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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 \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.

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

3

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19

20

21 **Abstract**

22 Characterisation of the physiologic equine amniotic fluid (AF) proteome is a prerequisite to
23 study its changes during diseases and discover new biomarkers. The aim of this study was to
24 identify by a proteomic approach the most abundant proteins of equine AF. AF samples were
25 collected at parturition from 24 healthy mares that delivered healthy foals. All samples were
26 subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-
27 12% gels. A pool of the 24 samples, after SDS-PAGE, was cut in 25 slices, trypsin-digested
28 and analysed by mass spectrometry (MS) for protein identification. Mean AF protein
29 concentration was 1.96 ± 1.12 g/L. Thirty-four proteins were successfully identified by MS and
30 subsequently categorised according to Gene Ontology (GO). Twelve proteins (e.g.
31 fibronectin, lumican, thrombospondin and fibulin) belonged to or interacted with the
32 extracellular matrix (ECM) playing an important role in the development of foetal tissues.
33 Most of the remaining proteins were classified as transport (e.g. albumin, major allergen Equ
34 c1 and alpha-fetoprotein) delivering nutrients, ions and lipids essential for foetal growth and
35 development. Among these proteins, major allergen Equ c1 is widely studied in human
36 medicine because it induces Ig-E mediated type I allergic reaction. The absence of
37 immunoglobulins in equine AF was also confirmed.

38

39 **Keywords**

40 Horse, pregnancy, electrophoresis, proteome.

41

42

43

44 **Introduction**

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

57 Unlike the allantoic fluid, equine AF can be easily collected during parturition without
58 stressing the animal and avoiding any contamination (Castagnetti et al. 2007, Pirrone et al.
59 2012). As reported in women, the biochemical composition of AF, including proteins, is
60 primarily representative of the foetal profile and reflects its physiological status (Tong 2013),
61 thus it could be potentially useful to evaluate the high-risk foal born attended.

62 Proteomics is a powerful analytical approach providing a profile of proteins present in
63 a biological sample at a given time. The high potential of this approach has been recently
64 found to have a major role in different areas of veterinary medicine, from farm (Almeida et al.
65 2015) to companion (Ferlizza et al. 2015; Miller et al. 2014) animals. Proteomic techniques
66 have recently been applied to the characterisation of horse amniotic membrane (Galera et al.
67 2015) and bovine conceptus fluids (Riding et al. 2008), whereas the equine AF proteome
68 remains uncharacterised. Therefore, the aims of this study were to identify the most abundant

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

71

72 **Materials and Methods**

73 *Animal selection and data collection*

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

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

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

100

101 *Sample collection*

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

107

108 *SDS-PAGE*

109 To optimise protein separation, different protocols were tested including 4-12% and
110 12% polyacrylamide gels (NuPage/Thermo Fisher Scientific, Waltham, Massachusetts, USA)
111 in 2-(N-morpholino) propanesulfonic acid buffer (MOPS; NuPage/Thermo Fisher Scientific)
112 or 2-(N-morpholino) ethanesulfonic acid buffer (MES; NuPage/Thermo Fisher Scientific)
113 with sodium dodecyl sulphate (SDS). Each AF sample (n=24) was analysed at least twice
114 with the protocol assuring the best protein separation in our experimental conditions (4-12%
115 gels, in MOPS buffer). Twenty µg of proteins were loaded for each sample and the gels were
116 stained with Coomassie G250 compatible with mass spectrometry analysis. After staining,
117 each gel was digitalised by ChemiDocTMMP (BioRad, Hercules, California, USA) and its
118 pherogram was obtained using ImageLab 5.2.1 software (BioRad). The software determines
119 the volume of each protein band through the analysis of the pixel values in the digital image,

