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Manuscript Draft

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

REVISION NOTE

Animal Reproduction Science
Ms. No. ANIREP-D-16-6583
Identification of the most abundant proteins in equine amniotic fluid by a proteomic approach.

Dear Editor,

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

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

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

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

RESPONSE TO REVIEWER 1

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

The requested data have been added.

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

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

Highlights

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

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

2   **approach**

3

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Abstract

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

Keywords

Horse, pregnancy, electrophoresis, proteome.

Introduction

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

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

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

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

70 identification.

71

Materials and Methods

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 ChemiDoc[TM]MP (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,

5

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 ± 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

7

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**

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 ± standard deviation. AF amniotic fluid; TP total proteins.

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

317

318

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

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

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

15

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

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

[b] Protein entry name from UniProt knowledge database.

[c] Theoretical protein molecular weight.

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

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

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

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

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

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

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

Entry name [a]	Protein full name	MW (kDa) [b]	Biol. Proc. [c]	Mol. Funct. [d]	Cell. Comp. [e]
HPT_BOVIN	Haptoglobin	45.6	Acute-phase response	HB binding	Extracellular
BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3	75.3	Cell communication/ signal transduction	Receptor binding	Extracellular
CO6A1_HUMAN	Collagen alpha-1(VI) chain	109.6	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
CO6A3_HUMAN	Collagen alpha-3(VI) chain	345.2	Cell growth/ maintenance	peptidase inhibitor	Extracellular
CSPG2_BOV	Versican core protein	371.8	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
ECM1_HUMAN	Extracellular matrix protein 1	62.2	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
FBLN1_HUMAN	Fibulin-1	81.3	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
FINC_HORSE	Fibronectin	58.1	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
GELS_HORSE	Gelsolin	81.1	Cell growth/ maintenance	Structural constituent of cytoskeleton	Extracellular
IBP3_BOVIN	Insulin-like growth factor-binding protein	32.6	Cell growth/ maintenance	protein binding	Extracellular
LUM_MOUSE	Lumican	38.6	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
PRG4_HUMAN	Proteoglycan 4	152.2	Cell growth/ maintenance	Binding/Cell adhesion molecule	Extracellular
TIMP1_HORSE	Metalloproteinase inhibitor 1	23.7	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
TSP1_HUMAN	Thrombospondin-1	133.3	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
VINC_HUMAN	Vinculin	124.3	Cell growth/ maintenance	Cytoskeletal protein binding	Cytoplasm
CLUS_HORSE	Clusterin	52.7	Cell morphogenesis/ cell death	protein binding-chaperone	Cytoplasm
THIO_HORSE	Thioredoxin	12.0	Metabolism/ energy pathways	Catalytic activity	Cytoplasm
FETUA_BOVIN	Alpha-2-HS-glycoprotein	39.2	Mineral balance	protein binding	Extracellular
ACTB_BOVIN	Actin cytoplasmic 1	42.1	Protein folding	protein binding	Cytoskeleton
ACE2_PAGLA	Angiotensin-converting enzyme 2	93.0	Protein metabolism	Carboxylpeptidase	Plasma membrane
MMP2_BOVIN	72 kDa type IV collagenase	74.8	Protein metabolism	Metallopeptidase	Extracellular
PLMN_HORSE	Plasminogen	38.1	Protein metabolism	Peptidase	Extracellular
SFTPA_HORSE	Pulmonary surfactant-associated protein A	26.5	Respiratory gaseous exchange	carbohydrate/metal ion binding	Extracellular
ALBU_HORSE	Serum albumin	70.5	Transport	Transporter	Extracellular
ALL1_HORSE	Major allergen Equ c 1	21.9	Transport	Transporter	Extracellular
APOA1_CANFA	Apolipoprotein A-I	30.2	Transport	Binding	Extracellular/ HDL
FETA_HORSE	Alpha-fetoprotein	70.1	Transport	Transporter	Extracellular
HBA_HORSE	Haemoglobin subunit alpha	15.3	Transport	Transporter	Extracellular
HBB_HORSE	Haemoglobin subunit beta	16.1	Transport	Transporter	Extracellular
PLTP_HUMAN	Phospholipid transfer protein	54.9	Transport	Transporter	Extracellular
TRFE_HORSE	Serotransferrin	80.3	Transport	Transporter	Extracellular
TRFL_HORSE	Lactotransferrin	77.9	Transport	Transporter	Secretory

