

This is the peer reviewed version of the following article:

Identification of the most abundant proteins in equine amniotic fluid by a proteomic approach / Isani, Gloria; Ferlizza, Enea; Cuoghi, Aurora; Bellei, Elisa; Monari, Emanuela; Bianchin Butina, Barbara; Castagnetti, Carolina. - In: ANIMAL REPRODUCTION SCIENCE. - ISSN 0378-4320. - STAMPA. - 174:(2016), pp. 150-160. [10.1016/j.anireprosci.2016.10.003]

Terms of use:

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.

12/01/2026 15:35

Manuscript Number: ANIREP-D-16-6583R1

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

Article Type: Research paper

Keywords: Horse, pregnancy, electrophoresis, proteome

Corresponding Author: Dr. Enea Ferlizza, Ph.D., DVM

Corresponding Author's Institution: University of Bologna

First Author: Gloria Isani

Order of Authors: Gloria Isani; Enea Ferlizza, Ph.D., DVM; Aurora Cuoghi; Elisa Bellei; Emanuela Monari; Barbara Bianchin Butina; Carolina Castagnetti

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.

REVISION NOTE

Animal Reproduction Science

Ms. No. ANIREP-D-16-6583

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

Dear Editor,

thank you for considering the present paper for publication in Animal Reproduction Science and for the opportunity to revise our manuscript.

As you can see in the following pages, we answered all the questions raised by the reviewer. Following the reviewer suggestions, some sentences with more details have been added improving the clarity and the quality of the manuscript. In particular, more details on the statistical analysis performed were added and the discussion section was partially modified.

As requested, the added paragraph/words are written in red. Moreover, we highlighted the deleted parts in yellow. We upload two version of the revised manuscript: one with the changes highlighted and a second version corrected and edited.

In addition, after an accurate revision of the manuscript, we did also other minor corrections to improve the editing according to Animal Reproduction Science author's guidelines.

RESPONSE TO REVIEWER 1

- *Reviewer #1: In general, this is a well-written manuscript. However, it is merely descriptive and does not follow any hypothesis. Nevertheless, it is based on a sufficient number of experimental animals and data appear to be reliable. It is therefore recommended for publication after moderate revision. The following points of criticism should be taken into account:*

This research can be considered as pure, fundamental research, curiosity-driven and aimed to improve our knowledge on the complexity of equine amniotic fluid. The description of its proteome in healthy mares is a prerequisite for further investigations focused on specific diseases and/or pathologic conditions.

- *Information in the abstract is very general, please add some data.*

As suggested by the reviewer, some more data and information have been added.

- *In the introduction the authors should explain why they choose to analyze the protein of amniotic and not of allantoic fluid.*

As suggested by the reviewer, a sentence explaining the motivation of analysing AF instead of allantoic fluid was added.

- *Line 63: "and discuss their function" should be deleted.*

As suggested by the reviewer, the words have been deleted.

- *Lines 74-75: It is stated that all mares were healthy based on clinical and ultrasonographic evaluation. Please add details on which parameters have been accessed at what times before and after parturition.*

As suggested by the reviewer, more details on clinical and ultrasonographic evaluation have been added.

- *Information on statistical analysis is not detailed enough.*

As suggested by the reviewer, more information have been added. Correlation analysis between AF total proteins and the other evaluated parameters was added. Nevertheless, to the author opinion, since the main objective of the present paper was the characterization/description of the AF proteome in healthy samples and it was not a comparison between different experimental groups, the statistics are mainly descriptive.

- *The discussion does not start properly. It is always annoying for the reader if he/she has to deal with limitations of the study and has to wait for the more exciting points of the discussion. Please start with the most important results and shift the present beginning of the discussion to the end.*

Following the reviewer suggestion, the discussion has been modified, moving the first paragraphs to the end of the section. In the author's opinion the non-depletion of albumin and of the other proteins is not a limitation, but a decision driven by the intention to maintain the integrity of the sample.

- *The authors refer to the point that protein concentration of amniotic fluid analyzed in the present study differs from results of other studies and try to explain this discrepancy by the use of different methods. However, also in the present study wide variations were found. How can such an "inter-individual variability" be explained. What are the possible underlying causes?*

As underlined by the reviewer, the inter-individual variability was quite high. However, to the authors knowledge, no publications are present in the literature that evaluate possible source of inter-individual variability in AF protein concentration. Therefore, to the authors opinion, the possible explanation for the variability reported in the present paper could rely on different pre-hospitalization factors, such as differences in feed and hydration status and in housing conditions. A sentence was added at lines 326-331.

