed to contribute to hydrops is hypoalbuminemia. Our research question was whether hypoalbuminemia in immune hydrops is causative or a secondary effect.

Study design

Between 1987 and 2005, fetal blood samples were taken at the first fetal blood transfusion in 224 Rh-D alloimmunized pregnancies. We measured hemoglobin concentration and albumin concentration and assessed the severity of hydrops.

Results

A decrease in albumin concentration occurred only below a hemoglobin deficit of >8 SDs in 27 fetuses. In 161 nonhydropic, 44 mildly hydropic, and 19 severely hydropic fetuses, albumin concentrations were >2 SDs below the mean for gestational age in 6%, 14%, and 63%, respectively.

Conclusion

Our finding that most fetuses with immune hydrops have an albumin concentration within the normal range (71%) suggests that hypoalbuminemia is unlikely to cause the initial development of immune hydrops.

Introduction

The overall prognosis of fetal hydrops is poor, with a perinatal mortality rate between 50% and 98% [1]. In fetuses with severe alloimmune anemia, the severity of hydrops is a major determinant for the prognosis [2]. Also, in many other fetal diseases, the presence or absence of hydrops has a major influence on the chances for survival. However, the mechanisms in fetal disease that lead to hydrops remain unclear.

Several hypotheses regarding the pathophysiologic condition of fetal hydrops have been suggested [3]. Extravascular accumulation of fluid can be caused by decreased intravascular osmotic pressure, increased intravascular hydrostatic pressure, or lymphatic flow compromise.

Decreased intravascular colloid osmotic pressure can be caused by hypoalbuminemia. Leaking of albumin through endothelium may occur as a result of hypoxia-mediated damage [4]. Alternatively, decreased albumin production could be the result of fetal liver dysfunction (eg, in chronically anemic fetuses with increased extramedullary erythropoiesis or portal hypertension) [5]. Phibbs et al. [6] found, in severely anemic neonates, a relatively increased plasma volume that is associated with low albumin concentrations (Albs) and hydrops. Furthermore, elevation of umbilical venous pressure in the presence of decreased colloid osmotic pressure might be the onset of extravasation of fluid [7]. Fetal cardiac decompensation or increased intrathoracic pressures (eg, because of lung tumors or chylothorax) lead to increased central venous pressures and obstruction of lymphatic emptying, which results in the development of hydrops [8-10]. Finally, various mediators, such as atrial natriuretic factor, influence cardiovascular adaptation to anemia and hypoxia [11].

With the assumption that hypoalbuminemia is a major contributor to the development of hydrops, several investigators have tried to treat fetal hydrops using albumin infusions [12,13]. A better understanding of the cascade of events that lead to the development of hydrops may allow much needed advances in prenatal preventive or therapeutic modalities. This study was designed to evaluate the role of hypoalbuminemia in the development of fetal hydrops.

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Material and methods

We searched our fetal database for all Rh-D alloimmunized pregnancies that underwent intrauterine blood transfusion between 1987 and June 2005. Fetal hemoglobin concentration (Hb) and Alb from the first fetal blood sampling in each pregnancy were recorded prospectively. We excluded fetuses with structural or chromosomal anomalies and intrauterine growth restriction or infection and pregnancies with incomplete data.

From the ultrasound report at the time of fetal blood sampling, the presence or absence of hydrops was obtained. Hydropic fetuses were classified as mild or severe, by the criteria described by van Kamp et al. [2]. Briefly, mild hydrops was defined as the presence of a distinct rim of ascites, with or without pericardial effusion; severe hydrops was defined as the presence of a more abundant amount of fluid collection, usually ascites, with skin edema.

The measured values for Hb and Alb were plotted on previously published standard reference ranges. For Hb, the reference range of Nicolaides et al. [14] was used. The nomogram that we used for fetal Alb was from Takagi et al. [15]. We calculated gestational age independent Z-values to evaluate the correlation between Hb and Alb. Linear and cubic regression were used to analyze the data. The Kruskal Wallis test was used for comparison of groups. A probability value of < .05 was considered statistically significant. The percentage of fetuses with an Alb below 2 standard deviations (SD) were calculated in each subpopulation to evaluate whether the role of hypoalbuminemia in evolving hydrops is more likely to be the primary cause or a secondary effect. These percentages were compared in a chi-square (Fisher's exact) test. Ordinal logistic regression was performed to analyze the dependency of the decrease in Alb and the decrease in Hb for the presence of hydrops.

Results

A total of 224 fetuses could be included from which 161 fetuses were nonhydropic, 44 fetuses were mildly hydropic, and 19 fetuses were severely hydropic. Gestational age at the time of the first fetal blood sampling ranged from 17 to 38 weeks.

Hbs in fetal blood that were plotted against gestational age are shown in Figure 1. All fetuses except 1 were anemic, which was defined as an Hb of >2 SD below the

mean for gestational age (Hb deficit ranged from -1.2 to -11.8 SD). In the nonhydropic group, the mean Hb deficit was 7.1 SD (range, -1.2 to -10.5 SD). Mild hydrops was observed in fetuses with a mean Hb deficit of 9.2 SD (range, -3.5 to -11.8 SD). Severe hydrops was present only in fetuses with a Hb deficit of >9.4 SD. In this group, the mean Hb deficit was 10.3 SD (range, -9.4 to -11.4 SD). Mean Hb deficit among the 3 groups was statistically significantly different (p< 0.001).

Albs in fetal blood that was plotted against gestational age are shown in Figure 2. In the nonhydropic group, the mean Alb deficit was 0.6 SD (95%CI, -0.8 to -0.5; range, +2.9 to -3.2 SD). Mildly hydropic fetuses had a mean Alb deficit of 1.1 SD (95%CI, -1.3 to -0.8; range, +0.8 to -3.0 SD). Severely hydropic fetuses had a mean Alb deficit of 2.1 SD (95%CI, -2.6 to -1.6; range, -0.1 to -4.6 SD). The mean Alb deficit among the 3 groups was statistically significantly different (p< 0.001).

Only 27 of the 244 fetuses were found to have hypoalbuminemia. Of the nonhydropic fetuses, 5.6% had an Alb outside the normal range; of the mildly hydropic fetuses, 13.6% had an Alb outside the normal range, and of the severely hydropic fetuses, 63.2% had an Alb outside the normal range. Of all hydropic fetuses combined, mild and severe together, 28.6% had an Alb <2 SD. The difference in percentage of fetuses with hypoalbuminemia between nonhydropic and mildly hydropic fetuses is not statistically significant (p= 0.097, Fisher's exact test). The difference in percentage of hypoalbuminemia between severely hydropic fetuses and both nonhydropic and mildly hydropic fetuses is statistically significant (p< 0.001 and p< 0.001, chi-square and Fisher's exact test).

Gestational age independent Z-values of Alb and Hb were compared (Figure 3). A cubic regression line fitted the data best for the total population. Ordinal logistic regression showed that a decrease in Alb and a decrease in Hb independently of each other are predictive of the presence and severity of hydrops (p< 0.001).
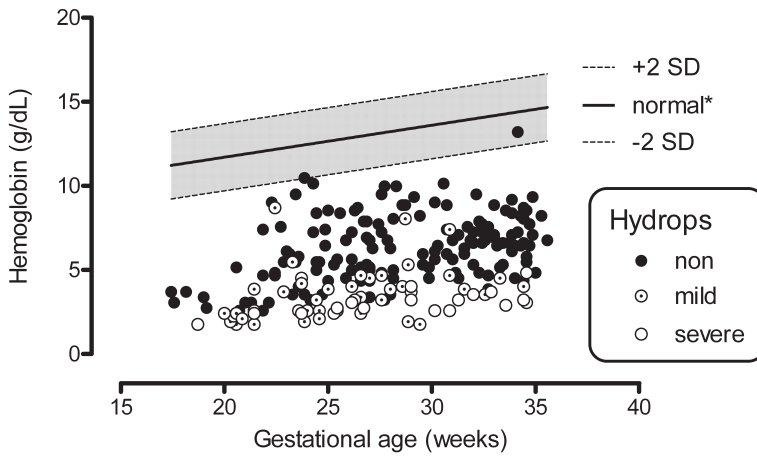
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Figure 1 Hbs in nonhydropic, mildly hydropic, and severely hydropic fetuses are plotted with the normal range (* adapted from Nicolaides [14]).
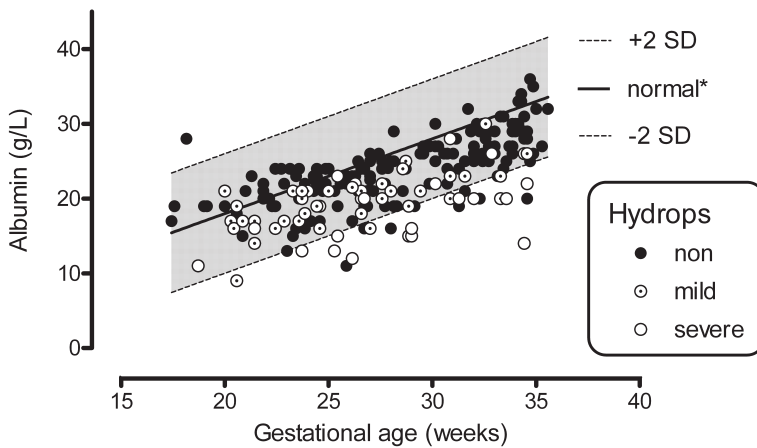

Figure 2 Albs in nonhydropic, mildly hydropic, and severely hydropic fetuses are plotted with the normal range (* adapted from Takagi [15]).
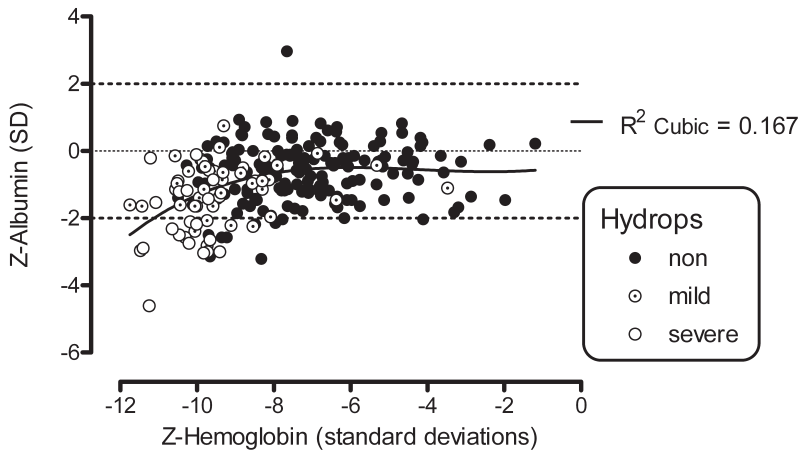
Figure 3 Z-albumin plotted against Z-hemoglobin that compares nonhydropic, mildly hydropic, and severely hydropic fetuses. The normal ranges that were used to calculate Z-values were adapted from Nicolaides [14] and Takagi [15].

