Original Research

Title. High-risk pregnancy is associated with increased alpha-fetoprotein concentrations in the amniotic fluid and foal plasma

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Abstract

This study aimed to determine alpha-fetoprotein (AFP) concentrations in amniotic fluid, plasma of mares and respective foals: carrying normal pregnancies and delivering healthy foals (n=20; Group 1); carrying apparently normal pregnancies and delivering sick foals (n=15; Group 2); carrying high-risk pregnancies and delivering sick foals (n=14; Group 3). High-risk pregnancy was defined by a history of premature udder development/lactation or increased of the combined thickness of the uterus and placenta, or vulvar discharge and/or mares' systemic illness. Sick foals were affected by neonatal encephalopathy, sepsis, prematurity/dysmaturity, or hypoxic-ischemic encephalopathy. Based on histological examination of the chorioallantois, AFP trend was analyzed in pregnancies with pathologic (PFM) and normal fetal membranes (NFM). Concentrations of AFP were measured using a commercially available immunoassay previously validated for horses. Mares' plasma AFP did not change during the last 15-20 days of pregnancy in the three groups, and there was no difference among them. Amniotic fluid AFP was higher in Group 3 ($p=0.014$). Foals' plasma AFP concentration was higher from birth to 72h in foals of Group 2 and 3 than in healthy ones, and foals of Group 3 had the highest value. The strong association ($r=0.84$; $p<0.0001$) between AFP in amniotic fluid and foals' plasma at birth is likely due to the presence of AFP in fetal urine. AFP was higher in pregnancy with PFM than with NFM in mare's plasma at admission ($p=0.031$), amniotic fluid ($p=0.004$), foal's plasma at birth ($p=0.002$), at 24 ($p=0.005$) and at 72 hours of life ($p=0.004$). AFP is higher in pregnancy with histopathological lesions of the chorioallantois providing the evidence of the differences between pregnancy with a normal placental barrier and the more compromised ones. The increased AFP concentration in the amniotic fluid and plasma of high-risk foals suggests upregulation.

Keywords: Alpha-fetoprotein; mare; neonatal foal; amniotic fluid; high-risk pregnancy

1. Introduction

Alpha-fetoprotein (AFP) is a glycoprotein first discovered in human fetuses in 1956, and then its presence and the putative role was significantly expanded in the following decades across mammalian species [1]. Early in pregnancy, AFP is produced by the yolk sac, and then the fetal liver and gastrointestinal tract system take over AFP production after regression of the yolk sac [2].

AFP is a member of the albuminoid superfamily associated with estrogen binding, heavy metals, and immuno-modulation [3-5]. During human pregnancy, AFP begins to rise from the end of the first trimester, peaks during the second trimester, and then begins to fall after 32 weeks of gestation [6]. In women, AFP concentrations have high predictive values for preterm placenta-mediated adverse pregnancy outcomes [7]. In addition, high AFP levels are associated with multiple pregnancies, pathologic conditions such as neural tube defects [8], abortion [9], congenital nephrosis [10], intrauterine growth retardation [11], preeclampsia [12], and preterm birth in the asymptomatic woman [13].

In horses, AFP was first described in the plasma of early pregnant mares [14]. The same study also reported that twin pregnancies have greater AFP concentrations than singleton pregnancies [14]. Of interest, concentrations of AFP were increased in plasma of mares experiencing pregnancy loss [14]. Thereafter, AFP was demonstrated to be present in high concentrations in the fetal fluids of pregnant mares and to be increased in plasma of mares with experimentally induced placentitis when compared to gestationally age-matched healthy mares [15]. Subsequently, AFP was investigated throughout pregnancy in Lipizzaner mares carrying normal pregnancies [16]. Thereafter, a study demonstrated that AFP is present in the plasma of foals in high concentrations, and there was a decline in the first week of life [17]; the same study determined that healthy Thoroughbred foals have lower concentrations than foals becoming sick during the first week of life [17].

This study aimed to evaluate the AFP concentration in mares' plasma, amniotic fluid, and foal plasma in both normal and high-risk pregnancy to understand if AFP could be used as a marker of high-risk