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

124

125 *Protein identification by mass spectrometry*

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

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

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

147

148 *Statistical analysis*

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

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

159

160 **Results**

161 *Clinical data*

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

166

167 *SDS-PAGE and protein identification by mass spectrometry*

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

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

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

183

184 **Discussion**

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

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

197 membrane (Galera et al. 2015). Among the ECM structural proteins, fibronectin is a
198 multifunctional glycoprotein known to participate in the organisation of ECM binding to
199 integrins. During pregnancy, fibronectin is expressed in the junction between maternal and
200 foetal membranes as well as in the uterus and placenta (Mogami et al. 2013). Lumican, a
201 member of the family of small leucine-rich proteoglycans, is the major keratan sulphate
202 proteoglycan of the cornea and is also present in the ECM throughout the body, including
203 human and equine amniotic membrane (Galera et al. 2015; Kao et al. 2006; Mavrou et al.
204 2008). Based on its interaction with fibrillar collagen and its ability to modulate cell
205 proliferation and migration, lumican could play a role in the maturation of foetal tissues
206 (Mavrou et al. 2008). Regarding the non-structural proteins involved in ECM development
207 and organisation, thrombospondin and fibulin are regulatory proteins belonging to the group
208 of the matricellular proteins. These proteins represent a bridge between matrix proteins and
209 cell surface receptors, or other molecules such as cytokines that can interact with the cell
210 surface (Bornstein, 1995). They are typically expressed at low levels in adult tissues, but are
211 strongly expressed during development or following injury or pathology (Morris and
212 Kyriakides, 2014). Gelsolin is a multifunctional actin regulatory protein involved in
213 cytoskeleton dynamics and structure. In addition to its role in aiding chemotaxis and
214 movement of intracellular structures, plasma gelsolin binds to a variety of proinflammatory
215 and bioactive molecules including fibronectin, platelet activating factor and the bacterial
216 surface lipids lipoteichoic acid and lipopolysaccharide (Peddada et al. 2012). The role of
217 gelsolin in AF is still unknown, but it has been suggested to modulate inflammation and
218 bacterial infections in human AF (Sezen et al. 2009). In association with gelsolin, vinculin is
219 also a component of the actine cytoskeleton and is involved in integrin-mediated focal
220 adhesion, cell motility and other cellular functions such as migration, proliferation and
221 differentiation (Wu et al. 2014). Other proteins interacting with ECM that could play
222 important roles in the development of foetal tissue are type IV collagenase (MMP2) and

223 metalloproteinase inhibitor 1 (TIMP1) belonging to the matrix metalloproteinases (MMPs)
224 and tissue inhibitors respectively. The MMPs are a family of over 20 enzymes acting on the
225 ECM components, regulated at different levels via their activators, inhibitors and localization
226 on the cell surface (Sternlicht and Werb, 2001). The biological functions of these enzymes
227 and their inhibitors have been widely studied, in particular MMP2 is important for bone
228 development and angiogenesis regulation and has been identified and studied in plasma and
229 AF of pregnant women (Anumba et al. 2010; Turner et al. 2014). MMPs activity was also
230 studied in amniotic and allantoic fluid from mares that delivered live term foals and from
231 mares with preterm delivery, suggesting that MMPs may have a role as markers for high risk
232 pregnancy in the mare (Oddsdóttir et al. 2011).

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

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

260 The proteomic approach applied in the present study led to the successful
261 identification of the most abundant proteins, even though a few additional points should be
262 taken into consideration. The first one regards the choice of non-depleting albumin and other
263 major proteins before electrophoresis and MS identification. Complex biological samples
264 contain thousands of different protein species, few of them characterised by high abundance
265 and many others by low or very low abundance. The presence of very high abundance
266 proteins like albumin and immunoglobulins often hampers the separation and characterisation
267 of serum and AF proteomes, therefore the depletion of these major components has been
268 applied in human proteomics (Cho et al. 2007; Michaels et al. 2007). However, this approach
269 can lead to the loss of some low abundance proteins due to the “sponge effect” of albumin
270 that can bind a variety of other proteins or peptides (Bellei et al. 2011). From this point of
271 view, equine AF is a preferential sample due to the absence of immunoglobulins. In domestic
272 animals, the passage of immunoglobulins is influenced by the placental structure: in horses,
273 pigs and ruminants the placenta is epitheliochorial, thus impermeable to immunoglobulins
274 (Furukawa et al. 2014), whereas in dogs, the endotheliochorial placenta allows only 5% to

275 10% transfer of maternal antibodies to the foetus (Dall'Ara et al. 2015). From an analytical
276 point of view, the absence of immunoglobulins in equine AF, as confirmed by this study, can
277 be considered an advantage, allowing to perform SDS-PAGE and MS identification without
278 affecting proteome integrity and complexity.

