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Title: The sheep conceptus modulates proteome profiles in caruncular endometrium during early pregnancy

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Abstract: The stage-specific expression of functional proteins within the endometrium, and their regulation by conceptus-derived signals, are crucial for conceptus development and successful establishment of pregnancy. Accurate knowledge of endometrium-conceptus interactions is key for the development of effective strategies to improve conceptus implantation rates both following natural conception and/or assisted reproductive technologies. The unilateral pregnant ewe provides a powerful experimental model for the study of endometrial function in the presence or absence of conceptuses during the peri-implantation period. Two-dimensional gel electrophoresis and mass spectrometry-based proteomics were used to compare and identify differentially expressed proteins in caruncular endometrium collected from the gravid uterine horns and the non-gravid uterine horns at the time of conceptus attachment (day 16 of pregnancy) and early post-implantation period (day 20 of pregnancy). Fifty seven protein spots were up-regulated in the gravid horn at day 16 of pregnancy and twenty seven protein spots were up-regulated in the gravid horn at day 20 of pregnancy. Sixteen proteins with different functions such as protein metabolism, cholesterol and ion transport and cell adhesion were identified. In conclusion, the use of the unilaterally pregnant ewe model provides evidence that the early implantation and post-implanting conceptus-derived signals up-regulate caruncle endometrial proteins, including carbonic anhydrase 2 (CA-II) and apolipoprotein A-1 (APOA1) and down-regulate caruncle endometrial proteins, including adenosylhomocysteinase (AHCY) and heat shock 60kDa protein 1 (HSP60). These regulated proteins are likely involved in providing a suitable intra-uterine environment required for conceptus attachment, implantation, early post-implantation development and the successful establishment of pregnancy in sheep.

Anim Reprod Sci: Ms # ANIREP-D-6441

Title: The sheep conceptus modulates proteome profiles in caruncular endometrium during early pregnancy.

Authors: Arianmanesh M, Fowler PA and Al-Gubory KH.

Point-by-point response to the reviewer comments.

Reviewer 1: The paper has been improved by the revisions made.

**Authors' comment**

We thank the reviewer for this positive feedback. The reviewer understood the main goal of our manuscript and the implications of our method.

The fact that several of the spots in Table 2 are associated with more than one protein ID is still not explained anywhere.

We apologies for this oversight and have addressed this in Table 2 and its footnotes with respect to the 3 protein spots showing a secondary identification.

Line 222 should be associated with

Line 264 should be involved

Line 286 should be increased

Line 287 should be evidence

**Authors' comment**

We apologize for these errors, and we have corrected the text as suggested.

Reviewer 2:

Reviewer's response to revised manuscript.

In their response to reviewer's comments, the authors admit that in their study, data on PGE2 and its receptor are sadly lacking. The authors even cite the 2011 reference by Dorniak et al (Biol Reprod 84:1127) which clearly shows the critical role of PGE2 the early establishment of early pregnancy in sheep but they do not include this reference in their revised Ms. A more recent comprehensive review confirms the critical role of PGE2 and its receptor in the early establishment of pregnancy in ruminants (Arosh et al, 2016 J Dairy Sci 99:5926).

So the bottom line is that this article is incomplete because it does not include data on PGE2 and its receptor in the two models that they employed. The study would be greatly improved by including two additional controls with non-pregnant ligated uterine horns in addition to the ovariectomized non-pregnant ligated uterine horn that they originally employed. First, in addition to their original control of the ovariectomized non-pregnant ligated uterine horn, they should also include the ligated non-pregnant uterine horn but with its ovary included. Second, they should also include animals with a ligated uterine horn but this time with a corpus luteum in the ovary. This is important because it is established in sheep that progesterone from the corpus luteum reaches its adjacent uterine horn locally at a much higher concentration than reaches it via the systemic circulation.

Such an improved experimental design together with the inclusion of measurement of PGE2 and its receptor would make an important contribution to our knowledge of the early establishment of pregnancy in ruminants

#### Authors' comment

We thank the reviewer for his/her in-depth analysis, useful comments, valuable time and useful contribution.

The reviewer asked to perform significant amount of experiments by using different sheep models together with the inclusion of measurement of PGE2 and its receptor to answer a specific point, which falls outside the scope of this study and is far from the rational of our study clearly stated in the introduction by the following paragraph (page 3, lines 53-57):

“Although a multitude of molecular pathways involved in extraembryonic membrane-endometrium crosstalk during conceptus implantation and post-implantation periods have been identified through studies of gene expression, a comprehensive understanding of changes in many endometrium proteins expressed in the presence of conceptuses is currently lacking. “

We believe that our paper, whose conclusions are not in doubt, is complete as it is, and as often is the case, more focused researches are still needed. But the paper is designed to answer an important outstanding question, and it does so. These requested experiments would not change the conclusion of the paper.

The unilateral pregnant sheep model used in the present study provides a new understanding about the role of conceptus-derived signals in the regulation of functional endometrial proteins involved in iron transport and homeostasis, hydrolysis, protein chaperoning and degradation, amino acid metabolism, cholesterol transport and cell adhesion.

We strongly think that the findings reported in our study establish a new reference database and will open the avenue for future follow-up mechanistic studies toward understanding the role, if any, of known (INFtau and/or PGE2) and unknown conceptus-derived factors in the regulation of caruncle endometrial proteins, including carbonic anhydrase 2, apolipoprotein A-1 (APOA1), adenosylhomocysteinase and heat shock 60kDa protein 1.

Directions for future research are now open such that the present study provides a stimulus for further research.

Dear Editor,

We acknowledge with thanks receipt of your e-mail of 21 September 2016 concerning our manuscript:

Ms. No. ANIREP-D-16-6441R1

The sheep conceptus modulates proteome profiles in caruncular endometrium during early pregnancy

We are pleased to inform you that the manuscript was revised as requested.

Thank you very much for giving us the opportunity to publish our study in Animal Reproduction Science.

Yours sincerely,

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## Highlights

1. The unilateral pregnant ewes were employed to investigate proteome changes during the peri-implantation period.
2. Conceptus-derived signals regulate multiple functional proteins in caruncular endometrium.
3. These proteins likely provide a suitable environment required for conceptus implantation and development.

1 **The sheep conceptus modulates proteome profiles in caruncular endometrium during**  
2 **early pregnancy**

3

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11

12 Abbreviated title: Conceptus control of endometrium protein expression

13 Keywords: sheep, endometrium, proteome, gravid and non-gravid uterine horns, early pregnancy

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23 **Abstract**

24 The stage-specific expression of functional proteins within the endometrium, and their regulation  
25 by conceptus-derived signals, are crucial for conceptus development and successful establishment  
26 of pregnancy. Accurate knowledge of endometrium-conceptus interactions is key for the  
27 development of effective strategies to improve conceptus implantation rates both following natural  
28 conception and/or assisted reproductive technologies. The unilateral pregnant ewe provides a  
29 powerful experimental model for the study of endometrial function in the presence or absence of  
30 conceptuses during the peri-implantation period. Two-dimensional gel electrophoresis and mass  
31 spectrometry-based proteomics were used to compare and identify differentially expressed proteins  
32 in caruncular endometrium collected from the gravid uterine horns and the non-gravid uterine  
33 horns at the time of conceptus attachment (day 16 of pregnancy) and early post-implantation period  
34 (day 20 of pregnancy). Fifty seven protein spots were up-regulated in the gravid horn at day 16 of  
35 pregnancy and twenty seven protein spots were up-regulated in the gravid horn at day 20 of  
36 pregnancy. Sixteen proteins with different functions such as protein metabolism, cholesterol and  
37 ion transport and cell adhesion were identified. In conclusion, the use of the unilaterally pregnant  
38 ewe model provides evidence that the early implantation and post-implanting conceptus-derived  
39 signals up-regulate caruncle endometrial proteins, including carbonic anhydrase 2 (CA-II) and  
40 apolipoprotein A-1 (APOA1) and down-regulate caruncle endometrial proteins, including  
41 adenosylhomocysteinase (AHCY) and heat shock 60kDa protein 1 (HSP60). These regulated  
42 proteins are likely involved in providing a suitable intra-uterine environment required for conceptus  
43 attachment, implantation, early post-implantation development and the successful establishment of  
44 pregnancy in sheep.

45

## 46 **Introduction**

47 In sheep, goats and cattle, successful conceptus (embryo and associated extraembryonic  
48 membranes) implantation relies on elaborate cellular, biochemical and molecular cross-talk  
49 between the extraembryonic membranes and receptive uterine endometrial tissues that ensures  
50 corpus luteum (CL) progesterone production and optimal post-implantation conceptus development  
51 and survival (Paria et al., 2001; Imakawa et al., 2004). During early pregnancy, a high rate of  
52 embryonic mortality occurs due to abnormal conceptus signalling (Goff, 2002; Dixon et al., 2007;  
53 Diskin and Morris, 2008). Although a multitude of molecular pathways involved in extraembryonic  
54 membrane-endometrium crosstalk during conceptus implantation and post-implantation periods  
55 have been identified through studies of gene expression, a comprehensive understanding of  
56 changes in many endometrium proteins expressed in the presence of conceptuses is currently  
57 lacking.

58

59 Our previous studies provided original evidence that several endometrial proteins with different  
60 functions, including protein synthesis and degradation, antioxidant defence, cell structural integrity,  
61 adhesion and signal transduction, play important roles in the establishment of early pregnancy in  
62 sheep (Al-Gubory et al., 2014). Of particular interest were proteins that were highly expressed in  
63 response to the presence of conceptuses at attachment and early post-implantation periods and,  
64 using our unilaterally pregnant ewe model, we demonstrated that the early implantation and post-  
65 implanting conceptus-derived signals up-regulate the expression of cytoplasmic tryptophanyl tRNA  
66 synthetase and the mitochondrial superoxide dismutase (Al-Gubory et al., 2014). The former is a  
67 key catalytic enzyme for the first step reaction in protein synthesis (Sallafranque et al., 1986) and  
68 the latter the first antioxidant defence enzyme against reactive oxygen species-induced  
69 mitochondrial oxidative damage (Orrenius et al., 2007), in sheep caruncular endometrium (Al-  
70 Gubory et al., 2015). In sheep, the endometrium caruncles (CAR) are highly vascularized stromal  
71 protuberances covered by a simple luminal epithelium. CAR areas are specialized sites of  
72 attachment of the outer covering extraembryonic membrane, the trophoctoderm, and are privileged



73 endometrial tissues for conceptus-uterine communication. Our hypothesis is that the early  
74 developing sheep conceptus modulates protein expression profiles in CAR endometrium during  
75 early pregnancy.