78 pregnancy in field condition. We hypothesized AFP is higher in mares with a high- risk pregnancy,
79 particularly in mares with placenta-mediated adverse pregnancy outcome, as described in women [7]
80 and that these higher concentrations are the reflection of the high concentration in their respective
81 foal and amniotic fluid.
82
83 **2. Materials and Methods**
84 2.1 Animals
85 The study was carried out as a prospective observational study with client-owned mares presented for
86 foaling management to the Equine Perinatology and Reproduction Unit of the Department of
87 Veterinary Medical Sciences of the University of Bologna during the 2018 and 2019 foaling seasons.
88 Mare's breed, age and parity were recorded.
89 The mares were divided into three groups: mares carrying normal pregnancies and delivering healthy
90 foals (Group 1); mares carrying apparently normal pregnancies and delivering sick foals (Group 2);
91 and mares carrying high-risk pregnancies and delivering sick foals (Group 3). High-risk pregnancy
92 was defined as a history of premature udder development/lactation, increase of the combined
93 thickness of the uterus and placenta, vulvar discharge, and/or mare's systemic illness. Mares with
94 dystocic delivery due to fetal or maternal causes were excluded from the study.
95 The mares were admitted due to apparent clinical problems (n= 14), or owners' concern for unattended
96 foaling (n= 33), or history of clinical problems in the previous pregnancies (n= 2). All mares were
97 admitted approximately by 310 days of gestation and remained on around-the-clock observation until
98 at least 7 days postpartum. The mares were kept in stalls (4 x 4 m) and fed hay ad libitum and
99 concentrate twice a day. All the mares received a complete physical examination twice a day during
100 the hospitalization and a complete blood cell count and blood chemistry at admission. Additionally,
101 transrectal palpation and ultrasonographic examination were performed to evaluate the combined
102 thickness of the uterus and placenta (CTUP) at admission and every ten days until parturition. The
103 reference ranges of CTUP were considered related to gestational age, as reported elsewhere [18,19].

The time from admission to foaling were recorded as Days Before Parturition (DBP). After parturition, the foals were classified as healthy when they had a normal clinical evaluation during hospitalization, including a complete blood count and serum biochemistry at birth and an IgG serum concentration ≥ of 800 mg/dL at 24 h of life [20]. Foals affected by Hypoxic-Ischemic Encephalopathy (HIE) with evidence of dystocic parturition were excluded. Foals with the same clinical presentation but without evidence of a hypoxic insult were classified as affected by Neonatal Syndrome (NS) [24]. Foals were defined as premature when born prior to 320 days of gestation and dysmature when born after 320 days both with immature physical characteristics: low body weight or small for gestational age respectively, inability to maintain body homeostasis and to suckle, hyperextension of flexor tendons in the, or both, incomplete carpal and tarsal bone ossification. Laboratory findings in premature foals can show a narrower neutrophil-lymphocyte ratio than in healthy term foal, with a higher lymphocyte count [25]. Foals were classified as septic in the presence of both infections, confirmed based on positive blood culture, culture of pathogens from local sites of suspected infection, or based on postmortem examination, and systemic inflammatory response [26].

Fetal membranes were grossly evaluated immediately after delivery. For histological evaluation, samples were collected, fixed in formalin, and then embedded in paraffin and routine histological hematoxylin-eosin (HE) stained slides were obtained. Diagnosis of placental insufficiency was performed retrospectively after macroscopic and histopathologic examination of the placenta [21-23]. Based on chorioallantois histological exam results and independently from the type of pregnancy, mares were also divided into 2 Groups: pathologic fetal membranes Group (PFM Group) and normal fetal membranes Group (NFM Group).

2.2. Samples collection and analysis

All samples were harvested as part of the clinical program of peripartum monitoring; owners gave consent to use samples for research. All blood samples were collected by jugular venipuncture into plastic tubes containing anticoagulant for routine CBC and biochemistry analysis. For the study

purpose an aliquot of plasma sample for each subject was centrifuged at 600 g/10 min within 30 minutes of collection, stored at -20°C, and then analyzed at the end of each foaling season. Mares' plasma was collected at admission, then every ten days, and at foaling. Amniotic fluid was collected by direct puncture of the amniotic membrane after its projection through the vulva. Foals' plasma was collected soon after delivery (T0), at 24 (T24) and 72 (T72) hours after birth. Concentrations of AFP were determined using a heterologous commercially available immunoassay on a chemiluminescence platform (Immulite® 2000, Siemens), previously validated for horses as described elsewhere [15]. The AFP assay has a range of 0.2 to 300 units/mL. The samples above the upper detection limit were diluted with the diluent of the commercial kit. According to the manufacturer, a conversion factor of 1.21 was applied for conversion of IU/mL to ng/mL of human AFP.

2.3. Statistical analysis

The Kolmogorov–Smirnov test was used to assess data for normal Gaussian distribution. Since data were non-normally distributed, they were assessed with non-parametric tests. Correlations of AFP concentrations between mare plasma, foal plasma, and amniotic fluid were assessed with Spearman's correlation test. Differences among sampling times were assessed with Kruskal-Wallis test followed by post-hoc analysis. Differences between males and females in foal’s plasma AFP concentration at birth were assessed with the Mann-Whitney test. Differences between the group NFM and PFM were assessed with the Mann-Whitney-U-test.

Spearman’s correlation test was used to assess the associations of AFP concentrations (amniotic fluid, mare, and foal plasma), gestational length, foals' weight, and complete blood count and blood chemistry parameters at birth. Data were presented as median and interquartile ranges. Significance was set as $P < 0.05$. All the data analysis was performed with a statistic software (SPSS).

