This is the final peer-reviewed accepted manuscript of:

Isani G, Ferlizza E, Cuoghi A, Bellei E, Monari E, Bianchin Butina B, Castagnetti C.
Identification of the most abundant proteins in equine amniotic fluid by a proteomic
approach. *Animal Reproduction Science*. 2016, 174, 150-160.

The final published version is available online at:

<https://doi.org/10.1016/j.anireprosci.2016.10.003>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in
the publishing policy. For all terms of use and more information see the publisher's
website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Identification of the most abundant proteins in equine amniotic fluid by a proteomic approach

Isani Gloria ^a, Ferlizza Enea ^{a,*}, Cuoghi Aurora ^b, Bellei Elisa ^b, Monari Emanuela ^b, Bianchin Butina Barbara ^a, and Castagnetti Carolina ^a

^a Department of Veterinary Medical Sciences, University of Bologna, via Tolara di sopra 50, 40064 Ozzano, Italy

^b Department of Diagnostic, Clinical and Public Health Medicine, University of Modena and Reggio Emilia, via del Pozzo 71, 41124 Modena, Italy

*** Corresponding author**

Enea Ferlizza

Dept. of Veterinary Medical Sciences, University of Bologna

Via Tolara di sopra, 50

40064 Ozzano (BO)

e-mail: enea.ferlizza2@unibo.it

Tel.: +39 051 2097023

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Abstract

Characterisation of the physiologic equine amniotic fluid (AF) proteome is a prerequisite to study its changes during diseases and discover new biomarkers. The aim of this study was to identify by a proteomic approach the most abundant proteins of equine AF. AF samples were collected at parturition from 24 healthy mares that delivered healthy foals. All samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% gels. A pool of the 24 samples, after SDS-PAGE, was cut in 25 slices, trypsin-digested and analysed by mass spectrometry (MS) for protein identification. Mean AF protein concentration was 1.96 ± 1.12 g/L. Thirty-four proteins were successfully identified by MS and subsequently categorised according to Gene Ontology (GO). Twelve proteins (e.g. fibronectin, lumican, thrombospondin and fibulin) belonged to or interacted with the extracellular matrix (ECM) playing an important role in the development of foetal tissues. Most of the remaining proteins were classified as transport (e.g. albumin, major allergen Equ c1 and alpha-fetoprotein) delivering nutrients, ions and lipids essential for foetal growth and development. Among these proteins, major allergen Equ c1 is widely studied in human medicine because it induces Ig-E mediated type I allergic reaction. The absence of immunoglobulins in equine AF was also confirmed.

Keywords

Horse, pregnancy, electrophoresis, proteome.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Introduction

Amniotic fluid (AF) is a complex dynamic milieu that changes as pregnancy progresses. AF contains nutrients and growth factors that facilitate foetal growth, provides mechanical cushioning and antimicrobial effectors to protect the foetus and allows assessment of foetal maturity and disease (Underwood et al. 2005). In comparison to humans, the physiology and pathophysiology of foetal fluids in domestic mammals are poorly understood (Canisso et al. 2015). In horses, some studies have investigated biochemical composition, particularly enzymes and electrolytes, in AF collected by ultrasound-guided transabdominal amniocentesis, at delivery or at slaughter (Holdstock et al. 1995; Lyle et al. 2006; Williams et al. 1993; Zanella et al. 2014). AF was also studied for evaluation of foal's lung maturity at birth through lecithin/sphingomyelin ratio and lamellar body count (Castagnetti et al. 2007). More recently, significantly higher levels of lactate were found in AF collected during parturition in mares delivering healthy foals (Pirrone et al. 2012).

Unlike the allantoic fluid, equine AF can be easily collected during parturition without stressing the animal and avoiding any contamination (Castagnetti et al. 2007, Pirrone et al. 2012). As reported in women, the biochemical composition of AF, including proteins, is primarily representative of the foetal profile and reflects its physiological status (Tong 2013), thus it could be potentially useful to evaluate the high-risk foal born attended.

Proteomics is a powerful analytical approach providing a profile of proteins present in a biological sample at a given time. The high potential of this approach has been recently found to have a major role in different areas of veterinary medicine, from farm (Almeida et al. 2015) to companion (Ferlizza et al. 2015; Miller et al. 2014) animals. Proteomic techniques have recently been applied to the characterisation of horse amniotic membrane (Galera et al.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

2015) and bovine conceptus fluids (Riding et al. 2008), whereas the equine AF proteome remains uncharacterised. Therefore, the aims of this study were to identify the most abundant proteins in equine AF by SDS-PAGE separation followed by mass spectrometry identification.

Materials and Methods

Animal selection and data collection

Twenty-four mares admitted for assisted delivery during three breeding seasons at the S. Belluzzi Equine Perinatology Unit of the Department of Veterinary Medical Sciences, University of Bologna, were included. The mares were hospitalised at about 310 days of pregnancy because the owners requested an attended parturition, and remained under observation for at least 7 days *postpartum*. They were housed in wide straw bedding boxes and fed with hay *ad libitum* and concentrates twice a day. During the day, the mares were allowed to go to pasture.

All the mares included in the study were healthy based on clinical and ultrasonographic evaluation. At admission, a complete clinical evaluation, including complete blood count, serum biochemistry and transrectal ultrasonography, was performed. Severe maternal illness, uterine discharge, premature lactation, twinning, abnormal foetal presentation, placenta oedema, and signs of foetal distress were ruled out. During the course of hospitalisation, mares were clinically evaluated twice a day and by transrectal ultrasonography every 10 days until parturition. The following ultrasonographic parameters were evaluated: combined thickness of the uterus and placenta, foetal fluids echogenicity, foetal activity, and foetal orbital area. Foals were born between 320 and 365 days of

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

pregnancy by normal delivery, had an Apgar score ≥ 8 recorded within 5 minutes from birth (Vaala et al. 2002) and had a normal clinical evaluation during the course of hospitalisation, including a complete blood count and serum biochemistry at birth and an immunoglobulin G (IgG) serum concentration ≥ 800 mg/dL at 18-24 hours of life.