					granule
A1AT2_HORSE	Alpha-1-antiproteinase 2	47.1	Unknown	Protease inhibitor	NA
PIP_HYLSY	Prolactin-inducible protein homolog	16.9	Unknown	Binding	Extracellular

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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.

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Figure Legends

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

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

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

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

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

2   **approach**

3

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20

1

Abstract

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

Keywords

Horse, pregnancy, electrophoresis, proteome.

Introduction

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

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

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

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

72 ~~and discuss their functions~~.

73

Materials and Methods

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 ChemiDoc^{TM}MP (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 ± 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

8

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

9

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

differentiation (Wu et al. 2014). Other proteins interacting with ECM that could play

important roles in the development of foetal tissue are type IV collagenase (MMP2) and

metalloproteinase inhibitor 1 (TIMP1) belonging to the matrix metalloproteinases (MMPs)

and tissue inhibitors respectively. The MMPs are a family of over 20 enzymes acting on the

ECM components, regulated at different levels via their activators, inhibitors and localization

on the cell surface (Sternlicht and Werb, 2001). The biological functions of these enzymes

and their inhibitors have been widely studied, in particular MMP2 is important for bone

development and angiogenesis regulation and has been identified and studied in plasma and

AF of pregnant women (Anumba et al. 2010; Turner et al. 2014). MMPs activity was also

studied in amniotic and allantoic fluid from mares that delivered live term foals and from

mares with preterm delivery, suggesting that MMPs may have a role as markers for high risk

pregnancy in the mare (Oddsdóttir et al. 2011).

Among transport proteins, albumin, transferrin, alpha-fetoprotein, apolipoprotein A1

and phospholipid transfer protein (PLTP) transport nutrients, ions and lipids essential for

foetal growth and development and have been identified as common components of AF also

in humans (Cho et al. 2007; Michaels et al. 2007). Alpha-fetoprotein is member of the

albuminoid superfamily~~,~~ and is present in the allantoic ~~fluid~~ and ~~AF~~ amniotic fluids of

domestic animals (Luft et al. 1984; Smith et al. 1979). In mammalian foetuses alpha-

fetoprotein is associated with oestrogen-binding, anti-oxidative properties and

immunoregulation (DeMees et al. 2006; Mizejewski 2001) and ~~In horses, alpha-fetoprotein~~ it

is highly expressed during early pregnancy by the equine conceptus (Simpson et al. 2000) ~~and~~

~~in mammalian foetuses, it is associated with estrogen-binding, anti-oxidative properties and~~

~~immunoregulation (DeMees et al. 2006; Mizejewski 2001).~~ In women, AF alpha-fetoprotein

is actively investigated for pathologies such as Down syndrome, trisomies 13 and 18, intra-

amniotic infection, preterm delivery, pre-eclampsia, membrane rupture, and foetoplacental

hypoxia (Cho et al. 2007). Recently, Canisso et al. (2015) confirmed the presence of alpha-

11

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

12

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 applyied 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 case of disease.

349

350 **Acknowledgements**

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 ± standard deviation. AF amniotic fluid; TP total proteins.

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

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

a				c	d	e	f	g	h	i
20	PRG4_HUMAN	Proteoglycan 4		152.2	130	18	8	3	2	88.6
	ALBU_EQUAS	Serum albumin		70.5	75	18	10	4	3	98.5

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

[b] Protein entry name from UniProt knowledge database.

[c] Theoretical protein molecular weight.

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

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

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

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

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

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

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

380   amniotic fluid. Identified proteins are listed according to the Biological Process category.