3. Results

Forty-nine mares were included in the study. Twenty mares were included in Group 1 (normal pregnancy and healthy foal), 15 mares in Group 2 (apparently normal pregnancy and sick foal), and 14 mares in Group 3 (high-risk pregnancy and sick foal) (Table 1). Foals in Group 2 had neonatal encephalopathy (n= 11) and HIE (n= 4), defined as described elsewhere [24]. Mares included in Group 3, presented: premature udder development and increased CTUP (n= 11), laminitis (n= 1), colic surgery at 282 d of gestation (n= 1), and prepubic tendon rupture with severe ventral abdominal hernia (n= 1). Foals born from these mares had a variety of clinical diagnosis: prematurity/dysmaturity (n= 4) and sepsis (n= 2), neonatal encephalopathy (n= 3) and HIE (n= 3). Two foals were stillborn. Mares suffering from dystocia and their foals were excluded from the study. Foal complete blood cell count and chemistry parameters at birth are depicted below (Table 2 and 3). Foals in Group 3 had lower hemoglobin (p=0.0222), erythrocyte (p=0.0071), lymphocyte (p=0.0072), and monocyte (p=0.0284) count in comparison with foals in the other two groups. On the other hand, ionized calcium (p=0.0065) was greater in foals of Group 3 than in the others. In Group 1, all fetal membranes were grossly normal. Four out of 15 fetal membranes in Group 2 and 12 out of 13 in Group 3 presented a variety of abnormalities. Fetal membranes in Group 2 presented chorionic villi hypoplasia (n=4); in Group 3, fetal membranes presented chorionic villi hypoplasia (n= 2), severe edema (n= 7), thickness of the chorioallantois with exudate, necrotic and avillous area (n= 3). In Group 3, one fetal membrane was not evaluated as the mare was euthanized before placenta expulsion due to prepubic tendon rupture and severe ventral abdominal hernia. The gross and histopathological findings of the chorioallantois in three representative subjects of the Groups 2 and 3 were described in Fig.1. Data about mare and pregnancy of Group 1, Group 2 and Group 3 are illustrated in Table 3, 4 and 5, respectively. The average of DBP was 19 ± 3 days in Group 1, 20 ± 9 days in Group 2, and 19 ± 10 in Group 3.

Data about AFP concentration in mares' plasma, amniotic fluid and foals' plasma are reported in Table 6. Mares' plasma AFP did not change from admission to foaling in the three Groups. There were no statistical differences between males and females in foal's plasma AFP concentration at birth.

In Group 1, Spearman correlation test found a significant correlation between AFP concentration in amniotic fluid and in foals' plasma at birth ($r=0.76$; $p<0.001$), in foals' plasma at 24h ($r=0.78$; $p<0.001$) and in foal's plasma at 72h ($r=0.79$; $p<0.001$). In Group 2, Spearman correlation test found a significant correlation between foals' plasma AFP at birth and foals' plasma AFP at 24h ($r=0.82$, $p<0.01$). In all the three Groups, AFP concentrations followed the same pattern during the first 72 hours of life with the highest concentration at birth and the decline over 72 h. In Group 1, foals' plasma concentrations of AFP were different among the three different sampling times ($p<0.001$). In Group 2, AFP concentration followed the same pattern, and a significant difference was found between foals' plasma AFP at birth and after 24 hours ($p<0.001$), between foals' plasma AFP at 24h and at 72h ($p<0.05$), between foals' plasma AFP at birth and at 72 h ($p<0.01$). In Group 3, a significant difference was found between foals' plasma AFP at birth and after 24 hours ($p<0.001$), between foals' plasma AFP at 24h and at 72h ($p<0.05$), between foals' plasma AFP at birth and at 72 h ($p<0.001$). In Group 3, Spearman correlation test found a significant correlation between foals' plasma AFP at birth and at 24h ($r=0.83$, $p=0.003$). The analysis of differences among Groups found a significant difference among AFP concentration in amniotic fluid ($p=0.014$), in particular the post-hoc analysis revealed differences of both Group 1 ($p=0.04$) and Group 2 ($p=0.027$) with Group 3. A similar trend was found among the AFP concentrations in foals' plasma at birth ($p<0.001$) and in particular the post-hoc analysis revealed differences of both Group 1 ($p<0.001$) and Group 2 ($p=0.005$) with Group 3. Also, the foals' plasma AFP at 24h and 72h were different among the three groups ($p=0.002$) with the same trend between Group 1 ($p<0.001$) and Group 2 ($p=0.023$) with Group 3 at 24h of life; at 72 h Group 1 ($p<0.001$) and Group 2 ($p=0.004$) were different from Group 3.

