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

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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 Ottsdottir 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 ± 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

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Abstract

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

Keywords

Horse, pregnancy, electrophoresis, proteome.

Introduction

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

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

Proteomics is a powerful analytical approach providing a profile of proteins present in a biological sample at a given time. The high potential of this approach has been recently found to have a major role in different areas of veterinary medicine, from farm (Almeida et al. 2015) to companion (Ferlizza et al. 2015; Miller et al. 2014) animals. Proteomic techniques have recently been applied to the characterisation of horse amniotic membrane (Galera et al. 2015) and bovine conceptus fluids (Riding et al. 2008), whereas the equine AF proteome remains uncharacterised. Therefore, the aims of this study were to identify the most abundant

proteins in equine AF by SDS-PAGE separation followed by mass spectrometry identification.

Materials and Methods

Animal selection and data collection

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

All the mares included in the study were healthy based on clinical and ultrasonographic evaluation. At admission, a complete clinical evaluation, including complete blood count, serum biochemistry and transrectal ultrasonography, was performed. Severe maternal illness, uterine discharge, premature lactation, twinning, abnormal foetal presentation, placenta oedema, and signs of foetal distress were ruled out. During the course of hospitalisation, mares were clinically evaluated twice a day and by transrectal ultrasonography every 10 days until parturition. The following ultrasonographic parameters were evaluated: combined thickness of the uterus and placenta, foetal fluids echogenicity, foetal activity, and foetal orbital area. Foals were born between 320 and 365 days of pregnancy by normal delivery, had an Apgar score ≥8 recorded within 5 minutes from birth (Vaala et al. 2002) and had a normal clinical evaluation during the course of hospitalisation, including a complete blood count and serum biochemistry at birth and an immunoglobulin G (IgG) serum concentration ≥800 mg/dL at 18-24 hours of life.

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

Sample collection

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

SDS-PAGE

To optimise protein separation, different protocols were tested including 4-12% and 12% polyacrylamide gels (NuPage/Thermo Fisher Scientific, Waltham, Massachusetts, USA) in 2-(N-morpholino) propanesulfonic acid buffer (MOPS; NuPage/Thermo Fisher Scientific) or 2-(N-morpholino) ethanesulfonic acid buffer (MES; NuPage/Thermo Fisher Scientific) with sodium dodecyl sulphate (SDS). Each AF sample (n=24) was analysed at least twice with the protocol assuring the best protein separation in our experimental conditions (4-12% gels, in MOPS buffer). Twenty µg of proteins were loaded for each sample and the gels were stained with Coomassie G250 compatible with mass spectrometry analysis. After staining, each gel was digitalised by ChemiDoc^{TM}MP (BioRad, Hercules, California, USA) and its pherogram was obtained using ImageLab 5.2.1 software (BioRad). The software determines the volume of each protein band through the analysis of the pixel values in the digital image,

meaning as volume the sum of all the pixels intensities within the band boundaries. The band volumes are subsequently compared to the entire volume of the lane and the relative abundances reported in percentage. A pool was prepared by collecting and mixing 50 µg of protein from each AF (n=24) and analysed twice with the same protocol used for each sample.

Protein identification by mass spectrometry

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

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

obtain a false discovery rate <5% (5% probability of false match for each protein with a score above 80).

Statistical analysis

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

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

Results

Clinical data

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

SDS-PAGE and protein identification by mass spectrometry

Representative gel and pherogram of AF are reported in Figure 1A and 1B. The mean number of bands was 23±1.5. All samples presented a similar pattern characterised by two clusters of bands: the first with molecular weights (MW) higher than 62 kDa and the second

with MW lower than 34 kDa. In the middle, very few faint bands were present. Figure 2 reports the relative abundance in percentage of AF protein bands. Out of the 25 slices cut from the gel (Figure 3), 20 yielded significant results leading to the unambiguous identification of 34 proteins (Table 2). Serum albumin and major allergen Equ c1 (ALL1) were the two most abundant proteins, followed by fibronectin, transferrin and haemoglobin; these five proteins represented >60% of the equine AF proteome. Fibronectin, versican and albumin were also identified in bands characterised by different MW.