- *Lines 221 to 229: The publication by Ottisdottir et al. 2010 (Theriogenology 75 (2011) 1130-1138) on dynamics of MMP in equine fetal fluids has not been considered.*

As suggested by the reviewer, the citation has been added.

- *The conclusion has to be rewritten. At present it is another summary but does not present a "take home message" for the reader which would be preferable.*

Following the suggestion of the reviewer the conclusion has been rewritten. However, as previously reported, since the study is mainly descriptive, also the "take home message" cannot have a direct practical/clinical application.

- *Table 1: please add bodyweight of mares and their fetuses*

The requested data have been added.

- *All Tables and Figures should "stand alone" with complete descriptions of what is being presented as for example mean \pm sem, numbers of animals, etc. so that the reader does not have to refer to the text.*

As suggested by the reviewer, some more information have been added to the figures and tables legends. In legend of figure 1 the sample number of the analysed AF as reported in table 1 was also added. We also decided to remove table 2, since the data reported were already present in figure 2. However, if the reviewer considers the table as useful we will add it again.

Highlights

- Equine amniotic fluid was preliminary characterised for the first time.
- SDS-PAGE coupled to MS allowed the identification of the most abundant proteins.
- The three most abundant proteins were albumin, major allergen Eqc1 and fibronectin.
- The role of the extracellular matrix component in fetal maturation was highlighted.
- The importance of transport proteins like alfa-fetoprotein and PLTP was evidenced.

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 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.

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).

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 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 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, 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.

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.

315 **Table 1.** Clinical data collected from the 24 mares included in the study. Data are reported as
 316 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

317

318

319 **Table 2.** Proteins identified in equine amniotic fluid by mass spectrometry. Identified proteins
320 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
	TSP1_HUMAN	Thrombospondin-1	133.3	751	60	49	12	11	98
15	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
	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.

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	Carboxypeptidase	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	

334

335 ^a Protein entry name from UniProt knowledge database.

336 ^b Theoretical protein molecular weight.

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

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

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

340

341

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.

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.
- Bornstein, P., 1995. Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin 1. *J. Cell Biol.* 130, 503–506.
- Botros, H.G., Poncet, P., Rabillon, J., Fontaine, T., Laval, J.M., David, B., 2001. Biochemical

388 characterization and surfactant properties of horse allergens. *Eur. J. Biochem.* 268,
 389 3126–3136.

390 Brehm, A., Geraghty, P., Campos, M., Garcia-Arcos, I., Dabo, A.J., Gaffney, A., Eden, E.,
 391 Jiang, X.C., D’Armiento, J., Foronjy, R., 2014. Cathepsin G degradation of phospholipid
 392 transfer protein (PLTP) augments pulmonary inflammation. *FASEB J.* 28, 2318–2331.

393 Canisso, I.F., Ball, B.A., Squires, E.L., Troedsson, M.H., 2014. How to perform
 394 transabdominal ultrasound-guided fetal fluid sampling in mares. *J. Equine Vet. Sci.* 34,
 395 1143–1147.

396 Canisso, I.F., Ball, B.A., Scoggin, K.E., Squires, E.L., Williams, N.M., Troedsson, M.H.,
 397 2015. Alpha-fetoprotein is present in the fetal fluids and is increased in plasma of mares
 398 with experimentally induced ascending placentitis. *Anim. Reprod. Sci.* 154, 48–55.

399 Castagnetti, C., Mariella, J., Serrazanetti, G.P., Grandis, A., Merlo, B., Fabbri, M., Mari, G.,
 400 2007. Evaluation of lung maturity by amniotic fluid analysis in equine neonate.
 401 *Theriogenology* 67, 1455–1462.

402 Cho, C.K.J., Shan, S.J., Winsor, E.J., Diamandis, E.P., 2007. Proteomics analysis of human
 403 amniotic fluid. *Mol. Cell. Proteomics* 6, 1406–1415.

404 Dall’Ara, P., Meloni, T., Rota, A., Servida, F., Filipe, J., Veronesi, M.C., 2015.
 405 Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of
 406 pregnancy. *Theriogenology* 83, 766–771.

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

411 Ferlizza, E., Campos, A., Neagu, A., Cuoghi, A., Bellei, E., Monari, E., Dondi, F., Almeida,

412 A.M., Isani, G., 2015. The effect of chronic kidney disease on the urine proteome in the
 413 domestic cat (*Felis catus*). *Vet. J.* 204, 73–81.