At birth, AFP concentration in sick foals' plasma of Group 2 and 3 was positively correlated to lymphocytes counts ($p= 0.0275$, $r =0.45$) and negatively correlated with erythrocytes counts ($p= 0.0092$, $r=- 0.52$), total bilirubin ($p= 0.0405$, $r=- 0.43$), and albumin ($p= 0.0069$, $r=- 0.55$) concentrations. Moreover, AFP concentration at birth in sick foals was negatively associated with foal birthweight ($p= 0.0019$, $r=-0.63$) and gestational length ($p= 0.0139$, $r=-0.50$).

On the basis of histological findings, 31 mares were included in group NFM and 14 in group PFM. Data were showed in Table 8. Unfortunately, for few mares with placental macroscopic alterations, the histological findings were not available, and were not included in the statistical analysis. The Mann-Whitney-U-test found a significant difference as regard AFP concentration in mares' plasma at admission ($p=0.031$), but not at foaling, between the NFM and PFM group, with a higher concentration in the latter. The AFP concentration in amniotic fluid ($p=0.004$) and in foals' plasma at birth ($p=0.002$), at 24 h ($p=0.005$) and at 72 h of life ($p=0.004$) was higher in PFM group than in NFM group.

4. Discussion

The present study was conducted to determine AFP's usefulness as a biomarker in normal and high-risk pregnancies and in their respective neonatal foals. This is the first study to document that AFP is increased in plasma of foals born from high-risk pregnancies. Mares at term have low plasma AFP concentrations. This finding is consistent with two other studies [15,27]. Concentrations of AFP did not change in plasma of mares with high-risk pregnancies herein. A previous study demonstrated that AFP increased in plasma of mares with experimentally induced ascending placentitis [15,28] and another field study demonstrated that AFP increased in plasma of mares with ascending and focal mucoid placentitis [29]. The lack of change in AFP concentration in the present study could be due to an heterogenous population of mares included or because the plasma sampling was too spread out, not allowing us to detect differences between groups. It is possible that if more frequent sampling were used, we could have observed differences. In addition, it is possible that placentitis may alter AFP concentrations more profoundly than other high-risk conditions [29]. The hypothesis of the present study that AFP is higher in mares with high- risk pregnancies has not been confirmed. In the 14 mares with a high-risk pregnancy, AFP's mean value was similar to those found in mares with normal pregnancy. However, our sampling herein was too infrequent, so it is possible that we could have missed critical changes in AFP concentrations. As proposed in humans, the fetal membranes are

not a site of AFP production, but when they are compromised, a greater amount of AFP gets transferred from the fetoplacental unit to the maternal circulation [7-8]. Although an increase in AFP concentrations has been documented in mares with placentitis [15,28], this is of much lower magnitude than in human compromised pregnancy, probably due to the differences in the type of placentation between the two species. Primates have hemochorial placentation, which facilitates the exchanges of molecules between the fetoplacental unit and maternal systemic circulation; conversely, mares have epitheliochorial placentation, which makes the exchange of molecules more limited. It is worth noting that in the present study, comparing AFP in mares grouped on the basis of the histological examination of chorioallantois, the difference between pregnancies with normal and compromised placental barrier became more evident, with mares presenting the more severe placenta changes having the greatest AFP plasma values. A study about human term placenta demonstrated that the expression pattern of AFP and its receptor is indicative of a transport of AFP from the fetal into the maternal circulation across the fetal vessel endothelium, the vessel muscle wall, the villous stroma and the syncytiotrophoblast [30]. The presence of AFP receptor has never been investigated in equine chorionic villi, but it can be assumed that a similar transport may also be present in this species and that every condition which alters the placental barrier may increase the concentration of AFP in maternal plasma. It is worth noting that AFP in human medicine is included in a list of maternal circulating biomarkers which reflect placental insufficiency and predict fetal growth restriction [31]. In equine medicine, several factors contribute to placental insufficiency such as premature placental separation, placental villous hypoplasia, placental thickening and especially placentitis. The result of this condition is inadequate fetal nutrition resulting in intrauterine growth retardation, premature delivery or abortion [21-23]Since in the present study not every mare with pathological histological findings was affected by placentitis, it is possible that the higher concentration of AFP in mare's circulation was related to other causes of placental insufficiency which implies an altered utero-placental blood perfusion and impaired materno-fetal exchange of nutrient, gases and waste products.

Immunoassays have been primarily used to measure AFP in horses, an early used immunosorbent enzyme-linked assay to determine AFP concentrations in serum of pregnant mares [14]. More recently, AFP was measured in equine plasma using a heterologous chemiluminescence assay using a platform (Immulite 1000) and kit widely available throughout the world [15,28]. Another study has also used a human immunosorbent enzyme-linked assay to horse pregnancy [16]. The chemiluminescence assay appears to have a more direct application to clinical practice as the results being readily available, and the platform has highly standardized quality control. Thus, the latter assay was used herein to assess AFP concentrations.