For each mare, the following data were recorded: breed, age, parity, days of pregnancy, body weight, length of stage II labour (minutes), and foal's body weight and Apgar score. All procedures on the animals were carried out with the approval of the Ethical Committee, in accordance with DL 116/92, approved by the Ministry of Health (approval number: n.18/64/11; date of approval 22/02/2011). Oral informed consent was given by the owners.

Sample collection

At foaling, a sample of AF was collected from each mare with a 50 mL syringe by needle puncture of the amniotic sac within few minutes of its appearance through the vulva during stage II of labour. The AF was then immediately transferred to 5 mL test tubes and stored at -80°C until SDS-PAGE and protein identification were performed. AF protein concentration was determined by the Biuret method using bovine serum albumin as standard.

SDS-PAGE

To optimise protein separation, different protocols were tested including 4-12% and 12% polyacrylamide gels (NuPage/Thermo Fisher Scientific, Waltham, Massachusetts, USA) in 2-(N-morpholino) propanesulfonic acid buffer (MOPS; NuPage/Thermo Fisher Scientific) or 2-(N-morpholino) ethanesulfonic acid buffer (MES; NuPage/Thermo Fisher Scientific)

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

with sodium dodecyl sulphate (SDS). Each AF sample (n=24) was analysed at least twice with the protocol assuring the best protein separation in our experimental conditions (4-12% gels, in MOPS buffer). Twenty µg of proteins were loaded for each sample and the gels were stained with Coomassie G250 compatible with mass spectrometry analysis. After staining, each gel was digitalised by ChemiDocTMMP (BioRad, Hercules, California, USA) and its pherogram was obtained using ImageLab 5.2.1 software (BioRad). The software determines the volume of each protein band through the analysis of the pixel values in the digital image, meaning as volume the sum of all the pixels intensities within the band boundaries. The band volumes are subsequently compared to the entire volume of the lane and the relative abundances reported in percentage. A pool was prepared by collecting and mixing 50 µg of protein from each AF (n=24) and analysed twice with the same protocol used for each sample.

Protein identification by mass spectrometry

The pool lanes were divided manually into 25 slices and subjected to in-gel tryptic digestion as previously described (Bellei et al. 2013). Digested dried samples were then re-suspended in 97% Water/3% ACN added of 1% formic acid (Buffer A) and analysed by a Nano LC-CHIP-MS system (ESI-Q-TOF 6520; Agilent Technologies, Santa Clara, California, USA). Four microliters of each sample were loaded into the system and transported to the Chip enrichment column (Zorbax C18, 4 mm x 5 µm i.d., Agilent Technologies) by a capillary pump, with a loading flow of 4 µL/min, using 95% ACN/5% water added of 0.1% formic acid (buffer B) as mobile phase. Nitrogen was used as the nebulising gas. A separation column (Zorbax C18, 43 mm x 75 µm i.d., Agilent Technologies), at flow rate of 0.4 µL, was used for peptide separation.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Since the horse protein database is not well annotated, a broader taxonomy, namely “all mammals”, was selected for identification to be based on sequence homology. Protein-identification peak lists were generated using the Mascot search engine (<http://mascot.cigs.unimo.it/mascot>) against the UniProt database (UniProt.org) specifying the following parameters: mammalian taxonomy, parent ion tolerance ± 20 ppm, MS/MS error tolerance ± 0.12 Da, alkylated cysteine as fixed modification and oxidised methionine as variable modification, and two potential missed trypsin cleavages, as previously described (Bertoldi et al., 2013). Proteins with a score >80 or identified with at least two or more significant sequences were selected. The significant threshold in Mascot searches was set to obtain a false discovery rate $<5\%$ (5% probability of false match for each protein with a score above 80).

Statistical analysis

Data (AF total proteins, mare’s age, mare’s and foal’s body weight, parity, days of pregnancy, length of stage II labour (minutes), foal’s Apgar score and number of bands) were analysed with statistical software (R version 2.15.1) and reported as mean $\pm$ standard deviation (SD). Shapiro-Wilk normality test was performed to evaluate data normal distribution. Pearson coefficient of correlation was calculated between AF total proteins and the other data recorded for each mare (mare’s age, mare’s and foal’s body weight, parity, days of pregnancy, length of stage II labour (minutes), foal’s Apgar score and number of bands).

The identified proteins were categorised by biological process, molecular function and cellular component with Gene Ontology terms according to Gene Ontology (GO) and Human Protein Reference Database (HPRD).

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Results

Clinical data

Clinical data collected from the 24 mares included in the study are shown in Table 1. Mean AF total protein concentration was 1.96 ± 1.12 g/L, ranging from 0.36 to 4.16 g/L. No significant correlation was found between AF protein concentration and the other data recorded.

SDS-PAGE and protein identification by mass spectrometry

Representative gel and pherogram of AF are reported in Figure 1A and 1B. The mean number of bands was 23 ± 1.5 . All samples presented a similar pattern characterised by two clusters of bands: the first with molecular weights (MW) higher than 62 kDa and the second with MW lower than 34 kDa. In the middle, very few faint bands were present. Figure 2 reports the relative abundance in percentage of AF protein bands. Out of the 25 slices cut from the gel (Figure 3), 20 yielded significant results leading to the unambiguous identification of 34 proteins (Table 2). Serum albumin and major allergen Equ c1 (ALL1) were the two most abundant proteins, followed by fibronectin, transferrin and haemoglobin; these five proteins represented >60% of the equine AF proteome. Fibronectin, versican and albumin were also identified in bands characterised by different MW.

The identified proteins categorised by their molecular function and biological process according to Gene Ontology (GO) and Human Protein Reference Database (HPRD) are shown in Table 3 and Figure 4. Most of the proteins were involved in cellular growth and/or

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

201 maintenance (38%), transport (26%) and protein metabolism (9%). The vast majority of the
202 identified proteins were classified as extracellular (79%).

204 **Discussion**

205 The present paper aimed to explore the complexity of equine AF proteome and to
206 identify its most abundant proteins. The study was carried out on 24 mares of different breed,
207 age and parity referred to the Equine Perinatology Unit, and they can be considered
208 representative of a typical equine hospital population. Therefore, the proteomic profile
209 described can be considered a useful starting point for further applied studies on the equine
210 AF proteins.