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

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

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

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

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

427 Lascombe, M.B., Grégoire, C., Poncet, P., Tavares, G.A., Rosinski-Chupin, I., Rabillon, J.,
 428 Goubran-Botros, H., Mazié, J.C., David, B., Alzari, P.M., 2000. Crystal structure of the
 429 allergen Equ c 1. A dimeric lipocalin with restricted IgE-reactive epitopes. *J. Biol.*
 430 *Chem.* 275, 21572–21577.

431 Luft, A.J., Lai, P.C., Robertson, H.A., Saunders, N.R., Lorscheider, F.L., 1984. Distribution
 432 of alpha-fetoprotein in fetal plasma and in amniotic and allantoic fluids of the pig. *J.*
 433 *Reprod. Fertil.* 70, 605–607.

434 Lyle, S.K., Paccamonti, D.L., Hubert, J.D., Schlafer, D.H., Causey, R.C., Eilts, B.E., Johnson,
 435 J.R., 2006. Laparoscopic placement of an indwelling allantoic catheter in the mare:

436 biochemical, cytologic, histologic, and microbiologic findings. *Anim. Reprod. Sci.* 94,
 437 428–431.

438 Mavrou, A., Anagnostopoulos, A.K., Kolialexi, A., Vougas, K., Papantoniou, N., Antsaklis,
 439 A., Kanavakis, E., Fountoulakis, M., Tsangaris, G.T., 2008. Proteomic analysis of
 440 amniotic fluid in pregnancies with Turner syndrome fetuses. *J. Proteomics* 7, 1863–
 441 1866.

442 Michaels, J.E., Dasari, S., Pereira, L., Reddy, A.P., Lapidus, J.A., Lu, X., Jacob, T., Thomas,
 443 A., Rodland, M., Roberts, C.T., Gravett, M.G., Nagalla, S.R., 2007. Comprehensive
 444 proteomic analysis of the human amniotic fluid proteome: gestational age-dependent
 445 changes. *J. Proteome Res.* 6, 1277–1285.

446 Miller, I., Preßlmayer-Hartler, A., Wait, R., Hummel, K., Sensi, C., Eberini, I., Razzazi-
 447 Fazeli, E., Gianazza, E., 2014. In between - Proteomics of dog biological fluids. *J.*
 448 *Proteomics* 106, 30–45.

449 Mizejewski, G.J., 2001. Alpha-fetoprotein structure and function: relevance to isoforms,
 450 epitopes, and conformational variants. *Exp. Biol. Med.* 226, 377–408.

451 Mogami, H., Kishore, A.H., Shi, H., Keller, P.W., Akgul, Y., Word, R.A., 2013. Fetal
 452 fibronectin signaling induces matrix metalloproteinases and cyclooxygenase-2 (COX-2) in
 453 amnion cells and preterm birth in mice. *J. Biol. Chem.* 288, 1953–1966.

454 Morris, A.H., Kyriakides, T.R., 2014. Matricellular proteins and biomaterials. *Matrix Biol.*
 455 37, 183–191.

456 Oddsdóttir, C., Riley, S.C., Leask, R., Shaw, D.J., Aurich, C., Palm, F., Fowden, A.L.,
 457 Ricketts, S.W., Watson, E.D., 2011. Dynamics of activities of matrix metalloproteinases-
 458 9 and -2, and the tissue inhibitors of MMPs in fetal fluid compartments during gestation
 459 and at parturition in the mare. *Theriogenology* 75, 1130–1138.

460 Paccamonti, D., Swiderski, C., Marx, B., Gaunt, S., Blouin, D., 1995. Electrolytes and
 461 biochemical enzymes in amniotic and allantoic fluid of the equine fetus during late
 462 gestation. *Biol. Reprod. Monogr. Ser.* 1, 39–48.

463 Peddada, N., Sagar, A., Ashish, Garg, R., 2012. Plasma gelsolin: A general prognostic marker
 464 of health. *Med. Hypotheses* 78, 203–210.

465 Pirrone, A., Mariella, J., Gentilini, F., Castagnetti, C., 2012. Amniotic fluid and blood lactate
 466 concentrations in mares and foals in the early postpartum period. *Theriogenology* 78,
 467 1182–1189.

468 Riding, G.A., Hill, J.R., Jones, A., Holland, M.K., Josh, P.F., Lehnert, S.A., 2008. Differential
 469 proteomic analysis of bovine conceptus fluid proteins in pregnancies generated by
 470 assisted reproductive technologies. *Proteomics* 8, 2967–2982.