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

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

295

296 **Conclusions**

297 Applying a qualitative proteomic approach, this study identified the 34 most abundant
298 proteins of the AF proteome from healthy mares that delivered live term foals. GO
299 categorisation demonstrated that these proteins are involved in different biological processes
300 and molecular functions including cell growth/maintenance and transport. Some of these

301 proteins belonged to or interacted with the extracellular matrix, highlighting the role of its
302 components in foetal maturation. The study confirmed also the importance of transport
303 proteins like alpha-fetoprotein and PLTP, and reported for the first time the presence of ALL1
304 in AF. Though entirely descriptive, these findings can be considered valuable context for
305 further investigations to gain insights into the function of the proteins identified and to
306 discover potential biomarkers of foetal disease at birth or during pregnancy.

307

308 **Acknowledgements**

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

311

312 **Conflict of interest**

313 The authors have no conflict of interests to declare.

314

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
		\pm 9.5	\pm 74.1	\pm 11.4	\pm 3	\pm 10.7	\pm 5.7	\pm 1	\pm 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 ^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
7	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
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
12	BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3	75.3	53	12	6	4	3	92.9
	A1AT2_HORSE	Alpha-1-antitrypsin 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
14	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
	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
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
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
17	APOA1_CANFA	Apolipoprotein A-1	30.2	124	24	11	4	3	80.8
	HBB_HORSE	Haemoglobin sub. beta	16.1	2153	159	114	14	12	100
17	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

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

322 ^b Protein entry name from UniProt knowledge database.

323 ^c Theoretical protein molecular weight.

324 ^d The highest scores obtained with Mascot search engine.

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

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

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

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

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

332 **Table 3.** Biological and functional classification of the proteins identified in equine amniotic
 333 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-antitrypsin	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

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 \pm 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 μ g 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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364 **References**

- 365 Albers, J.J., Vuletic, S., Cheung, M.C., 2012. Role of plasma phospholipid transfer protein in
366 lipid and lipoprotein metabolism. *Biochim. Biophys. Acta* 1821, 345–357.
- 367 Almeida, A.M., Bassols, A., Bendixen, E., Bhide, M., Ceciliani, F., Cristobal, S., Eckersall,
368 P.D., Hollung, K., Lisacek, F., Mazzucchelli, G., McLaughlin, M., Miller, I., Nally, J.E.,
369 Plowman, J., Renaut, J., Rodrigues, P., Roncada, P., Staric, J., Turk, R., 2015. Animal
370 board invited review: advances in proteomics for animal and food sciences. *Animal* 9, 1–
371 17.
- 372 Anumba, D.O.C., Gelany, S.E., Elliott, S.L., Li, T.C., 2010. Circulating levels of matrix
373 proteases and their inhibitors in pregnant women with and without a history of recurrent
374 pregnancy loss. *Reprod. Biol. Endocrinol.* 8, 62. doi:10.1186/1477-7827-8-62
- 375 Bellei, E., Bergamini, S., Monari, E., Fantoni, L.I., Cuoghi, A., Ozben, T., Tomasi, A., 2011.
376 High-abundance proteins depletion for serum proteomic analysis: concomitant removal
377 of non-targeted proteins. *Amino Acids* 40, 145–156.
- 378 Bellei, E., Monari, E., Cuoghi, A., Bergamini, S., Guerzoni, S., Ciccarese, M., Ozben, T.,
379 Tomasi, A., Pini, L.A., 2013. Discovery by a proteomic approach of possible early
380 biomarkers of drug-induced nephrotoxicity in medication-overuse headache. *J. Headache
381 Pain* 14, 6. doi:10.1186/1129-2377-14-6
- 382 Bertoldi, C., Bellei, E., Pellacani, C., Ferrari, D., Lucchi, A., Cuoghi, A., Bergamini, S.,
383 Cortellini, P., Tomasi, A., Zaffe, D., Monari, E., 2013. Non-bacterial protein expression
384 in periodontal pockets by proteome analysis. *J. Clin. Periodontol.* 40, 573–82.
- 385 Bornstein, P., 1995. Diversity of function is inherent in matricellular proteins: an appraisal of
386 thrombospondin 1. *J. Cell Biol.* 130, 503–506.
- 387 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., Smitz, 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

484 stem cells during the embryonic development of amniotes. *J. Chin. Med. Assoc.* 76, 606-
485 610.

486 Turner, S.W., Carter, J., Danielian, P., Chalmers, I., McConaghy, L., Pacitti, N., Booth, N.,
487 2014. Protease concentration in amniotic fluid at term and early childhood respiratory
488 symptoms. *J. Matern. Fetal. Neonatal Med.* 27, 416–420.