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Comment

In this study of a large cohort of anemic human fetuses, we found a significant negative correlation between the fetal serum Alb and the degree of fetal hydrops. However, most of the fetuses with hydrops had albumin levels within the normal range. These results suggest that hypoalbuminemia is unlikely to cause the primary onset of fetal immune hydrops. This conclusion is supported by the observation that there was little difference between nonhydropic and mildly hydropic fetuses. Only in severe hydrops was hypoalbuminemia present in more than one half of the cases. Hypoalbuminemia thus seems to occur as a secondary effect in the cascade of hydrops (eg, because of a reduced re-uptake of albumin from the interstitial compartment). Hypoalbuminemia might even be the trigger for mild hydrops to evolve into severe hydrops.

The result of our study also warrants caution in drawing conclusions about the relationship between the presence of hydrops and the Alb, because the severity of anemia seems to be a confounding factor in this relationship. Hypoalbuminemia was observed only in fetuses with an Hb deficit of >8 SDs. Severe anemia could be associated, for example, with a relatively large plasma volume, which could cause a dilution of plasma proteins. Besides a decrease in Alb, a decrease in Hb was independently predictive for the presence of hydrops. Therefore, the development

of hydrops cannot be explained solely by either the severe anemia or hypoalbuminemia. Our data do not permit us to draw conclusions about the reasons that some anemic fetuses become hydropic when others remain without hydrops.

Our study confirms results from animal experiments in which fetal lambs were made anemic, with hydrops developing in some lambs and not in others. Both groups of fetal lambs were found to have the same level of plasma protein [16]. Our findings seem to be in contrast, however, to results from a study by Nicolaides et al. [17]. They compared albumin levels from 10 nonhydropic anemic fetuses and 7 hydropic anemic fetuses with normal control fetuses. They found that most hydropic fetuses (6/7) and only a few nonhydropic fetuses (2/10) had an Alb of <2 SDs. However, from a graph in their paper, it appears that, in only 3 hydropic fetuses, the albumin values clearly fell below the normal limits. The degree of anemia was the most severe in these 3 fetuses.

This is the first study to explore the possible role of hypoalbuminemia with a large number of severely anemic and hydropic fetuses. An obvious limitation is that our conclusions are based only on fetuses with alloimmune anemia. Other conditions that lead to hydrops may have a different pathophysiologic condition. In some of these conditions, hypoalbuminemia may play a more important role. However, many other causes of hydrops (such as viral infections, vascular tumors, hematologic conditions, and several metabolic conditions) are anemia related and thereby likely to have a pathophysiologic condition that is similar to alloimmune hemolytic disease. We speculate therefore that, in most nonimmune fetal conditions that lead to hydrops, hypoalbuminemia is unlikely to play a causative role.

In our study, we measured fetal albumin levels and assumed a close correlation with intravascular colloid osmotic pressures. Experimental protein reduction in fetal lambs was shown to decrease protein levels and colloid osmotic pressures to the same extent without causing edema [18]. Lumbers et al. [19] showed, again in fetal lambs, a close correlation between plasma protein levels and colloid osmotic pressures.

In conclusion, hypoalbuminemia was not found in most hydropic anemic fetuses. In the chain of events that leads to hydrops, other mechanisms (such as cardiac failure and lymphatic flow obstruction) are likely to be more important. In the search for better understanding that would lead eventually to effective treatment strategies for immune and nonimmune hydrops, the focus will have to be on these mechanisms.

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Addendum: Letter to the editors
(of American Journal of Obstetrics and Gynecology)

Alfa-fetoprotein and albumin levels together are more predictive of severe fetal hydrops

To the editors

The recent paper by Pasman et al. [1] concludes that "hypoalbuminemia is unlikely to cause the initial development of immune hydrops", yet in severe hydrops, hypoalbuminemia was present in more than half the cases. Alfa-fetoprotein, a fetal liver product, may be involved in (extramedullary) fetal hematopoiesis and in the development of severe allo-immune hydrops [2]. Alfa-fetoprotein levels vary by compartment (fetal, placental, maternal), gestational age, and physiologic variables [3], so that both low and high maternal serum values have been described as clinically important fetal markers [4]. We suggest that concomitant assessment of albumin and alfa-fetoprotein may be predictive of severe fetal hydrops (rather than the absolute value of fetal albumin or alfa-fetoprotein alone) because together they are reflective of the sequence of events in red blood cell allo-immunized pregnancies and fetuses. We believe that findings of overt fetal hypoalbuminemia together with elevated fetal alfa-fetoprotein may differentiate the fetus with severe hydrops from the fetus whose course will be milder.

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Reply

We appreciate the interest of Dr Elstein in our work. We apparently succeeded in one of our main goals, which was to stimulate discussion and development of new hypotheses. Dr Elstein speculates on the possible relation of alpha-fetoprotein (AFP) to albumin with the severity of fetal hydrops. She suggests that albumin and AFP together are reflective of the sequence of events in red blood cell allo-immunized pregnancies. First, one of the important factors, oncotic pressure, is unlikely to be influenced significantly by AFP changes, since the concentration of AFP in fetal serum is about 10 times lower than albumin concentration, with similar molecular size. Only when a large difference in negative charges would be present, which is unlikely to be the case in these similar proteins, would a more important contribution of AFP be understandable.

Second, AFP may be a regulating factor in hematopoiesis. Bartha et al. showed that AFP in maternal serum correlates with fetal MCA Doppler measurements and hemoglobin concentrations [1]. However, in a previous study by the same group, a decrease of maternal serum AFP in severely anemic and often hydropic fetuses was found [2]. Strikingly, this is very similar to our observation that fetal serum albumin concentration was decreased only in severely anemic fetuses that were often, but not always, hydropic. It is compelling to hypothesize that the decrease in albumin and AFP in severe anemia has a common etiology. Zhang et al. showed that hepatoblast cells express both albumin and AFP and seem to facilitate hematopoiesis in the human fetal liver [3]. Increased venous pressure and excessive erythropoiesis are thought to affect fetal liver function, which could result in decreased AFP and albumin production. In turn, these changes could aggrevate anemia and fluid shifts. It would be of interest to assess associations between maternal serum AFP, fetal albumin concentrations, and severity of fetal anemia and hydrops. Because we studied human fetuses during treatment, we were only able to obtain fetal blood samples at one moment in time. To provide more insight in the roles of AFP and albumin in the development of hydrops, frequent serial blood sampling without giving any treatment would be needed. Such an invasive study is obviously not justifiable in human pregnancies.

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Total blood volume is maintained in nonhydropic fetuses with severe hemolytic anemia.

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Abstract

Objective

Fetal alloimmune anemia is associated with increased blood flow velocities and cardiomegaly. In severe cases, hydrops can develop. We investigated whether the decrease of red blood cell volume is associated with a reduction or expansion of plasma volume.

Methods

In 86 alloimmunized fetuses that received a first intrauterine transfusion, we calculated fetal total blood volumes (i.e. fetoplacental blood volumes) using a dilutional principle of fetal hemoglobin with adult hemoglobin. The relation between total blood volume and estimated fetal weight, severity of anemia and hydrops was analyzed.

Results

Gestational age ranged from 17 to 35 weeks. Mean hemoglobin deficit was 6.8 standard deviations (range 2.1–11.7) below the normal mean. Fetal total blood volume was significantly related to estimated fetal weight ($p < 0.001$). Mean total blood volume in nonhydropic fetuses was 123 ml/kg ($n = 74$) and in hydropic fetuses 144 ml/kg ($n = 12$). There was a significant relation between total blood volume per kg body weight and hydrops ($p = 0.035$); however, there was no relation with severity of anemia ($p = 0.94$).

Conclusion

In the human nonhydropic fetus with severe hemolytic anemia, total blood volume is maintained: the decrease in red blood cell volume is thus compensated by an increase in plasma volume. In hydropic fetuses, however, total blood volume seems to be increased. This is in accordance with the hypothesis that congestive heart failure plays a role in the pathophysiology of hydrops in anemic fetuses.

Introduction

In the human fetus, hemolytic anemia is most frequently caused by maternal allo-antibodies against fetal red cells. Intrauterine transfusion of red blood cells has become an established therapy. Nowadays, the diagnosis of fetal anemia is mainly based on ultrasound and Doppler findings, such as cardio-, hepato- and splenomegaly, and increased arterial and venous flow velocities. In severe cases, hydrops can be observed, usually beginning with discrete ascites and pericardial effusion, followed by massive ascites and skin edema in a more advanced stage [1].

Fetal total blood volume, also referred to as fetoplacental blood volume, can be regarded as the combination of red cell volume and plasma volume, circulating in the fetus and the placenta. Theoretically, a considerable decrease of red cell volume may be associated with a similar reduction in plasma volume, or a compensatory increase, or even an overcompensating expansion in plasma volume. Adults with chronic anemia commonly have an increase in plasma volume with a slightly decreased total blood volume [2]. In neonates with alloimmune anemia, the decrease in red cell volume is mostly found to be compensated by the increase in plasma volume [3,4]. In the fetus, however, there are indications that the capillary filtration coefficient and vascular compliance are different from that in the neonate or adult [5]. Thus, the fetus may react differently compared to neonates or adults. Also, in the severely anemic fetus, cardiomegaly, raised umbilical venous flow and hydrops are possible signs of congestive heart failure. High-output cardiac failure is thought to play a role in the pathophysiology of hydrops. Raised cardiac output indeed has been found in anemic fetuses [6–8] and in anemic neonates [9, 10]. In adults, congestive heart failure is often associated with increased total blood volume [11]. Therefore, we sought to determine whether the loss of red cells is associated with a reduction or expansion of total blood volume in the fetus.

Several methods have been used to estimate fetal total blood volume. Most methods used the increase in hematocrit during an intrauterine transfusion to calculate the initial total blood volume. However, these methods either overestimated the blood volume by assuming that the final blood volume equals the initial blood volume [12], or they underestimated the blood volume by assuming that the final blood volume equals the sum of the initial blood volume with the added donor blood volume [13–16]. In animal experiments, both assumptions were shown to be incorrect, as around 30% of the volume given during transfusion immediately leaves the circulation [17]. Rapid plasma loss during transfusion was also suggested in a study in human fetuses

6

[18]. Hoogeveen et al. [19] therefore proposed another method to estimate fetal total blood volume: a calculation based on the dilution of fetal hemoglobin with adult (donor) hemoglobin during an intrauterine transfusion. We calculated fetal blood volume using this formula, with minor adaptations, and investigated the relation between fetal total blood volume and severity of anemia or presence of hydrops.

Methods

Measurements and Inclusion Criteria

Leiden University Medical Center is the national referral centre for the treatment of fetal anemia in the Netherlands. Our methods for diagnosis and treatment of severe fetal alloimmune anemia with intrauterine transfusion have been described previously [20]. In short, a pre-transfusion sample is taken to measure the hemoglobin concentration and hematocrit, to determine the required amount of donor blood. After the transfusion and a 2-min waiting period to allow even distribution of the donor blood, a post-transfusion sample is taken to check if the desired level of hematocrit is reached. The hemoglobin concentration and the hematocrit of the fetal blood samples are measured using a Sysmex XE 2100 hematology analyzer (Sysmex, Kobe, Japan). The hematocrit of the donor blood is determined by capillary high-spin centrifugation. Also, fetal total blood volume is routinely calculated at every transfusion. In the initial and the final sample, the percentage of fetal hemoglobin is measured using high-performance liquid chromatography (HPLC, Primus Ultra 2 ; Siemens, The Netherlands). The measurement is the same as for routine HbA1c determination. The fetal hemoglobin and the derived (glycated or acetylated) fetal hemoglobin peak are combined to determine the total amount of fetal hemoglobin. During the study period, from January 2002 to April 2006, we included all first intrauterine transfusions for fetal anemia due to red cell allo-immunization. At subsequent transfusions, there usually is only a small amount of fetal hemoglobin left, making measurement of the dilution of fetal hemoglobin less accurate.