Entry name [a]	Protein full name	MW (kDa) [b]	Biol. Proc. [c]	Mol. Funct. [d]	Cell. Comp. [e]
HPT_BOVIN	Haptoglobin	45.6	Acute-phase response	HB binding	Extracellular
BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3	75.3	Cell communication/ signal transduction	Receptor binding	Extracellular
CO6A1_HUMAN	Collagen alpha-1(VI) chain	109.6	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
CO6A3_HUMAN	Collagen alpha-3(VI) chain	345.2	Cell growth/ maintenance	peptidase inhibitor	Extracellular
CSPG2_BOV	Versican core protein	371.8	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
ECM1_HUMAN	Extracellular matrix protein 1	62.2	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
FBLN1_HUMAN	Fibulin-1	81.3	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
FINC_HORSE	Fibronectin	58.1	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
GELS_HORSE	Gelsolin	81.1	Cell growth/ maintenance	Structural constituent of cytoskeleton	Extracellular
IBP3_BOVIN	Insulin-like growth factor-binding protein	32.6	Cell growth/ maintenance	protein binding	Extracellular
LUM_MOUSE	Lumican	38.6	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
PRG4_HUMAN	Proteoglycan 4	152.2	Cell growth/ maintenance	Binding/Cell adhesion molecule	Extracellular
TIMP1_HORSE	Metalloproteinase inhibitor 1	23.7	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
TSP1_HUMAN	Thrombospondin-1	133.3	Cell growth/ maintenance	Extracellular matrix structural constituent	Extracellular
VINC_HUMAN	Vinculin	124.3	Cell growth/ maintenance	Cytoskeletal protein binding	Cytoplasm
CLUS_HORSE	Clusterin	52.7	Cell morphogenesis/ cell death	protein binding-chaperone	Cytoplasm
THIO_HORSE	Thioredoxin	12.0	Metabolism/ energy pathways	Catalytic activity	Cytoplasm
FETUA_BOVIN	Alpha-2-HS-glycoprotein	39.2	Mineral balance	protein binding	Extracellular
ACTB_BOVIN	Actin cytoplasmic 1	42.1	Protein folding	protein binding	Cytoskeleton
ACE2_PAGLA	Angiotensin-converting enzyme 2	93.0	Protein metabolism	Carboxylpeptidase	Plasma membrane
MMP2_BOVIN	72 kDa type IV collagenase	74.8	Protein metabolism	Metallopeptidase	Extracellular
PLMN_HORSE	Plasminogen	38.1	Protein metabolism	Peptidase	Extracellular
SFTPA_HORSE	Pulmonary surfactant-associated protein A	26.5	Respiratory gaseous exchange	carbohydrate/metal ion binding	Extracellular
ALBU_HORSE	Serum albumin	70.5	Transport	Transporter	Extracellular
ALL1_HORSE	Major allergen Equ c 1	21.9	Transport	Transporter	Extracellular
APOA1_CANFA	Apolipoprotein A-I	30.2	Transport	Binding	Extracellular/ HDL
FETA_HORSE	Alpha-fetoprotein	70.1	Transport	Transporter	Extracellular
HBA_HORSE	Haemoglobin subunit alpha	15.3	Transport	Transporter	Extracellular
HBB_HORSE	Haemoglobin subunit beta	16.1	Transport	Transporter	Extracellular
PLTP_HUMAN	Phospholipid transfer protein	54.9	Transport	Transporter	Extracellular
TRFE_HORSE	Serotransferrin	80.3	Transport	Transporter	Extracellular
TRFL_HORSE	Lactotransferrin	77.9	Transport	Transporter	Secretory

20

					granule
A1AT2_HORSE	Alpha-1-antiproteinase 2	47.1	Unknown	Protease inhibitor	NA
PIP_HYLSY	Prolactin-inducible protein homolog	16.9	Unknown	Binding	Extracellular

381

382   [a] Protein entry name from UniProt knowledge database.

383   [b] Theoretical protein molecular weight.

