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

# 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

**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.

## 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.

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

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)

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 ChemiDoc$^{TM}$MP (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.

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 ± 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).

## 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

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

## Discussion

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

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

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

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

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

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,

293 pigs and ruminants the placenta is epitheliochorial, thus impermeable to immunoglobulins
294 (Furukawa et al. 2014), whereas in dogs, the endotheliochorial placenta allows only 5% to
295 10% transfer of maternal antibodies to the foetus (Dall'Ara et al. 2015). From an analytical
296 point of view, the absence of immunoglobulins in equine AF, as confirmed by this study, can
297 be considered an advantage, allowing to perform SDS-PAGE and MS identification without
298 affecting proteome integrity and complexity.

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

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

315

## 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.

335 **Table 1.** Clinical data collected from the 24 mares included in the study. Data are reported as

336 mean ± 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 ± 9.5** | **583.8 ± 74.1** | **48.7 ± 11.4** | **3 ± 3** | **342 ± 10.7** | **15 ± 5.7** | **9 ± 1** | **1.96 ± 1.12** |

337

338

339 **Table 2.** Proteins identified in equine amniotic fluid by mass spectrometry. Identified proteins

340 are listed according to the number of the band as marked in Figure 3.

| Band [a] | 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 |
| 6 | ACE2_PAGLA | Angiotensin-converting enzyme 2 | 93.0 | 380 | 43 | 18 | 10 | 6 | 86 |
| | PLMN_HORSE | Plasminogen | 38.1 | 288 | 36 | 18 | 8 | 5 | 100 |
| | FINC_CANFA | Fibronectin | 58.2 | 148 | 18 | 11 | 7 | 5 | 96.4 |
| 7 | GELS_HORSE | Gelsolin | 81.1 | 1911 | 193 | 122 | 27 | 21 | 100 |
| | 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 |
| 10 | 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 |
| | 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 |
| 11 | ALBU_HORSE | Serum albumin | 70.5 | 17663 | 1154 | 849 | 70 | 56 | 100 |
| | FETA_HORSE | Alpha-fetoprotein | 70.1 | 407 | 59 | 34 | 23 | 14 | 100 |
| | 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-antiproteinase 2 | 47.1 | 195 | 11 | 8 | 4 | 2 | 100 |
| 13 | 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 |
| | 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 |
| 14 | CLUS_HORSE | Clusterin | 52.7 | 42 | 18 | 4 | 7 | 3 | 100 |
| | 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 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | TIMP1_HORSE | Metalloproteinase inhibitor 1 | 23.7 | 189 | 15 | 7 | 5 | 3 | 100 |
| 16 | ALL1_HORSE | Major allergen Equ c 1 | 21.9 | 1620 | 193 | 107 | 17 | 11 | 100 |
| | 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.

352 **Table 3.** Biological and functional classification of the proteins identified in equine amniotic

353 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 |

| | | | | | |
|---|---|---|---|---|---|
| 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

362 **Figure Legends**

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

368

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

371

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

377

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

381

382

383

## 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.

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

pregnancy. Theriogenology 83, 766–771.

DeMees, C., Bakker, J., Smitz, J., VanVooren, P., Gabant, P., Szpirer, J., Szpirer, C., 2006. Alpha-fetoprotein controls female fertility and prenatal development of the gonadotropin-releasing hormone pathway through an antiestrogenic action. Mol. Cell. Biol. 26, 2012–2018.

Ferlizza, E., Campos, A., Neagu, A., Cuoghi, A., Bellei, E., Monari, E., Dondi, F., Almeida, A.M., Isani, G., 2015. The effect of chronic kidney disease on the urine proteome in the domestic cat (*Felis catus*). Vet. J. 204, 73–81.

Furukawa, S., Kuroda, Y., Sugiyama, A., 2014. A comparison of the histological structure of the placenta in experimental animals. J. Toxicol. Pathol. 27, 11–18.

Galera, P.D., Ribeiro, C.R., Sapp, H.L., Coleman, J., Fontes, W., Brooks, D.E., 2015. Proteomic analysis of equine amniotic membrane: characterization of proteins. Vet. Ophthalmol. 18, 198–209.

Holdstock, N.B., McGladdery, A.J., Ousey, J.C., Rossdale, P.D., 1995. Assessing methods of collection and changes of selected biochemical constituents in amniotic and allantoic fluid throughout equine pregnancy. Biol. Reprod. Monogr. 1, 21–38.

Kao, W.W., Funderburgh, J.L., Xia, Y., Liu, C.Y., Conrad, G.W., 2006. Focus on molecules: Lumican. Exp. Eye Res. 82, 3–4.

Kochhar, H.P.S., Simran, P.S., Nanda, A.S., Kaur, R., 1997. Comparative biochemical indices of fetal fluids in normal foaling and stressful delivery in Indian thoroughbred mares. J. Equine Vet. Sci. 17, 206–210.

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 foetuses. 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.

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

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

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

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

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

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

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

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

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

anymore. J. Perinatol. 25, 341–348.

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

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

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

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