The identified proteins categorised by their molecular function and biological process according to Gene Ontology (GO) and Human Protein Reference Database (HPRD) are shown in Table 3 and Figure 4. Most of the proteins were involved in cellular growth and/or maintenance (38%), transport (26%) and protein metabolism (9%). The vast majority of the identified proteins were classified as extracellular (79%).

Discussion

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

Most of the 34 proteins identified were involved in cellular growth and maintenance, transport and protein metabolism reflecting the dynamic biological functions of AF. Regarding cellular growth and/or maintenance, 12 of the proteins identified belonged to or interacted with the extracellular matrix (ECM) that plays an important role in the development of foetal tissues. All these proteins, with the exception of versican and proteoglycan 4, were also identified in human AF (Cho et al. 2007; Michaels et al. 2007) and/or in equine amniotic

membrane (Galera et al. 2015). Among the ECM structural proteins, fibronectin is a multifunctional glycoprotein known to participate in the organisation of ECM binding to integrins. During pregnancy, fibronectin is expressed in the junction between maternal and foetal membranes as well as in the uterus and placenta (Mogami et al. 2013). Lumican, a member of the family of small leucine-rich proteoglycans, is the major keratan sulphate proteoglycan of the cornea and is also present in the ECM throughout the body, including human and equine amniotic membrane (Galera et al. 2015; Kao et al. 2006; Mavrou et al. 2008). Based on its interaction with fibrillar collagen and its ability to modulate cell proliferation and migration, lumican could play a role in the maturation of foetal tissues (Mavrou et al. 2008). Regarding the non-structural proteins involved in ECM development and organisation, thrombospondin and fibulin are regulatory proteins belonging to the group of the matricellular proteins. These proteins represent a bridge between matrix proteins and cell surface receptors, or other molecules such as cytokines that can interact with the cell surface (Bornstein, 1995). They are typically expressed at low levels in adult tissues, but are strongly expressed during development or following injury or pathology (Morris and Kyriakides, 2014). Gelsolin is a multifunctional actin regulatory protein involved in cytoskeleton dynamics and structure. In addition to its role in aiding chemotaxis and movement of intracellular structures, plasma gelsolin binds to a variety of proinflammatory and bioactive molecules including fibronectin, platelet activating factor and the bacterial surface lipids lipoteichoic acid and lipopolysaccharide (Peddada et al. 2012). The role of gelsolin in AF is still unknown, but it has been suggested to modulate inflammation and bacterial infections in human AF (Sezen et al. 2009). In association with gelsolin, vinculin is also a component of the actine cytoskeleton and is involved in integrin-mediated focal adhesion, cell motility and other cellular functions such as migration, proliferation and differentiation (Wu et al. 2014). Other proteins interacting with ECM that could play important roles in the development of foetal tissue are type IV collagenase (MMP2) and

metalloproteinase inhibitor 1 (TIMP1) belonging to the matrix metalloproteinases (MMPs) and tissue inhibitors respectively. The MMPs are a family of over 20 enzymes acting on the ECM components, regulated at different levels via their activators, inhibitors and localization on the cell surface (Sternlicht and Werb, 2001). The biological functions of these enzymes 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 ± standard deviation. AF amniotic fluid; TP total proteins.