211 Most of the 34 proteins identified were involved in cellular growth and maintenance,
212 transport and protein metabolism reflecting the dynamic biological functions of AF.
213 Regarding cellular growth and/or maintenance, 12 of the proteins identified belonged to or
214 interacted with the extracellular matrix (ECM) that plays an important role in the development
215 of foetal tissues. All these proteins, with the exception of versican and proteoglycan 4, were
216 also identified in human AF (Cho et al. 2007; Michaels et al. 2007) and/or in equine amniotic
217 membrane (Galera et al. 2015). Among the ECM structural proteins, fibronectin is a
218 multifunctional glycoprotein known to participate in the organisation of ECM binding to
219 integrins. During pregnancy, fibronectin is expressed in the junction between maternal and
220 foetal membranes as well as in the uterus and placenta (Mogami et al. 2013). Lumican, a
221 member of the family of small leucine-rich proteoglycans, is the major keratan sulphate
222 proteoglycan of the cornea and is also present in the ECM throughout the body, including
223 human and equine amniotic membrane (Galera et al. 2015; Kao et al. 2006; Mavrou et al.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

2008). Based on its interaction with fibrillar collagen and its ability to modulate cell proliferation and migration, lumican could play a role in the maturation of foetal tissues (Mavrou et al. 2008). Regarding the non-structural proteins involved in ECM development and organisation, thrombospondin and fibulin are regulatory proteins belonging to the group of the matricellular proteins. These proteins represent a bridge between matrix proteins and cell surface receptors, or other molecules such as cytokines that can interact with the cell surface (Bornstein, 1995). They are typically expressed at low levels in adult tissues, but are strongly expressed during development or following injury or pathology (Morris and Kyriakides, 2014). Gelsolin is a multifunctional actin regulatory protein involved in cytoskeleton dynamics and structure. In addition to its role in aiding chemotaxis and movement of intracellular structures, plasma gelsolin binds to a variety of proinflammatory and bioactive molecules including fibronectin, platelet activating factor and the bacterial surface lipids lipoteichoic acid and lipopolysaccharide (Peddada et al. 2012). The role of gelsolin in AF is still unknown, but it has been suggested to modulate inflammation and bacterial infections in human AF (Sezen et al. 2009). In association with gelsolin, vinculin is also a component of the actine cytoskeleton and is involved in integrin-mediated focal adhesion, cell motility and other cellular functions such as migration, proliferation and differentiation (Wu et al. 2014). Other proteins interacting with ECM that could play important roles in the development of foetal tissue are type IV collagenase (MMP2) and metalloproteinase inhibitor 1 (TIMP1) belonging to the matrix metalloproteinases (MMPs) and tissue inhibitors respectively. The MMPs are a family of over 20 enzymes acting on the ECM components, regulated at different levels via their activators, inhibitors and localization on the cell surface (Sternlicht and Werb, 2001). The biological functions of these enzymes

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

and their inhibitors have been widely studied, in particular MMP2 is important for bone development and angiogenesis regulation and has been identified and studied in plasma and AF of pregnant women (Anumba et al. 2010; Turner et al. 2014). MMPs activity was also studied in amniotic and allantoic fluid from mares that delivered live term foals and from mares with preterm delivery, suggesting that MMPs may have a role as markers for high risk pregnancy in the mare (Oddsdóttir et al. 2011).

Among transport proteins, albumin, transferrin, alpha-fetoprotein, apolipoprotein A1 and phospholipid transfer protein (PLTP) transport nutrients, ions and lipids essential for foetal growth and development and have been identified as common components of AF also in humans (Cho et al. 2007; Michaels et al. 2007). Alpha-fetoprotein is member of the albuminoid superfamily and is present in the allantoic and amniotic fluids of domestic animals (Luft et al. 1984; Smith et al. 1979). In mammalian foetuses alpha-fetoprotein is associated with oestrogen-binding, anti-oxidative properties and immunoregulation (DeMees et al. 2006; Mizejewski 2001) and it is highly expressed during early pregnancy by the equine conceptus (Simpson et al. 2000). In women, AF alpha-fetoprotein is actively investigated for pathologies such as Down syndrome, trisomies 13 and 18, intra-amniotic infection, preterm delivery, pre-eclampsia, membrane rupture, and foetoplacental hypoxia (Cho et al. 2007). Recently, Canisso et al. (2015) confirmed the presence of alpha-fetoprotein in equine foetal fluids during the third trimester of pregnancy and found increased maternal plasma concentrations of the protein in mares with experimentally induced placentitis. The presence of ALL1 in AF is challenging. ALL1 is a glycoprotein of 21.7 kDa belonging to the family of lipocalins, whose function is to carry small hydrophobic molecules such as odorants, steroids and pheromones. This protein is expressed in salivary glands and in the

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

liver and is highly concentrated in secretory fluids such as saliva and urine as well as in hair and dander (Botros et al. 2001). ALL1 is widely studied in human medicine because it induces an IgE-mediated type I allergic reaction in the majority of patients allergic to horses (Lascombe et al. 2000). The physiological role of this protein is still unknown and to our knowledge, this is the first study reporting the presence of ALL1 in AF. PLTP is a monomeric glycoprotein involved in lipid transport, lipoprotein metabolism and lipopolysaccharide binding. It is ubiquitously expressed in human tissues and is secreted into the plasma, where its central role has been well established (Albers et al. 2012). PLTP is highly expressed in lung epithelial cells, and may play a role in surfactant metabolism during foetus lung development (Brehm et al. 2014).