76

77 The unilateral pregnant sheep model enables changes in the expression of endometrial proteins in  
78 the presence or absence of conceptuses to be studied, providing a powerful model for the  
79 investigation of proteome changes during the peri-implantation period (Al-Gubory et al., 2015).

80 The benefit of this model is that both uterine horns are exposed to similar concentrations of  
81 circulating hormones such as progesterone but only the gravid horn is under the direct action of  
82 local signalling molecules produced by the conceptus. In the present study, the unilateral pregnant  
83 ewes with functional ovaries were therefore employed to test our hypothesis. Two-dimensional gel  
84 electrophoresis (2DE) based proteomics (Fowler et al., 2007; Arianmanesh et al., 2011; Al-Gubory  
85 et al., 2014) was used to characterize specific alterations in the proteome of CAR endometrial  
86 tissues collected from the gravid uterine horns (GH) and the non-gravid uterine horns (NG) at the  
87 time of conceptus attachment (day 16 of pregnancy) and early post-implantation period (day 20 of  
88 pregnancy).

89

## 90 **Materials and Methods**

### 91 **Experimental animals**

92 All procedures relating to care and use of animals were approved by the French Ministry of  
93 Agriculture according to the French regulation for animal experimentation (authorization no° 78-  
94 34). Ewes of the Préalpes-du-Sud breed (18 months of age) were used in this study. Unilaterally  
95 pregnant ewes were prepared surgically as described previously (Payne and Lamming, 1994;  
96 Lamming et al., 1995). Briefly, ewes were initially anesthetized with a mixture of pentobarbital  
97 (Sanofi, Paris, France) and thiopentone (Abbott, Aubervilliers, France). After endotracheal  
98 intubation, general anaesthesia was maintained by constant inhalation of a mixture of oxygen and  
99 halothane. Reproductive organs were exposed via midventral laparotomy and one ovary was  
100 removed. One uterine horn was ligated close to the uterine bifurcation so that after mating the

101 conceptus is confined to the non-ligated pregnant horn. Three weeks after surgery, the ewes were  
102 treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet,  
103 Angers, France) to synchronize oestrous. Ewes were mated twice with fertile rams of the same  
104 breed, at an interval of 12 h during the synchronized oestrus. The ewes were housed under  
105 conditions of natural day-length and temperature and had free access to mineral licks and water.

#### 106 **Endometrial tissue collection**

107 The ewes were slaughtered at a local abattoir in accordance with protocols approved by the local  
108 institutional animal use committee at the Institut National de la Recherche Agronomique (INRA,  
109 Jouy-en-Josas, France). Pregnant ewes were randomly allocated for slaughter at two specific stages  
110 of early pregnancy corresponding to the initial conceptus attachment to CAR areas (day 16, n=4  
111 ewes) and the early conceptus post-implantation period (day 20, n=4 ewes). The stages of  
112 pregnancy were confirmed by the presence and the morphology of the conceptus in uterine  
113 flushings. Immediately after slaughter of the ewes, the reproductive tracts were collected, placed on  
114 crushed ice and transported to the laboratory. Endometrial CAR areas were collected from the  
115 entire GH (ipsilateral uterine horn) and NG (contralateral uterine horn) of each ewe, snap-frozen in  
116 liquid nitrogen and stored at -80 °C until processed for 2DE gel electrophoresis and Western blot.

117

#### 118 **Protein extraction and quantification for electrophoretic analysis**

119 CAR from the gravid horns on days 16 (GH16) and 20 (GH20), non-gravid horns on days 16  
120 (NG16) and 20 (NG20) were processed separately for 1DE and 2DE gel electrophoresis as  
121 described previously (Fowler et al., 2007). Briefly, tissues were combined with 5 ml lysis buffer/1  
122 mg wet weight of tissue. The lysis buffer (0.01 M Tris-HCl, pH 7.4) contained 1 mM EDTA, 8 M  
123 urea, 0.05 M dithiothreitol, 10% (v/v) glycerol 5% (v/v), NP40, 6% (w/v), pH 3–10, resolyte  
124 (Merck Eurolab Ltd, Poole, Dorset, UK) and protease inhibitor cocktail (Roche Diagnostics). The  
125 tissues were disrupted using a Tissue Lyser (Qiagen Ltd) for 4 min at 30 Hz. Insoluble materials  
126 were removed from the lysates by centrifugation (50,000 g at 4°C) for 30 min. The protein content

127 of the final supernatant had been determined by RC-DC assay (Bio-Rad Laboratories Ltd). The  
128 protein extracts were stored at -80°C until required for further analysis.

129

### 130 **Two dimensional gel electrophoresis (2DE) analysis**

131 Equal amounts of protein from CAR of each ewe in each group were combined to make 4 protein  
132 pools (800 µg protein in each pool): gravid horn on days 16 (GH16) and 20 (GH20), non-gravid  
133 horn on days 16 (NG16) and 20 (NG20). 2DE was performed as described (Cash et al., 2003). As a  
134 first dimension separation, 70 µg of total protein from each pool was loaded onto 7 cm  
135 immobiline™ DryStrip non-linear pH gradient (IPG) strips of pH 3-10 (GE Healthcare, UK). The  
136 second dimension was carried out using 13 cm NUPAGE® Novex 4-12%, Bis-Tris Zoom® gels  
137 (Invitrogen Ltd, Paisley, UK). Quadruplicate 2DE gels were prepared for each of the 4 groups  
138 (representative gel is shown in Figure 1). Proteins were visualized using Colloidal CBB G-250 and  
139 scanned using an ImageScanner™ III (GE Healthcare). Protein spot profiles were analysed using  
140 Progenesis SameSpots version 3 software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) as  
141 described (Arianmanesh et al., 2011). Briefly, reference gel was selected and the other gels were  
142 aligned to be closely matched to this reference gel. Background was subtracted individually from  
143 each gel and spot volumes were normalised relative to total spot volume individually for each gel.  
144 Ultimately, 15 were selected for identification by LC-MS/MS on the basis of significance (log-  
145 normalised spot volumes had to differ between two groups at the level of  $P < 0.05$  by ANOVA and  
146 post-hoc testing), spot volume (a difference of a  $\geq 1.25$ -fold increase or decrease between two  
147 groups), concentrating on the most abundant proteins with the most stable expression across the 4  
148 replicate gels for each group.

149

### 150 **Mass spectrometry**

151 To identify proteins, 15 selected spots were excised from stained gels and subjected to in-gel  
152 trypsin digestion as described previously (Uwins et al. 2006). The peptide fragment mass spectra

153 were acquired on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer  
154 operated in the reflection delayed extraction mode. Tryptic peptides from the MS/MS spectra were  
155 applied to search the NCBI (National Centre for Biotechnology Information) database with the  
156 MASCOT program (<http://www.matrixscience.com>). Search parameters for the programme  
157 included maximum allowed error of peptide mass 250 ppm, cysteine as S-carbamidomethyl-  
158 derivative and oxidation of methionine were allowed.

159

### 160 **Statistical Analysis**

161 Normality of data was tested with the Shapiro-Wilk test. Normally distributed data were subjected  
162 to one- and two-way ANOVA and Bonferroni post-hoc test using SPSS 17.0 software to assess  
163 significance of differences. Statistical comparisons between specific groups were carried out by  
164 student's t-test. Differences were considered significant at  $P < 0.05$ .

165

### 166 **Results**

167 Overall, 998 protein spots were included (on the basis of clear, reproducible expression and  
168 absence of noise in all four gels for each group) for analysis from a total of 1482 distinct protein  
169 spots detected by automatic detection with Progenesis SameSpots Software. The number of spots  
170 showing statistically significant differences in normalized spot volumes between groups is shown  
171 in Table 1.

172

173 Comparison between the GH and the NG uterine horns at day 16 of pregnancy revealed that 47  
174 (3%) of protein spots were significantly changed ( $P < 0.05$ ). Among these, 35 normalized spot  
175 volumes were up-regulated and 12 normalized spot volumes were down-regulated (Table 1).

176 Comparison between the GH and the NG uterine horns at day 20 of pregnancy revealed that 27  
177 (2%) of protein spots were significantly changed ( $P < 0.05$ ). 25 of these normalized spot volumes  
178 were up-regulated and 2 normalized spot volumes were down-regulated (Table 1). In GH uterine  
179 horns, 48 (3%) of protein spots were significantly changed ( $P < 0.05$ ) between days 16 and 20 of

180 pregnancy. In this category, 17 normalized spot volumes were up-regulated and 31 normalized spot  
181 volumes were down-regulated (Table 1). In NG uterine horns, 48 (3%) of protein spots were  
182 significantly changed ( $P<0.05$ ) between days 16 and 20 of pregnancy. Among these, 30 normalized  
183 spot volumes were up-regulated and 18 normalized spot volumes were down-regulated (Table 1).

184

185 The proteins spots in GH and NG uterine horns exhibiting significant differences in expression at  
186 implantation day and early post-implantation period and identified are shown in Table 2.  
187 Adenosylhomocysteinase (AHCY, Figure 2A) increased ( $P<0.05$ ) in NG uterine horns compared to  
188 GH uterine horns at both days 16 and 20 of pregnancy. (Table 2). Carbonic anhydrase 2 (CA-II,  
189 Figure 2B), increased ( $P<0.05$ ) in GH uterine horns compared to NG uterine horns at both days 16  
190 and 20 of pregnancy (Table 2). Heat shock 60 kDa protein 1 (HSP60, Figure 2C), increased  
191 ( $P<0.05$ ) in NG uterine horns compared to GH uterine horns at day 20 of pregnancy. (Table 2). In  
192 GH uterine horns, HSP60 decreased (Figure 2C,  $P<0.05$ ) at day 20 when compared with day 16 of  
193 pregnancy (Table 2).

194

195 In GH and NG uterine horns, proteasome activator subunit 2 (PA28beta/PSME2, Figure 3A)  
196 increased ( $P<0.05$ ) at day 20 when compared with day 16 of pregnancy (Table 2). In GH uterine  
197 horns, apolipoprotein A-1 (APOA1, Figure 3B) increased ( $P<0.05$ ) at day 20 when compared with  
198 day 16 of pregnancy (Table 2). In GH and NG uterine horns, transferrin (TF, Figure 3C) decreased  
199 ( $P<0.05$ ) at day 20 when compared with the day 16 of pregnancy (Table 2). In GH uterine horns,  
200 galectin 15 (LGALS15, Figure 3D) decreased ( $P<0.05$ ) at day 20 when compared with day 16 of  
201 pregnancy (Table 2).