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

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

Band [a]	Entry name [b]	Protein full name	MW (kDa) [c]	Score [d]	Pept. [e]	Sign. Pept. [f]	Seq. [g]	Sign Seq. [h]	% id. [i]
1	CSPG2_BOVIN	Versican core protein	371.8	257	76	21	15	6	77.2
2	FINC_HORSE	Fibronectin	58.1	1451	155	89	19	18	100
3	FINC_HORSE	Fibronectin	58.1	440	76	34	15	10	100
4	FINC_BOVIN	Fibronectin	275.5	155	46	16	16	7	95.4
	CO6A1_HUMAN	Collagen alpha-1(VI) chain	109.6	168	41	16	10	7	89
5	VINC_HUMAN	Vinculin	124.3	148	53	18	26	10	99.5
6	ACE2_PAGLA	Angiotensin-converting enzyme 2	93.0	380	43	18	10	6	86
	PLMN_HORSE	Plasminogen	38.1	288	36	18	8	5	100
	FINC_CANFA	Fibronectin	58.2	148	18	11	7	5	96.4
7	GELS_HORSE	Gelsolin	81.1	1911	193	122	27	21	100
	FBLN1_HUMAN	Fibulin-1	81.3	434	80	47	11	9	91.2
	FINC_HORSE	Fibronectin	58.1	360	36	23	11	7	100
	LUM_MOUSE	Lumican	38.6	159	27	14	6	4	87.4
8	TRFE_HORSE	Serotransferrin	80.3	2201	263	153	43	32	100
	TRFL_HORSE	Lactotransferrin	77.9	268	61	30	19	11	100
9	TRFE_HORSE	Serotransferrin	80.3	1642	194	106	34	24	100
10	ALBU_HORSE	Serum albumin	70.5	2863	244	148	44	33	100
	ECM1_HUMAN	Extracellular matrix protein 1	62.2	188	53	22	4	4	78.4
	MMP2_BOVIN	72 kDa type IV collagenase	74.8	468	50	27	14	9	95.4
	PLTP_HUMAN	Phospholipid transfer protein	54.9	400	24	19	3	3	89.1
	LUM_MOUSE	Lumican	38.6	153	23	12	6	4	87.4
	FETA_HORSE	Alpha-fetoprotein	70.1	145	17	10	7	4	100
	CSPG2_MACNE	Versican core protein	96.8	101	13	7	4	3	82
11	ALBU_HORSE	Serum albumin	70.5	17663	1154	849	70	56	100
	FETA_HORSE	Alpha-fetoprotein	70.1	407	59	34	23	14	100
	CSPG2_MACNE	Versican core protein	96.8	347	13	10	5	4	82
	BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3	75.3	53	12	6	4	3	92.9
12	A1AT2_HORSE	Alpha-1-antiproteinase 2	47.1	195	11	8	4	2	100
13	ALBU_EQUAS	Serum albumin	70.5	160	53	13	23	9	98.5
	CLUS_HORSE	Clusterin	52.7	453	48	28	15	10	100
	ACTB_BOVIN	Actin cytoplasmic 1	42.1	118	36	11	14	6	100
	GELS_HORSE	Gelsolin	81.1	40	12	4	7	4	100
	IBP3_BOVIN	Insulin-like growth factor-binding protein	32.6	79	11	9	5	5	86.7
	FETUA_BOVIN	Alpha-2-HS-glycoprotein	39.2	65	11	6	2	2	71.3
	CO6A3_HUMAN	Collagen alpha-3(VI) chain	345.2	93	5	4	3	2	88.5
	HPT_BOVIN	Haptoglobin	45.6	52	3	2	3	2	78.9
14	CLUS_HORSE	Clusterin	52.7	42	18	4	7	3	100
	TSP1_BOVIN	Thrombospondin-1	133.4	29	13	3	9	3	96.8
	SFTPA_HORSE	Pulmonary surfactant-associated protein A	26.5	71	4	3	3	2	100
15	TSP1_HUMAN	Thrombospondin-1	133.3	751	60	49	12	11	98
	ALL1_HORSE	Major allergen Equ c 1	21.9	642	58	33	10	8	100
	TIMP1_HORSE	Metalloproteinase inhibitor 1	23.7	189	15	7	5	3	100
16	ALL1_HORSE	Major allergen Equ c 1	21.9	1620	193	107	17	11	100
	TSP1_HUMAN	Thrombospondin-1	133.3	351	58	26	13	9	98
	APOA1_CANFA	Apolipoprotein A-1	30.2	124	24	11	4	3	80.8
17	HBB_HORSE	Haemoglobin sub. beta	16.1	2153	159	114	14	12	100
	HBA_HORSE	Haemoglobin sub. alpha	15.3	824	87	53	8	7	100
18	PIP_HYLSY	Prolactin-inducible protein homolog	16.9	122	9	8	1	1	64.3

19	THIO_HORSE	Thioredoxin	12.0	80	12	2	4	1	100
20	PRG4_HUMAN	Proteoglycan 4	152.2	130	18	8	3	2	88.6
	ALBU_EQUAS	Serum albumin	70.5	75	18	10	4	3	98.5

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

[b] Protein entry name from UniProt knowledge database.

[c] Theoretical protein molecular weight.

[d] The highest scores obtained with Mascot search engine.