The proteomic approach applied in the present study led to the successful identification of the most abundant proteins, even though a few additional points should be taken into consideration. The first one regards the choice of non-depleting albumin and other major proteins before electrophoresis and MS identification. Complex biological samples contain thousands of different protein species, few of them characterised by high abundance and many others by low or very low abundance. The presence of very high abundance proteins like albumin and immunoglobulins often hampers the separation and characterisation of serum and AF proteomes, therefore the depletion of these major components has been applied in human proteomics (Cho et al. 2007; Michaels et al. 2007). However, this approach can lead to the loss of some low abundance proteins due to the “sponge effect” of albumin that can bind a variety of other proteins or peptides (Bellei et al. 2011). From this point of view, equine AF is a preferential sample due to the absence of immunoglobulins. In domestic animals, the passage of immunoglobulins is influenced by the placental structure: in horses,

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

pigs and ruminants the placenta is epitheliochorial, thus impermeable to immunoglobulins (Furukawa et al. 2014), whereas in dogs, the endotheliochorial placenta allows only 5% to 10% transfer of maternal antibodies to the foetus (Dall'Ara et al. 2015). From an analytical point of view, the absence of immunoglobulins in equine AF, as confirmed by this study, can be considered an advantage, allowing to perform SDS-PAGE and MS identification without affecting proteome integrity and complexity.

The second point is related to the sample collection. This study collected AF only at parturition because mares were client-owned and transabdominal amniocentesis is still not recommended for clinical use. Recently, Canisso et al. (2014) described a safe technique to perform multiple ultrasound-guided foetal fluid samplings during the last trimester of gestation in mares. On this basis, abdominal amniocentesis will probably be preferred more frequently also in a clinical setting and to evaluate gestational changes in the AF proteome as reported in women (Michaels et al. 2007).

The last point regards AF total protein concentrations, which were similar to those reported by Williams et al. (1993) (3.1 ± 2.6 g/L) and Paccamonti et al. (1995) (1-2 g/L), but lower than those reported by Kochhar et al. (1997) (9.1 ± 2 g/L), and higher than those of Zanella et al. (2014) (0.3 ± 0.1 g/L). The reported discrepancies could be related to the use of quantification methods characterised by different analytical performances; also the influence of wide inter-individual variability cannot be excluded. Many environmental and physiological factors can contribute to this variability; in particular, since the mares were client-owned, pre-hospitalisation conditions, such as housing, feeding, nutrition and hydration status, might have affected AF total protein concentration.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Conclusions

Applying a qualitative proteomic approach, this study identified the 34 most abundant proteins of the AF proteome from healthy mares that delivered live term foals. GO categorisation demonstrated that these proteins are involved in different biological processes and molecular functions including cell growth/maintenance and transport. Some of these proteins belonged to or interacted with the extracellular matrix, highlighting the role of its components in foetal maturation. The study confirmed also the importance of transport proteins like alpha-fetoprotein and PLTP, and reported for the first time the presence of ALL1 in AF. Though entirely descriptive, these findings can be considered valuable context for further investigations to gain insights into the function of the proteins identified and to discover potential biomarkers of foetal disease at birth or during pregnancy.

Acknowledgements

This study was supported by a grant from the University of Bologna (RFO) to Isani G and Castagnetti C.

Conflict of interest

The authors have no conflict of interests to declare.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

335 **Table 1.** Clinical data collected from the 24 mares included in the study. Data are reported as
 336 mean $\pm$ standard deviation. AF amniotic fluid; TP total proteins.

Sample	Breed	Age	Mare weight	Foal weight	Parity	Length of pregnancy	Length of stage II	Foal's Apgar score	AF TP
		years	Kg	Kg		days	minutes		g/L
1	Saddlebred	11	660	51.7	1	355	25	10	2.74
2	Thoroughbred	6	645	51	1	344	20	10	1.66
3	Standardbred	5	565	50	2	335	14	8	1.51
4	Standardbred	6	585	44	1	329	8	8	3.07
5	Saddlebred	7	660	53	2	330	16	9	2.64
6	Saddlebred	12	500	40	1	360	20	9	1.01
7	Saddlebred	17	650	58	3	330	13	9	0.55
8	Quarter Horse	11	560	42.6	5	333	14	9	0.58
9	Saddlebred	14	660	53.5	1	354	20	10	0.36
10	Arabian	5	450	42	2	335	15	10	0.90
11	Standardbred	7	565	45.3	1	328	11	10	1.39
12	Standardbred	19	578	43.5	3	349	12	8	1.25
13	Standardbred	12	546	45	2	352	15	8	1.30
14	Standardbred	16	590	42.5	11	347	9	8	1.73
15	Standardbred	10	535	50	6	343	8	10	0.58
16	Standardbred	14	610	45	6	336	21	9	2.50
17	Arabian	12	430	50	6	326	20	10	2.29
18	Standardbred	6	620	59	1	341	18	10	1.66
19	Standardbred	20	606	45	12	338	12	10	4.01
20	Saddlebred	16	650	47	2	360	5	8	3.01
21	Standardbred	18	680	50	4	332	9	9	4.16
22	Thoroughbred	13	580	58	4	354	12	8	2.14
23	Quarter Horse	16	425	41	3	357	17	9	4.16
24	Standardbred	19	660	56	3	345	25	8	1.75
		12	583.8	48.7	3	342	15	9	1.96
		± 9.5	± 74.1	± 11.4	± 3	± 10.7	± 5.7	± 1	± 1.12

337

338

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Table 2. Proteins identified in equine amniotic fluid by mass spectrometry. Identified proteins are listed according to the number of the band as marked in Figure 3.