In farm animals such as cattle, pigs, and sheep, AFP is primarily produced by the fetal liver and secondarily in low levels by the gastrointestinal tract [32-34]; thus, AFP production in horses occurs in these sites. It is possible that high-risk pregnancy resulted in AFP upregulation in the liver and/or gastrointestinal tract of equine fetus. The peripheral increase in AFP observed in mares with experimentally induced placentitis is either due to upregulation by the fetal liver or leakage of this protein in the mares' plasma [15,28]. The increased AFP concentration in the amniotic fluid and plasma of high-risk foals suggests upregulation.

It is thought that the presence of AFP in fetal fluids is related to its secretion in the fetal urine, as suggested in humans and in cows [32,35]. It seems possible that AFP enters both amniotic and allantoic fluid as a component of fetal urine since other plasma proteins of similar molecular weight do not appear to cross fetal membranes [35]. In mares, during the third trimester of normal pregnancy, AFP is present in amniotic fluid at a greater concentration than during parturition, as reported elsewhere [15] and by the present study.

Concentrations of AFP are detected in mare's plasma from mid to late gestation, although 100-1000-fold lower than in fetus, fetal fluids and newborn foal [15]. On the contrary, newborn foals' plasma had a high concentration of AFP, as happening in the newborn of other species [34,36-37]. Alpha-fetoprotein decreased 72 h after birth as previously reported for other species [32,33], but

concentration remained remarkably greater than in adults. In the human newborn, AFP half-life is approximately five days after birth [36]; in the equine neonate, AFP half-life has not been determined. As in other species, AFP in the amniotic fluid of high-risk pregnancy was higher than in healthy pregnancy, and this is probably due to the higher concentrations in the fetal circulation [32,33]. Both healthy and sick foals had a reduction in AFP concentrations leading to 72 h after birth, though sick foals still had greater concentrations. A similar trend was reported in a recent study in septic foals born from mares with experimentally induced ascending placentitis [39]. Septic foals had greater AFP concentrations than healthy foals. It has been suggested that AFP behaves as a positive acute-phase protein in the fetus [40].

The weak but significant associations between AFP concentrations and lymphocytes, erythrocytes, bilirubin, and albumin could suggest a response to intrauterine inflammation, as proposed in humans [41]. Alpha-fetoprotein is also negatively associated with erythrocyte count in human fetuses [41]. The negative correlation with albumin is not surprising since albumin is considered a negative acute-phase protein, and its production by the liver is down-regulated by positive acute-phase proteins, such as AFP [42]. The intrauterine inflammatory environment could be responsible for the lower values of hemoglobin concentration and erythrocyte, lymphocyte, and monocyte number in foals born from high-risk pregnancies [43]. In addition, the negative correlation between AFP and total bilirubin could be since the latter may function as a carrier [44]. The negative correlation between foal's plasma AFP at birth and foal's birth weight and gestational length concurs with that reported in humans, where high values of AFP are found in low-birth-weight newborns and preterm birth babies [38]. Despite the weakness of the correlations obtained in the present study could be a limitation and could result in speculative conclusions, blood parameters need to be critically evaluated with a larger and more homogeneous population, particularly for high-risk pregnancies. This could clarify the clinical role of AFP in equine perinatal period. A previous study suggested that AFP can be a useful screening tool for newborn foals needing further care in the first week of life [17]. As suggested, this could be

309 added in the biomarkers panel of the transitioning phase between intrauterine and extrauterine life in
310 foals [15,28].
311
312 **5. Conclusions**
313 In conclusion, in the studied population of high-risk mares the lack of change in AFP plasma
314 concentration could be due to several conditions presented, ranging from severe placentitis to
315 systemic illness. On the other hand, it is evident that AFP is higher in chorioallantois alterations. The
316 role of AFP and the pathogenesis of its increase in plasma concentration remain to be clarified in
317 newborn foals needing further care.
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433 **Table 1.** Mares (n= 49) assigned to three groups.

Group	Gestational length (d) (Mean ± SD)	BCS	Age	Male foals	Female foals	Breeds
1	341±10	8±1	10±5	7/20	13/20	Standardbred (n=18) Saddlebred (n=2)
2	346±12	7±1	8±5	10/15	5/15	Standardbred (n=14) Saddlebred (n=1)
3	327±12	7±1	11±5	8/14	6/14	Standardbred (n=7) Saddlebred (n=5) Quarter Horse (n=2)

434 BCS: Body Condition Score

435

436 **Table 2.** Foal complete blood cell counts at birth (median and interquartile ranges).