## 202 **Discussion**

203 The establishment of pregnancy requires correctly timed, exquisitely coordinated, adaptive,  
204 responses of the uterine endometrium to the presence of conceptuses. Some of these responses will  
205 be via modification of the expression of functional proteins during early pregnancy. The proteomic  
206 profile of sheep CAR endometrium reported here provided a new understanding about the role of

207 conceptus-derived signals in the regulation of a substantial number of functional proteins involved  
208 in iron transport and homeostasis, hydrolysis, protein chaperoning and degradation, amino acid  
209 metabolism, cholesterol transport and cell adhesion.

210

211 Conceptus-derived factors reduce the expression of AHCY protein as evidenced by the down-  
212 regulation of this protein in endometrial caruncular tissues of the gravid uterine horns compared  
213 with the non-gravid uterine horns at both implantation and post-implantation periods (present  
214 study). AHCY catalyzes the breakdown of S-adenosylhomocysteine (AdoHcy) to adenosine (Ado)  
215 and L-homocysteine (Hcy) (Turner et al., 2000). It is important to note that hyperhomocysteinemia  
216 (HHcy) exerts adverse effects through the induction of inflammation pathways, including  
217 endothelial monocyte adhesion and infiltration (Wang et al., 2002), oxidative stress, activation of  
218 pro-inflammatory factors and endothelial dysfunction (Lawrence de Koning et al. 2003). Hcy  
219 activates NADPH oxidase and increases reactive oxygen species in human umbilical vein  
220 endothelial cells (Dong et al., 2005). Elevated level of Hcy within organs and tissues is therefore a  
221 potentially pathophysiological risk factor for uterine endothelial function via an enhancement of  
222 oxidative stress and inflammation. Maternal Hhcy should be associated with placental abruption and  
223 spontaneous abortion (Ray and Laskin, 1999). Increased Hcy levels and oxidative stress represent a  
224 risk factor for the establishment and maintenance of pregnancy (Micle et al. 2012). Therefore, the  
225 conceptus must hold Hcy in check within CAR endometrium at conceptus attachment (Day 16) and  
226 early post-implantation period (Day 20) of pregnancy through down-regulation of AHCY protein  
227 expression (present study). We suggest that the conceptus-derived factors exert local effects within  
228 the endometrium to counteract peri-implantation oxidative stress through the control of Hcy  
229 production and thereby support the establishment of pregnancy.

230

231 Upregulation of CA-II protein expression by conceptus-derived signals observed here on the day of  
232 conceptus implantation and during early post-implantation period had not been reported previously  
233 in endometrium of any mammalian species. Identification of these signaling molecules is essential  
234 in our understanding of the molecular mechanisms that should be involved in the establishment of

235 pregnancy. CAII catalyzes the reversible hydration of carbon dioxide to bicarbonate and plays an  
236 important role in acid-base homeostasis within tissues of biological systems (Khalifah, 1971).  
237 These reactions are requisite for cancer development, invasion and progression. Interestingly, CA  
238 II is highly expressed in tumours of different organs, including brain (Parkkila et al., 1995a),  
239 pancreas (Parkkila et al., 1995b) and kidney (Parkkila et al., 2000), where it favourably induces an  
240 environment necessary for the growth and spread of the tumour by changing acidity of the  
241 extracellular medium surrounding cancer cells. In the neonatal mouse uterus, where members of  
242 the CA family are expressed (Hu et al., 2004), CAII mRNAs were localized in epithelial and  
243 stromal cells of the endometrium suggesting a functional role for CAII in endometrial gland  
244 development during postnatal uterine development (Hu and Spencer, 2005). The expression of CA  
245 II in the bovine (Nishita et al., 1990) and human (Aliakbar et al., 1990; Muhlhauser et al., 1994)  
246 placentas supports the suggestion that this enzyme is required for endometrial tissue remodelling.  
247 Endometrium structural remodelling in ruminants, including sheep, plays crucial role in  
248 implantation, placentation and conceptus nutrition (Igwebuike, 2009). On the day of conceptus  
249 attachment (day 16 of pregnancy), there is close contact between trophoblast, the extra-embryonic  
250 membrane of the conceptus, and the epithelium overlying CAR endometrium, over raised areas of  
251 the endometrium, to allow implantation and early placental development. The high level of CA-II  
252 protein expression in CAR endometrium of the gravid uterine horns (present study) likely suggests  
253 an important role for this regulated protein in promoting trophoblast attachment, invasion and  
254 fusion with endometrial epithelium and/or remodelling the endometrium for successful early  
255 conceptus implantation and, consequently, formation of the maternal-fetal interface during  
256 placental development.

257

258 HSP60 has multiple functions in the normal cells, including inter-organelle transport, inhibition of  
259 aggregation of denatured polypeptides, antigen presentation, pro-apoptotic activity (Yu et al., 2012)  
260 and promotion of the proper folding of polypeptides (Witkin et al., 1996). Moreover, it stimulates  
261 human sperm capacitation in the fallopian tube (Lachance et al., 2007). In human endometrium,  
262 HSP60 increased during the late proliferative and early secretory phase then decreased in the mid

263 to late secretory phase while other members of this family probably protect endometrial proteins  
264 against factors involvement in denaturant activity such as TNF- $\alpha$ , particularly in the implantation  
265 window (Tabibzadeh et al., 1999). Therefore, a significant reduction in HSP60 expression in CAR  
266 endometrium of the gravid horns during the early conceptus post-implantation period (present  
267 study) may be due to protein redundancy in which the other members of HSP family take over the  
268 role of HSP60 in the chaperon activity that is assumed to be required at the time of conceptus  
269 implantation.

270

271 A balance between protein synthesis and degradation of abnormal, damaged and short-lived  
272 proteins by proteasomes (Hochstrasser, 1995) is essential for several cellular processes, including  
273 cell cycle and division (King et al., 1996), proliferation and apoptosis (Naujokat and Hoffmann,  
274 2002) and gene transcription (Muratani and Tansey, 2003). The turnover of proteins within cells by  
275 the ubiquitin-proteasome system depend on proteasome activators (Zhang et al., 1998). The  
276 proteasome activator 28 (PA28 or PSME) consists of two homologous subunits, PA28-alpha (or  
277 PSME1) and PA28-beta (or PSME2), each of which activates the proteasome (Zhang et al., 1998).  
278 Up-regulation of PA28- $\beta$  protein expression observed in the present study in sheep CAR  
279 endometrium during the early conceptus post-implantion period had not been reported previously.  
280 Of note is that PA28-beta protein expression increased dramatically in both the gravid and non  
281 gravid uterine horns suggesting a systemic rather than a local effect on endometrium PA28- $\beta$   
282 protein expression. These results suggest that factors present and associated with early pregnancy  
283 enhance PA28- $\beta$  protein expression. The high level of PA28- $\beta$  protein expression in CAR  
284 endometrium of the gravid and non gravid uterine horns (present study) likely suggests an  
285 important role for this regulated protein in protein-turnover since one can expect that the  
286 endometrium protein synthesis should be increased during the early post-implantation period.  
287 There should be evidence to suggest that proteasomes are parts of cellular defense mechanism  
288 against oxidative stress and protein oxidative damage by controlling the degradation of oxidatively  
289 damaged proteins (Ding et al. 2006; Poppek and Grune, 2006; Squier, 2006). Beside the high  
290 antioxidative capacity of the sheep CAR endometrium in the early conceptus post-implantation



291 period (Al-Gubory and Garrel, 2012), the dramatic post-implantation increase in PA28- $\beta$  protein  
292 expression observed in the present study probably plays an important role in degradation of  
293 oxidised endometrium proteins during early pregnancy.

294

295 APOA1 is a main component of HDL synthesized by the liver and intestine (Zannis et al., 1985). In  
296 fertile women, APOA1 was down-regulated in secretory endometrium compared to proliferative  
297 endometrium (Brosens et al., 2010). In infertile women, Apo-A1 increased in mid-secretory phase  
298 endometrium as compared to early-secretory phase endometrium (Manohar et al., 2014).  
299 Deregulations of endometrial APOA1 protein (Fowler et al., 2007) and mRNA (Brosens et al.,  
300 2010) expression are important features of endometriosis in women. These findings suggest a role  
301 of Apo-A1 in endometrium preparation for conceptus implantation and development. HDL  
302 cholesterol and APOA1 play a crucial role in human embryo development (Baardman et al., 2013).  
303 Of note, the increased level of APOA1 secretion by blastocysts in spent media from cultures of  
304 high quality blastocysts compared to low quality blastocysts and this may be associated with  
305 implantation potential (Mains et al., 2011). Moreover, APOA1 is a source of nutrients for the early  
306 post-implanted conceptus (Assemat et al., 2005). In the present study, we showed that APOA1 was  
307 highly expressed in CAR endometrium from the gravid uterine horns at the early conceptus post-  
308 implantation period. Given APOA1 functions, it may be assumed that the implantaing conceptus  
309 exerts local effects on CAR areas of the sheep endometrium of the gravid horns to increase the  
310 production of apoA-I-containing lipoproteins necessary for early conceptus development and  
311 survival.

312

313 Transferrin (TF), an iron-binding and transport protein, is detected in sheep intrauterine luminal  
314 fluid between days 17 and 18 of pregnancy suggesting that TF is a conceptus-synthesized protein  
315 (Lee et al., 1998). However, it is unlikely that TF is synthesied and secreted solely by the  
316 developing conceptuses during the peri-implantatiuon periods. Indeed, it has been reported that  
317 porcine intrauterine fluid on day 16 of the oestous cycle or pregnancy contains high amount of TF  
318 (Vallet et al., 1996). In addition, TF protein expression in sheep CAR endometrium increased at

319 day 16 of the oestrous cycle as compared to the matching day of pregnancy (Al-Gubory et al.,  
320 2014). Interestingly, TF protein was highly expressed in CAR endometrium of the gravid and non  
321 gravid uterine horns on the day of conceptus attachment when compared with the early post-  
322 implantation period (present study). Therefore, under the physiologically relevant in vivo  
323 conditions of a unilaterally pregnant ewes and conceptus development, it is likely that the sheep  
324 endometrium is major source of TF during early pregnancy. Considering the role of TF in the  
325 proliferation and differentiation of mouse embryonic tissues in culture (Ekblom et al. 1981;  
326 Thesleff and Ekblom, 1985), it is likely that TF could be required for sheep conceptus  
327 development during early pregnancy.