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

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

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

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

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

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

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

[a] Protein entry name from UniProt knowledge database.

[b] Theoretical protein molecular weight.

[c] Biological Process according to Gene Ontology and Human Protein Reference Database.

[d] Molecular Function according to Gene Ontology and Human Protein Reference Database.

[e] Cellular Component according to Gene Ontology and Human Protein Reference Database.

342 **Figure Legends**

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

348

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

351

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

357

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

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505

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

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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 r~~R~~ecently, 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 ChemiDoc^{TM}MP (BioRad, Hercules, California, USA) and its pherogram was obtained using ImageLab 5.2.1 software (BioRad). The software determines the volume of each protein band through the analysis of the pixel values in the digital image,

meaning as volume the sum of all the pixels intensities within the band boundaries. The band volumes are subsequently compared to the entire volume of the lane and the relative abundances reported in percentage. A pool was prepared by collecting and mixing 50 µg of protein from each AF (n=24) and analysed twice with the same protocol used for each sample.

Protein identification by mass spectrometry

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

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

147 obtain a false discovery rate <5% (5% probability of false match for each protein with a score
148 above 80).
149
150 *Statistical analysis*
151 Data (AF total proteins, mare's age, mare's and foal's body weight, parity, days of
152 pregnancy, length of stage II labour (minutes), foal's Apgar score and number of bands) were
153 analysed with statistical software (R version 2.15.1) and reported as mean ± standard
154 deviation (SD). Shapiro-Wilk normality test was performed to evaluate data normal
155 distribution. Pearson coefficient of correlation was calculated between AF total proteins and
156 the other data recorded for each mare (mare's age, mare's and foal's body weight, parity, days
157 of pregnancy, length of stage II labour (minutes), foal's Apgar score and number of bands).
158 The identified proteins were categorised by biological process, molecular function and
159 cellular component with Gene Ontology terms according to Gene Ontology (GO) and Human
160 Protein Reference Database (HPRD).
161
162 **Results**
163 *Clinical data*
164 Clinical data collected from the 24 mares included in the study are shown in Table 1. Mean
165 AF total protein concentration was 1.96±1.12 g/L, ranging ~~varied~~ from 0.36 to 4.16 g/L. No
166 significant correlations were found between AF protein concentration and the other data
167 recorded.
168
169 *SDS-PAGE and protein identification by mass spectrometry*
170 Representative gel and pherogram of AF are reported in Figure 1A and 1B. The mean
171 number of bands was 23±1.5. All samples presented a similar pattern characterised by two
172 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 2 ~~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 ~~4~~ 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~~

evaluate gestational changes in the AF proteome as in women (Michaels et al. 2007). Regarding total proteins, values found in the present research (1.96±1.12 g/L) are 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.

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 or immunoglobulins often hampers the separation and characterisation of serum and AF proteomes, therefore the depletion of these major components has been widely 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). 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.

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 ~~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-

277 fetoprotein in equine foetal fluids during the third trimester of pregnancy and found increased
278 maternal plasma concentrations of the protein in mares with experimentally induced
279 placentitis. The presence of ALL1 in AF is challenging. ALL1 is a glycoprotein of 21.7 kDa
280 belonging to the family of lipocalins, whose function is to carry small hydrophobic molecules
281 such as odorants, steroids and pheromones. This protein is expressed in salivary glands and in
282 the liver and is highly concentrated in secretory fluids such as saliva and urine as well as in
283 hair and dander (Botros et al. 2001). ALL1 is widely studied in human medicine because it
284 induces an IgE-mediated type I allergic reaction in the majority of patients allergic to horses
285 (Lascombe et al. 2000). The physiological role of this protein is still unknown and to our
286 knowledge, this is the first study reporting the presence of ALL1 in AF. PLTP is a monomeric
287 glycoprotein involved in lipid transport, lipoprotein metabolism and lipopolysaccharide
288 binding. It is ubiquitously expressed in human tissues and is secreted into the plasma, where
289 its central role has been well established (Albers et al. 2012). PLTP is highly expressed in
290 lung epithelial cells, and may play a role in surfactant metabolism during foetus lung
291 development (Brehm et al. 2014).