Parameters	**Group 1**	**Group 2**	**Group 3**
Hemoglobin (g/L)	152 (147-163) [a]	154 (148-160) [a]	138 (125-148) [b]
Hematocrit (L/L)	0.47 (0.45-0.49)	0.46 (0.45-0.49)	0.43 (0.41-0.48)
Erythrocytes (10^{12}/L)	10.7 (10.3-11.9) [a]	10.8 (10.4-11.2) [a]	9.9 (9.1-10.5) [b]
Platelets (10^9/L)	196.5 (167.3-222.7)	191.0 (177.7-197.8)	196.0 (178.1-229.2)
Leucocytes (10^9/L)	7260 (6199-8255)	7700 (6962-8973)	6815 (5325-9071)
Lymphocytes (10^9/L)	1260 (1142-1437) [a]	1350 (1205-1518) [a]	1995 (1388-3005) [b]
Monocytes (10^9/L)	180 (115-256) [a]	210 (115-240) [a]	90 (64-178) [b]
Neutrophils (10^9/L)	5970 (4638-6705)	6000 (5387-7280)	4725 (2440-6354)
Eosinophils (10^9/L)	10 (0-10)	10 (10-22)	15 (10-20)
Basophils (10^9/L)	30 (30-50)	30 (30-40)	40 (19-85)

437 (a-b) Different superscript letters in row indicate differences ($P < 0.05$) among groups with Kruskal-
438 Wallis test.

439

440 **Table 3.** Foal blood chemistry at birth (median and interquartile ranges).

Parameters	Group 1	Group 2	Group 3
Creatine kinase (µkat/L)	3.7 (2.3-4.8)	3 (2.6-4)	4.4 (3.4-11.9)
Total bilirubin (µmol/L)	41 (32.5-47.9)	37.6 (29.1-42.7)	30.8 (23.9-49.6)
Total protein (g/L)	24 (19-28)	22 (17-25)	18 (13-29)
Albumin (g/L)	33 (30-35)	34 (31-34)	32 (27-33)
Alb/Glob (g/L)	40 (34-50)	37 (31-41)	31 (28-38)
BUN (mmol/L)	13 (11.7-14.7)	12.6 (11.1-13.9)	11.6 (9.5-15.2)
Creatinine (µmol/L)	212.2 (176.8-256.4)	238.7 (185.6-274)	265.2 (203.3-353.6)
Calcium (mmol/L)	3.3 (3.1-3.3) [a]	3.2 (3-3.4) [a]	4.3 (3.3-4.2) [b]
Magnesium (mmol/L)	0.7 (0.7-0.8)	0.8 (0.7-0.8)	0.8 (0.7-0.9)
Fibrinogen (g/L)	1.6 (1.5-1.9)	1.7 (1.4-2.7)	2.9 (1.7-4.1)

441 (a-b) Different superscript letters in row indicate significant differences ($P < 0.05$) among groups
442 with Kruskal-Wallis test.

443 Table 4. Data about mares carrying normal pregnancy and delivering healthy foals (Group 1).

ID	Days of gest. at admission	Plasma AFP at admission (µg/mL)	DBP	Gest. lenght (days)	Plasma AFP at parturition (µg/mL)	Amniotic Fluid AFP (µg/mL)
1	323	0.24	22	345	0.24	4.17
2	320	0.53	18	338	0	21.30
3	308	0.24	23	331	0.24	30.25
4	306	0.43	19	325	0.66	11.72
5	325	0.49	22	347	0.45	7.21
6	334	0.24	23	357	0.37	5.83
7	326	0.24	15	341	0.67	6.32
8	326	0.30	16	342	0.32	9.37
9	323	0.64	16	339	0.54	8.11
10	318	0.84	18	336	0.51	2.81
11	308	0.24	23	331	0.24	5.28
12	338	0.46	20	358	0.42	4.34
13	343	0.24	16	359	0.31	7.77
14	325	0.37	16	341	0.31	17.18

15	312	0.88	16	328	0.67	10.10
16	311	0.60	21	332	0.42	6.50
17	332	0.24	20	352	0.37	8.08
18	324	0.46	15	339	0.64	5.14
19	329	0.38	19	348	0.37	5.06
20	316	0.24	17	333	0.46	13.67