328

329 Galectins are a family of beta-galactoside-binding lectins. In the endometrial luminal epithelium of  
330 pregnant ewes, galectin-15 mRNA expression increased between days 12 and 16, and galectin-15  
331 (LGALS15) protein in the uterine lumen increased between days 14 and 16 of pregnancy (Gray et  
332 al., 2004). LGALS15 is expressed uniquely in the endometrium of sheep and goats and plays an  
333 important role in trophoblast attachment (Lewis et al., 2007; Farmer et al., 2008). It is important to  
334 note that LGALS15 protein expression was not different between the gravid and non gravid uterine  
335 horns at the day of conceptus attachment and early post-implantation period (present study).  
336 Moreover, in the gravid uterine horns, LGALS15 decreased at post-implantation period when  
337 compared with the attachment day. These results suggest that the regulation of LGALS15  
338 expression in sheep CAR endometrium likely does not depend on factors produced by the  
339 conceptus during early pregnancy.

340

341 In conclusion, our study provide evidence that conceptus-derived signals play key roles in the  
342 regulation of multiple functional proteins in sheep CAR endometrium, importantly AHCY, CA-II,  
343 HSP60 and APOA1 during conceptus implantation and the early post-implantation periods. These  
344 regulated proteins likely involved in providing a suitable intra-uterine environment required for  
345 conceptus attachment, implantation, early post-implantation development and successful  
346 establishment of pregnancy in sheep.



348 **Declaration of interest**

349 The authors declare that there is no conflict of interest that could be perceived as prejudicing the  
350 impartiality of the research reported.

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358 **Author contributions**

359 KHA jointly conceived and designed the study with PAF. KHA prepared the animal model,  
360 performed surgery and tissue collection. MA carried out the proteomic analysis, performed  
361 production and acquisition of data. KHA and MA wrote the manuscript. KHA and PAF contributed  
362 reagents and materials and helped in data interpretation. PAF made critical revisions of the  
363 manuscript for important intellectual content. All authors approved the final version of manuscript.

364

365 **Figure Legends**

366

367 **Figure 1.** Sheep caruncular endometrial proteome separated by 2DE gel using a 3-10 pH gradient.368 A representative 2DE gel of the caruncle proteins from sheep non-gravid (NG) horn on day 20 of  
369 pregnancy (NG20) is shown, indicating selected spots for cutting by arrows.

370

371 **Figure 2.** Expression changes of (A) adenosylhomocysteinase (AHCY), (B) carbonic anhydrase 2  
372 (CA-II), and (C) heat shock 60kDa protein 1 (HSP60) in sheep caruncular endometrial tissues  
373 collected from gravid horns (GH) and non-gravid horns (NG) of uteri at implantation (day 16 of  
374 pregnancy) and post implantation (day 20 of pregnancy) periods. Normalised protein spot volumes  
375 are shown as means  $\pm$  SEM (n=4 ewes per group). Zoom boxes from the 2D gels showing the  
376 identified proteins (arrows) are shown (right panels). The acceptable level of significance was set at  
377  $P < 0.05$ .

378

379 **Figure 3.** Expression changes of (A) proteasome activator 28 beta (PA28beta), (B) apolipoprotein  
380 A-1 (APOA1), (C), transferrin (TF) and (D) galectin 15 (LGALS15) in sheep caruncular  
381 endometrial tissues collected from gravid horns (GH) and non-gravid horns (NG) of uteri at  
382 implantation (day 16 of pregnancy) and post implantation (day 20 of pregnancy) periods.  
383 Normalised protein spot volumes are shown as means  $\pm$  SEM (n=4 ewes per group). Zoom boxes  
384 from the 2D gels showing the identified proteins (arrows) are shown (right panels). The acceptable  
385 level of significance was set at  $P < 0.05$ .

386

387

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536 **Table 1.** Numbers of protein spots significantly ( $P < 0.05$ ) differing between caruncle of gravid  
 537 horns (GH) and caruncle of non-gravid horns (NG) of sheep endometrium at the time of  
 538 conceptus implantation (Day 16) and early post-implantation (Day 20) periods of pregnancy.  
 539

540 Groups compared

541	Group	Compared with	Total number	Up-regulated	Down-regulated	% of total spots
542			of spots			
543	NG16	GH16	47	35	12	3
544	NG20	GH20	27	25	2	2
545	GH20	GH16	48	17	31	3
546	NG20	NG16	48	30	18	3

547

548 **Table 2** Caruncle proteins of gravid (GH) and non-gravid (NG) uterine horns demonstrating significant differences in expression at conceptus implantation  
 549 (Day 16) and early post-implantation (Day 20) periods of pregnancy. The significant fold changes are shown in bold with their corresponding P values  
 550 (P<0.05). Increases in spot volumes are denoted by a “+” and decreases by a “-“ prefix to the fold-change values. The comparisons between groups follow the  
 551 rule that the fold-changes are calculated on the basis that the first group is being compared with the second group. Accession number is written regarding to  
 552 bovine species. The accession number specific for ovine protein is shown in brackets if available.

Protein	Spot no.	MW (KDa)	PI	MOWSE score (MASCOT)	Swiss-Prot	Fold change (P value)			
						NG16 vs. GH16	NG20 vs. GH20	GH20 vs. GH16	NG20 vs. NG16
<b>Actin binding protein</b>									
Gelsolin isoform b (GSN)	520	80.9	5.54	413	<b>Q3SX14</b>	+1.07	<b>+1.28 (0.031)</b>	-1.1	+1.08
<b>Iron transport and homeostasis</b>									
Transferrin (TF)	1463	79.8	6.75	473	<b>Q29443</b>	+1.04	+1.18	<b>-1.96 (0.001)</b>	<b>-1.73 (0.0001)</b>
<b>Hydrolase</b>									
Adenosylhomocysteinase (AdoHcyase) (AHCY)	964	48.1	5.88	932	<b>Q3MHL4</b>	<b>+1.27 (0.008)</b>	<b>+1.18 (0.03)</b>	-1.00	-1.08
<b>Cytokine and nucleotide binding protein</b>									
<sup>P</sup> High mobility group box 1 protein (HMGB1)		25.0	5.75	416	<b>P63158</b>				
<sup>S</sup> Cytokine induced protein 29 KDa (CIP29)	1242	23.6	5.98	202	<b>Q2TBX1</b>	<b>+1.32 (0.003)</b>	+1.07	+1.03	-1.20
<b>Metalloenzyme</b>									
Carbonic anhydrase 2 (CA-II)	1267	29.1	6.41	546	<b>P00922</b>	<b>-1.34 (0.0004)</b>	<b>-1.39 (0.049)</b>	+1.02	+1.06
<b>Actin binding protein, heparin binding protein</b>									
<sup>P</sup> Tropomyosin alpha-1 chain (TPM1)		32.7	4.74	309	<b>Q91XN6</b>				
<sup>S</sup> Hepatoma derived growth factor (HDGF)	1102	26.3	4.84	176	<b>Q9XSK7</b>	-1.11	+1.06	+1.07	<b>+1.27 (0.013)</b>

<b>Chaperones</b>									
Heat shock 60kDa protein 1 (HSP60)	791	61.1	5.71	1660	<b>P31081</b>	-1.02	<b>+1.27 (0.01)</b>	<b>-1.19 (0.044)</b>	+1.08
<b>Amino acid metabolism, Metabolism</b>									
<sup>P</sup> Glycine amidinotransferase, mitochondrial (GATM)	979	48.8	8	448	<b>Q2HJ74</b>	<b>+1.41 (0.04)</b>	+1.21	-1.12	-1.32
<sup>S</sup> Isocitrate dehydrogenase 1 (NADP+), soluble (IDH1)		47.1	6.34	365	<b>Q9XSG3</b>				
<b>Cholesterol transport</b>									
Apolipoprotein A-1 (APOA1)	1320	28.4	5.57	457	P02647	+1.11	-1.40	<b>+2.26 (0.01)</b>	+1.45
<b>Protein degradation</b>									
Proteasome activator subunit 2 (PA28beta) (PSME2)	1253	27.5	5.31	376	<b>Q5E9G3</b>	-1.12	+1.14	<b>+2.7 (0.0001)</b>	<b>+3.46 (0.0006)</b>
<b>Ion transport</b>									
Chloride intracellular channel protein 1 (CLIC1)	1231	23.8	5.12	169	<b>O00299</b>	-1.25	+1.07	+1.12	<b>+1.28 (0.03)</b>
Potassium channel tetramerisation domain containing 12 (KCTD12)	1109	47	5.68	260	616416 (NCBI)	-1.15	+1.04	+1.06	<b>+1.35 (0.0002)</b>
<b>Cell adhesion</b>									
Galectin 15 (LGALS15/OVGAL11)	1456	15.5	5.22	405	<b>Q19MU7*</b>	-1.19	+1.52	<b>-1.91 (0.003)</b>	-1.05

553 \* The accession number is for ovine species as the accession number for bovine was not found.

554

555 For 3 spots, peptide fragments were identified that belonged to more than one protein and the primary protein in the spot was identified based on 1) highest

556 Mascot score, 2) best agreement between estimated (ie, from electrophoretic gel mobility) and calculated molecular weight and isoelectric point, and 3)

557 highest peptide coverage.

558 <sup>P</sup> = primary protein in the spot ; <sup>S</sup> = secondary protein in the spot





1 **The sheep conceptus modulates proteome profiles in caruncular endometrium during**  
2 **early pregnancy**

3

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11

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13 Keywords: sheep, endometrium, proteome, gravid and non-gravid uterine horns, early pregnancy

14

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23 **Abstract**

24 The stage-specific expression of functional proteins within the endometrium, and their regulation  
25 by conceptus-derived signals, are crucial for conceptus development and successful establishment  
26 of pregnancy. Accurate knowledge of endometrium-conceptus interactions is key for the  
27 development of effective strategies to improve conceptus implantation rates both following natural  
28 conception and/or assisted reproductive technologies. The unilateral pregnant ewe provides a  
29 powerful experimental model for the study of endometrial function in the presence or absence of  
30 conceptuses during the peri-implantation period. Two-dimensional gel electrophoresis and mass  
31 spectrometry-based proteomics were used to compare and identify differentially expressed proteins  
32 in caruncular endometrium collected from the gravid uterine horns and the non-gravid uterine  
33 horns at the time of conceptus attachment (day 16 of pregnancy) and early post-implantation period  
34 (day 20 of pregnancy). Fifty seven protein spots were up-regulated in the gravid horn at day 16 of  
35 pregnancy and twenty seven protein spots were up-regulated in the gravid horn at day 20 of  
36 pregnancy. Sixteen proteins with different functions such as protein metabolism, cholesterol and  
37 ion transport and cell adhesion were identified. In conclusion, the use of the unilaterally pregnant  
38 ewe model provides evidence that the early implantation and post-implanting conceptus-derived  
39 signals up-regulate caruncle endometrial proteins, including carbonic anhydrase 2 (CA-II) and  
40 apolipoprotein A-1 (APOA1) and down-regulate caruncle endometrial proteins, including  
41 adenosylhomocysteinase (AHCY) and heat shock 60kDa protein 1 (HSP60). These regulated  
42 proteins are likely involved in providing a suitable intra-uterine environment required for conceptus  
43 attachment, implantation, early post-implantation development and the successful establishment of  
44 pregnancy in sheep.