Estimated fetal weight was determined with the formula of Hadlock et al. [21], using sonographically measured biparietal diameter, head circumference, abdominal circumference and femur length, within 2 days before or at the time of transfusion. Hydropic fetuses were classified as mild or severe using criteria described by van Kamp et al. [22]. Briefly, mild hydrops was defined as the presence of a distinct rim of ascites, with or without pericardial effusion, while severe hydrops was defined as

the presence of a more abundant amount of fluid collection, usually ascites, with skin edema. In severely hydropic fetuses, fetal weight was also estimated using the formula of Hadlock et al. [21], but with a measurement of the abdominal circumference that excluded the abundant amount of ascites. For this purpose, the tracing elipse, in the usual transverse plane, included all fetal organs, except the intra-abdominal collection of fluid and the anterior abdominal wall. In this way an attempt was made to estimate the nonhydropic size of the fetus. We excluded fetuses with non-immune hydrops, structural or chromosomal anomalies or congenital infection.

Calculations

The initial total blood volume was calculated with a formula based on the dilutional principle as described by Hoogeveen et al. [19]. Basically, with the known amount of adult hemoglobin in the donor blood, and the dilution of fetal hemoglobin with adult hemoglobin, the initial red cell volume can be calculated (formula 1). The assumption herein is that the mean corpuscular hemoglobin concentration (MCHC) in the fetal and the donor blood is approximately the same. In that case, the change in hemoglobin concentration during transfusion is representative for the change in red cell volume during transfusion.

(1) RCV $initial$ = [(V $donor$ x Ht $donor$) x HbF $final$] / (HbF $initial$ – HbF $final$)

RCV $initial$ is the initial red cell volume, V $donor$ is the volume of transfused donor blood, Ht $donor$ is the hematocrit of the donor blood and HbF $initial$ and HbF $final$ are the pre-transfusion and post-transfusion percentages of fetal hemoglobin. With the initial red cell volume and the initial hematocrit (Ht $initial$), the initial total blood volume (FBV $initial$) can be determined (formula 2). The volume of the initial sample (V $sample$) is taken into account, to determine the entire initial total blood volume.

(2) FBV $initial$ = [(V $sample$ x Ht $initial$) + RCV $initial$] / Ht $initial$

Data Analysis

First, severity of anemia was expressed as the standardized hemoglobin deficit, defined as the number of standard deviations that an actual value deviated from the normal mean for gestational age. Reference values for hemoglobin were derived

6

from the literature [23]. Then, the variation in MCHC was analyzed and a paired samples t test was performed to rule out the possibility of a large difference between MCHC before and after transfusion. Further, it was possible to estimate the measurement error of our calculation, since in some cases an interim blood sample was taken during transfusion. This gave us the opportunity to compare 2 measurements of total blood volume in the same fetus. These 2 measurements were compared in a Bland-Altman plot. Next, linear regression was performed to study the relation between estimated fetal weight and fetal total blood volume. The standard deviations of fetal total blood volume were given per weight category. Pearson's correlation coefficient was calculated after log transformation of estimated fetal weight and total blood volume, since these variables both grow exponentially during gestation, to analyze the correlation between estimated fetal weight and total blood volume. Then the average fetal total blood volume per kg body weight was determined in nonhydropic, mildly hydropic and severely hydropic fetuses. To study the additional influence of the severity of anemia and presence and severity of hydrops on fetal blood volume, multivariate regression analysis was performed, with fetal blood volume per kg body weight as dependent and severity of anemia and hydrops (as an ordinal variable) as independent variables. The null hypothesis was that there was no significant correlation between fetal total blood volume per kg body weight and severity of anemia or hydrops. We considered $p < 0.05$ to be significant. The statistical software SPSS 14.0.1 and Graphpad Prism 5.0 were used.

Results

From January 2002 to April 2006, we performed 112 first intrauterine transfusions in alloimmunized anemic fetuses. We included 86 transfusions with complete data. Alloimmunization, at the time of transfusion, was caused by anti-D or anti-D+C (n= 69), anti-Kell (n= 10), anti-c (n= 4), anti-Jka (n= 1), anti-Kpa (n= 1) and anti-Verdegaal (n= 1). Patient baseline characteristics are shown in table 1. Mean hemoglobin deficit was 6.8 standard deviations below the normal mean, with a range of –2.1 to –11.7 standard deviations.

The mean MCHC before transfusion was 20.4 (n= 85, SD= 1.2), the mean MCHC of the donor blood was 20.9 (n= 19, SD= 0.5) and the mean MCHC after transfusion was 21.1 (n= 81, SD= 0.8). There was a mean increase of 0.7 in MCHC after transfusion, and this was a significant, albeit small, difference (n= 80, SD= 1.02, $p < 0.01$).

In 9 nonhydropic cases an interim blood sample was taken. In these cases the measurements of the blood volumes, calculated with the interim and the final blood samples, were compared in a Bland-Altman plot. The bias between the 2 methods was small: the interim measurement was on average 1.1 ml higher. The SD of the differences, however, was 15.8 ml (95% limits of agreement= –32.1 to 29.9 ml; percent SD= 10.2%, with 95% limits of agreement= –21.7% to 18.4%). Figure 1 shows that the average fetal total blood volume had no influence on the extent of the difference between the 2 methods.

Figure 2 shows that there is a strong relation between fetal total blood volume and estimated fetal weight, both in nonhydropic and hydropic fetuses. Variance of fetal total blood volume increased with increasing estimated fetal weight. Under 1 kg, the SD was 30 ml; between 1 and 2 kg, the SD was 53 ml; above 2 kg the SD was 61 ml. Since estimated fetal weight and fetal blood volume increase exponentially during gestation, a log transformation was performed. There was a significant correlation between log(estimated fetal weight) and log(total blood volume) (p< 0.001, Pearson correlation coefficient= 0.96).

6

Figure 3 shows the relation between severity of anemia and fetal blood volume per kg body weight. The mildly hydropic fetuses had a Z-hemoglobin of less than –6.2 SD and the severely hydropic cases of less than –9.8 SD. The average total blood volume per kg body weight in nonhydropic fetuses was 123 ml/kg (SD= 23 ml/kg). However, in hydropic fetuses the average total blood volume per kg body weight was higher: 144 ml/kg (SD= 29 ml/kg) (average in mildly hydropic fetuses 144 ml/kg, and in severely hydropic fetuses 142 ml/kg). Multivariate regression analysis showed that there was a significant relation between fetal total blood volume per kg body weight and the presence of hydrops (p= 0.035, r= 14.6, 95%CI= 1.1–28.1); however, there was no relation with severity of anemia (Z-hemoglobin; p= 0.94, r= 0.12, 95%CI= –3.1 to 3.3).

Table 1 Patient baseline characteristics.

	first intrauterine transfusion, initial values (n=86)
Gestational age (weeks)	27.5 (17.1 – 35.4) *
Estimated fetal weight (g)	1272 (167 – 3033) *
Hematocrit	0.19 (0.05 - 0.36) *
Hemoglobin (g/dl)	6.3 (1.8 – 11.8) *
Fetal hemoglobin (%)	91 (81 - 96) *
Presence of hydrops	74 no hydrops,
	9 mild hydrops,
	3 severe hydrops

* mean (range)
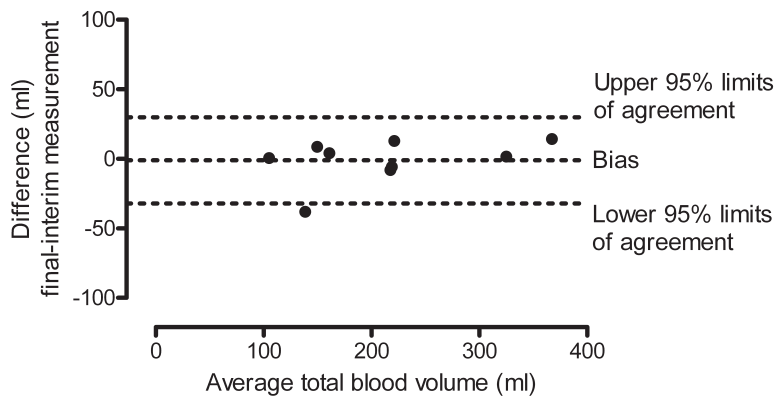
Figure 1 The differences between the interim and final blood volumes are compared with their average in a Bland-Altman plot. The bias and 95% limits of agreement are depicted.
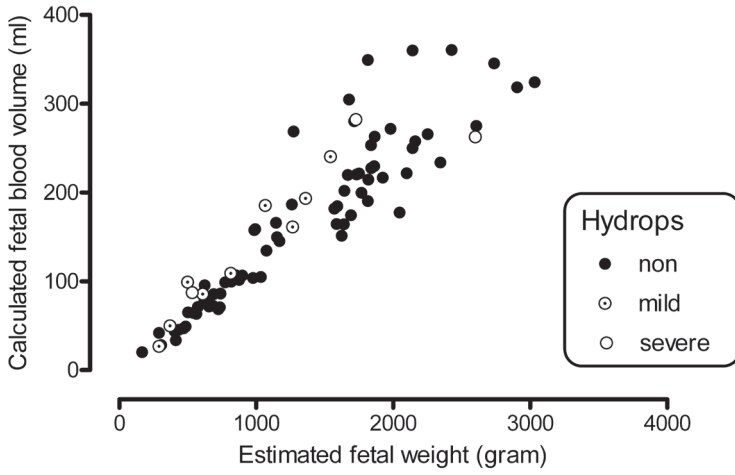
Figure 2 Relation between calculated fetal total blood volume (ml) and estimated fetal weight (g). Nonhydropic, mildly hydropic and severely hydropic cases are depicted.

6
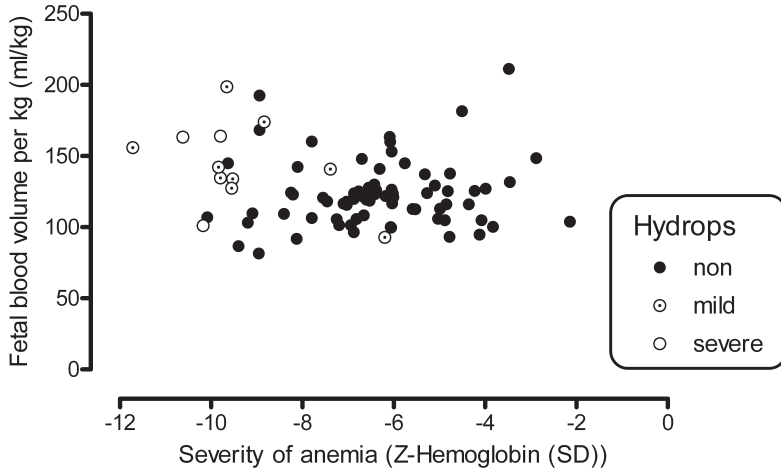
Figure 3 Relation between fetal total blood volume per kg body weight (ml/kg) and severity of anemia (Z-hemoglobin). Z-values (in standard deviations) were calculated with normal values adapted from Nicolaides et al. [23]. Nonhydropic, mildly hydropic and severely hydropic cases are depicted.