Band	Entry name ^b	Protein full name	MW (kDa) ^c	Score ^d	Pept. ^e	Sign. Pept. ^f	Seq. ^g	Sign. Seq. ^h	% id. ⁱ
1	CSPG2_BOVIN	Versican core protein	371.8	257	76	21	15	6	77.2
2	FINC_HORSE	Fibronectin	58.1	1451	155	89	19	18	100
3	FINC_HORSE	Fibronectin	58.1	440	76	34	15	10	100
4	FINC_BOVIN	Fibronectin	275.5	155	46	16	16	7	95.4
	CO6A1_HUMAN	Collagen alpha-1(VI) chain	109.6	168	41	16	10	7	89
5	VINC_HUMAN	Vinculin	124.3	148	53	18	26	10	99.5
	ACE2_PAGLA	Angiotensin-converting enzyme 2	93.0	380	43	18	10	6	86
6	PLMN_HORSE	Plasminogen	38.1	288	36	18	8	5	100
	FINC_CANFA	Fibronectin	58.2	148	18	11	7	5	96.4
	GELS_HORSE	Gelsolin	81.1	1911	193	122	27	21	100
7	FBLN1_HUMAN	Fibulin-1	81.3	434	80	47	11	9	91.2
	FINC_HORSE	Fibronectin	58.1	360	36	23	11	7	100
	LUM_MOUSE	Lumican	38.6	159	27	14	6	4	87.4
8	TRFE_HORSE	Serotransferrin	80.3	2201	263	153	43	32	100
	TRFL_HORSE	Lactotransferrin	77.9	268	61	30	19	11	100
9	TRFE_HORSE	Serotransferrin	80.3	1642	194	106	34	24	100
	ALBU_HORSE	Serum albumin	70.5	2863	244	148	44	33	100
	ECM1_HUMAN	Extracellular matrix protein 1	62.2	188	53	22	4	4	78.4
	MMP2_BOVIN	72 kDa type IV collagenase	74.8	468	50	27	14	9	95.4
10	PLTP_HUMAN	Phospholipid transfer protein	54.9	400	24	19	3	3	89.1
	LUM_MOUSE	Lumican	38.6	153	23	12	6	4	87.4
	FETA_HORSE	Alpha-fetoprotein	70.1	145	17	10	7	4	100
	CSPG2_MACNE	Versican core protein	96.8	101	13	7	4	3	82
	ALBU_HORSE	Serum albumin	70.5	17663	1154	849	70	56	100
	FETA_HORSE	Alpha-fetoprotein	70.1	407	59	34	23	14	100
11	CSPG2_MACNE	Versican core protein	96.8	347	13	10	5	4	82
	BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3	75.3	53	12	6	4	3	92.9
12	A1AT2_HORSE	Alpha-1-antitrypsin	47.1	195	11	8	4	2	100
	ALBU_EQUAS	Serum albumin	70.5	160	53	13	23	9	98.5
	CLUS_HORSE	Clusterin	52.7	453	48	28	15	10	100
	ACTB_BOVIN	Actin cytoplasmic 1	42.1	118	36	11	14	6	100
	GELS_HORSE	Gelsolin	81.1	40	12	4	7	4	100
13	IBP3_BOVIN	Insulin-like growth factor-binding protein	32.6	79	11	9	5	5	86.7
	FETUA_BOVIN	Alpha-2-HS-glycoprotein	39.2	65	11	6	2	2	71.3
	CO6A3_HUMAN	Collagen alpha-3(VI) chain	345.2	93	5	4	3	2	88.5
	HPT_BOVIN	Haptoglobin	45.6	52	3	2	3	2	78.9
	CLUS_HORSE	Clusterin	52.7	42	18	4	7	3	100
14	TSP1_BOVIN	Thrombospondin-1	133.4	29	13	3	9	3	96.8
	SFTPA_HORSE	Pulmonary surfactant-associated protein A	26.5	71	4	3	3	2	100
15	TSP1_HUMAN	Thrombospondin-1	133.3	751	60	49	12	11	98
	ALL1_HORSE	Major allergen Equ c 1	21.9	642	58	33	10	8	100

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

	TIMP1_HORSE	Metalloproteinase inhibitor 1	23.7	189	15	7	5	3	100
	ALL1_HORSE	Major allergen Equ c 1	21.9	1620	193	107	17	11	100
16	TSP1_HUMAN	Thrombospondin-1	133.3	351	58	26	13	9	98
	APOA1_CANFA	Apolipoprotein A-1	30.2	124	24	11	4	3	80.8
17	HBB_HORSE	Haemoglobin sub. beta	16.1	2153	159	114	14	12	100
	HBA_HORSE	Haemoglobin sub. alpha	15.3	824	87	53	8	7	100
18	PIP_HYLSY	Prolactin-inducible protein homolog	16.9	122	9	8	1	1	64.3
19	THIO_HORSE	Thioredoxin	12.0	80	12	2	4	1	100
20	PRG4_HUMAN	Proteoglycan 4	152.2	130	18	8	3	2	88.6
	ALBU_EQUAS	Serum albumin	70.5	75	18	10	4	3	98.5

^a Number of the identified band as marked in Figure 3.

^b Protein entry name from UniProt knowledge database.

^c Theoretical protein molecular weight.

^d The highest scores obtained with Mascot search engine.

^e Peptides: total number of peptides matching the identified proteins.

^f Significant peptides: total number of significant peptides matching the identified proteins.

^g Sequence: total number of distinct sequences matching the identified proteins.

^h Significant sequences: total number of significant distinct sequences matching the identified proteins.

ⁱ Percentage of identical amino acids between the identified protein and the respective horse protein.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Table 3. Biological and functional classification of the proteins identified in equine amniotic fluid. Identified proteins are listed according to the Biological Process category.