45

## 46 **Introduction**

47 In sheep, goats and cattle, successful conceptus (embryo and associated extraembryonic  
48 membranes) implantation relies on elaborate cellular, biochemical and molecular cross-talk  
49 between the extraembryonic membranes and receptive uterine endometrial tissues that ensures  
50 corpus luteum (CL) progesterone production and optimal post-implantation conceptus development  
51 and survival (Paria et al., 2001; Imakawa et al., 2004). During early pregnancy, a high rate of  
52 embryonic mortality occurs due to abnormal conceptus signalling (Goff, 2002; Dixon et al., 2007;  
53 Diskin and Morris, 2008). Although a multitude of molecular pathways involved in extraembryonic  
54 membrane-endometrium crosstalk during conceptus implantation and post-implantation periods  
55 have been identified through studies of gene expression, a comprehensive understanding of  
56 changes in many endometrium proteins expressed in the presence of conceptuses is currently  
57 lacking.

58

59 Our previous studies provided original evidence that several endometrial proteins with different  
60 functions, including protein synthesis and degradation, antioxidant defence, cell structural integrity,  
61 adhesion and signal transduction, play important roles in the establishment of early pregnancy in  
62 sheep (Al-Gubory et al., 2014). Of particular interest were proteins that were highly expressed in  
63 response to the presence of conceptuses at attachment and early post-implantation periods and,  
64 using our unilaterally pregnant ewe model, we demonstrated that the early implantation and post-  
65 implanting conceptus-derived signals up-regulate the expression of cytoplasmic tryptophanyl tRNA  
66 synthetase and the mitochondrial superoxide dismutase (Al-Gubory et al., 2014). The former is a  
67 key catalytic enzyme for the first step reaction in protein synthesis (Sallafranque et al., 1986) and  
68 the latter the first antioxidant defence enzyme against reactive oxygen species-induced  
69 mitochondrial oxidative damage (Orrenius et al., 2007), in sheep caruncular endometrium (Al-  
70 Gubory et al., 2015). In sheep, the endometrium caruncles (CAR) are highly vascularized stromal  
71 protuberances covered by a simple luminal epithelium. CAR areas are specialized sites of  
72 attachment of the outer covering extraembryonic membrane, the trophoctoderm, and are privileged

73 endometrial tissues for conceptus-uterine communication. Our hypothesis is that the early  
74 developing sheep conceptus modulates protein expression profiles in CAR endometrium during  
75 early pregnancy.

76

77 The unilateral pregnant sheep model enables changes in the expression of endometrial proteins in  
78 the presence or absence of conceptuses to be studied, providing a powerful model for the  
79 investigation of proteome changes during the peri-implantation period (Al-Gubory et al., 2015).

80 The benefit of this model is that both uterine horns are exposed to similar concentrations of  
81 circulating hormones such as progesterone but only the gravid horn is under the direct action of  
82 local signalling molecules produced by the conceptus. In the present study, the unilateral pregnant  
83 ewes with functional ovaries were therefore employed to test our hypothesis. Two-dimensional gel  
84 electrophoresis (2DE) based proteomics (Fowler et al., 2007; Arianmanesh et al., 2011; Al-Gubory  
85 et al., 2014) was used to characterize specific alterations in the proteome of CAR endometrial  
86 tissues collected from the gravid uterine horns (GH) and the non-gravid uterine horns (NG) at the  
87 time of conceptus attachment (day 16 of pregnancy) and early post-implantation period (day 20 of  
88 pregnancy).

89

## 90 **Materials and Methods**

### 91 **Experimental animals**

92 All procedures relating to care and use of animals were approved by the French Ministry of  
93 Agriculture according to the French regulation for animal experimentation (authorization no° 78-  
94 34). Ewes of the Préalpes-du-Sud breed (18 months of age) were used in this study. Unilaterally  
95 pregnant ewes were prepared surgically as described previously (Payne and Lamming, 1994;  
96 Lamming et al., 1995). Briefly, ewes were initially anesthetized with a mixture of pentobarbital  
97 (Sanofi, Paris, France) and thiopentone (Abbott, Aubervilliers, France). After endotracheal  
98 intubation, general anaesthesia was maintained by constant inhalation of a mixture of oxygen and  
99 halothane. Reproductive organs were exposed via midventral laparotomy and one ovary was  
100 removed. One uterine horn was ligated close to the uterine bifurcation so that after mating the

101 conceptus is confined to the non-ligated pregnant horn. Three weeks after surgery, the ewes were  
102 treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet,  
103 Angers, France) to synchronize oestrous. Ewes were mated twice with fertile rams of the same  
104 breed, at an interval of 12 h during the synchronized oestrus. The ewes were housed under  
105 conditions of natural day-length and temperature and had free access to mineral licks and water.

#### 106 **Endometrial tissue collection**

107 The ewes were slaughtered at a local abattoir in accordance with protocols approved by the local  
108 institutional animal use committee at the Institut National de la Recherche Agronomique (INRA,  
109 Jouy-en-Josas, France). Pregnant ewes were randomly allocated for slaughter at two specific stages  
110 of early pregnancy corresponding to the initial conceptus attachment to CAR areas (day 16, n=4  
111 ewes) and the early conceptus post-implantation period (day 20, n=4 ewes). The stages of  
112 pregnancy were confirmed by the presence and the morphology of the conceptus in uterine  
113 flushings. Immediately after slaughter of the ewes, the reproductive tracts were collected, placed on  
114 crushed ice and transported to the laboratory. Endometrial CAR areas were collected from the  
115 entire GH (ipsilateral uterine horn) and NG (contralateral uterine horn) of each ewe, snap-frozen in  
116 liquid nitrogen and stored at -80 °C until processed for 2DE gel electrophoresis and Western blot.

117

#### 118 **Protein extraction and quantification for electrophoretic analysis**

119 CAR from the gravid horns on days 16 (GH16) and 20 (GH20), non-gravid horns on days 16  
120 (NG16) and 20 (NG20) were processed separately for 1DE and 2DE gel electrophoresis as  
121 described previously (Fowler et al., 2007). Briefly, tissues were combined with 5 ml lysis buffer/1  
122 mg wet weight of tissue. The lysis buffer (0.01 M Tris-HCl, pH 7.4) contained 1 mM EDTA, 8 M  
123 urea, 0.05 M dithiothreitol, 10% (v/v) glycerol 5% (v/v), NP40, 6% (w/v), pH 3–10, resolyte  
124 (Merck Eurolab Ltd, Poole, Dorset, UK) and protease inhibitor cocktail (Roche Diagnostics). The  
125 tissues were disrupted using a Tissue Lyser (Qiagen Ltd) for 4 min at 30 Hz. Insoluble materials  
126 were removed from the lysates by centrifugation (50,000 g at 4°C) for 30 min. The protein content

127 of the final supernatant had been determined by RC-DC assay (Bio-Rad Laboratories Ltd). The  
128 protein extracts were stored at -80°C until required for further analysis.

129

### 130 **Two dimensional gel electrophoresis (2DE) analysis**

131 Equal amounts of protein from CAR of each ewe in each group were combined to make 4 protein  
132 pools (800 µg protein in each pool): gravid horn on days 16 (GH16) and 20 (GH20), non-gravid  
133 horn on days 16 (NG16) and 20 (NG20). 2DE was performed as described (Cash et al., 2003). As a  
134 first dimension separation, 70 µg of total protein from each pool was loaded onto 7 cm  
135 immobiline™ DryStrip non-linear pH gradient (IPG) strips of pH 3-10 (GE Healthcare, UK). The  
136 second dimension was carried out using 13 cm NUPAGE® Novex 4-12%, Bis-Tris Zoom® gels  
137 (Invitrogen Ltd, Paisley, UK). Quadruplicate 2DE gels were prepared for each of the 4 groups  
138 (representative gel is shown in Figure 1). Proteins were visualized using Colloidal CBB G-250 and  
139 scanned using an ImageScanner™ III (GE Healthcare). Protein spot profiles were analysed using  
140 Progenesis SameSpots version 3 software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) as  
141 described (Arianmanesh et al., 2011). Briefly, reference gel was selected and the other gels were  
142 aligned to be closely matched to this reference gel. Background was subtracted individually from  
143 each gel and spot volumes were normalised relative to total spot volume individually for each gel.  
144 Ultimately, 15 were selected for identification by LC-MS/MS on the basis of significance (log-  
145 normalised spot volumes had to differ between two groups at the level of  $P < 0.05$  by ANOVA and  
146 post-hoc testing), spot volume (a difference of a  $\geq 1.25$ -fold increase or decrease between two  
147 groups), concentrating on the most abundant proteins with the most stable expression across the 4  
148 replicate gels for each group.

149

### 150 **Mass spectrometry**

151 To identify proteins, 15 selected spots were excised from stained gels and subjected to in-gel  
152 trypsin digestion as described previously (Uwins et al. 2006). The peptide fragment mass spectra

153 were acquired on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer  
154 operated in the reflection delayed extraction mode. Tryptic peptides from the MS/MS spectra were  
155 applied to search the NCBI (National Centre for Biotechnology Information) database with the  
156 MASCOT program (<http://www.matrixscience.com>). Search parameters for the programme  
157 included maximum allowed error of peptide mass 250 ppm, cysteine as S-carbamidomethyl-  
158 derivative and oxidation of methionine were allowed.

159

### 160 **Statistical Analysis**

161 Normality of data was tested with the Shapiro-Wilk test. Normally distributed data were subjected  
162 to one- and two-way ANOVA and Bonferroni post-hoc test using SPSS 17.0 software to assess  
163 significance of differences. Statistical comparisons between specific groups were carried out by  
164 student's t-test. Differences were considered significant at  $P < 0.05$ .

165

### 166 **Results**

167 Overall, 998 protein spots were included (on the basis of clear, reproducible expression and  
168 absence of noise in all four gels for each group) for analysis from a total of 1482 distinct protein  
169 spots detected by automatic detection with Progenesis SameSpots Software. The number of spots  
170 showing statistically significant differences in normalized spot volumes between groups is shown  
171 in Table 1.