Discussion

In this study, we found that the calculated total blood volume was strongly correlated with the estimated fetal weight. We found no relation between total blood volume and severity of anemia. Therefore, we conclude that, with decreasing red cell volume, there is a compensating increase in plasma volume. However, there was on average a higher fetal total blood volume per kg body weight in hydropic fetuses.

Human fetal total blood volume has been estimated, in different studies, to lie between 94 and 176 ml/kg. Yao et al. [24] estimated the fetoplacental blood volume, using a radioactively labelled albumin dilution method, to be 105 ml/kg in the term newborn. This volume was determined from the sum of blood volume in the newborn plus the residual volume in the cord and placenta [24]. However, it is known that plasma is lost from the fetal circulation during labor, resulting in an increase of hematocrit and a lowering of fetal blood volume. The study of Yao et al. [24] is the only study that was performed in a non-anemic human population. Several investigators have used the change in hematocrit during transfusion to calculate the blood volume in anemic fetuses. In a first formula, the assumption was made that the transfusion volume was added to the pre-transfusion blood volume and that no volume is lost during transfusion. With this formula, the mean fetal blood volume was underestimated and calculated to be between 94 and 115 ml/kg [13–16]. With a second formula, the assumption was made that the blood volume before and after transfusion remained the same [12]. With this formula, the mean fetal blood volume was overestimated and calculated to be 176 ml/kg [19]. Another method was proposed by Hoogeveen et al. [19] to calculate total blood volume with the use of a dilutional principle (of fetal hemoglobin with adult hemoglobin), so the amount of plasma that is lost during transfusion does not influence calculations. With this method, which we also used in this study, a mean fetal blood volume of 121 ml/kg was found [19].

We believe our method to calculate the fetal blood volume is quite accurate, without using artificial red cell labelling. A few assumptions have to be made, however. First, we assume that the hemoglobin concentration differences are representational of the red cell volume differences. This is only true if there is no free hemoglobin in the circulation and if there are no differences in MCHC between fetal and donor blood. We tested the variation in MCHC before and after transfusion and found it to be small. Second, we neglected the small amount of fetal hemoglobin that can be found in donor blood, because in the study by Hoogeveen et al., fetal hemoglobin in the

donor blood was always lower than 1% [19]. Finally, we have to accept that errors can occur due to measurement errors of hemoglobin, hematocrit and percentage fetal hemoglobin. In 9 cases, an interim blood sample was taken, giving us the opportunity to verify the range of error that occurs, when using our method to determine the total blood volume. The difference between the interim measurement and the final measurement had an SD of 16 ml. When estimating the total blood volume per kg body weight, there is also a possible error in the sonographically estimated fetal weight. Recent literature showed that, when using the formula of Hadlock et al. [21] or closely related formulas, in only 86.5% of the cases, the prediction was within 15% of actual birth weight [25]. In case of hydrops fetalis, this measurement error may be even larger. With increasing fetal weight, the absolute error becomes larger, which might also explain the wider variance of fetal blood volume that we observed with increasing fetal weight.

Our finding that the severity of anemia has no influence on fetal total blood volume is in agreement with studies in adults and neonates [2–4]. Studies in human fetuses have also concluded that there is no evident relation between blood volume and severity of anemia or hydrops [13–16]. However, these studies all used the formula that underestimates blood volume and that does not take into account a plasma shift during transfusion. Our study is the first study that uses the more accurate calculation method to study the influence of anemia on total blood volume in a large number of human fetuses. In our study population, some very severely anemic fetuses were included, with a hemoglobin deficit of up to 12 SD below the normal mean, making it possible to draw conclusions on the entire scope of severity of anemia. We found it remarkable to find that even very young fetuses obviously adequately maintain their total blood volume.

Although there was a significant relation between hydrops and fetal total blood volume per kg body weight, the sample size of the hydropic fetuses was small, which warrants caution in drawing any conclusions. Hydropic fetuses on average had a 17% higher blood volume. This is in accordance with the hypothesis that hydrops is (in part) a consequence of congestive heart failure, or a state of high-output failure, with a rise in venous return. However, the increase in cardiac output that has been reported in anemic fetuses [6–8] does not always coincide with an increase in total blood volume, as nonhydropic fetuses apparently maintain their blood volume. This must result in a hyperdynamic circulation, where the same blood volume has a shorter circulation time. The increase in blood flow velocities corresponds to this phenomenon. The cardiomegaly that is usually observed can be explained by the increased stroke

6

volume, most probably resulting from the decrease in peripheral resistance due to the decreased viscosity of the blood and vasodilatation [2, 7, 26, 27].

It has been shown that the red cell volume is a better measure for the severity of anemia, or oxygen delivery capacity, in sick neonates, than hemoglobin concentration or hematocrit [28, 29]. Since we found that the decrease in red cell volume, on average, is compensated by an increase in plasma volume, the hemoglobin concentration or hematocrit is a representative measure for the severity of anemia, in the alloimmunized fetus. When performing an intrauterine transfusion, the hematocrit of the initial blood sample therefore is the best basic assumption on which the required amount of donor blood can be calculated. The hematocrit of the final blood sample can, however, be lower than what is actually achieved, because in the days after transfusion there probably will be a return to the initial total blood volume and thus a further increase in hematocrit.

In conclusion, we found an average fetal total blood volume of 123 ml/kg in nonhydropic fetuses and 144 ml/kg in hydropic fetuses. In the human nonhydropic fetus, total blood volume is maintained when severe hemolytic anemia develops. Thus, the decrease in red blood cell volume is compensated by an increase in plasma volume. In hydropic fetuses, however, total blood volume seems to be increased. Thus, there is an overcompensating expansion in plasma volume. This is in accordance with the hypothesis that congestive heart failure plays a role in the pathophysiology of hydrops in anemic fetuses.

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Fluid shift out of the fetal circulation during intrauterine red cell transfusion

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Abstract

Introduction

Intrauterine transfusion presents a considerable burden on the fetal circulation by increasing volume and pressure. Already during transfusion, a fluid shift out of the fetal circulation occurs. Aim of the study was to quantify the intraprocedural fluid shift, and the effect of procedural and fetal characteristics on this fluid shift.

Methods

In 95 alloimmunized pregnancies, we calculated fluid shift at first intrauterine transfusions, by determining initial and final blood volumes. We evaluated the association of the fluid shift with speed and volume of transfusion, severity of anemia and presence of hydrops.

Results

Of the included fetuses, 11 were mildly hydropic and 4 were severely hydropic. A mean fluid shift of 36% of transfused volume was found. Fluid shift related positively to transfused volume ($p<0.001$), and inversely to speed of transfusion (ml/kg/min) ($p<0.001$). The % fluid shift of transfused volume was not related to severity of anemia ($p=0.55$) or hydrops ($p=0.66$). At low gestational age, fetuses had been unintentionally burdened with relative high volume and speed of transfusion.

Discussion

Around one third of transfused volume is lost from the intravascular compartment already during intrauterine transfusion. However, a large variation, only partly explained by volume and speed of transfusion was observed. Severity of anemia or hydrops played no clear-cut role, therefore, other unknown factors that require further studies may explain the variation in fluid shift. The probability that hematocrit will still increase after transfusion, due to a continuing fluid shift, should be considered in transfusion policy.

Introduction

During intrauterine red cell transfusion, a considerable volume of donor blood is administered in a short period of time. The transfused volume is up to 20 to 60% of initial fetoplacental blood volume, while transfusion-times are merely 10 to 30 minutes. It has been shown that both arterial and venous blood pressure significantly increase after intrauterine transfusion [1-4].

Increase in fetoplacental blood volume after transfusion has been shown to be smaller than added donor blood volume [5;6]. Red cells cannot leave the intravascular compartment so this volume loss is believed to be the result of a loss of plasma. This immediate fluid shift can theoretically take place towards the interstitium of the fetus or that of the placenta, the maternal circulation or even towards amniotic fluid, for example through blood vessels on the surface of the placenta.

In animal experiments, Brace et al found that 37% of the transfused volume was shifted out of circulation already during the procedure [5]. One hour after transfusion, another 20% was lost. In a study in human fetuses, Hoogeveen et al found a similar mean fluid shift during transfusion of 31% of transfused volume [6].

Improved insight in pathophysiology and fluid dynamics around transfusion is important to further reduce fetal mortality and morbidity in these high risk pregnancies. Prevention of volume overload and prevention of unphysiological elevation of hematocrit in the period following transfusion, can be important steps in improving safety and decreasing morbidity as a result of intrauterine transfusion [7].

Purpose of this research was to investigate to what extent the fluid shift was influenced by transfusion rate and fetal condition. We hypothesised that the amount of fluid shift would be positively correlated with volume and speed of transfusion, and could be associated with severity of anemia and presence or absence of fetal hydrops.

Methods

Our methods for treatment of severe fetal alloimmune anemia with intrauterine transfusion have been described previously [8]. In short, a pre-transfusion sample is taken to measure the hemoglobin concentration and hematocrit (Ht). The required amount of donor blood (V_{donor}) is determined by the formula proposed by Rodeck et al. [9]: $V_{donor} = FP_{Vinitial} *$ (desired Ht- initial Ht)/ Ht donor blood

The initial fetoplacental blood volume (FPV$_{initial}$) is based on estimated fetal weight and normal values for fetoplacental blood volume as proposed by Nicolaides et al. [10]. Usually, Rodeck's formula underestimates the required donor volume, to reach desired hematocrit, since the formula does not take an increase in blood volume during transfusion into account. Furthermore, normal values found by Nicolaides were somewhat lower then we have calculated [6;11]. Packed red cells, with a hematocrit of around 80%, are usually transfused at 5 ml/min, depending on fetal heart rate stability. After the transfusion and a two minute waiting period, to allow even distribution of the donor blood, a post-transfusion sample is taken to check if the desired level of hematocrit (45-50%) is reached. Fetoplacental blood volume is routinely calculated at every transfusion.

During the study period, from January 2002 to September 2006, we included all first intrauterine transfusions for fetal anemia due to red cell alloimmunization. We excluded fetuses with non-immune hydrops, structural or chromosomal anomalies, or congenital infection. Estimated fetal weight was determined with the formula of Hadlock et al. [12], using sonographically measured biparietal diameter, head circumference, abdominal circumference and femur length, within two days before or at the time of transfusion. Hydropic fetuses were classified as mild or severe using criteria described by Van Kamp et al. [13]. Briefly, mild hydrops was defined as the presence of a distinct rim of ascites, with or without pericardial effusion, while severe hydrops was defined as the presence of a more abundant amount of fluid collection, usually ascites, with skin edema. In severely hydropic fetuses, fetal weight was also estimated using the formula of Hadlock, but with a measurement of the abdominal circumference that excluded the abundant amount of ascites. For this purpose, the tracing elipse, in the usual transverse plane, included all fetal organs, except the intra-abdominal collection of fluid and the anterior abdominal wall. In this way an attempt was made to estimate the nonhydropic size of the fetus.