471 Sezen, D., Bongiovanni, A.M., Gelber, S., Perni, U., Hutson, J.M., Skupski, D., Witkin, S.S.,
 472 2009. Gelsolin down-regulates lipopolysaccharide-induced intraamniotic tumor necrosis
 473 factor- α production in the midtrimester of pregnancy. *Am. J. Obstet. Gynecol.* 200, 191–
 474 192.

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

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

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

483 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.

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

3
4 Isani Gloria ^a, Ferlizza Enea ^{a,*}, Cuoghi Aurora ^b, Bellei Elisa ^b, Monari Emanuela ^b, Bianchin
5 Butina Barbara ^a, and Castagnetti Carolina ^a

6
7 ^a Department of Veterinary Medical Sciences, University of Bologna, via Tolara di sopra 50,
8 40064 Ozzano, Italy

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

11
12 *** Corresponding author**

13 Enea Ferlizza
14 Dept. of Veterinary Medical Sciences, University of Bologna
15 Via Tolara di sopra, 50
16 40064 Ozzano (BO)
17 e-mail: enea.ferlizza2@unibo.it
18 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. The present study successfully applied SDS-PAGE coupled to MS identifying the most abundant proteins of equine AF, highlighting the importance of ECM and transport proteins.

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 and discuss their functions.

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 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.

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).

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 varied from 0.36 to 4.16 g/L. No significant correlations were 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 and Table 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 3). 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. Since this The study was carried out on 24 mares of different breeds, age and parity referred to the Equine Perinatology Unit, the animals were of different breed, age and parity, and they can be considered representative of a typical equine hospital population reflecting the local equine population. Therefore, the proteomic profile described can be considered a useful starting point for further applied studies on the equine AF proteins.

This study collected AF 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

199 evaluate gestational changes in the AF proteome as in women (Michaels et al. 2007).
200 Regarding total proteins, values found in the present research (1.96 ± 1.12 g/L) are similar to
201 those reported by Williams et al. (1993) (3.1 ± 2.6 g/L) and Paccamonti et al. (1995) (1.2 g/L),
202 but lower than those reported by Kochhar et al. (1997) (9.1 ± 2 g/L) and higher than those of
203 Zanella et al. (2014) (0.3 ± 0.1 g/L). The use of different methods for AF total proteins
204 quantification or the wide inter-individual variability in concentration could be the cause of
205 the reported discrepancies.

206 Complex biological samples contain thousands of different protein species, few of
207 them characterised by high abundance and many others by low or very low abundance. The
208 presence of very high abundance proteins like albumin or immunoglobulins often hampers the
209 separation and characterisation of serum and AF proteomes, therefore the depletion of these
210 major components has been widely applied in human proteomics (Cho et al. 2007; Michaels
211 et al. 2007). However, this approach can lead to the loss of some low abundance proteins due
212 to the “sponge effect” of albumin that can bind a variety of other proteins or peptides (Bellei
213 et al. 2011). From this point of view, equine AF is a preferential sample due to the absence of
214 immunoglobulins. In domestic animals, the passage of immunoglobulins is influenced by the
215 placental structure: in horses, pigs and ruminants the placenta is epitheliochorial, thus
216 impermeable to immunoglobulins (Furukawa et al. 2014), whereas in dogs, the
217 endotheliochorial placenta allows only 5% to 10% transfer of maternal antibodies to the
218 foetus (Dall’Ara et al. 2015). The absence of immunoglobulins in equine AF, as confirmed by
219 this study, was advantageous for its more reliable characterisation, as a consequence to
220 preserve proteome integrity and complexity the most abundant proteins were not depleted.

221 Most of the 34 proteins identified were involved in cellular growth and maintenance,
222 transport and protein metabolism reflecting the dynamic biological functions of AF.
223 Regarding cellular growth and/or maintenance, 12 of the proteins identified belonged to or
224 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 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 fluid and AF 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 In horses, alpha-fetoprotein it is highly expressed during early pregnancy by the equine conceptus (Simpson et al. 2000) and in mammalian foetuses, it is associated with estrogen binding, anti-oxidative properties and immunoregulation (DeMees et al. 2006; Mizejewski 2001). 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 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, 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, was advantageous for its more reliable characterisation, as a consequence to preserve proteome integrity and complexity the most abundant proteins were not depleted. 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 Regarding AF total protein concentrations, which values found in the present research (1.96 ± 1.12 g/L) with a wide variability (range 0.36-4.16 g/L) are 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 use of different methods for AF total proteins quantification or the wide inter-individual variability in concentration could be the cause of the reported discrepancies. 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.