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

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

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

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

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

505

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

3

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20

21 **Abstract**

22 Characterisation of the physiologic equine amniotic fluid (AF) proteome is a prerequisite to
23 study its changes during diseases and discover new biomarkers. The aim of this study was to
24 identify by a proteomic approach the most abundant proteins of equine AF. AF samples were
25 collected at parturition from 24 healthy mares that delivered healthy foals. All samples were
26 subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-
27 12% gels. A pool of the 24 samples, after SDS-PAGE, was cut in 25 slices, trypsin-digested
28 and analysed by mass spectrometry (MS) for protein identification. Mean AF protein
29 concentration was 1.96 ± 1.12 g/L. Thirty-four proteins were successfully identified by MS and
30 subsequently categorised according to Gene Ontology (GO). Twelve proteins (e.g.
31 fibronectin, lumican, thrombospondin and fibulin) belonged to or interacted with the
32 extracellular matrix (ECM) playing an important role in the development of foetal tissues.
33 Most of the remaining proteins were classified as transport (e.g. albumin, major allergen Equ
34 c1 and alpha-fetoprotein) delivering nutrients, ions and lipids essential for foetal growth and
35 development. Among these proteins, major allergen Equ c1 is widely studied in human
36 medicine because it induces Ig-E mediated type I allergic reaction. The absence of
37 immunoglobulins in equine AF was also confirmed. The present study successfully applied
38 SDS-PAGE coupled to MS identifying the most abundant proteins of equine AF, highlighting
39 the importance of ECM and transport proteins.

40

41 **Keywords**

42 Horse, pregnancy, electrophoresis, proteome.

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45

46 **Introduction**

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

59 **Unlike the allantoic fluid, equine AF can be easily collected during parturition without**
60 **stressing the animal and avoiding any contamination (Castagnetti et al. 2007, Pirrone et al.**
61 **2012). As reported in women, the biochemical composition of AF, including proteins, is**
62 **primarily representative of the foetal profile and reflects its physiological status (Tong 2013),**
63 **thus it could be potentially useful to evaluate the high-risk foal born attended.**

64 Proteomics is a powerful analytical approach providing a profile of proteins present in
65 a biological sample at a given time. The high potential of this approach has been recently
66 found to have a major role in different areas of veterinary medicine, from farm (Almeida et al.
67 2015) to companion (Ferlizza et al. 2015; Miller et al. 2014) animals. Proteomic techniques
68 have recently been applied to the characterisation of horse amniotic membrane (Galera et al.
69 2015) and bovine conceptus fluids (Riding et al. 2008), whereas the equine AF proteome
70 remains uncharacterised. Therefore, the aims of this study were to identify the most abundant

71 proteins in equine AF by SDS-PAGE separation followed by mass spectrometry identification
72 and discuss their functions.

73

74 **Materials and Methods**

75 *Animal selection and data collection*

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

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

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

102

103 *Sample collection*

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

109

110 *SDS-PAGE*

111 To optimise protein separation, different protocols were tested including 4-12% and
112 12% polyacrylamide gels (NuPage/Thermo Fisher Scientific, Waltham, Massachusetts, USA)
113 in 2-(N-morpholino) propanesulfonic acid buffer (MOPS; NuPage/Thermo Fisher Scientific)
114 or 2-(N-morpholino) ethanesulfonic acid buffer (MES; NuPage/Thermo Fisher Scientific)
115 with sodium dodecyl sulphate (SDS). Each AF sample (n=24) was analysed at least twice
116 with the protocol assuring the best protein separation in our experimental conditions (4-12%
117 gels, in MOPS buffer). Twenty µg of proteins were loaded for each sample and the gels were
118 stained with Coomassie G250 compatible with mass spectrometry analysis. After staining,
119 each gel was digitalised by ChemiDocTMMP (BioRad, Hercules, California, USA) and its
120 pherogram was obtained using ImageLab 5.2.1 software (BioRad). The software determines
121 the volume of each protein band through the analysis of the pixel values in the digital image,

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

126

127 *Protein identification by mass spectrometry*

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

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

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

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

185

186 Discussion

187 The present paper aimed to explore the complexity of equine AF proteome and to
188 identify its most abundant proteins. Since this The study was carried out on 24 mares of
189 different breeds, age and parity referred to the Equine Perinatology Unit, the animals were of
190 different breed, age and parity, and they can be considered representative of a typical equine
191 hospital population reflecting the local equine population. Therefore, the proteomic profile
192 described can be considered a useful starting point for further applied studies on the equine
193 AF proteins.