Entry name ^a	Protein full name	MW (kDa) ^b	Biol. Proc. ^c	Mol. Funct. ^d	Cell. Comp. ^e
HPT_BOVIN	Haptoglobin	45.6	Acute-phase response	HB binding	Extracellular
BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3	75.3	Cell communication/signal transduction	Receptor binding	Extracellular
CO6A1_HUMAN	Collagen alpha-1(VI) chain	109.6	Cell growth/maintenance	Extracellular matrix structural constituent	Extracellular
CO6A3_HUMAN	Collagen alpha-3(VI) chain	345.2	Cell growth/maintenance	peptidase inhibitor	Extracellular
CSPG2_BOV	Versican core protein	371.8	Cell growth/maintenance	Extracellular matrix structural constituent	Extracellular
ECM1_HUMAN	Extracellular matrix protein 1	62.2	Cell growth/maintenance	Extracellular matrix structural constituent	Extracellular
FBLN1_HUMAN	Fibulin-1	81.3	Cell growth/maintenance	Extracellular matrix structural constituent	Extracellular
FINC_HORSE	Fibronectin	58.1	Cell growth/maintenance	Extracellular matrix structural constituent	Extracellular
GELS_HORSE	Gelsolin	81.1	Cell growth/maintenance	Structural constituent of cytoskeleton	Extracellular
IBP3_BOVIN	Insulin-like growth factor-binding protein	32.6	Cell growth/maintenance	protein binding	Extracellular
LUM_MOUSE	Lumican	38.6	Cell growth/maintenance	Extracellular matrix structural constituent	Extracellular
PRG4_HUMAN	Proteoglycan 4	152.2	Cell growth/maintenance	Binding/Cell adhesion molecule	Extracellular
TIMP1_HORSE	Metalloproteinase inhibitor 1	23.7	Cell growth/maintenance	Extracellular matrix structural constituent	Extracellular
TSP1_HUMAN	Thrombospondin-1	133.3	Cell growth/maintenance	Extracellular matrix structural constituent	Extracellular
VINC_HUMAN	Vinculin	124.3	Cell growth/maintenance	Cytoskeletal protein binding	Cytoplasm
CLUS_HORSE	Clusterin	52.7	Cell morphogenesis/cell death	protein binding-chaperone	Cytoplasm
THIO_HORSE	Thioredoxin	12.0	Metabolism/energy pathways	Catalytic activity	Cytoplasm
FETUA_BOVIN	Alpha-2-HS-glycoprotein	39.2	Mineral balance	protein binding	Extracellular
ACTB_BOVIN	Actin cytoplasmic 1	42.1	Protein folding	protein binding	Cytoskeleton
ACE2_PAGLA	Angiotensin-converting enzyme 2	93.0	Protein metabolism	Carboxylpeptidase	Plasma membrane
MMP2_BOVIN	72 kDa type IV collagenase	74.8	Protein metabolism	Metallopeptidase	Extracellular
PLMN_HORSE	Plasminogen	38.1	Protein metabolism	Peptidase	Extracellular
SFTPA_HORSE	Pulmonary surfactant-associated protein A	26.5	Respiratory gaseous exchange	carbohydrate/metal ion binding	Extracellular
ALBU_HORSE	Serum albumin	70.5	Transport	Transporter	Extracellular
ALL1_HORSE	Major allergen Equ c 1	21.9	Transport	Transporter	Extracellular
APOA1_CANFA	Apolipoprotein A-I	30.2	Transport	Binding	Extracellular/HDL

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

FETA_HORSE	Alpha-fetoprotein	70.1	Transport	Transporter	Extracellular
HBA_HORSE	Haemoglobin subunit alpha	15.3	Transport	Transporter	Extracellular
HBB_HORSE	Haemoglobin subunit beta	16.1	Transport	Transporter	Extracellular
PLTP_HUMAN	Phospholipid transfer protein	54.9	Transport	Transporter	Extracellular
TRFE_HORSE	Serotransferrin	80.3	Transport	Transporter	Extracellular
TRFL_HORSE	Lactotransferrin	77.9	Transport	Transporter	Secretory granule
A1AT2_HORSE	Alpha-1-antiproteinase 2	47.1	Unknown	Protease inhibitor	NA
PIP_HYLSY	Prolactin-inducible protein homolog	16.9	Unknown	Binding	Extracellular

354

355 ^a Protein entry name from UniProt knowledge database.

356 ^b Theoretical protein molecular weight.

357 ^c Biological Process according to Gene Ontology and Human Protein Reference Database.

358 ^d Molecular Function according to Gene Ontology and Human Protein Reference Database.

359 ^e Cellular Component according to Gene Ontology and Human Protein Reference Database.

360

361

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Figure Legends

Figure 1. Representative SDS-PAGE of 6 out of the 24 amniotic fluid samples on 4-12% gel in MOPS buffer. Twenty micrograms of proteins were loaded for each lane and the gel was stained with Coomassie Blue. A) Representative AF samples (lane 1, molecular weight marker; lanes 2-7, AF collected from six different healthy mares [samples 16-21]); B) representative pherogram obtained from lane 3

Figure 2. Relative abundance of each protein band compared to the entire volume of the lane; data are expressed as percentage (%) and reported as mean $\pm$ standard deviation (n=24).

Figure 3. SDS-PAGE of the amniotic fluid pool on 4-12% gel in MOPS buffer. The pool was prepared by collecting and mixing 50 μ g of proteins from each AF sample (n=24). Two replicates are reported. Arrows and numbers indicate the slices that have been excised and analysed by ESI-Q-TOF as listed in Table 2. Asterisk (*) indicates bands that did not give significant results by MS identification.

Figure 4. Distribution of amniotic fluid proteins in the Biological process category according to Gene Ontology (GO) and the Human Protein Reference Database (HPRD) as reported in Table 3.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

References

- Albers, J.J., Vuletic, S., Cheung, M.C., 2012. Role of plasma phospholipid transfer protein in lipid and lipoprotein metabolism. *Biochim. Biophys. Acta* 1821, 345–357.
- Almeida, A.M., Bassols, A., Bendixen, E., Bhide, M., Ceciliani, F., Cristobal, S., Eckersall, P.D., Hollung, K., Lisacek, F., Mazzucchelli, G., McLaughlin, M., Miller, I., Nally, J.E., Plowman, J., Renaut, J., Rodrigues, P., Roncada, P., Staric, J., Turk, R., 2015. Animal board invited review: advances in proteomics for animal and food sciences. *Animal* 9, 1–17.
- Anumba, D.O.C., Gelany, S.E., Elliott, S.L., Li, T.C., 2010. Circulating levels of matrix proteases and their inhibitors in pregnant women with and without a history of recurrent pregnancy loss. *Reprod. Biol. Endocrinol.* 8, 62. doi:10.1186/1477-7827-8-62
- Bellei, E., Bergamini, S., Monari, E., Fantoni, L.I., Cuoghi, A., Ozben, T., Tomasi, A., 2011. High-abundance proteins depletion for serum proteomic analysis: concomitant removal of non-targeted proteins. *Amino Acids* 40, 145–156.
- Bellei, E., Monari, E., Cuoghi, A., Bergamini, S., Guerzoni, S., Ciccarese, M., Ozben, T., Tomasi, A., Pini, L.A., 2013. Discovery by a proteomic approach of possible early biomarkers of drug-induced nephrotoxicity in medication-overuse headache. *J. Headache Pain* 14, 6. doi:10.1186/1129-2377-14-6
- Bertoldi, C., Bellei, E., Pellacani, C., Ferrari, D., Lucchi, A., Cuoghi, A., Bergamini, S., Cortellini, P., Tomasi, A., Zaffe, D., Monari, E., 2013. Non-bacterial protein expression in periodontal pockets by proteome analysis. *J. Clin. Periodontol.* 40, 573–82.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