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

303 view, equine AF is a preferential sample due to the absence of immunoglobulins. In domestic
304 animals, the passage of immunoglobulins is influenced by the placental structure: in horses,
305 pigs and ruminants the placenta is epitheliochorial, thus impermeable to immunoglobulins
306 (Furukawa et al. 2014), whereas in dogs, the endotheliochorial placenta allows only 5% to
307 10% transfer of maternal antibodies to the foetus (Dall'Ara et al. 2015). From an analytical
308 point of view, the absence of immunoglobulins in equine AF, as confirmed by this study, ~~was~~
309 ~~advantageous for its more reliable characterisation, as a consequence to preserve proteome~~
310 ~~integrity and complexity the most abundant proteins were not depleted.~~ can be considered an
311 advantage, allowing to perform SDS-PAGE and MS identification without affecting proteome
312 integrity and complexity.
313 The second point is related to the sample collection. This study collected AF only at
314 parturition because mares were client-owned and transabdominal amniocentesis is still not
315 recommended for clinical use. Recently, Canisso et al. (2014) described a safe technique to
316 perform multiple ultrasound-guided foetal fluid samplings during the last trimester of
317 gestation in mares. On this basis, abdominal amniocentesis will probably be preferred more
318 frequently also in a clinical setting and to evaluate gestational changes in the AF proteome as
319 reported in women (Michaels et al. 2007).
320 The last point regards ~~Regarding~~ AF total protein concentrations, which ~~values found~~
321 ~~in the present research (1.96±1.12 g/L) with a wide variability (range 0.36-4.16 g/L) are~~ were
322 similar to those reported by Williams et al. (1993) (3.1±2.6 g/L) and Paccamonti et al. (1995)
323 (1-2 g/L), but lower than those reported by Kochhar et al. (1997) (9.1±2 g/L), and higher than
324 those of Zanella et al. (2014) (0.3±0.1 g/L). ~~The use of different methods for AF total proteins~~
325 ~~quantification or the wide inter-individual variability in concentration could be the cause of~~
326 ~~the reported discrepancies.~~ The reported discrepancies could be related to the use of
327 quantification methods characterised by different analytical performances; also the influence
328 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 applyied a qualitative proteomic approach to characterise the AF proteome from healthy mares. The 34 proteins identified~~ 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 ~~the ECM~~ 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.~~

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Conflict of interest

The authors have no conflict of interests to declare.

Table 1. Clinical data collected from the 24 mares included in the study. Data are reported as mean ± standard deviation. AF amniotic fluid; TP total proteins.