444 DBP: days before parturition (admission – foaling).

445

446 Table 5: Data about mares carrying apparently normal pregnancies and delivering sick foals (Group 2).

ID	Days of gest. at admission	Plasma AFP at admission (µg/mL)	DBP	Gest. lenght (days)	Plasma AFP at parturition (µg/mL)	Amniotic Fluid AFP (µg/mL)	Foal's weight (kg)	Placenta weight (kg)	Placenta macroscopical alterations (Y/N)	Histopathologic placenta alterations	Mare's diagnosis	Foal's diagnosis
1	320	0.26	33	353	0.57	NA	46	5.7	N	NA	/	Neonatal encephalopathy
2	320	NA	26	346	0.62	8.80	45	4.4	N	NA	/	Neonatal encephalopathy
3	327	0.48	24	351	0.93	4.60	45	5.5	N	NA	/	Neonatal encephalopathy
4	326	0.52	14	340	0.46	19.72	38	3.9	N	NA	/	Neonatal encephalopathy
5	326	1.07	9	335	0.42	16.21	42	3.8	N	NA	/	HIE
6	320	0.68	11	331	0.43	NA	39	3.1	Y	Severe hypoplasia of the chorionic villi	Placental insufficiency	Neonatal encephalopathy
7	332	0.64	16	348	0.61	9.47	50	6	N	NA	/	Neonatal encephalopathy
8	303	0.94	52	355	0.45	4.50	58	6.7	N	NA	/	Neonatal encephalopathy
9	368	0.25	3	371	0.47	5.35	40	4.1	Y	Severe hypoplasia of the chorionic villi	Placental insufficiency	Neonatal encephalopathy
10	322	0.99	14	336	0.52	8.22	43	4.2	N	NA	/	Neonatal encephalopathy
11	327	0.55	32	359	0.69	21.42	41	3.6	Y	Severe hypoplasia of the chorionic villi	Placental insufficiency	HIE

12	328	0.69	4	332	0.62	7.51	54	4.5	N	NA	/	HIE
13	335	0.24	20	355	0.31	NA	53	5.2	N	NA	/	Neonatal encephalopathy
14	332	0.42	23	355	0.38	13.67	43	5	N	NA	/	Neonatal encephalopathy
15	NA	NA	NA	329	0.61	20.45	46	7.7	Y	Severe hypoplasia of the chorionic villi and edema	Placental insufficiency	HIE

447 DBP: days before parturition (admission – foaling); HIE: Hypoxic-Ischemic Encephalopathy; NA: data not available

448

449 Table 6. Data about mares with high-risk pregnancy (Group 3)

ID	Days of gest. at admission	Clinical signs (N=none)	CTUP at admission (mm)	Plasma AFP at admission (µg/mL)	Cervical swab (Neg/Pos)	DBP	Gest. lenght (days)	Plasma AFP at parturition (µg/mL)	Amniotic fluid AFP (µg/mL)	Foal's weight (kg)	Placenta weight (kg)	Placenta macroscopical alterations (Y/N)	Histopathologic placenta alterations	Mare's Diagnosis	Foal's diagnosis
1	324	N	19	0.26	NA	13	337	0.70	NA	47	8.1	Y	Villous hypoplasia, chorionic lamina edema	Placental insufficiency	Neonatal encephalopathy
2	298	N	12	0.50	NA	22	320	0.75	13.79	41	4.5	Y	NA	Placental insufficiency	Neonatal encephalopathy
3	315	Vulvar discharge	8	0.74	Pos	25	340	0.83	NA	48	7.3	Y	Chorionic lamina edema	Placentitis/ placental insufficiency	Neonatal encephalopathy
4	296	N	14	0.30	NA	39	335	0.66	25.77	48	5.8	Y	Interstitial edema and hyperemia	Placental insufficiency	HIE
5	308	Vulvar discharge, premature lactation	9	2.0	Neg	9	317	0.42	NA	37	5.1	Y	Interstitial edema and hyperemia	Placentitis/ placental insufficiency	Prematurity
6	305	Premature lactation	8	0.50	Neg	9	314	0.55	11.40	23	2.3	Y	NA	Sistemic illness (laminitis)	Prematurity
7	315	N	47	0.65	Neg	15	330	NA	39.69	42	5.8	Y	Villous atrophy, microtrombosis, pigments deposition, chorionic lamina edema	Placental insufficiency	HIE
8	342	Vulvar discharge	13	NA	NA	2	344	0.40	NA	NA	NA	Y	NA	Placentitis/ placental insufficiency	Sepsis
9	309	N	17	0.36	Neg	15	324	7.37	87.85	37	14.8	Y	Chorionic lamina edema and hyperemia, villous hypoplasia, microvasculitis	Placental insufficiency	Stillborn
10	342	N	7.7	NA	NA	0	342	0.63	NA	40	3.35	Y	Villous atrophy, microvasal fibrosis/hyperplasia, microtrombosis, neutrophilic infiltration	Sistemic illness (laminitis), placentitis/ placental insufficiency	Dismaturity Sepsis
11	269	Vulvar discharge, premature lactation	10.3	0.52	Pos	30	299	1.51	NA	28	4.6	Y	Chorionic lamina edema, villous atrophy and necrosis	Placentitis/ placental insufficiency	Stillborn

12	319	N	11	0.66	Neg	13	332	0.37	NA	43	5.5	Y	Chorionic lamina edema, villous hypoplasia	Sistemic illness (surgical colic), placental insufficiency	HIE
13	313	Vulvar discharge	9.3	NA	Pos	2	315	0.59	9.83	35	3.15	Y	Villous hypoplasia, hyperemia	Placentitis/ placental insufficiency	Prematurity
14	322	N	NA	0.24	NA	1	323	NA	32.31	NA	NA	NA	NA	Systemic illness (prepubic tendon rupture, severe abdominal ventral hernia)	Dismaturity

DBP: days before parturition (admission – foaling); CTUP: combined thickness of the uterus and placenta (mm); HIE: Hypoxic-Ischemic Encephalopathy; NA: data not available.