- 405 Bornstein, P., 1995. Diversity of function is inherent in matricellular proteins: an appraisal of
406 thrombospondin 1. *J. Cell Biol.* 130, 503–506.
- 407 Botros, H.G., Poncet, P., Rabillon, J., Fontaine, T., Laval, J.M., David, B., 2001. Biochemical
408 characterization and surfactant properties of horse allergens. *Eur. J. Biochem.* 268,
409 3126–3136.
- 410 Brehm, A., Geraghty, P., Campos, M., Garcia-Arcos, I., Dabo, A.J., Gaffney, A., Eden, E.,
411 Jiang, X.C., D’Armiento, J., Foronjy, R., 2014. Cathepsin G degradation of phospholipid
412 transfer protein (PLTP) augments pulmonary inflammation. *FASEB J.* 28, 2318–2331.
- 413 Canisso, I.F., Ball, B.A., Squires, E.L., Troedsson, M.H., 2014. How to perform
414 transabdominal ultrasound-guided fetal fluid sampling in mares. *J. Equine Vet. Sci.* 34,
415 1143–1147.
- 416 Canisso, I.F., Ball, B.A., Scoggin, K.E., Squires, E.L., Williams, N.M., Troedsson, M.H.,
417 2015. Alpha-fetoprotein is present in the fetal fluids and is increased in plasma of mares
418 with experimentally induced ascending placentitis. *Anim. Reprod. Sci.* 154, 48–55.
- 419 Castagnetti, C., Mariella, J., Serrazanetti, G.P., Grandis, A., Merlo, B., Fabbri, M., Mari, G.,
420 2007. Evaluation of lung maturity by amniotic fluid analysis in equine neonate.
421 *Theriogenology* 67, 1455–1462.
- 422 Cho, C.K.J., Shan, S.J., Winsor, E.J., Diamandis, E.P., 2007. Proteomics analysis of human
423 amniotic fluid. *Mol. Cell. Proteomics* 6, 1406–1415.
- 424 Dall’Ara, P., Meloni, T., Rota, A., Servida, F., Filipe, J., Veronesi, M.C., 2015.
425 Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

- 426 pregnancy. Theriogenology 83, 766–771.
- 427 DeMees, C., Bakker, J., Smits, J., VanVooren, P., Gabant, P., Szpirer, J., Szpirer, C., 2006.
- 428 Alpha-fetoprotein controls female fertility and prenatal development of the
- 429 gonadotropin-releasing hormone pathway through an antiestrogenic action. Mol. Cell.
- 430 Biol. 26, 2012–2018.
- 431 Ferlizza, E., Campos, A., Neagu, A., Cuoghi, A., Bellei, E., Monari, E., Dondi, F., Almeida,
- 432 A.M., Isani, G., 2015. The effect of chronic kidney disease on the urine proteome in the
- 433 domestic cat (*Felis catus*). Vet. J. 204, 73–81.
- 434 Furukawa, S., Kuroda, Y., Sugiyama, A., 2014. A comparison of the histological structure of
- 435 the placenta in experimental animals. J. Toxicol. Pathol. 27, 11–18.
- 436 Galera, P.D., Ribeiro, C.R., Sapp, H.L., Coleman, J., Fontes, W., Brooks, D.E., 2015.
- 437 Proteomic analysis of equine amniotic membrane: characterization of proteins. Vet.
- 438 Ophthalmol. 18, 198–209.
- 439 Holdstock, N.B., McGladdery, A.J., Ousey, J.C., Rossdale, P.D., 1995. Assessing methods of
- 440 collection and changes of selected biochemical constituents in amniotic and allantoic
- 441 fluid throughout equine pregnancy. Biol. Reprod. Monogr. 1, 21–38.
- 442 Kao, W.W., Funderburgh, J.L., Xia, Y., Liu, C.Y., Conrad, G.W., 2006. Focus on molecules:
- 443 Lumican. Exp. Eye Res. 82, 3–4.
- 444 Kochhar, H.P.S., Simran, P.S., Nanda, A.S., Kaur, R., 1997. Comparative biochemical indices
- 445 of fetal fluids in normal foaling and stressful delivery in Indian thoroughbred mares. J.
- 446 Equine Vet. Sci. 17, 206–210.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

- 447 Lascombe, M.B., Grégoire, C., Poncet, P., Tavares, G.A., Rosinski-Chupin, I., Rabillon, J.,
 448 Goubran-Botros, H., Mazié, J.C., David, B., Alzari, P.M., 2000. Crystal structure of the
 449 allergen Equ c 1. A dimeric lipocalin with restricted IgE-reactive epitopes. J. Biol.
 450 Chem. 275, 21572–21577.
- 451 Luft, A.J., Lai, P.C., Robertson, H.A., Saunders, N.R., Lorscheider, F.L., 1984. Distribution
 452 of alpha-fetoprotein in fetal plasma and in amniotic and allantoic fluids of the pig. J.
 453 Reprod. Fertil. 70, 605–607.
- 454 Lyle, S.K., Paccamonti, D.L., Hubert, J.D., Schlafer, D.H., Causey, R.C., Eilts, B.E., Johnson,
 455 J.R., 2006. Laparoscopic placement of an indwelling allantoic catheter in the mare:
 456 biochemical, cytologic, histologic, and microbiologic findings. Anim. Reprod. Sci. 94,
 457 428–431.
- 458 Mavrou, A., Anagnostopoulos, A.K., Kolialexi, A., Vougas, K., Papantoniou, N., Antsaklis,
 459 A., Kanavakis, E., Fountoulakis, M., Tsangaris, G.T., 2008. Proteomic analysis of
 460 amniotic fluid in pregnancies with Turner syndrome fetuses. J. Proteomics 7, 1863–
 461 1866.
- 462 Michaels, J.E., Dasari, S., Pereira, L., Reddy, A.P., Lapidus, J.A., Lu, X., Jacob, T., Thomas,
 463 A., Rodland, M., Roberts, C.T., Gravett, M.G., Nagalla, S.R., 2007. Comprehensive
 464 proteomic analysis of the human amniotic fluid proteome: gestational age-dependent
 465 changes. J. Proteome Res. 6, 1277–1285.
- 466 Miller, I., Preßlmayer-Hartler, A., Wait, R., Hummel, K., Sensi, C., Eberini, I., Razzazi-
 467 Fazeli, E., Gianazza, E., 2014. In between - Proteomics of dog biological fluids. J.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