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. The present study applied a qualitative proteomic approach to characterise the AF proteome from healthy mares. The 34 proteins identified by 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. Further studies are needed to define reference intervals for AF total protein in healthy mares and. 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. In particular, 2DE could be applied to better characterise the physiologic AF proteome and to evaluate differentially expressed proteins in case of disease.

Acknowledgements

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

Conflict of interest

355 The authors have no conflict of interests to declare.

356

357 **Table 1.** Clinical data collected from the 24 mares included in the study. Data are reported as
 358 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

359

360

Table 2. Relative abundance in percentage of each protein band compared to the entire volume of the lane (n=24).

MW (kDa)	Mean (%)	SD (%)
>165	0.3	0.2
>165	1.5	0.4
>165	5.2	1.9
>165	1.4	0.3
145	0.3	0.2
131	0.1	0.1
117	0.6	0.1
102	0.6	0.2
91	0.7	0.2
83	3.6	0.7
73	2.0	0.9
64	25.7	2.8
53	1.4	0.5
41	1.4	0.5
35	0.7	0.2
29	1.6	0.5
24	19.2	3.6
21	0.7	0.2
18	4.2	1.2
14	3.0	2.9
<13	3.4	1.0
<13	2.8	1.2
<13	4.0	0.8

366 **Table 2 3.** Proteins identified in equine amniotic fluid by mass spectrometry. **Identified**
367 **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-antiproteinase 2	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
	TSP1_HUMAN	Thrombospondin-1	133.3	751	60	49	12	11	98
15	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
	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.

379 **Table 3 4.** Biological and functional classification of the proteins identified in equine

380 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
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

381

382 ^a Protein entry name from UniProt knowledge database.

383 ^b Theoretical protein molecular weight.

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

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

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

387

388

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 of lane 3

Figure 2. Relative abundance in percentage 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. Two replicates of 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 3. 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.

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.
- Bornstein, P., 1995. Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin 1. *J. Cell Biol.* 130, 503–506.
- Botros, H.G., Poncet, P., Rabillon, J., Fontaine, T., Laval, J.M., David, B., 2001. Biochemical

436 characterization and surfactant properties of horse allergens. *Eur. J. Biochem.* 268,
 437 3126–3136.

438 Brehm, A., Geraghty, P., Campos, M., Garcia-Arcos, I., Dabo, A.J., Gaffney, A., Eden, E.,
 439 Jiang, X.C., D’Armiento, J., Foronjy, R., 2014. Cathepsin G degradation of phospholipid
 440 transfer protein (PLTP) augments pulmonary inflammation. *FASEB J.* 28, 2318–2331.

441 Canisso, I.F., Ball, B.A., Squires, E.L., Troedsson, M.H., 2014. How to perform
 442 transabdominal ultrasound-guided fetal fluid sampling in mares. *J. Equine Vet. Sci.* 34,
 443 1143–1147.

444 Canisso, I.F., Ball, B.A., Scoggin, K.E., Squires, E.L., Williams, N.M., Troedsson, M.H.,
 445 2015. Alpha-fetoprotein is present in the fetal fluids and is increased in plasma of mares
 446 with experimentally induced ascending placentitis. *Anim. Reprod. Sci.* 154, 48–55.

447 Castagnetti, C., Mariella, J., Serrazanetti, G.P., Grandis, A., Merlo, B., Fabbri, M., Mari, G.,
 448 2007. Evaluation of lung maturity by amniotic fluid analysis in equine neonate.
 449 *Theriogenology* 67, 1455–1462.

450 Cho, C.K.J., Shan, S.J., Winsor, E.J., Diamandis, E.P., 2007. Proteomics analysis of human
 451 amniotic fluid. *Mol. Cell. Proteomics* 6, 1406–1415.

452 Dall’Ara, P., Meloni, T., Rota, A., Servida, F., Filipe, J., Veronesi, M.C., 2015.
 453 Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of
 454 pregnancy. *Theriogenology* 83, 766–771.

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

459 Ferlizza, E., Campos, A., Neagu, A., Cuoghi, A., Bellei, E., Monari, E., Dondi, F., Almeida,

460 A.M., Isani, G., 2015. The effect of chronic kidney disease on the urine proteome in the
 461 domestic cat (*Felis catus*). *Vet. J.* 204, 73–81.

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

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

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

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

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

475 Lascombe, M.B., Grégoire, C., Poncet, P., Tavares, G.A., Rosinski-Chupin, I., Rabillon, J.,
 476 Goubran-Botros, H., Mazié, J.C., David, B., Alzari, P.M., 2000. Crystal structure of the
 477 allergen Equ c 1. A dimeric lipocalin with restricted IgE-reactive epitopes. *J. Biol.*
 478 *Chem.* 275, 21572–21577.