172

173 Comparison between the GH and the NG uterine horns at day 16 of pregnancy revealed that 47  
174 (3%) of protein spots were significantly changed ( $P < 0.05$ ). Among these, 35 normalized spot  
175 volumes were up-regulated and 12 normalized spot volumes were down-regulated (Table 1).

176 Comparison between the GH and the NG uterine horns at day 20 of pregnancy revealed that 27  
177 (2%) of protein spots were significantly changed ( $P < 0.05$ ). 25 of these normalized spot volumes  
178 were up-regulated and 2 normalized spot volumes were down-regulated (Table 1). In GH uterine  
179 horns, 48 (3%) of protein spots were significantly changed ( $P < 0.05$ ) between days 16 and 20 of

180 pregnancy. In this category, 17 normalized spot volumes were up-regulated and 31 normalized spot  
181 volumes were down-regulated (Table 1). In NG uterine horns, 48 (3%) of protein spots were  
182 significantly changed ( $P<0.05$ ) between days 16 and 20 of pregnancy. Among these, 30 normalized  
183 spot volumes were up-regulated and 18 normalized spot volumes were down-regulated (Table 1).

184

185 The proteins spots in GH and NG uterine horns exhibiting significant differences in expression at  
186 implantation day and early post-implantation period and identified are shown in Table 2.  
187 Adenosylhomocysteinase (AHCY, Figure 2A) increased ( $P<0.05$ ) in NG uterine horns compared to  
188 GH uterine horns at both days 16 and 20 of pregnancy. (Table 2). Carbonic anhydrase 2 (CA-II,  
189 Figure 2B), increased ( $P<0.05$ ) in GH uterine horns compared to NG uterine horns at both days 16  
190 and 20 of pregnancy (Table 2). Heat shock 60 kDa protein 1 (HSP60, Figure 2C), increased  
191 ( $P<0.05$ ) in NG uterine horns compared to GH uterine horns at day 20 of pregnancy. (Table 2). In  
192 GH uterine horns, HSP60 decreased (Figure 2C,  $P<0.05$ ) at day 20 when compared with day 16 of  
193 pregnancy (Table 2).

194

195 In GH and NG uterine horns, proteasome activator subunit 2 (PA28beta/PSME2, Figure 3A)  
196 increased ( $P<0.05$ ) at day 20 when compared with day 16 of pregnancy (Table 2). In GH uterine  
197 horns, apolipoprotein A-1 (APOA1, Figure 3B) increased ( $P<0.05$ ) at day 20 when compared with  
198 day 16 of pregnancy (Table 2). In GH and NG uterine horns, transferrin (TF, Figure 3C) decreased  
199 ( $P<0.05$ ) at day 20 when compared with the day 16 of pregnancy (Table 2). In GH uterine horns,  
200 galectin 15 (LGALS15, Figure 3D) decreased ( $P<0.05$ ) at day 20 when compared with day 16 of  
201 pregnancy (Table 2).

## 202 **Discussion**

203 The establishment of pregnancy requires correctly timed, exquisitely coordinated, adaptive,  
204 responses of the uterine endometrium to the presence of conceptuses. Some of these responses will  
205 be via modification of the expression of functional proteins during early pregnancy. The proteomic  
206 profile of sheep CAR endometrium reported here provided a new understanding about the role of



207 conceptus-derived signals in the regulation of a substantial number of functional proteins involved  
208 in iron transport and homeostasis, hydrolysis, protein chaperoning and degradation, amino acid  
209 metabolism, cholesterol transport and cell adhesion.

210

211 Conceptus-derived factors reduce the expression of AHCY protein as evidenced by the down-  
212 regulation of this protein in endometrial caruncular tissues of the gravid uterine horns compared  
213 with the non-gravid uterine horns at both implantation and post-implantation periods (present  
214 study). AHCY catalyzes the breakdown of S-adenosylhomocysteine (AdoHcy) to adenosine (Ado)  
215 and L-homocysteine (Hcy) (Turner et al., 2000). It is important to note that hyperhomocysteinemia  
216 (HHcy) exerts adverse effects through the induction of inflammation pathways, including  
217 endothelial monocyte adhesion and infiltration (Wang et al., 2002), oxidative stress, activation of  
218 pro-inflammatory factors and endothelial dysfunction (Lawrence de Koning et al. 2003). Hcy  
219 activates NADPH oxidase and increases reactive oxygen species in human umbilical vein  
220 endothelial cells (Dong et al., 2005). Elevated level of Hcy within organs and tissues is therefore a  
221 potentially pathophysiological risk factor for uterine endothelial function via an enhancement of  
222 oxidative stress and inflammation. Maternal Hhcy **shoud be associated with** placental abruption and  
223 spontaneous abortion (Ray and Laskin, 1999). Increased Hcy levels and oxidative stress represent a  
224 risk factor for the establishment and maintenance of pregnancy (Micle et al. 2012). Therefore, the  
225 conceptus must hold Hcy in check within CAR endometrium at conceptus attachment (Day 16) and  
226 early post-implantation period (Day 20) of pregnancy through down-regulation of AHCY protein  
227 expression (present study). We suggest that the conceptus-derived factors exert local effects within  
228 the endometrium to counteract peri-implantation oxidative stress through the control of Hcy  
229 production and thereby support the establishment of pregnancy.

230

231 Upregulation of CA-II protein expression by conceptus-derived signals observed here on the day of  
232 conceptus implantation and during early post-implantation period had not been reported previously  
233 in endometrium of any mammalian species. Identification of these signaling molecules is essential  
234 in our understanding of the molecular mechanisms that should be involved in the establishment of

235 pregnancy. CAII catalyzes the reversible hydration of carbon dioxide to bicarbonate and plays an  
236 important role in acid-base homeostasis within tissues of biological systems (Khalifah, 1971).  
237 These reactions are requisite for cancer development, invasion and progression. Interestingly, CA  
238 II is highly expressed in tumours of different organs, including brain (Parkkila et al., 1995a),  
239 pancreas (Parkkila et al., 1995b) and kidney (Parkkila et al., 2000), where it favourably induces an  
240 environment necessary for the growth and spread of the tumour by changing acidity of the  
241 extracellular medium surrounding cancer cells. In the neonatal mouse uterus, where members of  
242 the CA family are expressed (Hu et al., 2004), CAII mRNAs were localized in epithelial and  
243 stromal cells of the endometrium suggesting a functional role for CAII in endometrial gland  
244 development during postnatal uterine development (Hu and Spencer, 2005). The expression of CA  
245 II in the bovine (Nishita et al., 1990) and human (Aliakbar et al., 1990; Muhlhauser et al., 1994)  
246 placentas supports the suggestion that this enzyme is required for endometrial tissue remodelling.  
247 Endometrium structural remodelling in ruminants, including sheep, plays crucial role in  
248 implantation, placentation and conceptus nutrition (Igwebuike, 2009). On the day of conceptus  
249 attachment (day 16 of pregnancy), there is close contact between trophoblast, the extra-embryonic  
250 membrane of the conceptus, and the epithelium overlying CAR endometrium, over raised areas of  
251 the endometrium, to allow implantation and early placental development. The high level of CA-II  
252 protein expression in CAR endometrium of the gravid uterine horns (present study) likely suggests  
253 an important role for this regulated protein in promoting trophoblast attachment, invasion and  
254 fusion with endometrial epithelium and/or remodelling the endometrium for successful early  
255 conceptus implantation and, consequently, formation of the maternal-fetal interface during  
256 placental development.

257

258 HSP60 has multiple functions in the normal cells, including inter-organelle transport, inhibition of  
259 aggregation of denatured polypeptides, antigen presentation, pro-apoptotic activity (Yu et al., 2012)  
260 and promotion of the proper folding of polypeptides (Witkin et al., 1996). Moreover, it stimulates  
261 human sperm capacitation in the fallopian tube (Lachance et al., 2007). In human endometrium,  
262 HSP60 increased during the late proliferative and early secretory phase then decreased in the mid

263 to late secretory phase while other members of this family probably protect endometrial proteins  
264 against factors involvement in denaturant activity such as TNF- $\alpha$ , particularly in the implantation  
265 window (Tabibzadeh et al., 1999). Therefore, a significant reduction in HSP60 expression in CAR  
266 endometrium of the gravid horns during the early conceptus post-implantation period (present  
267 study) may be due to protein redundancy in which the other members of HSP family take over the  
268 role of HSP60 in the chaperon activity that is assumed to be required at the time of conceptus  
269 implantation.

270

271 A balance between protein synthesis and degradation of abnormal, damaged and short-lived  
272 proteins by proteasomes (Hochstrasser, 1995) is essential for several cellular processes, including  
273 cell cycle and division (King et al., 1996), proliferation and apoptosis (Naujokat and Hoffmann,  
274 2002) and gene transcription (Muratani and Tansey, 2003). The turnover of proteins within cells by  
275 the ubiquitin-proteasome system depend on proteasome activators (Zhang et al., 1998). The  
276 proteasome activator 28 (PA28 or PSME) consists of two homologous subunits, PA28-alpha (or  
277 PSME1) and PA28-beta (or PSME2), each of which activates the proteasome (Zhang et al., 1998).  
278 Up-regulation of PA28- $\beta$  protein expression observed in the present study in sheep CAR  
279 endometrium during the early conceptus post-implantion period had not been reported previously.  
280 Of note is that PA28-beta protein expression increased dramatically in both the gravid and non  
281 gravid uterine horns suggesting a systemic rather than a local effect on endometrium PA28- $\beta$   
282 protein expression. These results suggest that factors present and associated with early pregnancy  
283 enhance PA28- $\beta$  protein expression. The high level of PA28- $\beta$  protein expression in CAR  
284 endometrium of the gravid and non gravid uterine horns (present study) likely suggests an  
285 important role for this regulated protein in protein-turnover since one can expect that the  
286 endometrium protein synthesis should be increased during the early post-implantation period.  
287 There **should be** evidence to suggest that proteasomes are parts of cellular defense mechanism  
288 against oxidative stress and protein oxidative damage by controlling the degradation of oxidatively  
289 damaged proteins (Ding et al. 2006; Poppek and Grune, 2006; Squier, 2006). Beside the high  
290 antioxidative capacity of the sheep CAR endometrium in the early conceptus post-implantation

291 period (Al-Gubory and Garrel, 2012), the dramatic post-implantation increase in PA28- $\beta$  protein  
292 expression observed in the present study probably plays an important role in degradation of  
293 oxidised endometrium proteins during early pregnancy.