194 This study collected AF at parturition because mares were client owned and
195 transabdominal amniocentesis is still not recommended for clinical use. Recently, Canisso et
196 al. (2014) described a safe technique to perform multiple ultrasound guided foetal fluid
197 samplings during the last trimester of gestation in mares. On this basis, abdominal
198 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

225 of foetal tissues. All these proteins, with the exception of versican and proteoglycan 4, were
226 also identified in human AF (Cho et al. 2007; Michaels et al. 2007) and/or in equine amniotic
227 membrane (Galera et al. 2015). Among the ECM structural proteins, fibronectin is a
228 multifunctional glycoprotein known to participate in the organisation of ECM binding to
229 integrins. During pregnancy, fibronectin is expressed in the junction between maternal and
230 foetal membranes as well as in the uterus and placenta (Mogami et al. 2013). Lumican, a
231 member of the family of small leucine-rich proteoglycans, is the major keratan sulphate
232 proteoglycan of the cornea and is also present in the ECM throughout the body, including
233 human and equine amniotic membrane (Galera et al. 2015; Kao et al. 2006; Mavrou et al.
234 2008). Based on its interaction with fibrillar collagen and its ability to modulate cell
235 proliferation and migration, lumican could play a role in the maturation of foetal tissues
236 (Mavrou et al. 2008). Regarding the non-structural proteins involved in ECM development
237 and organisation, thrombospondin and fibulin are regulatory proteins belonging to the group
238 of the matricellular proteins. These proteins represent a bridge between matrix proteins and
239 cell surface receptors, or other molecules such as cytokines that can interact with the cell
240 surface (Bornstein, 1995). They are typically expressed at low levels in adult tissues, but are
241 strongly expressed during development or following injury or pathology (Morris and
242 Kyriakides, 2014). Gelsolin is a multifunctional actin regulatory protein involved in
243 cytoskeleton dynamics and structure. In addition to its role in aiding chemotaxis and
244 movement of intracellular structures, plasma gelsolin binds to a variety of proinflammatory
245 and bioactive molecules including fibronectin, platelet activating factor and the bacterial
246 surface lipids lipoteichoic acid and lipopolysaccharide (Peddada et al. 2012). The role of
247 gelsolin in AF is still unknown, but it has been suggested to modulate inflammation and
248 bacterial infections in human AF (Sezen et al. 2009). In association with gelsolin, vinculin is
249 also a component of the actine cytoskeleton and is involved in integrin-mediated focal
250 adhesion, cell motility and other cellular functions such as migration, proliferation and

251 differentiation (Wu et al. 2014). Other proteins interacting with ECM that could play
252 important roles in the development of foetal tissue are type IV collagenase (MMP2) and
253 metalloproteinase inhibitor 1 (TIMP1) belonging to the matrix metalloproteinases (MMPs)
254 and tissue inhibitors respectively. The MMPs are a family of over 20 enzymes acting on the
255 ECM components, regulated at different levels via their activators, inhibitors and localization
256 on the cell surface (Sternlicht and Werb, 2001). The biological functions of these enzymes
257 and their inhibitors have been widely studied, in particular MMP2 is important for bone
258 development and angiogenesis regulation and has been identified and studied in plasma and
259 AF of pregnant women (Anumba et al. 2010; Turner et al. 2014). **MMPs activity was also
260 studied in amniotic and allantoic fluid from mares that delivered live term foals and from
261 mares with preterm delivery, suggesting that MMPs may have a role as markers for high risk
262 pregnancy in the mare (Oddsdóttir et al. 2011).**

263 Among transport proteins, albumin, transferrin, alpha-fetoprotein, apolipoprotein A1
264 and phospholipid transfer protein (PLTP) transport nutrients, ions and lipids essential for
265 foetal growth and development and have been identified as common components of AF also
266 in humans (Cho et al. 2007; Michaels et al. 2007). Alpha-fetoprotein is member of the
267 albuminoid superfamily, and is present in the allantoic fluid and AF amniotic fluids of
268 domestic animals (Luft et al. 1984; Smith et al. 1979). **In mammalian foetuses alpha-
269 fetoprotein is associated with oestrogen-binding, anti-oxidative properties and
270 immunoregulation (DeMees et al. 2006; Mizejewski 2001) and in horses, alpha-fetoprotein it
271 is highly expressed during early pregnancy by the equine conceptus (Simpson et al. 2000) and
272 in mammalian foetuses, it is associated with estrogen binding, anti-oxidative properties and
273 immunoregulation (DeMees et al. 2006; Mizejewski 2001).** In women, AF alpha-fetoprotein
274 is actively investigated for pathologies such as Down syndrome, trisomies 13 and 18, intra-
275 amniotic infection, preterm delivery, pre-eclampsia, membrane rupture, and foetoplacental
276 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

329 physiological factors can contribute to this variability; in particular, since the mares were
330 client-owned, pre-hospitalisation conditions, such as housing, feeding, nutrition and hydration
331 status, might have affected AF total protein concentration.