479 Luft, A.J., Lai, P.C., Robertson, H.A., Saunders, N.R., Lorscheider, F.L., 1984. Distribution
 480 of alpha-fetoprotein in fetal plasma and in amniotic and allantoic fluids of the pig. *J.*
 481 *Reprod. Fertil.* 70, 605–607.

482 Lyle, S.K., Paccamonti, D.L., Hubert, J.D., Schlafer, D.H., Causey, R.C., Eilts, B.E., Johnson,
 483 J.R., 2006. Laparoscopic placement of an indwelling allantoic catheter in the mare:

484 biochemical, cytologic, histologic, and microbiologic findings. *Anim. Reprod. Sci.* 94,
 485 428–431.

486 Mavrou, A., Anagnostopoulos, A.K., Kolialexi, A., Vougas, K., Papantoniou, N., Antsaklis,
 487 A., Kanavakis, E., Fountoulakis, M., Tsangaris, G.T., 2008. Proteomic analysis of
 488 amniotic fluid in pregnancies with Turner syndrome fetuses. *J. Proteomics* 7, 1863–
 489 1866.

490 Michaels, J.E., Dasari, S., Pereira, L., Reddy, A.P., Lapidus, J.A., Lu, X., Jacob, T., Thomas,
 491 A., Rodland, M., Roberts, C.T., Gravett, M.G., Nagalla, S.R., 2007. Comprehensive
 492 proteomic analysis of the human amniotic fluid proteome: gestational age-dependent
 493 changes. *J. Proteome Res.* 6, 1277–1285.

494 Miller, I., Preßlmayer-Hartler, A., Wait, R., Hummel, K., Sensi, C., Eberini, I., Razzazi-
 495 Fazeli, E., Gianazza, E., 2014. In between - Proteomics of dog biological fluids. *J.*
 496 *Proteomics* 106, 30–45.

497 Mizejewski, G.J., 2001. Alpha-fetoprotein structure and function: relevance to isoforms,
 498 epitopes, and conformational variants. *Exp. Biol. Med.* 226, 377–408.

499 Mogami, H., Kishore, A.H., Shi, H., Keller, P.W., Akgul, Y., Word, R.A., 2013. Fetal
 500 fibronectin signaling induces matrix metalloproteases and cyclooxygenase-2 (COX-2) in
 501 amnion cells and preterm birth in mice. *J. Biol. Chem.* 288, 1953–1966.

502 Morris, A.H., Kyriakides, T.R., 2014. Matricellular proteins and biomaterials. *Matrix Biol.*
 503 37, 183–191.

504 Oddsdóttir, C., Riley, S.C., Leask, R., Shaw, D.J., Aurich, C., Palm, F., Fowden, A.L.,
 505 Ricketts, S.W., Watson, E.D., 2011. Dynamics of activities of matrix metalloproteinases-
 506 9 and -2, and the tissue inhibitors of MMPs in fetal fluid compartments during gestation
 507 and at parturition in the mare. *Theriogenology* 75, 1130–1138.

508 Paccamonti, D., Swiderski, C., Marx, B., Gaunt, S., Blouin, D., 1995. Electrolytes and
509 biochemical enzymes in amniotic and allantoic fluid of the equine fetus during late
510 gestation. *Biol. Reprod. Monogr. Ser.* 1, 39–48.

511 Peddada, N., Sagar, A., Ashish, Garg, R., 2012. Plasma gelsolin: A general prognostic marker
512 of health. *Med. Hypotheses* 78, 203–210.

513 Pirrone, A., Mariella, J., Gentilini, F., Castagnetti, C., 2012. Amniotic fluid and blood lactate
514 concentrations in mares and foals in the early postpartum period. *Theriogenology* 78,
515 1182–1189.

516 Riding, G.A., Hill, J.R., Jones, A., Holland, M.K., Josh, P.F., Lehnert, S.A., 2008. Differential
517 proteomic analysis of bovine conceptus fluid proteins in pregnancies generated by
518 assisted reproductive technologies. *Proteomics* 8, 2967–2982.

519 Sezen, D., Bongiovanni, A.M., Gelber, S., Perni, U., Hutson, J.M., Skupski, D., Witkin, S.S.,
520 2009. Gelsolin down-regulates lipopolysaccharide-induced intraamniotic tumor necrosis
521 factor- α production in the midtrimester of pregnancy. *Am. J. Obstet. Gynecol.* 200, 191–
522 192.