Initial fetoplacental blood volume (FPV$_{initial}$) was calculated as described by our group previously [11]. In short, given the known amount of adult hemoglobin in the donor blood, and the dilution of fetal hemoglobin with adult hemoglobin, the initial red cell volume (RCV$_{initial}$) can be calculated. Since the initial hematocrit is known, initial fetoplacental blood volume can then be determined.

The volume (V) and hematocrit (Ht) of the initial sample and the transfused donor blood, are used for calculation of final red cell volume (RCV$_{final}$). The final hematocrit together with calculated final red cell volume determines final fetoplacental blood volume (FPV$_{final}$). Next, the difference between initial blood volume with added donor blood volume and the calculated final blood volume, determines the fluid shift. The

volume of atracurium (atr) and saline (NaCl) administered during transfusion are taken into account, to determine the most exact volume of fluid shift.

The following formulae where used to determine the final fetoplacental blood volume and subsequently the volume of fluid shift:

$$RCV_{final} = RCV_{initial} - (Vsample * Htinitial) + (V_{donor} * Ht_{donor})$$

$$FPV_{final} = RCV_{final} / Ht_{final}$$

$$V_{fluid\ shift} = FPV_{initial} - V_{sample} + V_{atr} + V_{donor} + V_{NaCl} - FPV_{final}$$

To calculate transfusion speed, the time at first sampling and at last sampling was noted. Thus, time during occasional needle dislodgement or complications otherwise delaying the procedure was included.

Linear regression analysis was used to investigate the relations between fluid shift and transfused volume, and speed of transfusion. Quadratic regression analysis was used to investigate the relation between speed of transfusion and gestational age. Severity of anemia was expressed as the standardized hemoglobin deficit (Z-hemoglobin), defined as the number of standard deviations that an actual value deviated from the normal mean for gestational age. Reference values for hemoglobin were derived from the literature [14]. Linear regression analysis was used to investigate the relation between the percentage of fluid shift of transfused volume and severity of anemia (Z-hemoglobin). Oneway anova was used to test the difference between the percentage of fluid shift of transfused volume in nonhydropic, mildly hydropic and severely hydropic fetuses. We considered a p-value of <0.05 to be significant. Statistical software programs SPSS 16.0.2 and Graphpad Prism 5.0 were used.

Results

During the study period, we performed 125 first intrauterine transfusions in red cell alloimmunized anemic fetuses. We included 95 transfusions with complete data. Alloimmunization, was caused by anti-D or anti-D+C (n=74), anti-Kell (n=12), anti-c (n=6), anti-Jka (n=1), anti-Kpa (n=1) and anti-Verdegaal (n=1). Gestational age ranged from 17 to 35 weeks and estimated fetal weight ranged from 167 to 3033 gr. There were 80 nonhydropic fetuses, 11 mildly hydropic and 4 severely hydropic fetuses

included. Hemoglobin concentration ranged from 1.8 tot 11.8 g/dl. The severity of anemia (Z-hemoglobin) ranged from -2.1 to -11.7 SD and was not related to gestational age (p=0.27).

The mean amount of transfused volume was 52 ml/kg estimated fetal weight. The mean amount of transfused volume was 43% of initial fetoplacental blood volume and this percentage was strongly correlated to gestational age (p<0.001). Notably, fetuses below 20 weeks gestation (with a mean Z-hemoglobin -7.5 SD) received a transfused volume of 48 to 99% of initial fetoplacental blood volume, while this was 11 to 55% in fetuses above 30 weeks gestation (with a mean Z-hemoglobin -6.7 SD).

A fluid shift with a range of 1 to 83 ml was found. The mean fluid shift was 14% of the initial fetoplacental blood volume (SE=0.7%, SD=7%) and 36% of the transfused volume (SE=2.2%, SD=21%). Figure 1 shows that the fluid shift was positively and linearly related to the amount of transfused volume (R^2=0.40, p<0.001).

As shown in figure 2, the fluid shift was inversely related to the speed of transfusion, expressed as transfused volume/ estimated fetal weight/ minute (R^2=0.18, p<0.001). However, there is no evident relation with amount of fluid shift at low transfusion speed. Surprisingly, as shown in figure 3, we found a strong decrease of relative transfusion speed with gestational age (R^2 quadratic=0.68, p<0.001).

Figure 4 shows the wide variation in fluid shift, without an evident influence of severity of anemia or the presence of hydrops. The relation between the percentage fluid shift of transfused volume and Z-hemoglobin was not significant (R^2=0.01, p=0.55). Finally, there was no significant difference, between the means of the nonhydropic, the mildly hydropic and the severely hydropic fetuses, in the percentage fluid shift of transfused volume (respectively 35%, 41% and 40%, p=0.66).
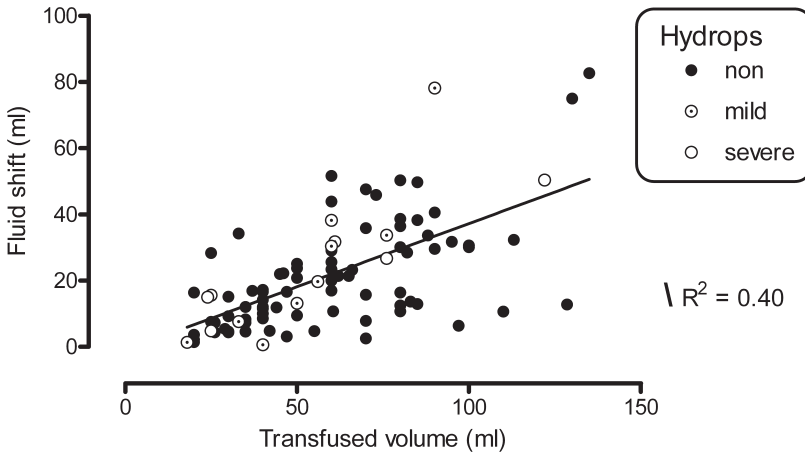
Figure 1 Correlation between transfused volume and extravascular fluid shift during intrauterine transfusion. Hydropic fetuses are depicted.

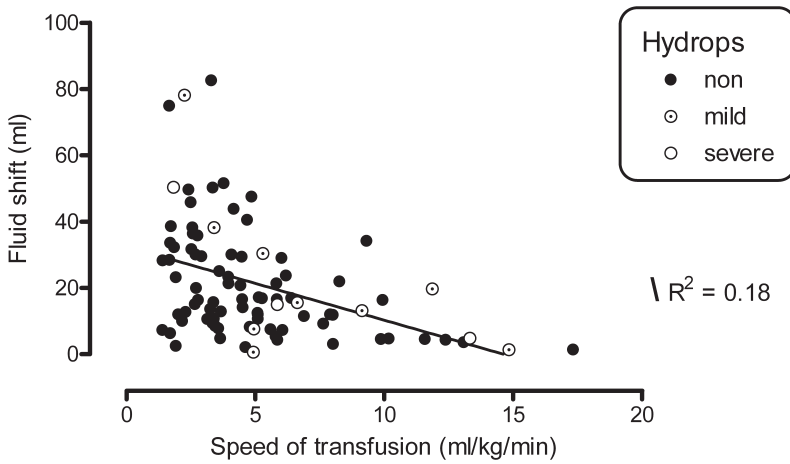**Figure 2** Correlation between transfusion speed and extravascular fluid shift during intrauterine transfusion. Hydropic fetuses are depicted.
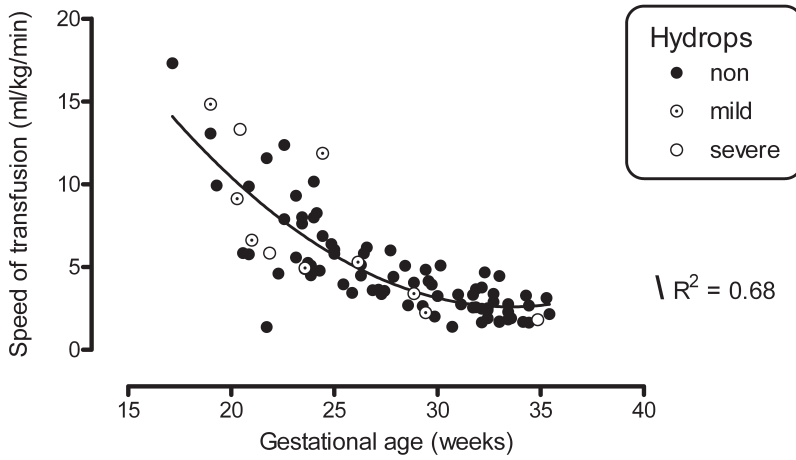
Figure 3 Relative transfusion speed in our patients as a function of gestational age. Hydropic fetuses are depicted.
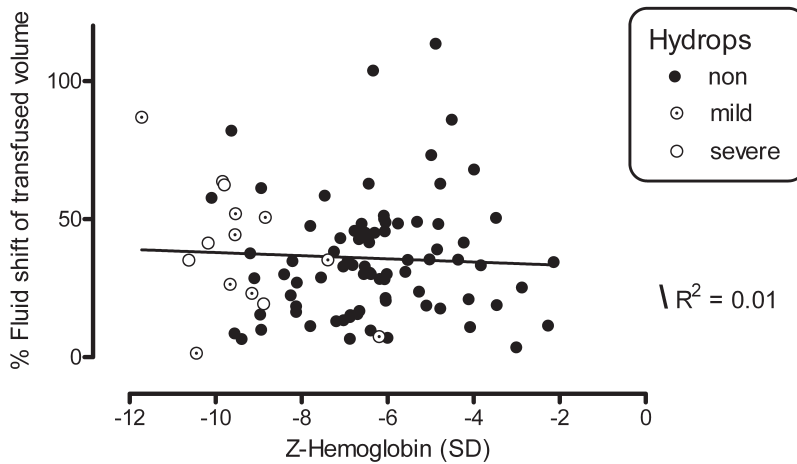

Figure 4 Correlation between severity of anemia (Z-hemoglobin) and % fluid shift of transfused volume during intrauterine transfusion. Hydropic fetuses are depicted.

Discussion

In this study, the volume of donor blood given to the fetus was approximately half of the initial fetoplacental bloodvolume. The mean extravascular fluid shift during intrauterine transfusion was 36% of the transfused volume. As expected, we found a positive linear relation between volume of fluid shift and transfused volume. In contrast to our hypothesis, the fluid shift was decreased at relatively high speed of transfusion. Also, we found no relation between severity of anemia or presence of hydrops, and the relative amount of fluid shift.

Our large human study confirms data from previous animal and human studies, showing that around one third of transfused volume leaves the intravascular space during transfusion [5;6]. A large interindividual variation was observed, though. This may in part be due to measurement error, since some of the measured values (that are expected to contain small errors) appear more than once in our formula, enlarging the over-all error in calculated fluid shift. Furthermore, biological variation in vascular compliance, interstitial compliance and capillary filtration coefficient may explain the variation in fluid movement between vascular and interstitial space. In general, the fetus is able to keep its blood volume closer to normal than adults after reduction or expansion in volume [15;16]. Besides these causes of variation, other factors may influence the amount of fluid shift, some of which were investigated by us.