332

333 **Conclusions**

334 Applying a qualitative proteomic approach, this study identified the 34 most abundant
335 proteins of the AF proteome from healthy mares that delivered live term foals. ~~The present~~
336 ~~applied a qualitative proteomic approach to characterise the AF proteome from healthy~~
337 ~~mares. The 34 proteins identified~~ GO categorisation demonstrated that these proteins are
338 involved in different biological processes and molecular functions including cell
339 growth/maintenance and transport. Some of these proteins belonged to or interacted with the
340 extracellular matrix, highlighting the role of its ~~the ECM~~ components in foetal maturation.
341 The study confirmed also the importance of transport proteins like alpha-fetoprotein and
342 PLTP, and reported for the first time the presence of ALL1 in AF. ~~Further studies are needed~~
343 ~~to define reference intervals for AF total protein in healthy mares and~~ Though entirely
344 descriptive, these findings can be considered valuable context for further investigations to
345 gain insights into the function of the proteins identified and to discover potential biomarkers
346 of foetal disease at birth or during pregnancy. ~~In particular, 2DE could be applied to better~~
347 ~~characterise the physiologic AF proteome and to evaluate differentially expressed proteins in~~
348 ~~ease of disease.~~

349

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353

354 **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

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

363

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

364

365 |

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
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-antitrypsin	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
14	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
15	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
16	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
17	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
18	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
19	PIP_HYLSY	Prolactin-inducible protein homolog	16.9	122	9	8	1	1	64.3
	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

- 368 ^a Number of the identified band as marked in Figure 3.
- 369 ^b Protein entry name from UniProt knowledge database.
- 370 ^c Theoretical protein molecular weight.
- 371 ^d The highest scores obtained with Mascot search engine.
- 372 ^e Peptides: total number of peptides matching the identified proteins.
- 373 ^f Significant peptides: total number of significant peptides matching the identified proteins.
- 374 ^g Sequence: total number of distinct sequences matching the identified proteins.
- 375 ^h Significant sequences: total number of significant distinct sequences matching the identified
- 376 proteins.
- 377 ⁱ Percentage of identical amino acids between the identified protein and the respective horse
- 378 | 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-antitrypsin	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

389 **Figure Legends**

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

395

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

399

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

405

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

409

410

411

412 **References**

- 413 Albers, J.J., Vuletic, S., Cheung, M.C., 2012. Role of plasma phospholipid transfer protein in
414 lipid and lipoprotein metabolism. *Biochim. Biophys. Acta* 1821, 345–357.
- 415 Almeida, A.M., Bassols, A., Bendixen, E., Bhide, M., Ceciliani, F., Cristobal, S., Eckersall,
416 P.D., Hollung, K., Lisacek, F., Mazzucchelli, G., McLaughlin, M., Miller, I., Nally, J.E.,
417 Plowman, J., Renaut, J., Rodrigues, P., Roncada, P., Staric, J., Turk, R., 2015. Animal
418 board invited review: advances in proteomics for animal and food sciences. *Animal* 9, 1–
419 17.
- 420 Anumba, D.O.C., Gelany, S.E., Elliott, S.L., Li, T.C., 2010. Circulating levels of matrix
421 proteases and their inhibitors in pregnant women with and without a history of recurrent
422 pregnancy loss. *Reprod. Biol. Endocrinol.* 8, 62. doi:10.1186/1477-7827-8-62
- 423 Bellei, E., Bergamini, S., Monari, E., Fantoni, L.I., Cuoghi, A., Ozben, T., Tomasi, A., 2011.
424 High-abundance proteins depletion for serum proteomic analysis: concomitant removal
425 of non-targeted proteins. *Amino Acids* 40, 145–156.
- 426 Bellei, E., Monari, E., Cuoghi, A., Bergamini, S., Guerzoni, S., Ciccarese, M., Ozben, T.,
427 Tomasi, A., Pini, L.A., 2013. Discovery by a proteomic approach of possible early
428 biomarkers of drug-induced nephrotoxicity in medication-overuse headache. *J. Headache
429 Pain* 14, 6. doi:10.1186/1129-2377-14-6
- 430 Bertoldi, C., Bellei, E., Pellacani, C., Ferrari, D., Lucchi, A., Cuoghi, A., Bergamini, S.,
431 Cortellini, P., Tomasi, A., Zaffe, D., Monari, E., 2013. Non-bacterial protein expression
432 in periodontal pockets by proteome analysis. *J. Clin. Periodontol.* 40, 573–82.
- 433 Bornstein, P., 1995. Diversity of function is inherent in matricellular proteins: an appraisal of
434 thrombospondin 1. *J. Cell Biol.* 130, 503–506.
- 435 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., Smitz, 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 metalloproteinases 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