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

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

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

387

388

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 ± 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 μg 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

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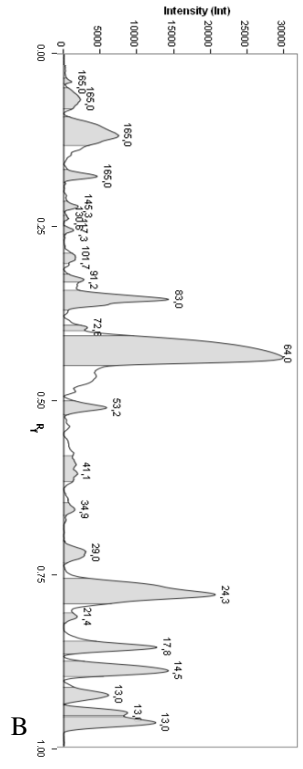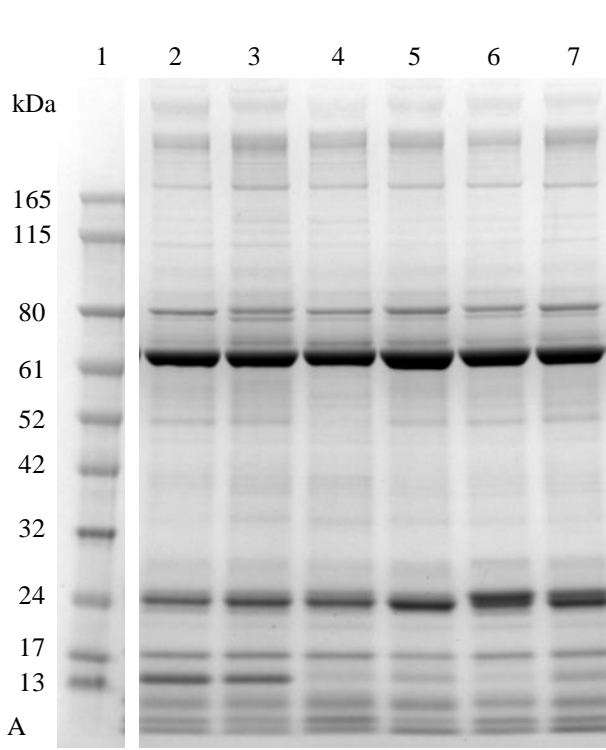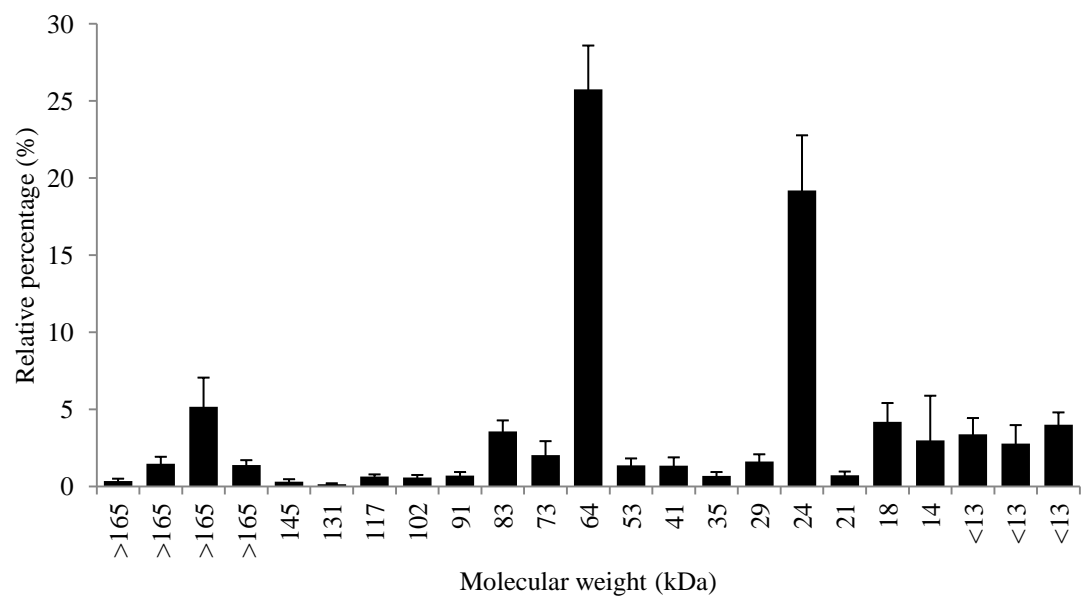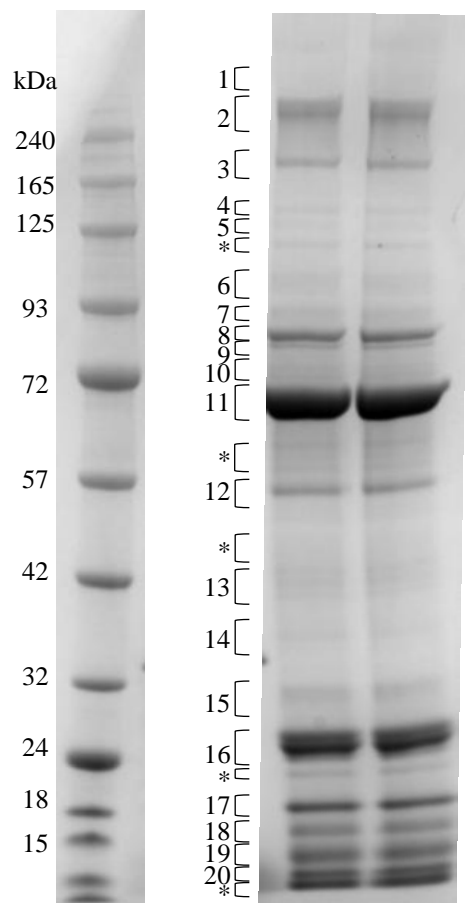
553