Table 7. AFP concentration (ng/mL) in mares' plasma, amniotic fluid, foals' plasma at birth (0h) and after 24 and 72 h in Group 1, 2 and 3.

	Group 1	**Group 2**	**Group 3**
Mare's plasma at admission	0.38 (0.24-0.52) 0.24-0.88 n= 20	0.30 (0.24-0.41) 0.24-0.9 n= 12	0.44 (0.29-0.70) 0.24-2.00 n= 10
Mare's plasma at foaling	0.40 (0.31-0.53) (0-0.67) n= 20	0.32 (0.26-0.43) 0.24-0.61 n= 13	0.54 (0.37-0.83) 0.25-7.37 n= 11
Amniotic fluid	7.49 (5.2-11.32) $^{\$}$ 2.81-30.25 n= 20	8.21 (5.35-16.5) $^{\$}$ 2.76-20.5 n= 12	25.8 (9.8-38.1) $^{\#}$ 10-88 n= 7
Foal's plasma at birth (0 h)	1111.4 (825.5-1476.2) $^{a\$}$ 335.2-1996.5 n = 20	1246.3 (1038.2-1391.5) $^{a\$}$ 865.2-2008.6 n = 13	1669.8 (1573-2808.6) $^{a\#}$ 1331-2771 n= 11
Foal's plasma after 24 h	811.91 (598.3-1145.3) $^{b\$}$ 246.8-1548.8 n= 20	960.7 (758.1-1294.7) $^{b\$}$ 659.5-1669.8 n= 14	1452 (1104.7-1633.5) $^{b\#}$ 982.5-2190.1 n= 11
Foal's plasma after 72 h	643.7 (503.7-930.5) $^{c\$}$ 1158.5-1476.2 n = 20	718.7 (540.9-883.3) $^{c\$}$ 97.4-1153.1 n = 11	1195.5 (929.3-1403.6) $^{c\#}$ 689.7-2262.7 n = 11

Data are expressed as median (interquartile range) and min-max value.

Different superscript letters in columns indicate a significant difference between each time points (Mann-Whitney-U-test).

Different superscript symbols in row indicate a significant difference among groups (Kruskal-Wallis test).

460 **Table 7.** AFP concentration (ng/mL) in mares' plasma, amniotic fluid, foals' plasma at birth (0h)
461 and after 24 and 72 h in NFM and PFM Group.

	NFM Group	PFM Group	p
Mare's plasma at admission	0.37 (0.24-0.46) 0.24-0.89 n=29	0.44 (0.32-0.72) * 0.24-2.0 n= 12	0.031
Mare's plasma at foaling	0.38 (0.31-0.47) (0.24-0.67) n= 29	0.50 (0.3-0.68) 0.24-7.37 n= 14	0.076
Amniotic fluid	7.77 (5.21-12.7) 2.76-30.25 n= 29	17.3 (9.7-31.94) * 4.49-87.85 n= 12	0.004
Foal's plasma at birth (0 h)	1150.7 (870-1409.7) 335.2-2008.6 n= 29	1657.7 (1367.3-1917.8) * 995.8-2770.9 n = 12	0.002
Foal's plasma after 24 h	819.2 (655.2-1185.8) 246.8-1669.8 n= 30	1385.5 (1074.2-1579.1) * 709.1.2190.1 n= 14	0.005
Foal's plasma after 72 h	697 (507.6-903.3) 97.41-1476.2 n = 29	1165.8 (806.8-1370.3) * 614.7-2262.7 n = 12	0.004

462 Data are expressed as median (interquartile range) and min-max value.
463 Different superscript symbols in row indicate a significant difference between two groups (Mann-Whitney-U-
464 test).
465
466

Figure 1. Placental examination of high-risk pregnancies and apparently normal pregnancies delivering high risk foals. (a) Generalized edematous and heavy fetal membranes (14.8 kg) with a placental/foal weight ratio of 40%. The chorioallantois had 2 cm thickness. (b) Chorioallantois histological preparation stained with HE showing hyperemia and edema of the chorionic connective lamina associated with mild hypoplasia of the chorionic villi. (c) An extensive area of transition is observed between the normal (cervical star and non-gravid horn) and hypoplastic/discolored (body and gravid horn) chorionic surface of the chorioallantois. (d) Histological section of the gravid horn showing severe hypoplasia of the chorionic villi. (e) Grossly, an extensive focal lesion is observed in the chorionic surface of the caudal pole of the chorioallantois. In detail, a brown tenacious mucoidal material covers the chorionic surface of the caudal pole. (f) Histological section of the caudal placental pole showing necrosis of the chorionic villi, mild fibrosis and edema of the connective lamina.