One of the factors influencing fluid shift was the speed of transfusion. We initially hypothesised that with increasing speed of transfusion, blood pressure is increased accordingly, thus enhancing fluid shift. However, there was a decrease in amount of fluid shift with higher transfusion speed. It is possible that vascular compliance increases with higher infusion pressure, or capillary filtration capacity restricts fluid shift velocity. Apparently fluid shift is a process requiring a certain amount of time. It is therefore likely that this process continues after transfusion.

Other factors, hypothesized to influence the amount of fluid shift, were severity of anemia and presence of hydrops. On average, fetuses with severe anemia have a decreased concentration of plasma albumin [17]. This may be the result of decreased synthesis in the fetal liver [18] or a relative increase in plasma volume [11]. Another possibility is that albumin is lost from the intravascular compartment due to endothelial damage [19;20], which could imply an increase in the capillary filtration coefficient. Furthermore, a smaller difference between the concentration of albumin in the intravascular compartment and the interstitial compartment results in a diminished

7

capacity to contain water in the vascular compartment. Cardiac backward failure, leading to an increase in central venous pressure, also could promote extravascular fluid shift. Moreover, fetuses are particularly susceptible to interstitial fluid accumulation because of their vulnerability to venous pressure on lymphatic return [21]. On the other hand, cardiac forward failure could result in a failure to increase arterial blood pressure during transfusion, thus relatively reducing fluid shift. Finally, congestion in anemia can also be the effect, not of myocardial failure, but of retention of fluid in the vascular compartment as a result of a kidney mediated neuro-humoral cascade, induced by a low arterial pressure as a consequence of the low hemoglobin level [22-24]. In our study, there was no evident difference in fluid shift between mildly and severely anemic fetuses and nonhydropic or severely hydropic fetuses. Caution in drawing conclusions should be made, since the number of severely hydropic fetuses was limited. Our findings, however, are in accordance with the findings by Brace et al. [5] and Hoogeveen et al. [6]. Both positive and negative influencing factors might be present, thus not resulting in a clear-cut effect on fluid shift.

We found that younger fetuses in our hospital were transfused with significantly higher relative volumes and speeds. In fact, fetuses below 20 weeks' gestation were transfused with relative speeds up to 5 times higher than fetuses above 32 weeks. This usually was an unintentional effect. With increasing gestational age, fetoplacental blood volume increases exponentially, since it is linearly correlated with fetal weight [11]. A much smaller amount of donor blood is thus required to achieve a desired rise in hematocrit in younger fetuses. Further, administering donor blood at the same speed is thus a much higher burden in younger fetuses. It is possible that this attributed to the fact that low gestational age is a risk factor for complication of intrauterine transfusion, besides the increased procedural difficulty [7]. Perinatal mortality was 5.6% per procedure between 16 and 20 weeks, compared to 0.8% between 32 en 36 weeks. Fetal mortality after intrauterine transfusion has already been shown to be associated with a large increase in venous pressure [2], as well as with a relative large increase in hematocrit [25].

In animal experiments, fetal hematocrit was further increased in 1 hour after transfusion [5]. Although fetoplacental blood volume was not normalized after 24 hours, it was found that human fetuses maintain their total blood volume at different degrees of anemia [11]. Thus, it can be expected that a return to initial fetoplacental blood volume will take place shortly. This has clinical implications, since it may be hazardous to let the hematocrit increase to polycythemic values. 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), possibly baring clinical consequences. This may be due not only to hypoxic but also to polycythemic mediated damage.

The hematocrit in the final sample after transfusion could be considered to be a temporary value. The formula of Rodeck [9], that seems to underestimate the required amount of donor blood, therefore could actually be a correct guideline, assuming that in the hours or days after transfusion fetoplacental blood volume returns to the initial value. Final hematocrit in the Rodeck formula would then become the desired level that is chosen, not directly after transfusion but after a few hours or days.

The decay of donor cells has been calculated to be around 2% per day [26], by comparing the final hemoglobin level after transfusion with the initial hemoglobin level at the subsequent transfusion. This probably is an underestimation of the adult red cell decay, assuming that hematocrit first rises in the period after transfusion, before it declines again. This problem was already addressed by Egberts et al. since they sometimes found a surprisingly low decline and even an increase of adult hemoglobin concentration at the second transfusion. Furthermore, it is likely that the donor red cells do not decline linearly in time [27-29]. The difference between the adult red cell volumes at the end of transfusions, as calculated in this study, and the adult red cell volumes at the beginning of the subsequent transfusions, measured at different time intervals, might learn us about the actual decay of donor red cells in human fetuses.

Future research could resolve what other factors influence the fetal cardiovascular reaction to intrauterine transfusion. Calculations as were performed in this study can be combined with measurements of blood pressure, colloid osmotic pressure or rheological measurements before and after transfusion, difference in placenta size, changes in ANF, angiotensin, AVP and other hormone concentrations and functional echocardiography. From the factors studied in this investigation, volume overload can not be easily predicted. Our advice is to be cautious with amount and speed of transfusion in younger fetuses and to strive for a hematocrit that is not higher than the desired level at the end of transfusion.

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7

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General discussion

insights in fetal (patho-)physiology
implications for current practice
implications for future research

Insights in fetal (patho-)physiology: old, current and acquired knowledge

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 syncytiotrofoblast 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 by-pass 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.

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.

8

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.

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.
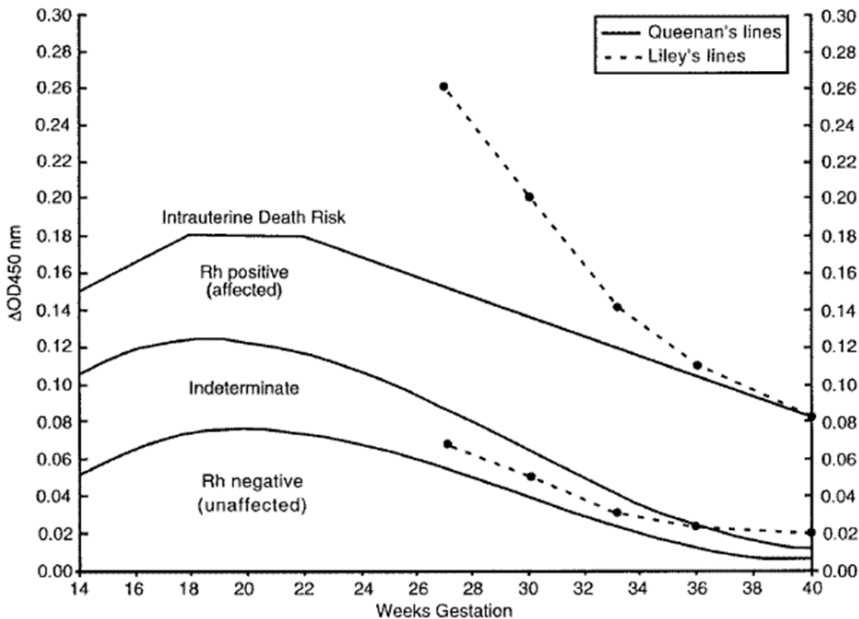

Figure 1 Queenan and Liley curve for amniotic fluid ΔOD450 (bilirubin) values

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.
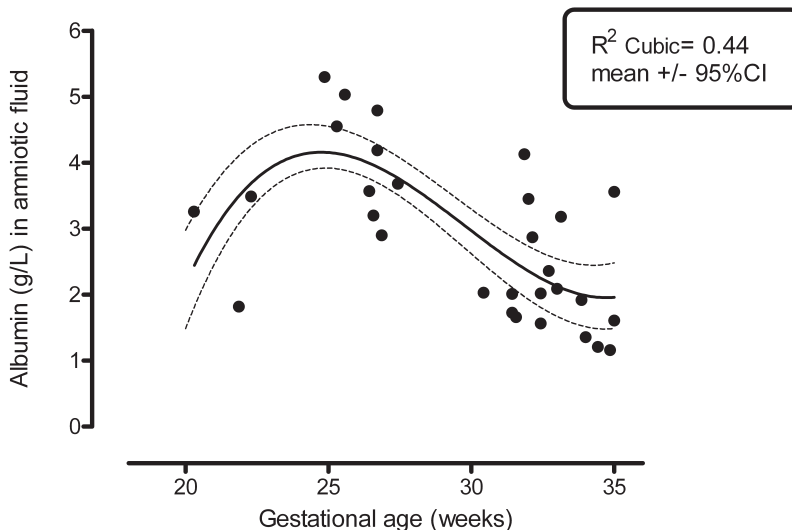

Figure 2 Albumin concentration in amniotic fluid during gestation

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 intramembraneous 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].
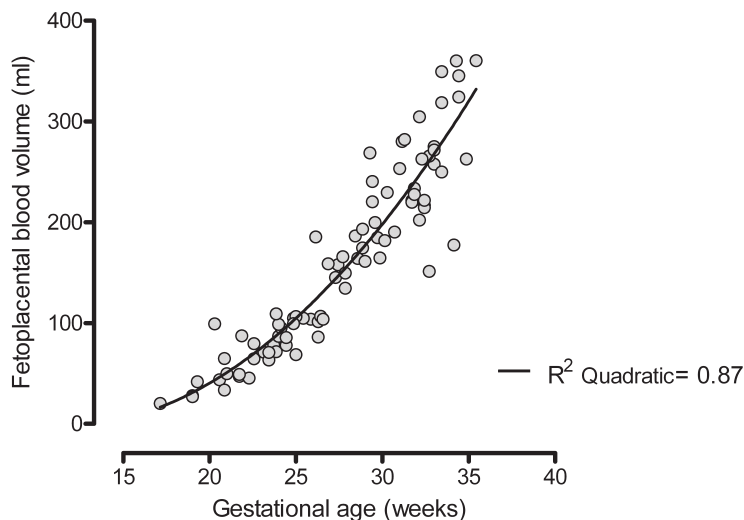
Figure 3 Fetoplacental blood volume in relation with gestational age, with the quadratic regression line shown (study population from chapter 6).

Tabel 1 Volume burden expressed as percentage of transfused volume/ fetoplacental blood volume (FPV)/ min, when using the standard 5 ml/min or the proposed transfusion speeds at different gestational ages.

Weeks	EFW*	FPV**	ml/min	%FPV/min	ml/min	% FPV/min
15	120	15	5	33,3%	1	6,7%
20	330	41	5	12,2%	2	4,9%
25	790	97	5	5,1%	3	3,1%
30	1550	190	5	2,6%	4	2,1%
35	2600	320	5	1,6%	5	1,6%

* EFW: fetal weight in g. [25], ** FPV 123 ml/kg (chapter 6)

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

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.

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. Postnatally, 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 accompany

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.

8

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 intraperitonal 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 acces 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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9

Summary

This thesis describes our research on fetal pathophysiology in hemolytic anemia and hydrops fetalis. Measurements performed in fetal blood as well as in amniotic fluid, before or during intrauterine red blood cell transfusion, where used for our studies.