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

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 [a]	Entry name [b]	Protein full name	MW (kDa) [c]	Score [d]	Pept. [e]	Sign. Pept. [f]	Seq. [g]	Sign Seq. [h]	% id. [i]
1	CSPG2_BOVIN	Versican core protein	371.8	257	76	21	15	6	77.2
2	FINC_HORSE	Fibronectin	58.1	1451	155	89	19	18	100
3	FINC_HORSE	Fibronectin	58.1	440	76	34	15	10	100
4	FINC_BOVIN	Fibronectin	275.5	155	46	16	16	7	95.4
	CO6A1_HUMAN	Collagen alpha-1(VI) chain	109.6	168	41	16	10	7	89
5	VINC_HUMAN	Vinculin	124.3	148	53	18	26	10	99.5
6	ACE2_PAGLA	Angiotensin-converting enzyme 2	93.0	380	43	18	10	6	86
	PLMN_HORSE	Plasminogen	38.1	288	36	18	8	5	100
	FINC_CANFA	Fibronectin	58.2	148	18	11	7	5	96.4
7	GELS_HORSE	Gelsolin	81.1	1911	193	122	27	21	100
	FBLN1_HUMAN	Fibulin-1	81.3	434	80	47	11	9	91.2
	FINC_HORSE	Fibronectin	58.1	360	36	23	11	7	100
	LUM_MOUSE	Lumican	38.6	159	27	14	6	4	87.4
8	TRFE_HORSE	Serotransferrin	80.3	2201	263	153	43	32	100
	TRFL_HORSE	Lactotransferrin	77.9	268	61	30	19	11	100
9	TRFE_HORSE	Serotransferrin	80.3	1642	194	106	34	24	100
10	ALBU_HORSE	Serum albumin	70.5	2863	244	148	44	33	100
	ECM1_HUMAN	Extracellular matrix protein 1	62.2	188	53	22	4	4	78.4
	MMP2_BOVIN	72 kDa type IV collagenase	74.8	468	50	27	14	9	95.4
	PLTP_HUMAN	Phospholipid transfer protein	54.9	400	24	19	3	3	89.1
	LUM_MOUSE	Lumican	38.6	153	23	12	6	4	87.4
	FETA_HORSE	Alpha-fetoprotein	70.1	145	17	10	7	4	100
	CSPG2_MACNE	Versican core protein	96.8	101	13	7	4	3	82
11	ALBU_HORSE	Serum albumin	70.5	17663	1154	849	70	56	100
	FETA_HORSE	Alpha-fetoprotein	70.1	407	59	34	23	14	100
	CSPG2_MACNE	Versican core protein	96.8	347	13	10	5	4	82
	BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3	75.3	53	12	6	4	3	92.9
12	A1AT2_HORSE	Alpha-1-antiproteinase 2	47.1	195	11	8	4	2	100
13	ALBU_EQUAS	Serum albumin	70.5	160	53	13	23	9	98.5
	CLUS_HORSE	Clusterin	52.7	453	48	28	15	10	100
	ACTB_BOVIN	Actin cytoplasmic 1	42.1	118	36	11	14	6	100
	GELS_HORSE	Gelsolin	81.1	40	12	4	7	4	100
	IBP3_BOVIN	Insulin-like growth factor-binding protein	32.6	79	11	9	5	5	86.7
	FETUA_BOVIN	Alpha-2-HS-glycoprotein	39.2	65	11	6	2	2	71.3
	CO6A3_HUMAN	Collagen alpha-3(VI) chain	345.2	93	5	4	3	2	88.5
	HPT_BOVIN	Haptoglobin	45.6	52	3	2	3	2	78.9
14	CLUS_HORSE	Clusterin	52.7	42	18	4	7	3	100
	TSP1_BOVIN	Thrombospondin-1	133.4	29	13	3	9	3	96.8
	SFTPA_HORSE	Pulmonary surfactant-associated protein A	26.5	71	4	3	3	2	100
15	TSP1_HUMAN	Thrombospondin-1	133.3	751	60	49	12	11	98
	ALL1_HORSE	Major allergen Equ c 1	21.9	642	58	33	10	8	100
	TIMP1_HORSE	Metalloproteinase inhibitor 1	23.7	189	15	7	5	3	100
16	ALL1_HORSE	Major allergen Equ c 1	21.9	1620	193	107	17	11	100
	TSP1_HUMAN	Thrombospondin-1	133.3	351	58	26	13	9	98
	APOA1_CANFA	Apolipoprotein A-1	30.2	124	24	11	4	3	80.8
17	HBB_HORSE	Haemoglobin sub. beta	16.1	2153	159	114	14	12	100
	HBA_HORSE	Haemoglobin sub. alpha	15.3	824	87	53	8	7	100
18	PIP_HYLSY	Prolactin-inducible protein homolog	16.9	122	9	8	1	1	64.3
19	THIO_HORSE	Thioredoxin	12.0	80	12	2	4	1	100

20	PRG4_HUMAN	Proteoglycan 4	152.2	130	18	8	3	2	88.6
	ALBU_EQUAS	Serum albumin	70.5	75	18	10	4	3	98.5

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

[b] Protein entry name from UniProt knowledge database.

[c] Theoretical protein molecular weight.

[d] The highest scores obtained with Mascot search engine.

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

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

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

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

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

Table 3 4. Biological and functional classification of the proteins identified in equine amniotic fluid. Identified proteins are listed according to the Biological Process category.

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

[a] Protein entry name from UniProt knowledge database.

[b] Theoretical protein molecular weight.

[c] Biological Process according to Gene Ontology and Human Protein Reference Database.

[d] Molecular Function according to Gene Ontology and Human Protein Reference Database.

[e] Cellular Component according to Gene Ontology and Human Protein Reference Database.

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 ± 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 µg 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.