- 468 Proteomics 106, 30–45.
- 469 Mizejewski, G.J., 2001. Alpha-fetoprotein structure and function: relevance to isoforms,
470 epitopes, and conformational variants. *Exp. Biol. Med.* 226, 377–408.
- 471 Mogami, H., Kishore, A.H., Shi, H., Keller, P.W., Akgul, Y., Word, R.A., 2013. Fetal
472 fibronectin signaling induces matrix metalloproteases and cyclooxygenase-2 (COX-2) in
473 amnion cells and preterm birth in mice. *J. Biol. Chem.* 288, 1953–1966.
- 474 Morris, A.H., Kyriakides, T.R., 2014. Matricellular proteins and biomaterials. *Matrix Biol.*
475 37, 183–191.
- 476 Oddsdóttir, C., Riley, S.C., Leask, R., Shaw, D.J., Aurich, C., Palm, F., Fowden, A.L.,
477 Ricketts, S.W., Watson, E.D., 2011. Dynamics of activities of matrix metalloproteinases-
478 9 and -2, and the tissue inhibitors of MMPs in fetal fluid compartments during gestation
479 and at parturition in the mare. *Theriogenology* 75, 1130–1138.
- 480 Paccamonti, D., Swiderski, C., Marx, B., Gaunt, S., Blouin, D., 1995. Electrolytes and
481 biochemical enzymes in amniotic and allantoic fluid of the equine fetus during late
482 gestation. *Biol. Reprod. Monogr. Ser.* 1, 39–48.
- 483 Peddada, N., Sagar, A., Ashish, Garg, R., 2012. Plasma gelsolin: A general prognostic marker
484 of health. *Med. Hypotheses* 78, 203–210.
- 485 Pirrone, A., Mariella, J., Gentilini, F., Castagnetti, C., 2012. Amniotic fluid and blood lactate
486 concentrations in mares and foals in the early postpartum period. *Theriogenology* 78,
487 1182–1189.
- 488 Riding, G.A., Hill, J.R., Jones, A., Holland, M.K., Josh, P.F., Lehnert, S.A., 2008. Differential

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

489 proteomic analysis of bovine conceptus fluid proteins in pregnancies generated by
 490 assisted reproductive technologies. *Proteomics* 8, 2967–2982.

491 Sezen, D., Bongiovanni, A.M., Gelber, S., Perni, U., Hutson, J.M., Skupski, D., Witkin, S.S.,
 492 2009. Gelsolin down-regulates lipopolysaccharide-induced intraamniotic tumor necrosis
 493 factor- α production in the midtrimester of pregnancy. *Am. J. Obstet. Gynecol.* 200, 191–
 494 192.

495 Simpson, K.S., Adams, M.H., Behrendt-Adam, C.Y., Baker, C.B., McDowell, K.J., 2000.
 496 Differential gene expression in day 12 and day 15 equine conceptuses. *J. Reprod. Fertil.*
 497 Suppl. 539–547.

498 Smith, K.M., Lai, P.C.W., Robertson, H.A., Church, R.B., Lorscheider, F.L., 1979.
 499 Distribution of alpha1-fetoprotein in fetal plasma, allantoic fluid, amniotic fluid and
 500 maternal plasma of cows. *J. Reprod. Fertil.* 57, 235–238.

501 Sternlicht, M.D., Werb, Z., 2001. How matrix metalloproteinases regulate cell behavior.
 502 *Annu. Rev. Cell Dev. Biol.* 17, 463–516.

503 Tong, X., 2013. Amniotic fluid may act as a transporting pathway for signaling molecules and
 504 stem cells during the embryonic development of amniotes. *J. Chin. Med. Assoc.* 76, 606–
 505 610.

506 Turner, S.W., Carter, J., Danielian, P., Chalmers, I., McConaghy, L., Pacitti, N., Booth, N.,
 507 2014. Protease concentration in amniotic fluid at term and early childhood respiratory
 508 symptoms. *J. Matern. Fetal. Neonatal Med.* 27, 416–420.

509 Underwood, M.A., Gilbert, W.M., Sherman, M.P., 2005. Amniotic fluid: not just fetal urine

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

510 anymore. J. Perinatol. 25, 341–348.

511 Vaala, W.E., House, J.K., Madigan, J.E., 2002. Initial management and physical examination
 512 of the neonate, in: Smith, B.P. (Ed.), Large Animal Internal Medicine. Mosby Inc., St.
 513 Louis, MO, USA, pp. 277–293.

514 Williams, M.A., Wallace, S.S., Tyler, J.W., McCall, C.A., Gutierrez, A., Spano, J.S., 1993.
 515 Biochemical characteristics of amniotic and allantoic fluid in late gestational mares.
 516 Theriogenology 40, 1251–1257.

517 Wu, X., Cao, M.P., Shen, Y.Y., Chu, K.P., Tao, W.B., Song, W.T., Liu, L.P., Wang, X.H.,
 518 Zheng, Y.F., Chen, S.D., Zeng, Q.L., Xia, R.H., 2014. Weak power frequency magnetic
 519 field acting similarly to EGF stimulation, induces acute activations of the EGFR
 520 sensitive actin cytoskeleton motility in human amniotic cells. PLoS One 9, e87626.
 521 doi:10.1371/journal.pone.0087626

522 Zanella, L.F., Takahira, R.K., Melo e Oña, C.M., Oña Magalhães, L.C., Prestes, N.C., 2014.
 523 Biochemical profile of amniotic and allantoic fluid during different gestational phases in
 524 mares. J. Equine Vet. Sci. 34, 403–406.

525

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.