In **chapter one**, the motives leading to the studies presented in this thesis are described. As a tertiary fetal therapy centre, the LUMC provides a unique possibility to study fetal pathophysiology. Many prenatal potentially treatable diseases are accompanied by amniotic fluid volume abnormalities and the final stages are almost inevitably associated with hydrops fetalis. The final common pathway of these pathophysiological phenomenons is a shift in fluids and proteins between the different fetal compartments (intra- and extravascular). One specific question that arose from clinical practice was to understand the background of the diagnostic use of the bilirubin concentration in amniotic fluid to predict fetal anemia. Another question was, to understand the fetal reaction to severe anemia and intrauterine blood transfusions, and thus to improve the safety of this procedure. Background information is presented about fetal hemolytic anemia, the diagnosis of fetal anemia, intrauterine transfusion and hydrops fetalis.

In **chapter two**, in order to understand the Queenan or Liley chart, and thus the relation between fetal anemia and the rise in bilirubin concentration in amniotic fluid, we studied the relationship between the bilirubin concentration in amniotic fluid and in fetal blood. In 68 nonhydropic fetuses that received a first intrauterine blood transfusion, we compared amniotic fluid, taken shortly before transfusion, with the initial blood sample taken at the commencing of transfusion. All fetuses were anemic and had an increase in bilirubin concentration in their blood, although there was not a strong correlation between this concentration and the severity of the anemia. Most important finding was that the amniotic fluid/ fetal blood ratio of bilirubin in these affected cases was in accordance with the amniotic fluid/ fetal blood ratio of normal physiological bilirubin values. This amniotic fluid/ fetal blood ratio was dependent on gestational age. This can be explained by the fact that this ratio is determined by the binding of unconjugated bilirubin to albumin and thus by the amniotic fluid/ fetal blood ratio of albumin, during gestation. We hypothesized that of all the possible pathways, it is most plausible that bilirubin exchanges between fetal blood and amniotic fluid over the intramembraneous pathway. Exception to the normal amniotic fluid/ fetal blood ratio of the bilirubin concentration is described in 6 severely hydropic fetuses.

In **chapter three**, we tested our hypothesis that the bilirubin concentration in amniotic fluid is determined by both the bilirubin concentration in fetal blood and the albumin concentration in fetal blood and in amniotic fluid. We measured bilirubin and albumin concentration in fetal blood and in amniotic fluid in 30 fetuses that received a first intrauterine blood transfusion. A strong correlation was found between the bilirubin to albumin ratio (BAR) in fetal blood and that in amniotic fluid, confirming our hypothesis. The BAR in fetal blood was consequently higher than the BAR in amniotic fluid though, possibly due to a difference in binding capacity of albumin in amniotic fluid compared to blood, for example due to a difference in pH. The slope in Queenan's and Liley's chart that has been used for years to predict the severity of fetal anemia, can now be understood: it can be explained by the corresponding slope of the albumin concentration in amniotic fluid during gestation. Exception to the consequent relation between the BAR in fetal blood and the BAR in amniotic fluid is described in 3 severely hydropic fetuses.

In **chapter four**, we focused on the question what the origin of albumin is in the amniotic fluid. We reviewed the literature to assess the available evidence on the fetal, the maternal, and the placental or membrane origin of amniotic fluid albumin. Also, we speculated on the function of albumin in amniotic fluid. From the available evidence, we concluded that a fetal contribution is minimal in second and third trimester, mainly because of the low concentration of protein in fetal urine and lung fluid. A maternal contribution seems plausible, since other large proteins in amniotic fluid were already shown to be of maternal origin. Transfer can take place directly through the fetal membranes. Finally, the amniotic membrane itself also contributes to amniotic fluid albumin, although it is not known in what quantity. The concentration of amniotic fluid albumin may influence the volume of amniotic fluid through maintenance of osmotic pressure or through regulation of receptors involved in amniotic fluid amount and composition. Albumin could have an important role as a carrier protein, for example for fatty acids. Considering its non-fetal origin it could also be an important addition to prenatal transplacental nutrition. Different suggestions are proposed to investigate the origin, the transport mechanisms, and the function of albumin in amniotic fluid.

In **chapter five**, we studied the role of a low albumin concentration in fetal blood on the development of hydrops fetalis. Data was collected from 224 fetuses that received a first intrauterine blood transfusion due to Rh-D alloimmunization. We included 161 nonhydropic, 44 mildly hydropic and 19 severely hydropic fetuses. Relative hemoglobin deficit and relative albumin deficit, both corrected for gestational age, were determined

9

at commencing of the procedure. A decrease in albumin concentration occurred only at a hemoglobin deficit below 8 standard deviations below the normal mean. Although the percentage of fetuses with a low albumin concentration was highest in severely hydropic fetuses (63%), it was much less in mildly hydropic fetuses (14%) and also occasionally present in nonhydropic fetuses (6%). Overall 73% of the hydropic fetuses had a normal albumin concentration. It therefore was concluded that a low albumin concentration in fetal blood is most likely a secondary effect and not the initial cause of hydrops in fetal anemia. In our study, both a low concentration of albumin and presence of severe anemia were independently predictive for the presence of hydrops.

In **chapter six**, the relationship between severity of anemia or presence of hydrops and the fetoplacental blood volume was assessed. We calculated fetal total blood volume in 86 fetuses that received a first intrauterine blood transfusion. The blood volume was calculated on the basis of a dilutional principle, namely of the fetal hemoglobin that was present at the beginning of the transfusion with the adult (donor) hemoglobin. The average fetal total blood volume was 123 ml/kg in nonhydropic fetuses and 144 ml/kg in hydropic fetuses. There was no relation between severity of anemia and blood volume, corrected for fetal weight. This implies that fetuses maintain their blood volume even in severe anemia and compensate for the loss of red cells with an equal increase in plasma volume. In hydropic fetuses, there even seems to take place an overcompensation of plasma volume. This is in accordance with the hypothesis that congestive heart failure plays a role in the pathophysiology of hydrops in anemic fetuses.

In **chapter seven**, we aimed to study the fluid shift that takes place out of the fetal circulation during an intrauterine blood transfusion. The effect of volume and speed of transfusion, and the severity of anemia and presence of hydrops were analyzed. In 95 fetuses, we calculated fluid shift at first intrauterine transfusions, by determining initial and final blood volumes. We found that on average 36% of the transfused volume already leaves the circulation during transfusion. Volume of fluid shift was related to the volume of donor blood that was administered. The fluid shift was however inversely related to the speed of transfusion, implying that this shift takes time, making it probable that this process continues in the hours after the transfusion. Severity of anemia and presence of hydrops surprisingly had no evident effect on the amount of fluid shift, possibly because they can have different contradictory effects on the cardiovascular system. Further, we found that at low gestational age, fetuses had been unintentionally burdened with relative high volume and speed of

transfusion. Transfusion policy should therefore be adjusted to gestational age. It should also be considered that the hematocrit still increases after transfusion, potentially leading to unintentional high hematocrit values and hyperviscosity of the fetal blood during the days following transfusion.

In **chapter eight**, the new insights acquired in this thesis are summarized. Among others, the differences between the fluid and protein dynamics in fetuses and in neonates are discussed. Our findings led to several implications for current practice. Finally, proposals for future research are described.

9

Dit proefschrift beschrijft onderzoek naar hemolytische foetale bloedarmoede en foetale hydrops. Hemolytische foetale bloedarmoede ontstaat door afbraak van rode bloedcellen. Foetale hydrops betreft het vasthouden van te veel vocht in het lichaam, die door chronische bloedarmoede kan ontstaan. Metingen in het kader van onze studies werden zowel in foetaal bloed als in vruchtwater verricht, vlak voor of tijdens intrauteriene bloedtransfusies (bloedtransfusies die, via een lange naald, gegeven worden aan kinderen in de baarmoeder).

In **hoofdstuk één** worden de achtergronden weergegeven die hebben geleid tot de onderzoeken, beschreven in dit proefschrift. Aangezien het LUMC een tertiair verwijscentrum voor foetale therapie is, bestaat hier een unieke mogelijkheid om ziektes van de ongeborene te bestuderen. Potentieel behandelbare prenatale ziektes gaan vaak gepaard met abnormale hoeveelheid vruchtwater. Over het algemeen is het eindstadium van deze ziektes foetale hydrops. Zowel bij abnormale vruchtwater hoeveelheden als bij hydrops is er sprake van een verschuiving in vocht en eiwitten tussen verschillende foetale compartimenten (binnen en buiten de bloedbaan). Een van de ziektes waarbij hydrops op kan treden is foetale bloedarmoede. Sinds enige decennia is bepaling van bilirubine (een afbraakproduct van hemoglobine in rode bloedcellen) in het vruchtwater de methode om bloedarmoede bij de foetus te voorspellen. Een vraag vanuit de kliniek was hoe deze relatie bepaald wordt. Een andere onderzoeksvraag was, hoe de foetus reageert op ernstige bloedarmoede en op intrauteriene bloedtransfusies, met het doel de veiligheid van deze behandeling te kunnen verbeteren. Tenslotte wordt achtergrondinformatie gegeven over foetale bloedarmoede door afbraak van rode bloedcellen, over de mogelijkheden van diagnostiek naar foetale bloedarmoede, over intrauteriene bloedtransfusie en over foetale hydrops.

In **hoofdstuk twee** wordt de relatie tussen de bilirubine concentratie in vruchtwater en die in foetaal bloed bestudeerd, met als doel de Queenan of Liley testen (die een relatie leggen tussen foetale bloedarmoede en de stijging van bilirubine in vruchtwater) te begrijpen. In 68 niet hydropische foetussen, die hun eerste intrauteriene bloedtransfusie ontvingen, werd het vruchtwater, afgenomen vlak voor de transfusie, vergeleken met het initiële bloedmonster, afgenomen bij aanvang van de transfusie. Alhoewel alle foetussen bloedarmoede en een verhoogd bilirubine gehalte in het bloed hadden, was er geen sterke relatie tussen de mate van bloedarmoede en de hoogte van de bilirubine concentratie. Belangrijkste bevinding was dat de verhouding van het bilirubine gehalte in vruchtwater ten opzichte van bloed in deze aangedane foetussen overeen kwam met de verhouding van bilirubine in vruchtwater ten opzicht

van foetaal bloed in niet aangedane zwangerschappen (referentiewaardes uit de literatuur). Deze vruchtwater/ bloed verhouding was afhankelijk van de zwangerschapsduur. Dit kan verklaard worden door het feit dat deze verhouding bepaald wordt door de binding van bilirubine aan albumine (een bindingseiwit) en dus door de verhouding van het albumine gehalte in vruchtwater ten opzicht van bloed, gedurende de zwangerschap. We formuleerden de hypothese dat van alle mogelijke transportwegen het meest waarschijnlijk is dat bilirubine uitwisselt tussen foetaal bloed en vruchtwater door de vliezen die het vruchtwater omringen (met name het oppervlakte van de placenta waar vruchtwater en veel foetale bloedvaten zich vlak naast elkaar bevinden). We beschrijven een uitzondering van de normale verhouding van bilirubine in vruchtwater ten opzichte van bloed in 6 foetussen die ernstig hydropisch waren.