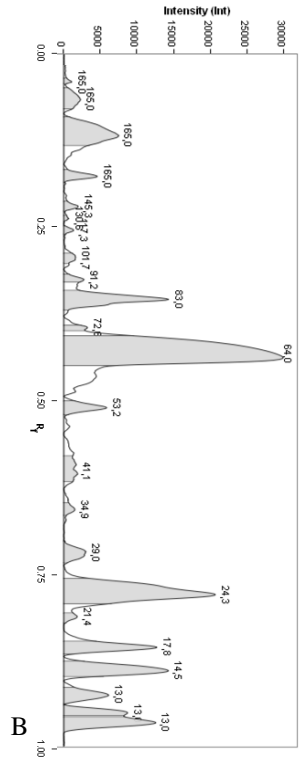
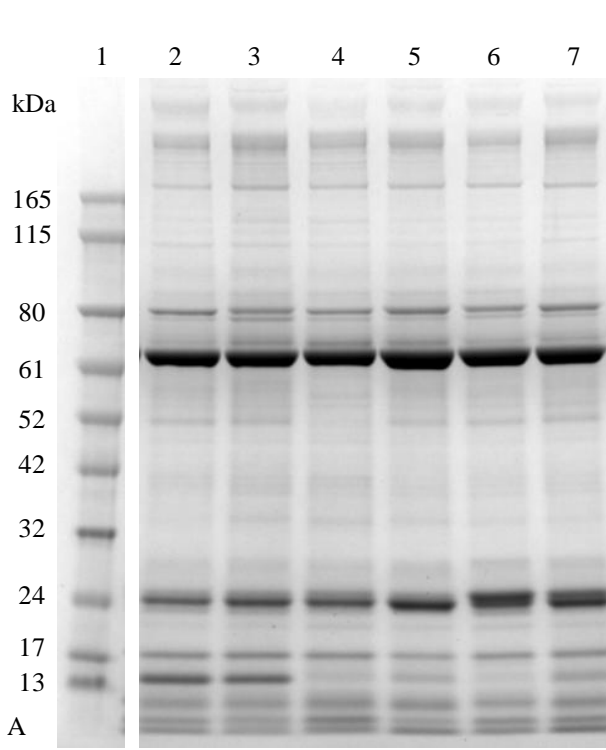