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Figure 1

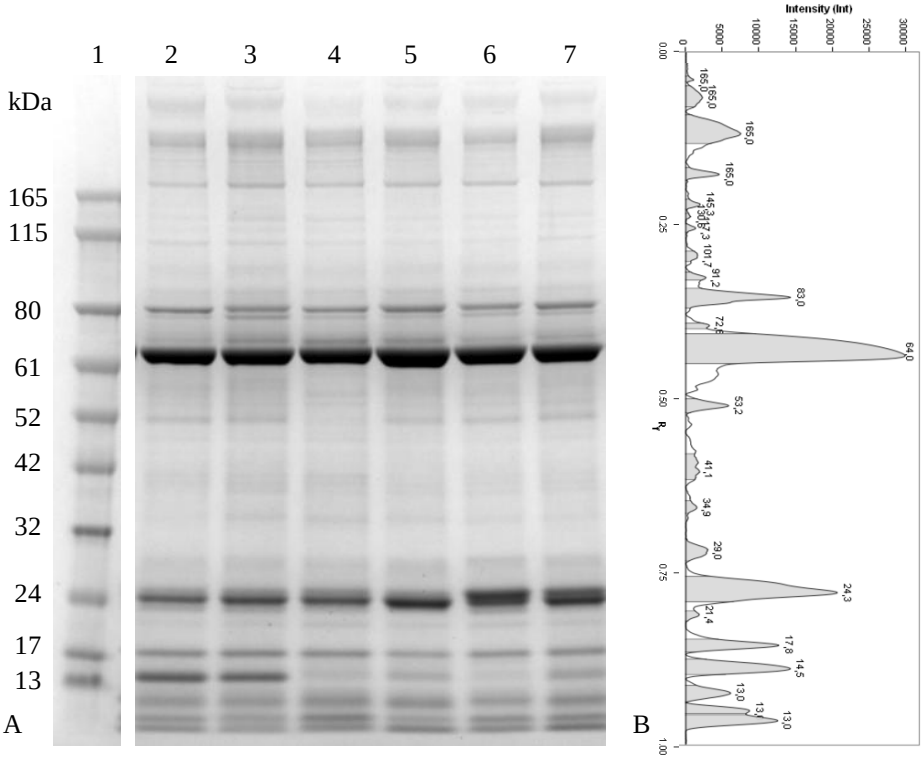
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Figure 2

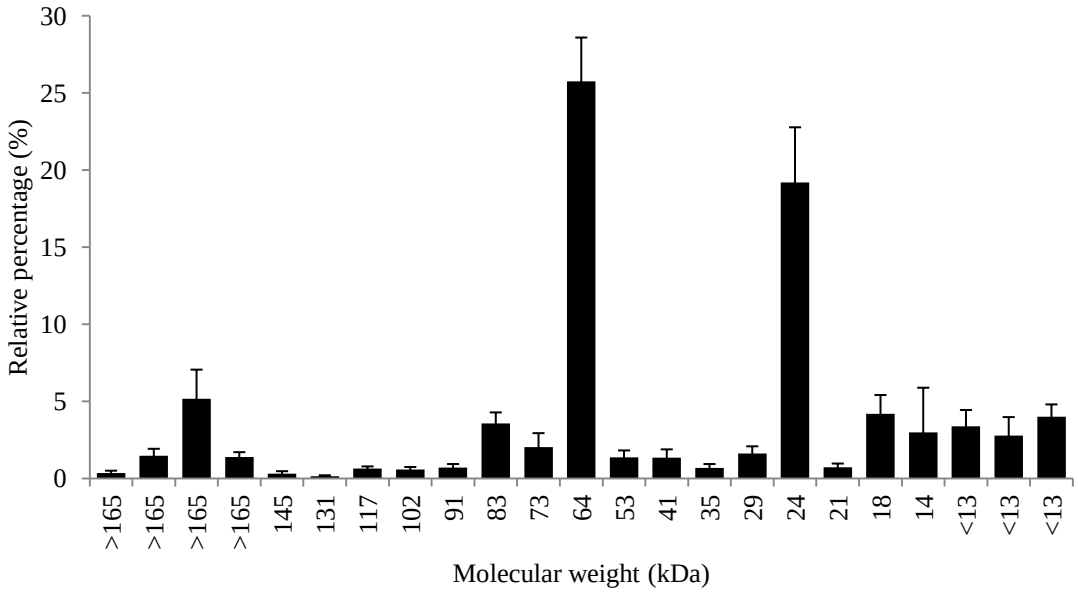
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Figure 3