294

295 APOA1 is a main component of HDL synthesized by the liver and intestine (Zannis et al., 1985). In  
296 fertile women, APOA1 was down-regulated in secretory endometrium compared to proliferative  
297 endometrium (Brosens et al., 2010). In infertile women, Apo-A1 increased in mid-secretory phase  
298 endometrium as compared to early-secretory phase endometrium (Manohar et al., 2014).  
299 Deregulations of endometrial APOA1 protein (Fowler et al., 2007) and mRNA (Brosens et al.,  
300 2010) expression are important features of endometriosis in women. These findings suggest a role  
301 of Apo-A1 in endometrium preparation for conceptus implantation and development. HDL  
302 cholesterol and APOA1 play a crucial role in human embryo development (Baardman et al., 2013).  
303 Of note, the increased level of APOA1 secretion by blastocysts in spent media from cultures of  
304 high quality blastocysts compared to low quality blastocysts and this may be associated with  
305 implantation potential (Mains et al., 2011). Moreover, APOA1 is a source of nutrients for the early  
306 post-implanted conceptus (Assemat et al., 2005). In the present study, we showed that APOA1 was  
307 highly expressed in CAR endometrium from the gravid uterine horns at the early conceptus post-  
308 implantation period. Given APOA1 functions, it may be assumed that the implantaing conceptus  
309 exerts local effects on CAR areas of the sheep endometrium of the gravid horns to increase the  
310 production of apoA-I-containing lipoproteins necessary for early conceptus development and  
311 survival.

312

313 Transferrin (TF), an iron-binding and transport protein, is detected in sheep intrauterine luminal  
314 fluid between days 17 and 18 of pregnancy suggesting that TF is a conceptus-synthesized protein  
315 (Lee et al., 1998). However, it is unlikely that TF is synthesied and secreted solely by the  
316 developing conceptuses during the peri-implantatiuon periods. Indeed, it has been reported that  
317 porcine intrauterine fluid on day 16 of the oestous cycle or pregnancy contains high amount of TF  
318 (Vallet et al., 1996). In addition, TF protein expression in sheep CAR endometrium increased at

319 day 16 of the oestrous cycle as compared to the matching day of pregnancy (Al-Gubory et al.,  
320 2014). Interestingly, TF protein was highly expressed in CAR endometrium of the gravid and non  
321 gravid uterine horns on the day of conceptus attachment when compared with the early post-  
322 implantation period (present study). Therefore, under the physiologically relevant in vivo  
323 conditions of a unilaterally pregnant ewes and conceptus development, it is likely that the sheep  
324 endometrium is major source of TF during early pregnancy. Considering the role of TF in the  
325 proliferation and differentiation of mouse embryonic tissues in culture (Ekblom et al. 1981;  
326 Thesleff and Ekblom, 1985), it is likely that TF could be required for sheep conceptus  
327 development during early pregnancy.

328

329 Galectins are a family of beta-galactoside-binding lectins. In the endometrial luminal epithelium of  
330 pregnant ewes, galectin-15 mRNA expression increased between days 12 and 16, and galectin-15  
331 (LGALS15) protein in the uterine lumen increased between days 14 and 16 of pregnancy (Gray et  
332 al., 2004). LGALS15 is expressed uniquely in the endometrium of sheep and goats and plays an  
333 important role in trophoblast attachment (Lewis et al., 2007; Farmer et al., 2008). It is important to  
334 note that LGALS15 protein expression was not different between the gravid and non gravid uterine  
335 horns at the day of conceptus attachment and early post-implantation period (present study).  
336 Moreover, in the gravid uterine horns, LGALS15 decreased at post-implantation period when  
337 compared with the attachment day. These results suggest that the regulation of LGALS15  
338 expression in sheep CAR endometrium likely does not depend on factors produced by the  
339 conceptus during early pregnancy.

340

341 In conclusion, our study provide evidence that conceptus-derived signals play key roles in the  
342 regulation of multiple functional proteins in sheep CAR endometrium, importantly AHCY, CA-II,  
343 HSP60 and APOA1 during conceptus implantation and the early post-implantation periods. These  
344 regulated proteins likely involved in providing a suitable intra-uterine environment required for  
345 conceptus attachment, implantation, early post-implantation development and successful  
346 establishment of pregnancy in sheep.



348 **Declaration of interest**

349 The authors declare that there is no conflict of interest that could be perceived as prejudicing the  
350 impartiality of the research reported.

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357 help and sheep management.

358 **Author contributions**

359 KHA jointly conceived and designed the study with PAF. KHA prepared the animal model,  
360 performed surgery and tissue collection. MA carried out the proteomic analysis, performed  
361 production and acquisition of data. KHA and MA wrote the manuscript. KHA and PAF contributed  
362 reagents and materials and helped in data interpretation. PAF made critical revisions of the  
363 manuscript for important intellectual content. All authors approved the final version of manuscript.

364

365 **Figure Legends**

366

367 **Figure 1.** Sheep caruncular endometrial proteome separated by 2DE gel using a 3-10 pH gradient.

368 A representative 2DE gel of the caruncle proteins from sheep non-gravid (NG) horn on day 20 of  
369 pregnancy (NG20) is shown, indicating selected spots for cutting by arrows.

370

371 **Figure 2.** Expression changes of (A) adenosylhomocysteinase (AHCY), (B) carbonic anhydrase 2  
372 (CA-II), and (C) heat shock 60kDa protein 1 (HSP60) in sheep caruncular endometrial tissues  
373 collected from gravid horns (GH) and non-gravid horns (NG) of uteri at implantation (day 16 of  
374 pregnancy) and post implantation (day 20 of pregnancy) periods. Normalised protein spot volumes  
375 are shown as means  $\pm$  SEM (n=4 ewes per group). Zoom boxes from the 2D gels showing the  
376 identified proteins (arrows) are shown (right panels). The acceptable level of significance was set at  
377  $P < 0.05$ .

378

379 **Figure 3.** Expression changes of (A) proteasome activator 28 beta (PA28beta), (B) apolipoprotein  
380 A-1 (APOA1), (C), transferrin (TF) and (D) galectin 15 (LGALS15) in sheep caruncular  
381 endometrial tissues collected from gravid horns (GH) and non-gravid horns (NG) of uteri at  
382 implantation (day 16 of pregnancy) and post implantation (day 20 of pregnancy) periods.  
383 Normalised protein spot volumes are shown as means  $\pm$  SEM (n=4 ewes per group). Zoom boxes  
384 from the 2D gels showing the identified proteins (arrows) are shown (right panels). The acceptable  
385 level of significance was set at  $P < 0.05$ .

386

387



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536 **Table 1.** Numbers of protein spots significantly ( $P < 0.05$ ) differing between caruncle of gravid  
 537 horns (GH) and caruncle of non-gravid horns (NG) of sheep endometrium at the time of  
 538 conceptus implantation (Day 16) and early post-implantation (Day 20) periods of pregnancy.  
 539

540 Groups compared

541	Group	Compared with	Total number	Up-regulated	Down-regulated	% of total spots
542			of spots			
543	NG16	GH16	47	35	12	3
544	NG20	GH20	27	25	2	2
545	GH20	GH16	48	17	31	3
546	NG20	NG16	48	30	18	3

547



548 **Table 2** Caruncle proteins of gravid (GH) and non-gravid (NG) uterine horns demonstrating significant differences in expression at conceptus implantation  
 549 (Day 16) and early post-implantation (Day 20) periods of pregnancy. The significant fold changes are shown in bold with their corresponding P values  
 550 (P<0.05). Increases in spot volumes are denoted by a “+” and decreases by a “-“ prefix to the fold-change values. The comparisons between groups follow the  
 551 rule that the fold-changes are calculated on the basis that the first group is being compared with the second group. Accession number is written regarding to  
 552 bovine species. The accession number specific for ovine protein is shown in brackets if available.

Protein	Spot no.	MW (KDa)	PI	MOWSE score (MASCOT)	Swiss-Prot	Fold change (P value)			
						NG16 vs. GH16	NG20 vs. GH20	GH20 vs. GH16	NG20 vs. NG16
<b>Actin binding protein</b>									
Gelsolin isoform b (GSN)	520	80.9	5.54	413	<b>Q3SX14</b>	+1.07	<b>+1.28 (0.031)</b>	-1.1	+1.08
<b>Iron transport and homeostasis</b>									
Transferrin (TF)	1463	79.8	6.75	473	<b>Q29443</b>	+1.04	+1.18	<b>-1.96 (0.001)</b>	<b>-1.73 (0.0001)</b>
<b>Hydrolase</b>									
Adenosylhomocysteinase (AdoHcyase) (AHCY)	964	48.1	5.88	932	<b>Q3MHL4</b>	<b>+1.27 (0.008)</b>	<b>+1.18 (0.03)</b>	-1.00	-1.08
<b>Cytokine and nucleotide binding protein</b>									
<sup>P</sup> High mobility group box 1 protein (HMGB1)	1242	25.0	5.75	416	<b>P63158</b>	<b>+1.32 (0.003)</b>	+1.07	+1.03	-1.20
<sup>S</sup> Cytokine induced protein 29 KDa (CIP29)		23.6	5.98	202	<b>Q2TBX1</b>				
<b>Metalloenzyme</b>									
Carbonic anhydrase 2 (CA-II)	1267	29.1	6.41	546	<b>P00922</b>	<b>-1.34 (0.0004)</b>	<b>-1.39 (0.049)</b>	+1.02	+1.06
<b>Actin binding protein, heparin binding protein</b>									
<sup>P</sup> Tropomyosin alpha-1 chain (TPM1)	1102	32.7	4.74	309	<b>Q91XN6</b>	-1.11	+1.06	+1.07	<b>+1.27 (0.013)</b>
<sup>S</sup> Hepatoma derived growth factor (HDGF)		26.3	4.84	176	<b>Q9XSK7</b>				

<b>Chaperones</b>									
Heat shock 60kDa protein 1 (HSP60)	791	61.1	5.71	1660	<b>P31081</b>	-1.02	<b>+1.27 (0.01)</b>	<b>-1.19 (0.044)</b>	+1.08
<b>Amino acid metabolism, Metabolism</b>									
<sup>P</sup> Glycine amidinotransferase, mitochondrial (GATM)	979	48.8	8	448	<b>Q2HJ74</b>	<b>+1.41 (0.04)</b>	+1.21	-1.12	-1.32
<sup>S</sup> Isocitrate dehydrogenase 1 (NADP+), soluble (IDH1)		47.1	6.34	365	<b>Q9XSG3</b>				
<b>Cholesterol transport</b>									
Apolipoprotein A-1 (APOA1)	1320	28.4	5.57	457	P02647	+1.11	-1.40	<b>+2.26 (0.01)</b>	+1.45
<b>Protein degradation</b>									
Proteasome activator subunit 2 (PA28beta) (PSME2)	1253	27.5	5.31	376	<b>Q5E9G3</b>	-1.12	+1.14	<b>+2.7 (0.0001)</b>	<b>+3.46 (0.0006)</b>
<b>Ion transport</b>									
Chloride intracellular channel protein 1 (CLIC1)	1231	23.8	5.12	169	<b>O00299</b>	-1.25	+1.07	+1.12	<b>+1.28 (0.03)</b>
Potassium channel tetramerisation domain containing 12 (KCTD12)	1109	47	5.68	260	616416 (NCBI)	-1.15	+1.04	+1.06	<b>+1.35 (0.0002)</b>
<b>Cell adhesion</b>									
Galectin 15 (LGALS15/OVGAL11)	1456	15.5	5.22	405	<b>Q19MU7*</b>	-1.19	+1.52	<b>-1.91 (0.003)</b>	-1.05

553 \* The accession number is for ovine species as the accession number for bovine was not found.