Figure 1

Figure 2

Figure 3

Figure 4

Biological process

- Cell growth/maintenance
- Transport
- Protein metabolism
- Other
- Unknown
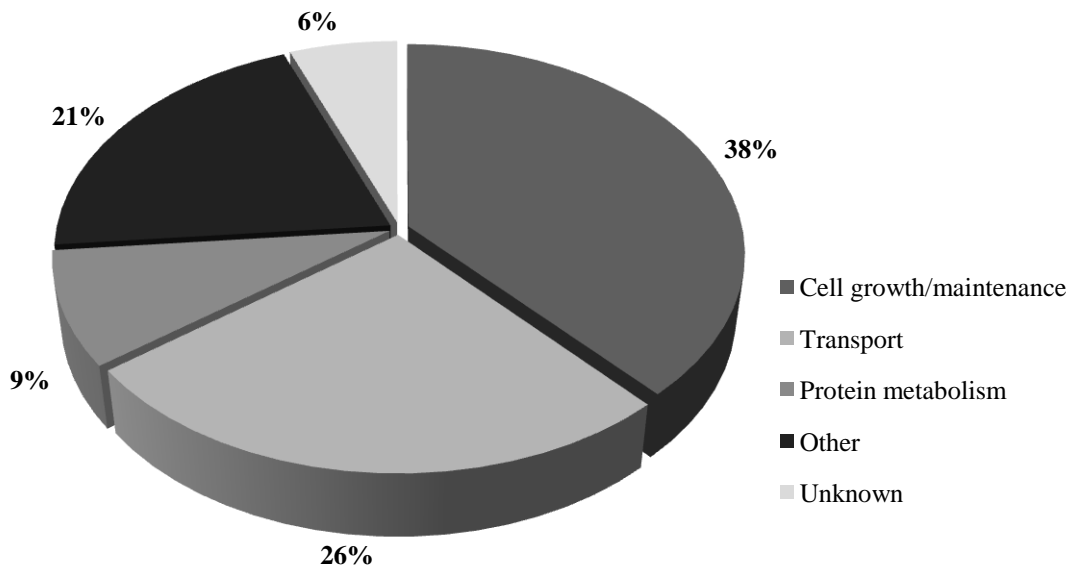
Conflict of interest: none

The authors have no conflict of interests to declare.