Figure 2

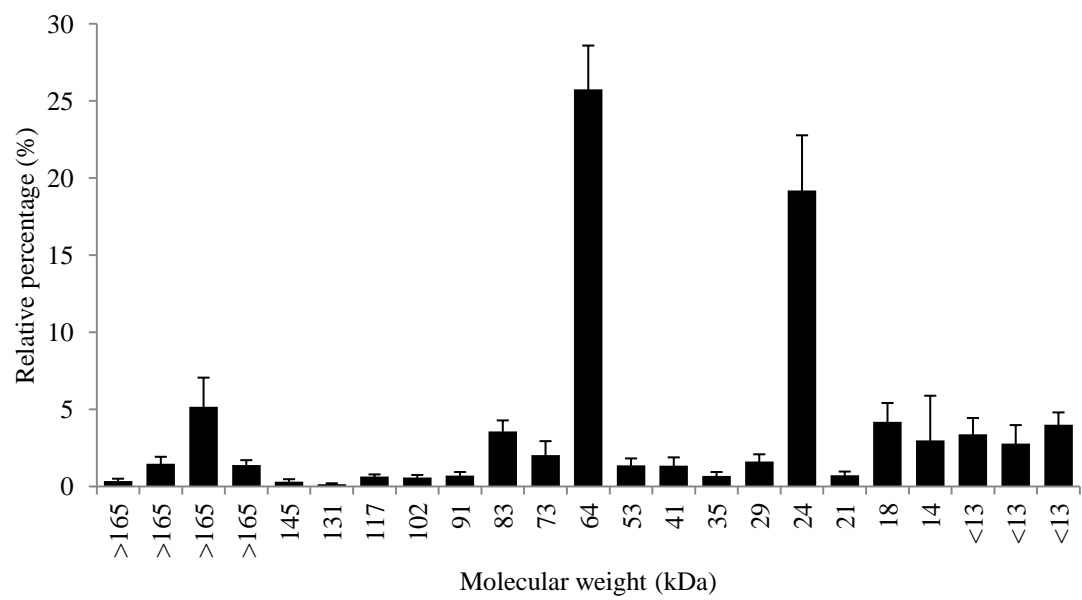


Figure 3

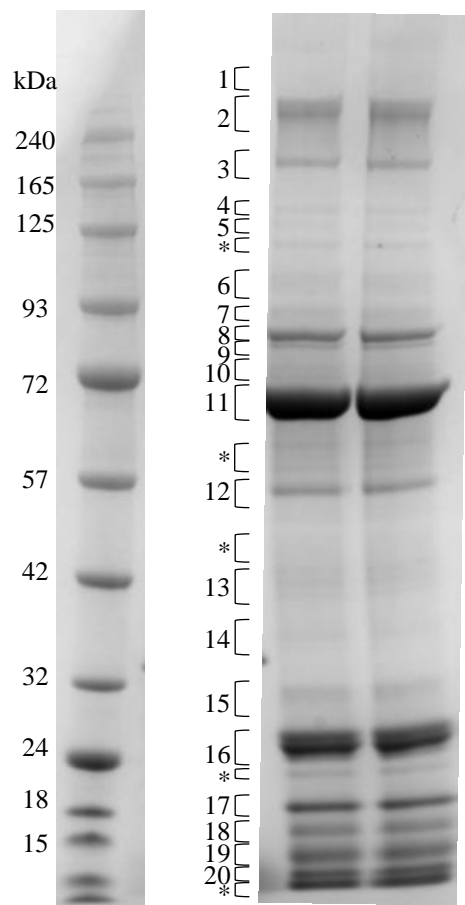
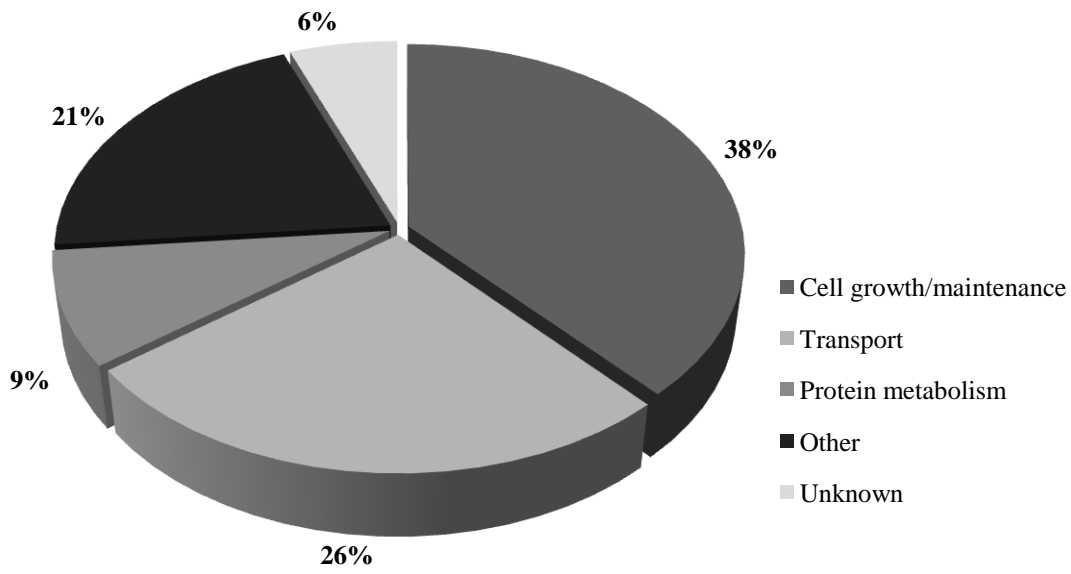


Figure 4

Biological process



Conflict of interest: none

The authors have no conflict of interests to declare.