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

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

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

531 Tong, X., 2013. Amniotic fluid may act as a transporting pathway for signaling molecules and

532 stem cells during the embryonic development of amniotes. *J. Chin. Med. Assoc.* 76, 606-
533 610.

534 Turner, S.W., Carter, J., Danielian, P., Chalmers, I., McConaghy, L., Pacitti, N., Booth, N.,
535 2014. Protease concentration in amniotic fluid at term and early childhood respiratory
536 symptoms. *J. Matern. Fetal. Neonatal Med.* 27, 416–420.

537 Underwood, M.A., Gilbert, W.M., Sherman, M.P., 2005. Amniotic fluid: not just fetal urine
538 anymore. *J. Perinatol.* 25, 341–348.

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

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

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

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

553

Figure 1

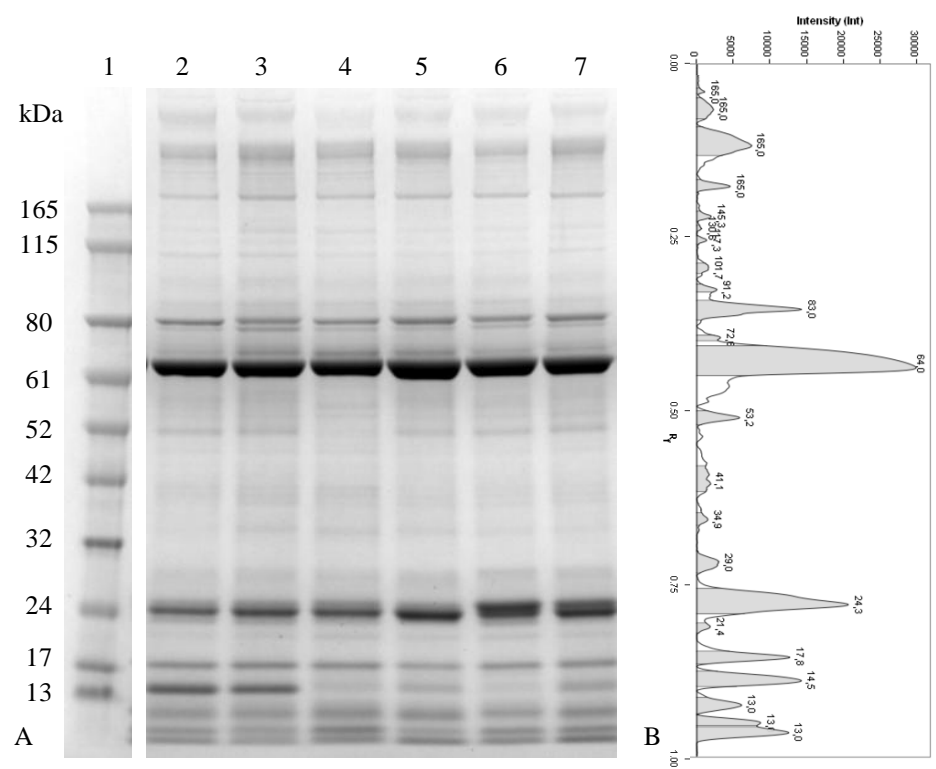


Figure 2

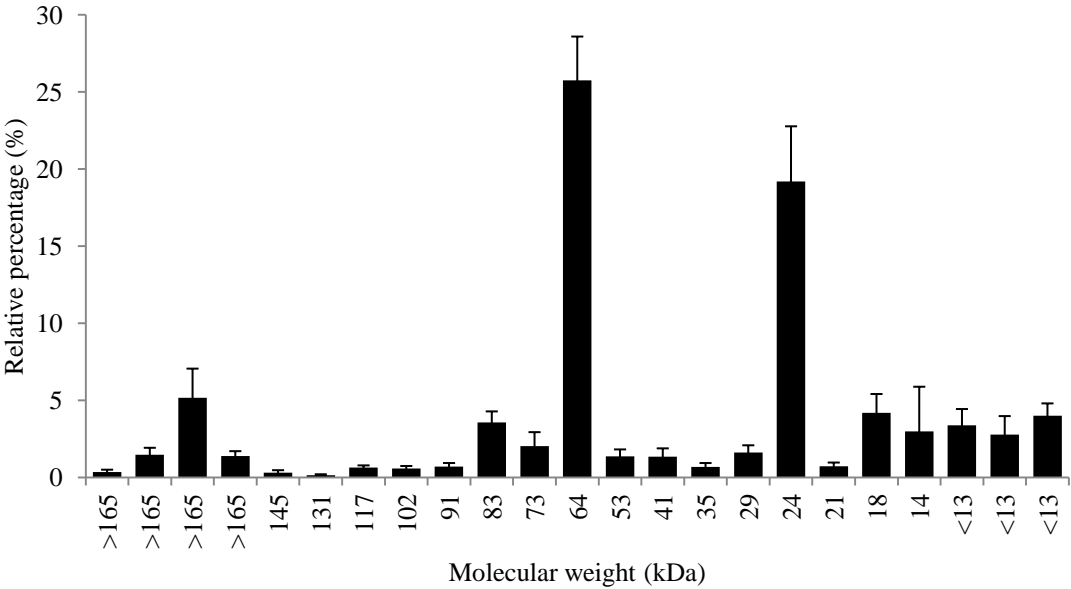


Figure 3

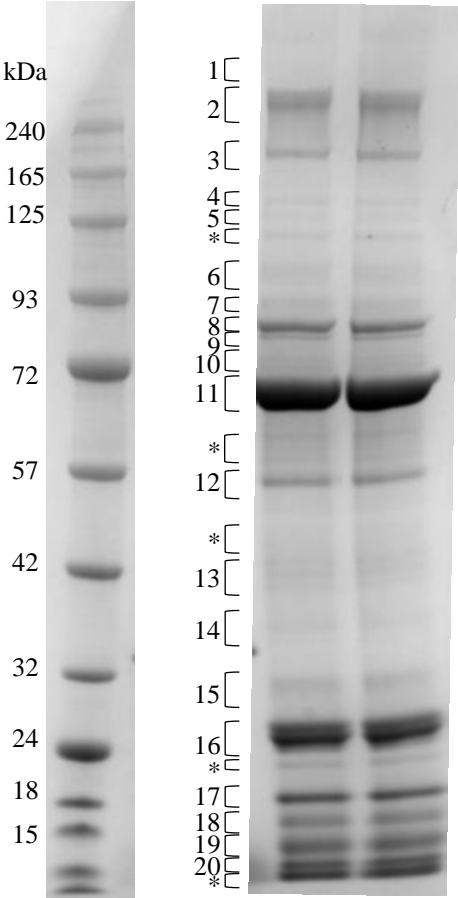
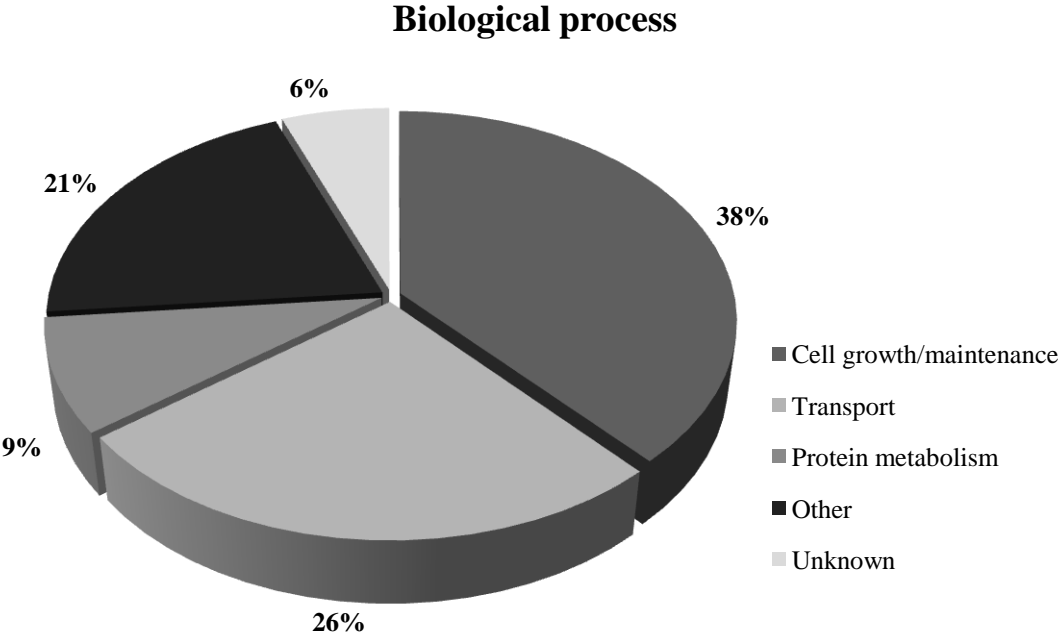


Figure 4



Conflict of interest: none

The authors have no conflict of interests to declare.