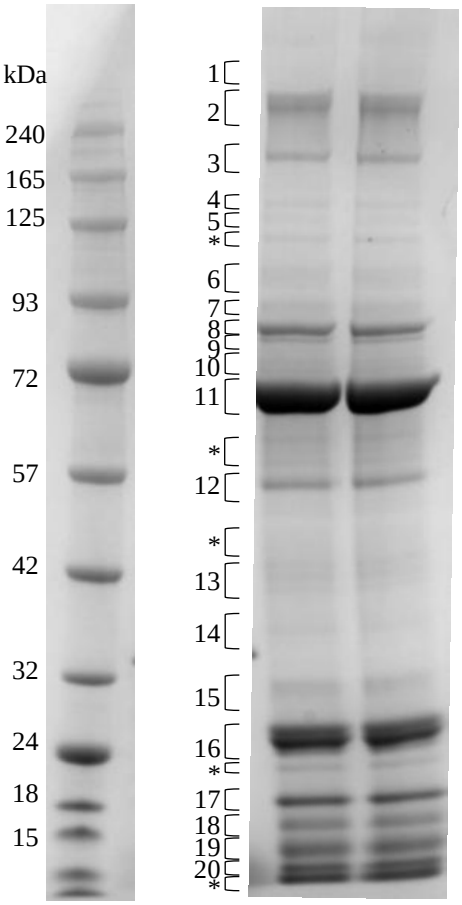
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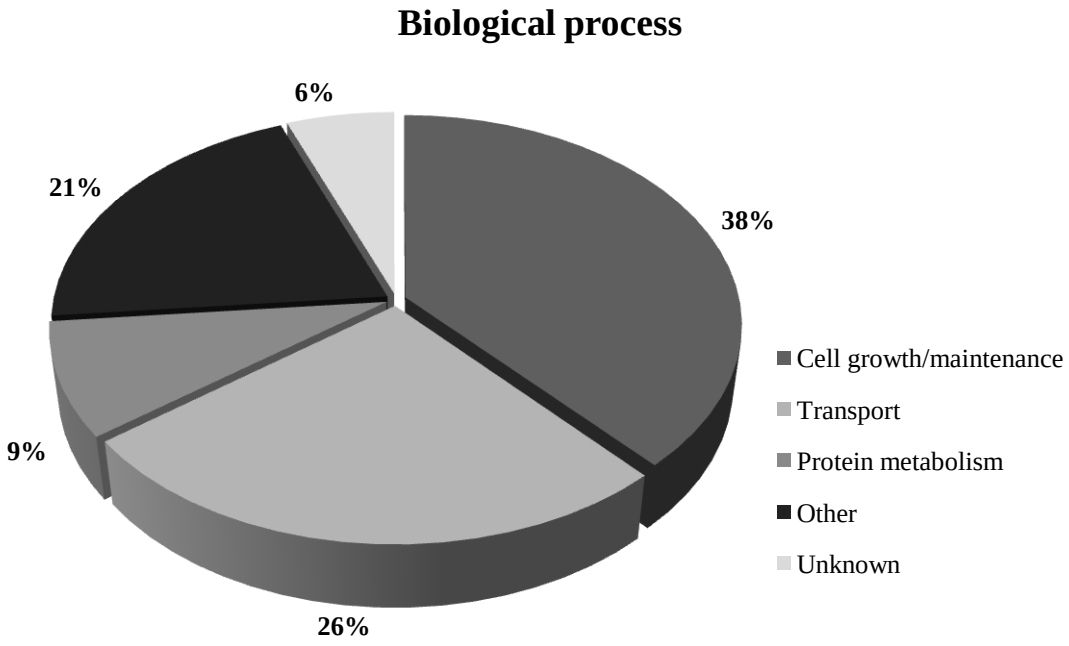
Figure 4


Biological process
6%
21%
38%
9%
26%
Cell growth/maintenance
Transport
Protein metabolism
Other
Unknown

Conflict of interest: none

The authors have no conflict of interests to declare.