554

555 For 3 spots, peptide fragments were identified that belonged to more than one protein and the primary protein in the spot was identified based on 1) highest

556 Mascot score, 2) best agreement between estimated (ie, from electrophoretic gel mobility) and calculated molecular weight and isoelectric point, and 3)

557 highest peptide coverage.

558 <sup>P</sup> = primary protein in the spot ; <sup>S</sup> = secondary protein in the spot



Figure 1

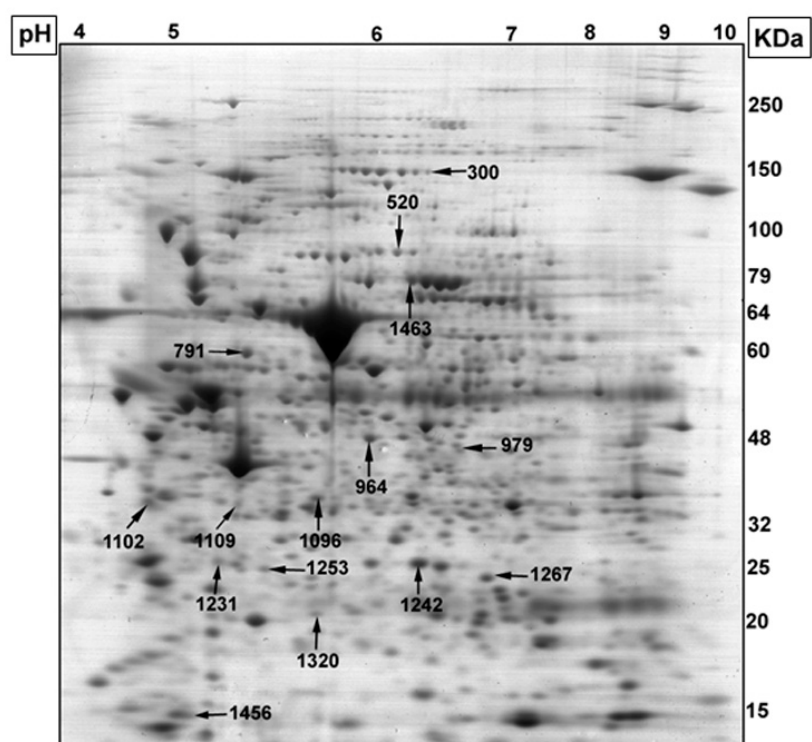


Figure 2

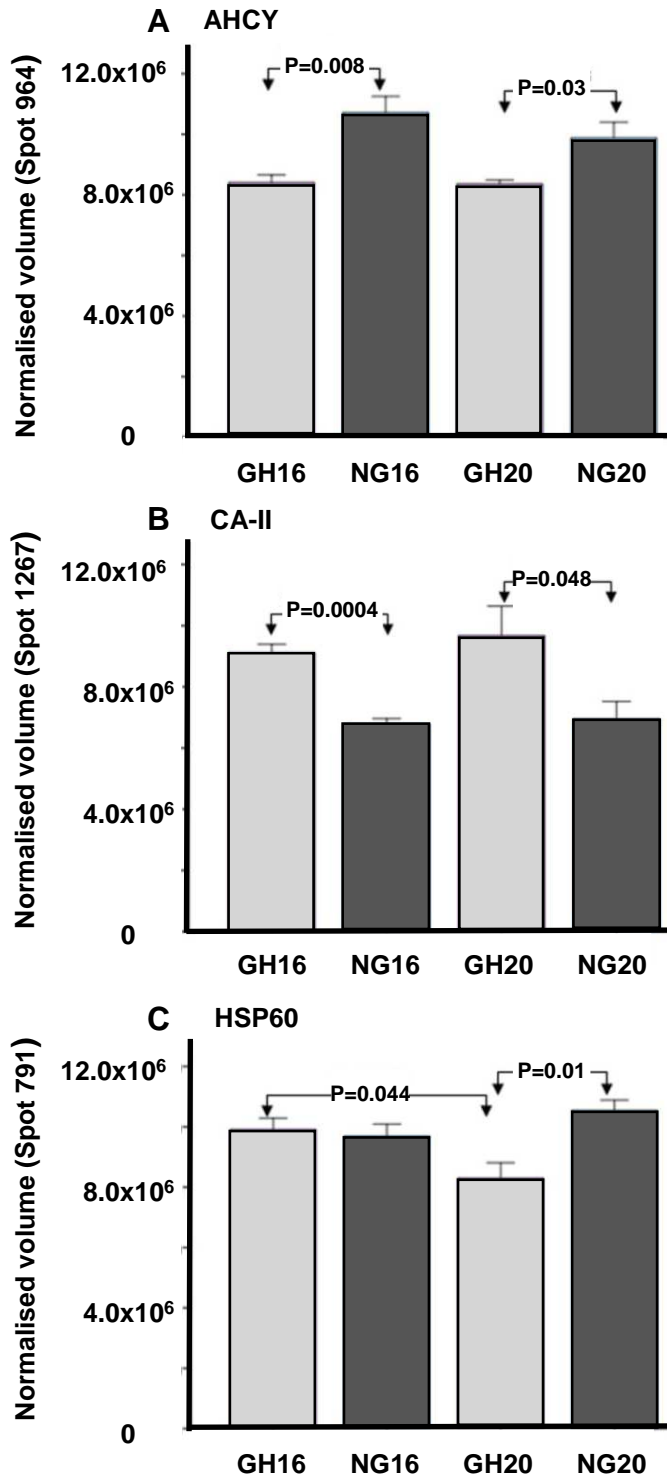
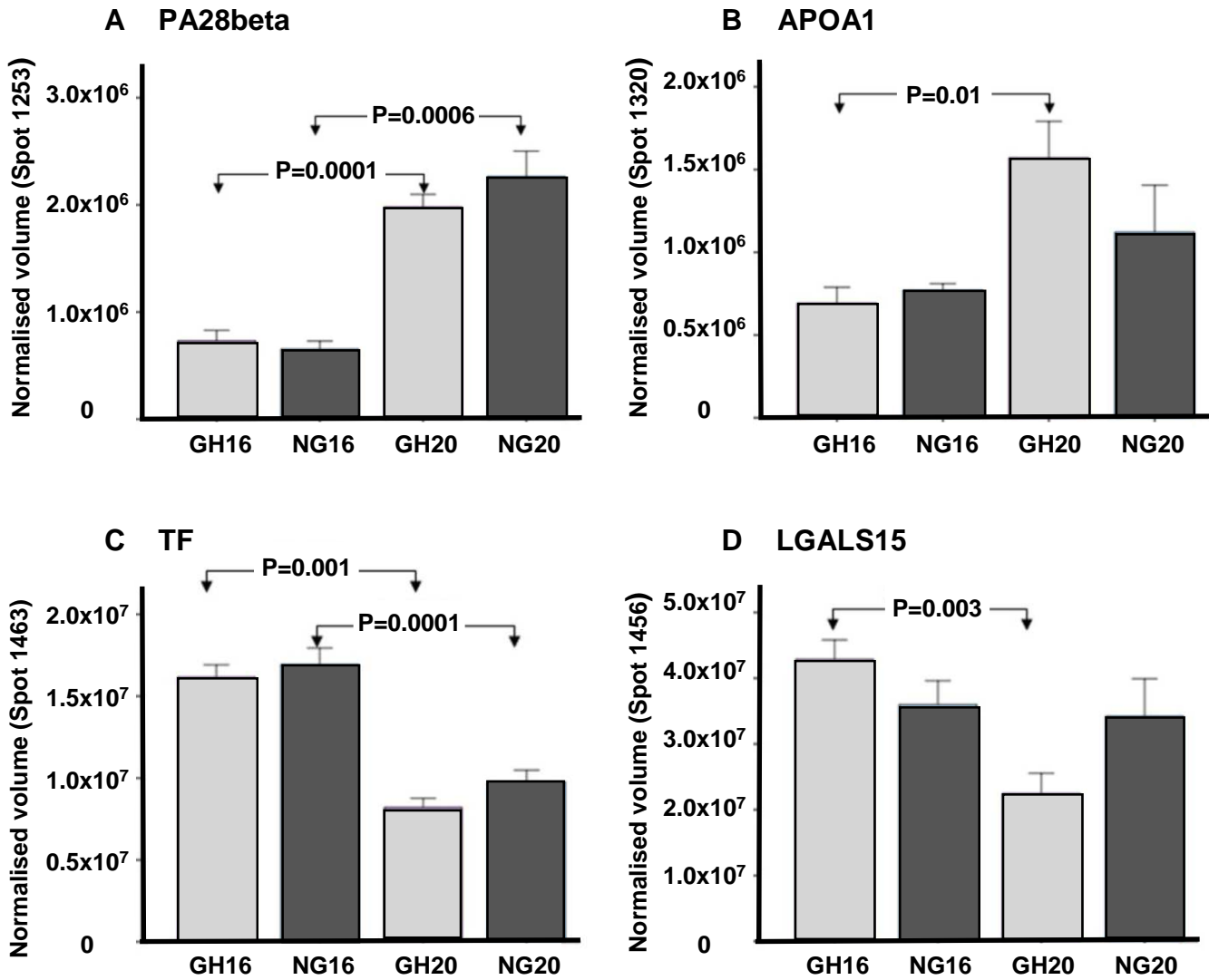


Figure 3



Conflict of Interest Statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.