In **hoofdstuk drie** testten we onze hypothese dat de bilirubine concentratie in vruchtwater bepaald wordt door zowel de bilirubine concentratie in het foetale bloed als de albumine concentraties in foetaal bloed en in vruchtwater. We maten de bilirubine en albumine concentratie in foetaal bloed en in vruchtwater in 30 foetussen die een eerste bloedtransfusie kregen. Er werd een sterke relatie gevonden tussen de bilirubine tot albumine ratio (BAR) in het foetale bloed en die in vruchtwater, wat onze hypothese bevestigde. De BAR in het foetale bloed was echter altijd hoger dan de BAR in vruchtwater, mogelijk door een verschil in bindingscapaciteit van albumine in vruchtwater ten opzicht van bloed, bijvoorbeeld als gevolg van het verschil in pH tussen bloed en vruchtwater. De curve in de grafiek van de Queenan of Liley test kan nu goed begrepen worden: deze wordt verklaard door het verloop van de normale albumine concentratie in vruchtwater gedurende de zwangerschap. We beschrijven een uitzondering op de constante relatie van de BAR in bloed ten opzichte van de BAR in vruchtwater bij 3 foetussen die ernstig hydropisch waren.

In **hoofdstuk vier** richtten we ons op de vraag hoe albumine in het vruchtwater terecht komt. We zochten in de literatuur naar bewijsmateriaal voor een foetale oorsprong, een moederlijke oorsprong of een oorsprong in de placenta of de vliezen, van het albumine dat in vruchtwater aanwezig is. We zochten ook naar aanwijzingen voor de mogelijke functie van albumine in vruchtwater. Uit de beschikbare gegevens concludeerden we dat een foetale bijdrage heel klein is in het tweede en derde trimester van de zwangerschap, voornamelijk omdat de concentratie van eiwit heel laag is in foetale urine en longvocht. Een moederlijke oorsprong van albumine in vruchtwater lijkt wel waarschijnlijk aangezien van andere grote eiwitten in vruchtwater reeds is aangetoond dat deze van moederlijke oorsprong zijn. Transport kan

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rechtstreeks door de foetale vliezen plaats vinden. Tenslotte levert het amnionvlies zelf een bijdrage aan albumine in vruchtwater, hoewel het onbekend is in welke hoeveelheid. De concentratie van albumine in vruchtwater zou het volume van het vruchtwater kunnen beïnvloeden door het behouden van osmotische druk of door regulatie van receptoren die betrokken zijn bij het bepalen van de vruchtwater hoeveelheid en samenstelling. Albumine zou een belangrijke rol kunnen hebben als transporteiwit, bijvoorbeeld voor vetzuren. Rekening houdend met de mogelijk niet foetale oorsprong van albumine zou het ook een belangrijke aanvulling kunnen zijn op prenatale, via de placenta verkregen, voeding. Verschillende suggesties worden gedaan om de oorsprong, de transportmechanismen en de functie van albumine in vruchtwater verder te onderzoeken.

In **hoofdstuk vijf** beschrijven we de rol die een lage albumine concentratie in het bloed zou kunnen hebben op de ontwikkeling van foetale hydrops. Gegevens van 224 foetussen die een eerste bloedtransfusie kregen in verband met Rhesus-D immunisatie, werden verzameld. Het betrof 161 niet hydropische, 44 mild hydropische en 19 ernstig hydropische foetussen. Het relatieve hemoglobine en albumine tekort, beide gecorrigeerd voor zwangerschapsduur, werden bepaald voor aanvang van de transfusie. Een verlaging van de albumine concentratie kwam alleen voor bij een hemoglobine tekort van meer dan 8 standaard deviaties onder het normale gemiddelde. Hoewel het percentage van een te laag albumine gehalte het hoogste was bij ernstig hydropische foetussen (63%), was dit veel minder bij mild hydropische foetussen (14%) doch ook incidenteel verlaagd bij niet hydropische foetussen (6%). Al met al hadden 73% van de hydropische foetussen een normaal albumine gehalte. We concludeerden daarom dat een te laag albumine meest waarschijnlijk een secundair effect is en niet de oorspronkelijke oorzaak van hydrops bij foetale bloedarmoede. Uit ons onderzoek bleek dat een laag albumine gehalte en een laag hemoglobine gehalte onafhankelijke voorspellers waren voor de aanwezigheid van hydrops.

In **hoofdstuk zes** onderzochten we de relatie tussen de ernst van de bloedarmoede en de aanwezigheid van hydrops met het foetoplacentaire bloedvolume (het bloed dat circuleert door de foetus, de navelstreng en de placenta). We berekenden het bloedvolume in 86 foetussen die een eerste bloedtransfusie ontvingen. Het bloedvolume werd berekend op basis van een verdunningsprincipe, van het reeds aanwezige foetaal hemoglobine met volwassen (donor) hemoglobine. Het gemiddelde foetale bloedvolume was 123 ml/kg in niet hydropische foetussen en 144 ml/kg in hydropische foetussen. Er was geen relatie tussen de ernst van de bloedarmoede

en het bloedvolume dat gecorrigeerd was voor foetaal gewicht. Dit impliceert dat foetussen hun bloedvolume constant kunnen houden, zelfs in geval van ernstige bloedarmoede, en het verlies van rode bloedcellen dus compenseren met een gelijke hoeveelheid plasma volume. In hydropische foetussen lijkt er zelfs een overcompensatie te zijn van het plasma volume. Dit past bij de hypothese dat hartfalen een rol speelt bij het ontstaan van hydrops bij foetussen met bloedarmoede.

In **hoofdstuk zeven** hebben we de vochtverplaatsing uit de foetale bloedbaan bestudeerd die plaats vindt tijdens een intrauteriene bloedtransfusie. Het effect van het volume van transfusie, de snelheid van transfusie, de ernst van bloedarmoede en de aanwezigheid van hydrops werden geanalyseerd. In 95 foetussen berekenden we de vochtverplaatsing tijdens een eerste intrauteriene bloedtransfusie, door het oorspronkelijke bloedvolume en het bloedvolume op het einde van de transfusie te bepalen. We vonden dat gemiddeld 36% van het volume dat gegeven werd tijdens de transfusie de foetale bloedbaan direct verlaat. De hoeveelheid vochtverplaatsing was gerelateerd aan de hoeveelheid transfusiebloed die werd gegeven. De hoeveelheid vochtverplaatsing was echter omgekeerd evenredig aan de snelheid van transfusie, implicerend dat dit proces tijd vergt, wat het waarschijnlijk maakt dat het proces van vochtverplaatsing nog verder gaat in de uren na de transfusie. De ernst van de bloedarmoede en de aanwezigheid van hydrops hadden verrassend genoeg geen evident effect op de hoeveelheid vochtverplaatsing, mogelijk omdat verschillende tegengestelde effecten op het cardiovasculaire systeem plaats kunnen vinden in deze situaties. Verder vonden we dat bij een lage zwangerschapsduur de foetussen met een relatief hoog volume en een hoge snelheid van transfusie werden belast. Het transfusie beleid zou dan ook aangepast moeten worden aan de hand van de zwangerschapsduur. Bovendien zou rekening gehouden moeten worden met de mogelijkheid dat het hematocriet (het bloedgehalte) nog doorstijgt na afloop van de transfusie, met als mogelijk gevolg een onbedoeld hoog hematocriet en hyperviscositeit van het foetale bloed in de dagen na de transfusie.

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In **hoofdstuk acht** worden de nieuwe inzichten die verkregen zijn in dit proefschrift, samengevat. Onder meer het verschil tussen de vocht- en eiwithuishouding bij foetussen en bij pasgeborenen wordt beschreven. Vervolgens worden verschillende implicaties voor de huidige praktijk beschreven. Tenslotte worden verschillende voorstellen voor toekomstig onderzoek besproken.

LUMC	=	Leiden University Medical Center
IgG	=	Immunoglobulin G
Rh D	=	Rhesus D (antibody)
Delta OD450	=	Delta Optical Density 450
		(spectrophotometric measurement)
CMV	=	Cytomegalovirus
IUT	=	Intrauterine transfusion
ADCC	=	Antibody-Dependent Cell-mediated Cytotoxicity assay
BAR	=	Bilirubin/ albumin ratio
FPV	=	Feto-placental blood volume
RCV	=	Red cell volume
Hb	=	Hemoglobin
Ht	=	Hematocrit
MCHC	=	Mean Corpuscular Hemoglobin Concentration
Alb	=	Albumin
AFP	=	Alpha-feto protein
SD	=	Standard deviation
SE	=	Standard error
95%CI	=	95% Confidence interval
ROC curve	=	Receiver operating characteristics curve
p	=	probability
R^2	=	Coefficient of determination
L	=	liter
dL	=	deciliter
ml	=	milliliter
kg	=	kilogram
g	=	gram
mg	=	milligram
mmol	=	millimol
nm	=	nanometer
kD	=	kiloDalton

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Suzanne A. Pasman werd op 10 augustus 1977 geboren te Nieuwegein. Zij groeide op in een gezin met één zus en twee broers. Na op diverse plekken te hebben gewoond, onder andere in Camberley te Engeland, bracht zij het grootste deel van haar jeugd door in Boxmeer. In 1995 behaalde zij er aan het Elzendaal college het Gymnasium diploma.

In 1996 behaalde zij de propedeuse Biomedische Wetenschappen te Leiden. Daarna vervolgde zij met de studie Geneeskunde. In dit jaar was zij tevens voorzitter van de gezelligheidsvereniging van de faculteit: "het Leids Medisch Dispuut Forestus" (250 leden). Voor en na deze bestuurstijd was zij tevens betrokken bij diverse serieuze en minder serieuze commissies binnen de faculteit. Op 9 april 1998 werd zoon Myrddin geboren. Vanaf dat moment studeerden zij en partner Sebastian Peters afwisselend. Zo doorliep zij in 2000 het eerste jaar van de co-schappen en in 2003 het tweede jaar. Op 3 februari 2002 vond de geboorte plaats van een tweede zoon: Thorquato. Tevens werd in deze periode het eerste contact gelegd met Dr. Frank Vandenbussche van de afdeling Verloskunde in het LUMC. Er werd in deeltijd gestart met het onderzoek dat uiteindelijk tot dit proefschrift heeft geleid.

In februari 2004 trad zij in dienst als "arts-echoscopist" bij de sectie prenatale diagnostiek en behandeling van de afdeling Verloskunde in het LUMC, tot vorig jaar onder leiding van Prof. Dr. Humphrey Kanhai. Daar is zij tot op heden werkzaam, inmiddels als "arts prenatale geneeskunde" onder leiding van Dr. Dick Oepkes en Prof. Dr. Jan van Lith. Binnen de foetale geneeskunde ontwikkelde zij niet alleen een voorliefde voor de patiëntenzorg en het onderzoek maar ook voor onderwijs.

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Bloed krijg én geef je met je hart!

**Voor alle hulp die ik gekregen heb in de afgelopen jaren,
wil ik graag mijn dank aan jullie geven uit het hart:**

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aan iedereen die mijn werk en onderzoek ondersteund heeft op de poli,
het secretariaat en op de afdeling verloskunde

aan iedereen die mijn werk en onderzoek ondersteund heeft op het Verloslab,
het CKCL en de Immunohematologie

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om dit werk te voltooien

aan mijn vrienden, familie en schoonfamilie

aan Myrddin en Thorquato (en iedereen die bij heeft gedragen aan hun opvang)

Sebastian, without you this would all still be nothing more than a figment of my imagination…

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